CRYOPRESERVATION OF RAT SPERMATOZOA: IMPACT OF FREEZING RATE INFLUENCED BY LIQUID NITROGEN VAPOR PHASE COOLING ON POST-THAW SPERM MOTILITY

by

KATRINA FOX

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Major Professor
Dr. Mark Weiss

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Abstract

Artificial insemination and cryopreservation of sperm are important components of any transgenic animal facility because they allow for the reduction in animal colony size and the safe storage of germplasm from valuable strains. In addition, they allow long-term storage of these strains and easy transportation of the genetic material to other research facilities internationally. Thus far, only one laboratory has created live rat pups after sperm cryopreservation and intrauterine insemination. Another laboratory made advances in cryopreservation media that improved sperm motility post-thawing, but no pups resulted from this work. In my study, these two cryopreservation media were utilized to perform intrauterine inseminations with both fresh samples of rat sperm as well as samples that were cryopreserved in liquid nitrogen to replicate and extend these studies. Pharmacoejaculation was tested as a means to obtain spermatozoa without euthanizing the male to collect the epididymis, but results were inconsistent and the samples were not useful for intrauterine inseminations or cryopreservation. Epidiymal sperm was then collected into the various media and frozen in liquid nitrogen. In my hands, the frozen/thawed rat sperm achieved motility of less than 1%. Next, the impact of altering the freezing rate on sperm motility was evaluated. Epididymal sperm was collected and processed using a modified protocol and were then frozen at 2, 4 or 6 cm above the level of liquid nitrogen. Four to six days after freezing, samples were thawed and post-thaw sperm motility was evaluated. Sperm motility was measured prior to freezing as well as after-thawing. The sperm motility was correlated with LIVE/DEAD® staining. Sperm motility did not differ between the groups as a result of the freezing rate (Friedman test $p=0.23$). The published techniques are not robust and require further development to improve the motility of rat sperm after cryopreservation and achieve pregnancy via intrauterine insemination.

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Dedication

I would like to dedicate this work to my husband, Bruce, and my family for their never ending support throughout this process. I couldn't have done it without them.

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Chapter 1 - Introduction

Historically, cryopreservation of spermatozoa has been utilized for several purposes. In livestock and domestic species, sperm freezing has allowed for transporting samples across many miles to produce offspring with superior genetics in order to improve the species. It also allows for preservation of the genetic material of a sire for many years after his reproductive capabilities have waned. This preserved material may also be used to increase genetic diversity and prevent extinction of endangered species (Leibo and Songsasen, 2002).

In laboratory animals, cryopreservation techniques have been well-developed in mice (*Mus musculus*). Despite this success, these protocols have not been translated to rats (*Rattus norvegicus*) and they remain a species that are difficult to work with. Rats are well-known for their reproductive capabilities. Their short generation time, large litters, and general ease of breeding have made it possible for facilities to maintain colonies of animals. Rats are commonly used in research and new strains and transgenic animals are being produced to meet the demand of various animal models and research projects. Unfortunately, a large amount of time and effort is needed to develop these animals and they may not be needed for several months to years at a time. To prevent loss of this work, these animals are housed and bred in order to maintain the strains. Maintaining rat strains uses valuable animal housing space, resources of the investigator as well as the animal care staff and creates many animals that are ultimately euthanized.

In the welfare of research animals, the "three R's" are often discussed (Zurlo *et al.*, 1996). These include the **refinement** of animal use to reduce potential pain and distress and **replacement** with non-animal models when possible. The last R is for **reduction** of animal numbers. Cryopreservation of sperm will help fulfill this goal. When a strain of rats is not needed for a current project, sperm can be collected from the males and frozen for storage in liquid nitrogen. In the event that an investigation is designed that requires recovery of that strain, the sperm can be thawed and used to produce new animals. The frozen straws can also be transported to other facilities without the need for shipping live animals and having them undergo quarantine. Having genetic material preserved will also be beneficial in the event of disease spread through a facility or other catastrophic event.

Intrauterine insemination is a technique that has low technological requirements unlike other artificial reproductive methods such as intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF) (Kashiwazaki *et al.*, 2010; Kaneko *et al.*, 2007). By evaluating the ability of the sperm samples to be utilized in this manner, we are ensuring that other facilities could easily reproduce the strains. This would further ease the sharing of valuable rat strains between investigators and help further research in multiple fields.

Through my preliminary work, I modified published cryopreservation protocols to obtain low sperm motility in post-thaw samples. One modification that achieved this sperm motility was altering the distance above the liquid nitrogen vapor phase stage of the cooling process to alter the freezing rate. By increasing this distance, the temperature was increased and the rate of freezing was slowed. To evaluate the effect of this stage of the freezing process, multiple trials were completed at various heights and post-thaw sperm motility was evaluated.

Chapter 2 - Literature Review

General Overview

Though man has been cryopreserving sperm since the 1600s, even today, few species can be cryopreserved with an efficiency of less than 50% of pre-freeze sperm motility being maintained (Walters *et al.*, 2009). In 1990, several groups published success with cryopreservation of mouse sperm (Tada *et al.*, 1990; Yokoyama *et al.*, 1990), with reported post-thaw sperm motility of up to 50% in both studies. The protocols developed were easily repeatable with consistent results of sperm motility maintenance within strains. Despite similarities between rat and mouse sperm, including their sensitivity to mechanical stress and membrane permeability, the mouse protocols that were developed were not successfully applied to rats (Critser and Mobraaten, 2000; Si *et al.*, 2006).

A report published in 2001 was the first time frozen-thawed rat sperm was used for successful production of rat pups via intrauterine insemination (IUI) (Nakatsukasa *et al.*, 2001). Nakatsukasa developed a freezing medium based upon Niwa and Sasaki Freezing extender (NSF-I) medium used for porcine sperm (Kikuchi *et al.*, 1999) and utilized it to freeze epididymal sperm samples from Sprague Dawley and Wistar rats. A post thaw sperm motility of approximately 9% (an 88% loss from pre-freezing sperm motility) was obtained and the procedure was successful in producing pups via intrauterine insemination (Nakatsukasa *et al.*, 2001). This work was repeated utilizing various strains of rats with similar results (Nakatsukasa *et al.*, 2003). These included representatives of closed colony, inbred, mutant and transgenic strains. Post-thaw sperm motility obtained ranged from 2 to 12% and pregnancies were produced from each of the samples (Nakatsukasa *et al.*, 2003).

In 2007, another group in Japan published the first article in their journey to develop a freezing medium that would net a higher post-thaw motility in sperm samples based upon the investigator's work in poodle dogs. The first article evaluated multiple buffers, including a modified Kreb's ringer bicarbonate solution (mKRB), phosphate-buffered saline (PBS) and distilled or deionized water (Yamashiro *et al.*, 2007). The addition of different sugars, varying concentrations of raffinose, glycerol, sodium dodecyl sulfate (SDS) and Equex was also evaluated. The solution with the best post-thaw sperm motility, ranging from 34 to 38%,

contained 0.1 M raffinose in mKRB with 0.75% Equex STM, 0.05% SDS and 20% egg yolk (Yamashiro *et al.*, 2007). This group's next publication occurred in 2010 when they used the previously defined media and experimented with the addition of lactate, glucose, pyruvate and adenosine triphosphate (ATP) to provide a source of energy for the sperm (Yamashiro *et al.*, 2010a). A combination of 32.35 mM lactate and 1.85 mM ATP was determined to increase the post-thaw sperm motility to 35%, which was greater than samples without those additions, but similar to results from the previous publication (Yamashiro *et al.*, 2010a). The final publication, also in 2010, evaluated the addition of cyclic adenosine monophosphate (cAMP) and ionomycin to increase calcium influx into the cell which will activate the Krebs cycle and provide the fuel needed to create sperm motility (Yamashiro *et al.*, 2010b). The final medium that gained the best post-thaw sperm motility result contained100 µM dibutyryl cAMP (dbcAMP) in addition to the above mentioned raffinose-mKRB-egg yolk extender with lactate and ATP. Though the post-thaw motility of the sperm samples increased to nearly 45%, attempts at obtaining pregnancy via IUI failed (Yamashiro *et al.*, 2010b).

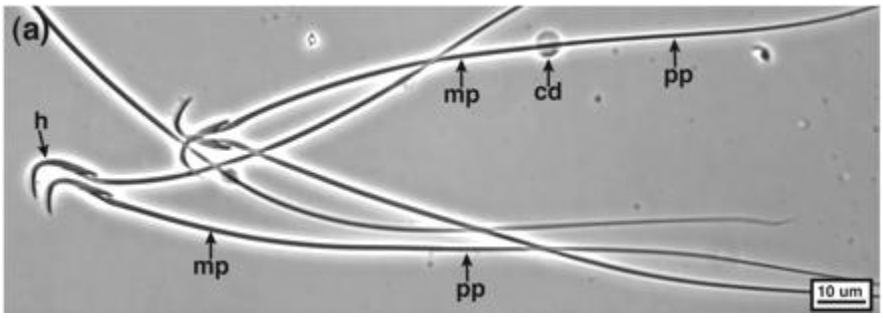
Sperm Structure

Mammalian sperm exhibit many similarities within anatomical structure across all species. The basic components of the sperm cell include a head and tail, or flagellum, which can be further divided into the midpiece, principal piece and end piece (McKinnon and Voss, 2011). All of these portions of the cell are surrounded by a plasma membrane.

The head of the sperm provides the genetic material for the male animal which is contained in the nucleus of the cell with a slight amount of cytoplasm. The acrosome is an organelle covering the rostral portion of the nucleus. It contains enzymes and other components necessary to adhere to the zona pellucida of the oocyte. Within the sperm flagellum is a structure called the axoneme. It is formed by microtubules surrounding a centriole at the base of the nucleus in the neck of the sperm. These microtubles are arranged in a 9+2 formation with nine doublet microtubules surrounding two singlet microtubules (Olson *et al.*, 2004). Surrounding the axoneme within the midpiece are mitochondria that provide the ATP required for motility of the cell. Dense fibers are located between the microtubules and mitochondria in the midpeice and extend through the length of the flagellum to provide structure and strength to this portion of the cell (McKinnon and Voss, 2011).

Rat sperm, like that found in other rodent species, has a unique anatomical structure when compared to other mammalian species. The sperm are longer in length, averaging 150 to 200 µm long in the rat (Krinke, 2000), compared to 61 to 86 µm in the horse (McKinnon and Voss, 2011). The head of the sperm cell is also uniquely formed into a hook-shape that is used to form sperm groups which is believed to be a component of sperm cooperation- groups of sperm joining together to increase the swimming velocity and thrusting force- present in many rodent species (Immler *et al.*, 2007). The volume of the rat sperm cell is approximately 36 to 37 μm^3, which is smaller than that of mice, but larger than boar, bull, stallion and human (Si *et al.*, 2006). The plasma membrane of rat sperm also has different lipid components than other species. In the tail of the epididymis (also called the *cauda epididymidis* or the caudal epididymis), phosphatidylethanolamine represents the largest percentage of membrane phospholipids, followed by phosphatidylcholine, phosphatidylserine and others (Hall *et al.*, 1991).

Figure 2.1 Rat sperm anatomy, figure from (Olson *et al.*, 2004).



Phase contrast photomicrograph. h- sperm head, mp- midpiece, pp- principal piece, cd- cytoplasmic droplet.

Sperm Collection

Sperm collection methods vary with each species. In domestic species, stallions are often collected utilizing an artificial vagina. Dogs and boars are manually stimulated for collection. Electrical stimulation of the pelvic nerves is utilized in bulls and small ruminants as well as in wildlife species being collected under general anesthesia. In all species, epididymal samples can be collected after death and ejaculated samples may be collected via flushing the female's reproductive tract. Erection, emission and ejaculation result from both parasympathetic and sympathetic actions on the nerve fibers originating in the pelvic plexus (Clement *et al.*, 2006). Stimulation of these nerve fibers can be provided with natural breeding activity, artificial collection, electrical stimulation or pharmacologically.

In rats, early research developed a method of using restraint and a modified electrical probe to stimulate ejaculation (Lawson *et al.*, 1967; Scott and Dziuk, 1959). A paper published in 1959 detailed a bipolar electrode that provided a 60-cycle alternating current that could be varied from 0 to 25 volts. After insertion into the rectum of a rat, the voltage was changed from zero to 2 to 3 volts and back to zero. The voltage peak was increased with each stimulus, providing a 5 to 10 second rest interval in between shocks, until it reached 10 to 15 volts. Stimuli at 10 to 15 volts were continued until the rat ejaculated or until a maximum of 25 stimuli were applied (Scott and Dziuk, 1959).

Due to the rats' accessory glands, a portion of the semen coagulates after ejaculation (Hart, 1970; Beil and Hart, 1973). This is responsible for forming the copulation plug that is necessary for conception during natural breeding (Carballada and Esponda, 1992; Ramm *et al.*, 2005). This created a challenge when collecting ejaculated sperm samples as they would often coagulate preventing their use for artificial breeding methods. In some males, the coagulated fluid also blocked the urethra causing the rats to develop uremia and die within 4 days of electroejaculation (Scott and Dziuk, 1959). Some researchers prepared the rats used for collection by surgically removing their coagulating glands and/or portions of the seminal vesicles (Scott and Dziuk, 1959; Lawson *et al.*, 1967; Carballada and Esponda, 1992). There was mixed results with this procedure as some animals still produced coagulating substances, likely from other accessory glands.

It was later discovered that by adjusting the strength and pattern of the electrical stimulation, it was possible to elicit expulsion of the sperm without coagulating factors (Birnbaum and Hall, 1961). A commercial audio oscillator to apply waves of frequency from 9 to 120,000 cycles per second with a voltage range of 0 to 6 volts was used as a stimulus. When it was applied to the rat's rectum at 30 cycles per second and a maximum of 2 volts, the ejaculate obtained had no coagulate (Birnbaum and Hall, 1961).

The number of sperm per ejaculated sample ranged from 415,000 to 342 million in one study. Sperm motility was graded on a scale of 0 to 6 with 0 indicating a sample with no motility and 6 being a sample with maximum motility. The average sperm motility obtained was 3.4 on this scale. No data on volume was provided (Scott and Dziuk, 1959). In another study, the volume of the ejaculate was reported to be one to two small drops containing an average of 7.94 million sperm (Lawson *et al.*, 1967).

There has been little research done since the 1960s with regard to electroejaculation. No research involving manual stimulation or the attempted use of an artificial vagina for sperm collection in rats was identified.

Another option for collecting an ejaculated semen sample without necessitating the euthanasia of the male utilized multiple female rats (Seita *et al.*, 2011). Males were allowed to naturally breed receptive females. After a sperm plug was visualized indicating breeding, the females were euthanized, their reproductive tracts were removed and flushed to collect the sperm sample. Samples collected in this manner had an average concentration of 32 to 45 million sperm per microliter when the uterus was flushed with 1 to 2 mL freezing medium (Seita *et al.*, 2011). While this preserves the male, it necessitates the euthanasia of multiple females to have a sample of enough volume to process (Seita *et al.*, 2011).

Ejaculation can also be stimulated pharmacologically (Clement *et al.*, 2006; Yonezawa *et al.*, 2005; Yonezawa *et al.*, 2000). A stimulating drug, such as p-chloroamphetamine (PCA), is injected into a rat, either intravenously or intraperitoneally, under general anesthesia. This amphetamine causes release of serotonin and catecholamines from monoaminergic nerve terminals, which stimulates the sympathetic nervous system causing ejaculation (Clement *et al.*, 2006). This often involves intense sympathetic nerve activity in the vas deferens nerve (Stafford *et al.*, 2006). This procedure typically occurs under general anesthesia and causes penile elongation and erection prior to ejaculation. The dosage of PCA most frequently used is 5 mg/kg and ejaculation is typically observed within 30 minutes of administration in 50 to 100% of the Wistar rats used in these studies (Yonezawa *et al.*, 2005; Clement *et al.*, 2006; Yonezawa *et al.*, 2000).

The most frequently used method of collecting sperm from rats is epididymal collection (Nakatsukasa *et al.*, 2003; Nakatsukasa *et al.*, 2001; Yamashiro *et al.*, 2007). This circumvents the addition of fluids from the accessory glands and the sperm cells located in the *cauda epididymidis* have matured to have fertilizing capabilities. The male is typically euthanized for this procedure, but it can also be performed on recently deceased animals or by unilateral surgical removal of an epididymis.

There are several methods for obtaining sperm from the *cauda epididymidis*. Slits may be cut into the tissue and allowing the sperm to swim out after being placed in solution (Nakatsukasa *et al.*, 2001). In another method, a portion of tissue is excised and the sperm

sample is allowed to well up and then be removed (Yamashiro *et al.*, 2007). Alternatively, a small gauge needle may be introduced and a few droplets aspirated (Kashiwazaki *et al.*, 2010). Retrograde flushing may also be used to force sperm out of the epididymis using a flushing medium instilled into the vas deference and allowed to exit an incision in the *cauda epididymidis* (McKinnon and Voss, 2011)

Sperm Motility and Capacitation

Motility of sperm cells is created by the propulsive force created by the axoneme located in the sperm flagellum. The microtubules utilize ATP created by mitochondria in the midpiece to create motion between the outer microtubules which causes flagellar bending (McKinnon and Voss, 2011). The motion between microtubules is facilitated by dynein arms extending between the doublet producing a sliding filament system. The dense fibers in the flagella provide structural support to the cell as motion occurs.

After the sperm leaves the epididymis in the course of ejaculation, it mixes with secretions from the accessory glands and is deposited in the female's reproductive tract. In rats, the sperm is deposited in the vagina, but transcervical transport to the uterus occurs within 6 to 20 minutes following ejaculation (Matthews and Adler, 1977). Once there, it undergoes many changes that allow it to fertilize the oocyte. This process is called capacitation and involves multiple steps including removal of cholesterol and other changes to the plasma membrane, acquisition of hyperactivated sperm motility and the ability to undergo the acrosome reaction (Roberts *et al.*, 2003).

There are many components involved in the capacitation process. Three of these are extracellular calcium, bicarbonate and a cholesterol-sequestering agent (Roberts *et al.*, 2003). During *in vitro* replications of this process, bovine serum albumin is often used as the protein source to remove cholesterol from the cell membrane (Zhou *et al.*, 2008). Once cholesterol is removed, calcium and bicarbonate can enter the cell and this increase in intracellular concentrations stimulates an increase in cyclic AMP. It has been shown that under *in vitro* conditions, the presence of cAMP is necessary for sperm motility (Lindemann *et al.*, 1987). When sperm have been capacitated, they exhibit a distinct pattern of movement consistent with hypermotility. A typical curvature of the flagellum takes place and the beat frequency is increased (Lindemann *et al.*, 1987).

The importance of this process in artificial reproduction is two-fold. When rat sperm are utilized for *in vitro* fertilization, this process must be artificially induced (Aoto *et al.*, 2011; Toyoda and Chang, 1974). It has also been shown that sperm that are capacitated prior to the cryopreservation process have a shortened life span and thus decrease their fertilization efficiency (Bailey *et al.*, 2000).

Artificial Reproduction Techniques

Once a sperm sample has been collected, there are multiple ways to create embryos and pregnancy. These range in levels of difficulty and technical requirements as well as success rates. The method selected will be dependent on the sperm sample obtained, the species of the animal and the skill of the operator. All methods require the female being at the proper stage of her reproductive cycle, which may be detected by monitoring or my synchronizing with various protocols. The timing of introduction of the sperm is dependent on the species. In rats, breeding takes place during late proestrus and early estrus (Krinke, 2000) and this should be taken into account when applying any artificial reproduction technique.

Artificial insemination (AI) involves direct placing of the sperm cells into the reproductive tract of the female animal. There are a variety of ways this can be accomplished, and the technique used is often specific to the species as well as the type of sperm sample being utilized (Faigl *et al.*, 2012). When preforming vaginal or pericervical AI, the semen sample is deposited in the vagina, as near to the cervix as possible. The sperm used for this route must be motile in order to penetrate the cervix. In dogs using fresh sperm samples, this technique has a 60 to 95% pregnancy rate (Makloski, 2012). In horses, a single insemination produced a 75% single cycle pregnancy rate (McKinnon and Voss, 2011)

The transcervical AI method requires more skill and appropriate tools and requires passing a catheter through the cervix to deposit the sperm into the uterus. This further reduces the distance the sperm have to travel, but there is some concern in small ruminants about the procedure damaging the cervix and reducing pregnancy rates (Faigl *et al.*, 2012). This has been successfully performed in rats, though supplemental oxytocin was administered to facilitate transport of the sperm through the uterine horns after insemination. There was a 7% pregnancy rate using this method that rose to 58% following administration of oxytocin when using frozen-thawed sperm samples (Nakata *et al.*, 2012). The samples utilized in this study had a post-thaw

sperm motility of at least 10%, and the volume of semen inseminated into each uterine horn contained 100,000 to 300,000 motile sperm cells.

Sperm can also be surgically introduced into the reproductive tract. Though this requires general anesthesia, or at minimum sedation and local anesthesia, to perform, laparoscopic techniques have been developed which reduces the size of surgical incision, but requires more specialized equipment (Makloski, 2012). The sperm is deposited directly into the uterus at either the body or the base of the uterine horn which decreases the distance the sperm must travel to reach the oocyte. With all methods of artificial insemination, the female reproductive tract fulfills the requirement of capacitating the sperm to allow for fertilization. Detection of the estrous cycle is needed to identify the correct timing for insemination and manipulation of the estrous cycle may be required to synchronize a group of females. In rats, insemination should occur at the same time as natural breeding would take place, which corresponds to late proestrus or early estrus (Krinke, 2000).

In vitro fertilization takes place when both the sperm and oocytes are collected and co-cultured in artificial medium to allow fertilization. After sperm is collected, it must be incubated for several hours to allow capacitation to take place to ensure fertilization capability (Kashiwazaki *et al.*, 2010). Superovulation protocols may be necessary and the female is often euthanized to collect the cumulus-oocyte complexes. A recipient animal must be prepared by inducing pseudopregnancy and must be timed to correspond with the age of the embryo that will be transplanted. The sperm and egg must be processed to prepare them for fertilization and culturing the embryo to at least the 2-cell stage is required (Toyoda and Chang, 1974; Aoto *et al.*, 2011). The embryos are then transferred into a recipient female's infundibulum, which is more technically difficult than the method of intrauterine insemination. The recipient female should be at the first day of diestrus, approximately 26 to 29 hours after induction of pseudopregnancy (Toyoda and Chang, 1974). If the embryo can be cultured to the morula or blastocyst stage, uterine transfer may be utilized, and the recipient female is allowed to progress further into her pseudopregnancy. Many IVF protocols are very time consuming, though one has been developed that may be completed within 12 hours (Aoto *et al.*, 2011).

Intracytoplasmic sperm injection (ICSI) can be performed with sperm that has low or even no motility (Kaneko *et al.*, 2007). Freeze-dried sperm can be utilized with this method, which eliminates the need for liquid nitrogen storage as well as removing the variability of

cryoprotective agents (Kaneko *et al.*, 2007; Kaneko and Serikawa, 2012). Intracytoplasmic sperm injection can also utilize spermatids obtained from frozen testicular biopsies (Jezek *et al.*, 2001). As with IVF, superovulation protocols may be necessary and the female is often euthanized to collect the cumulus-oocyte complexes. Oocytes still require treatment to prepare them for injection. The oocytes are freed from the cumulus cells using hyaluronidase, rinsed with an embryo culture medium and held at 37°C until the sperm are prepared for injection (Kaneko *et al.*, 2007). Sperm require damage to the membrane either via a cut on the tail or a decapitation procedure to detach the head from the tail. The sperm heads are then injected into the oocyte and they are cultured to the 2 cell stage prior to transfer into a pseudopregnant recipient female (Kaneko *et al.*, 2007). The advantages of this technique include the use of low to no motility sperm samples as well as only requiring one spermatozoa per oocyte (Kaneko *et al.*, 2007).

Rat Estrous Cycle

Rats begin to show regular estrous cycles approximately one week after opening of the vaginal canal, which occurs at 33 to 42 days after birth (Krinke, 2000). They typically have 4 or 5 day estrous cycles that consist of proestrus, estrus, metestrus and diestrus. Activity of the ovaries during the cycle is controlled by luteinizing hormone (LH) and gonadotropin-releasing hormone (GnRH). The female shows receptive behavior to the male in the late proestrus and early estrus phases of the cycle, which corresponds with the timing of ovulation. The LH surge typically occurs two hours prior to the dark phase during a fixed light-dark cycle and breeding may occur within several hours of the light transition time (Krinke, 2000).

The estrous cycle can be monitored via daily vaginal lavage or smears obtained at the same time each day (Krinke, 2000). The cell population of the vagina is influenced by circulating estrogen levels. During proestrus, nucleated cells are present in the cytology. Estrus is marked by cornified cells and metestrus/diestrus has large quantities of white blood cells. The transition time between these stages will have a mixture of the cell types. Due to the short cycle, the cytology will vary with time of day, which is why it should be performed at the same time each day for the most accurate mapping of the animal's cycle.

After fertilization occurs in the ampulla, the embryo begins its journey through the female's reproductive tract (Krinke, 2000). The embryo remains in the oviduct for the first three

to four days of development and enters the uterus by the end of day four. Implantation of the blastocyst occurs on day five of the pregnancy. Parturition occurs after 21-23 days of gestation.

Pseudopregnancy can be induced in rats when the female receives cervical stimulation (Krinke, 2000). This can be induced by breeding to a vasectomized male, or via mechanical stimulation either intentionally or unintentionally during the lavage process (De Feo, 1966). Pseudopregnancy is characterized by a 12 to 14 day duration of a lavage consistent with diestrus and is due to surges in prolactin (Krinke, 2000).

Cryopreservation

Cryopreservation, or the act of cooling samples to subzero temperatures in order to preserve their viability, has been performed on many types of cells from various species. Several protocols have been developed and optimized for spermatozoa of common species including livestock and man. These often include a step-wise cooling process that ends with submersion in liquid nitrogen for storage at a temperature of -196°C. There are several challenges to the process including both the cooling and warming protocols where tissue damage may occur.

When an unprotected cell is frozen to subzero temperatures, large ice crystals may form within the cell, causing membrane and organelle disruption leading to cell death (Shaw and Jones, 2003). One method of preventing this is to cause the cell to undergo dehydration. Cryopreservation solutions are designed to form ice crystals at temperatures warmer than those required to form ice crystals in the intracellular fluid. When cells are placed in these solutions prior to freezing, ice crystals will form within the extracellular fluid as the mixture is cooled. This causes the water found within the cell to exit into the extracellular fluid to maintain equilibrium of concentrations and preventing larger ice crystals from forming within the cell. Even with these solutions, if the temperature is dropped too rapidly, the cell's water is not given enough time to escape and larger ice crystals will form within the cell, causing damage. Cooling too slowly will result in osmotic damage (Critser and Mobraaten, 2000; Shaw and Jones, 2003; Walters *et al.*, 2009).

Aside from the dramatic effects of cell damage from ice crystals, the cryopreservation process has the possibility of affecting the plasma membrane of the cell in ways that reduce or eliminate the reproductive capability of that sperm cell. These changes typically take place during the rapid cooling to 0°C and is termed cold shock (Bailey *et al.*, 2000). The plasma

membrane of a sperm cell is composed of lipids and proteins that have a unique arrangement that is vital for sperm function (Hammerstedt *et al.*, 1990). The sperm cell must attach to oviductal epithelial cells in order to undergo capacitation and hyperactivation, proceed through the acrosome reaction and penetrate the zona pellucida (Bailey *et al.*, 2000). Damage to this membrane may prevent any number of these necessary functions.

As the cell is cooled, each temperature level transitions some membrane lipids from a fluid to a gel state. This alters the arrangement and association with membrane proteins which may not return to normal or a functional state following rewarming (McKinnon and Voss, 2011). Cooling may also induce phospholipids to flip from the inner leaflet of the membrane to the outer leaflet which also creates abnormal associations within the membrane (McKinnon and Voss, 2011). This altered arrangement may lead to phospholipid aggregates or ion-permeable gaps that lead to membrane fusion or rupture. These alterations to the plasma membrane may result in immediate reduction of cellular metabolism or may have delayed effects that reduces or eliminates the reproductive capability of the cell (McKinnon and Voss, 2011). Reduction of metabolism can reduce the amount of ATP formed and reduce the motility of the sperm cell which also has negative effects on reproduction.

If the cell survives the freezing process, it must then successfully undergo the thawing procedure. During slow cooling, the transfer of water out of the cell increases salt concentration within the cell. As the cells are thawed and rewarmed, this imbalance increases solute influx into the cell, possibly causing lysis (Walters *et al.*, 2009). Cell damage may also occur if the large ice crystals found in the extracellular fluid thaw too quickly, also inducing an osmotic change (Shaw and Jones, 2003). As the plasma membrane is rewarmed, the lipids transition back to a fluid phase from the gel phase. They may not resume the previous orientation with one another or with the plasma proteins. These changes may cause the cell to be unable to function normally (McKinnon and Voss, 2011). Another negative effect of the thawing process is sperm undergoing a partial capacitation. When sperm is capacitated *in vitro* prior to fertilization, it reduces the possibility of the sperm reaching the oocyte. (Bailey *et al.*, 2000)

These two challenges are balanced by determining the correct cooling rate for each cell utilizing different cryopreservative solutions (Walters *et al.*, 2009). For cryopreservation of sperm to be successful, each portion of the cell- acrosome, flagella, midpiece- needs to be functional and structurally sound following freezing and thawing (Walters *et al.*, 2009). The

cryopreservation solution selected must act as an antifreeze agent. Cryopreservation solutions are often based on physiological buffers to which a cryoprotectant, a sugar and protein are added. Each component plays an important role in the freezing and thawing process and must be tailored to each cell type, species and freezing method.

Permeating cryoprotectants will enter the cell or affect membrane water permeability and act to lower the freezing point. They may also act to take the place of bound water in the cell. This bound water provides structural support to DNA, proteins and other membranes within the cell, including those around the acrosome and mitochondria (Shaw and Jones, 2003). If this water is removed during the dehydration process and not replaced during the thaw process, damage and cell death will occur. The permeating cryoprotectant is able to cross the plasma membrane of the cell and form hydrogen bonds with proteins in place of this water, thus protecting the cell during freezing (Shaw and Jones, 2003). The rate of addition of these compounds to the sperm sample is important to ensure proper replacement rates of the water. Commonly used permeating cryoprotectants include dimethylsulphoxide (DMSO), glycerol, and ethylene glycol. These have been selected as they are small molecules that move quickly across membranes to equilibrate in the intracellular space. (Shaw and Jones, 2003)

Non-permeating cryoprotective agents, such as sugars (glucose, lactose, raffinose and others) and protein, are designed to remain extracellular and provide stabilization during the dehydration process. They can be used to increase osmotic pressure which will assist with removal of intracellular water (Varisli *et al.*, 2009a). Each cryopreservation solution that is developed will have a unique cooling and warming protocol that is optimized for its properties.

In rats, the cell volume of the sperm is approximately 36 to 37μm^3, which is smaller than mice but larger than boar, bull, stallion and human sperm (Si *et al.*, 2006), though they do have a longer tail than most other domestic animals (Kim *et al.*, 2012). A study comparing the cellular biophysics of rat and mouse sperm dehydration during freezing showed that rat sperm maintains the highest sperm motility when cooled at a rate of 53 to 70°C per minute, versus a rate of 28 to 36°C per minute in mice (Hagiwara *et al.*, 2009).

When various components of cryoprotective solutions were compared during the chilling of rat sperm, egg yolk was found to significantly reduce the negative effects of chilling and osmotic stress (Varisli *et al.*, 2009a). Equex STM is a product that has been used successfully in rat sperm cryopreservative solutions as well (Nakatsukasa *et al.*, 2001; Yamashiro *et al.*, 2007).

It is a detergent solution that serves to alter interactions between the lipoproteins in egg yolk and the plasma membrane of the cell (McKinnon and Voss, 2011). Concentrations of 0.5 to 2% were tested with the optimal concentration of Equex being 0.7 to 0.75% (Nakatsukasa *et al.*, 2001; Yamashiro *et al.*, 2007).

It has been shown that common permeating cryoprotective agents such as glycerol, in concentrations from 1 to 8%, are detrimental to rat sperm survival (Varisli *et al.*, 2009a; Nakatsukasa *et al.*, 2001; Yamashiro *et al.*, 2007). DMSO was also evaluated at concentrations of 0.4 M and 0.9 M with a large decrease in motility seen with the higher concentrations, though lower concentrations may prove beneficial as a permeating cryoprotectant (Varisli *et al.*, 2009a). Other factors that have a negative impact on rat sperm freezability is centrifugation (Nakatsukasa *et al.*, 2003; Varisli *et al.*, 2009b), even at speeds as low as 200 x *g* (Kim *et al.*, 2012), pH and osmotic stress (Si *et al.*, 2006). All of these factors contribute to the relative difficulty of successfully cryopreserving rat sperm.

Chapter 3 - Preparation of Cryopreservation Media

Methods and Materials

See Appendix A for a full list of materials and equipment used with source and catalog number information.

Preparing Chicken Egg Yolk

Chicken egg yolk is an ingredient included in both prepared cryopreservation media (Nakatsukasa and Yamashiro) utilized in this study. The eggs should be less than one week old at the time of use. After washing the egg with soap and water, it is sterilized by rinsing with 100% alcohol. The shell is carefully cracked and the egg yolk is transferred back and forth between the halves of the shell to remove the majority of the egg white. The egg yolk is then transferred to a petri dish containing a filter paper and rolled to remove the remaining white. A syringe is used to puncture the yolk membrane and aspirate the contents.

Nakatsukasa Media I and II

A solution of 8% (w/v) lactose, and 23% (v/v) chicken egg yolk, with 1000 units/mL of Penicillin G and 1 mg/mL streptomycin sulfate (Penicillin-Streptomycin liquid) was mixed in cell culture water. The pH is adjusted to 7.4 with 10% tris aminomethane solution. This is centrifuged at 1250 to 1750 x *g* for 15 minutes at room temperature and the upper layer of the solution is divided into aliquots and stored in -20°C for up to 4 months. Aliquots should be as close to the volume to be used in one session to avoid storage and refreezing after thawing. Excess may be stored at 4°C and used within 2 to 4 days (based upon recommended storage time for raw egg yolks put forth by food safety organizations, such as the American Egg Board). This is Nakatsukasa Medium I. To prepare medium II, a portion of medium I is utilized and 1.4% (v/v) Equex STM is added and mixed well on a stirring plate. This solution can also be divided into aliquots and stored at -20°C for up to 4 months. (Nakatsukasa *et al.*, 2001)

Yamashiro Media I and II

Modified Kreb's ringer bicarbonate (mKRB) solution is first prepared by mixing 94.6 mM sodium chloride, 4.78 mM potassium chloride, 1.71 mM calcium chloride dehydrate, 1.19 mM magnesium sulfate heptahydrate, 1.19 mM potassium phosphate monobasic, 25.07 mM sodium

bicarbonate, 32.37 mM sodium DL-lactate, 0.5 mM sodium pyruvate, and 5.56 mM D(+)glucose in sterile cell culture water. To this, 0.1 M D(+)raffinose pentahydrate, 750 units/mL penicillin G and 0.75 mg/mL streptomycin sulfate (Penicillin-Streptomycin liquid), 20% (v/v) egg yolk, 0.04% (w/v) sodium dodecyl sulfate and 1.85 mM adenosine triphosphate (ATP) are added. The solution is centrifuged twice at 7000 x *g* for 30 minutes at room temperature and filtered via filter syringe and 0.45µm filter paper. The pH is then adjusted to 7.3 with 3 M sodium hydroxide. This is medium I and can be divided into aliquots and stored in -20°C for up to 4 months. Aliquots should be as close to the volume to be used in one session to avoid storage and refreezing after thawing. Excess may be stored in 4°C and used within 2 to 4 days (based upon recommended storage time for raw egg yolks put forth by food safety organizations, such as the American Egg Board). To prepare medium II, 1.5% (v/v) Equex STM is added to medium I which can then also be divided into aliquots and stored at -20°C for up to 4 months. Dibutyryl cyclic-AMP (dbcAMP) solution is prepared by dissolving dbcAMP into sterile cell culture water to make a 1mM solution. This is added to the sperm solution at 0.1 mL dbcAMP solution to 1 mL sperm solution for a final concentration of 100 µM. (Yamashiro *et al.*, 2010b)

Chapter 4 - Epididymal Sperm Collection

Methods and Materials

See Appendix A for a full list of materials and equipment used with source and catalog number information.

Animals

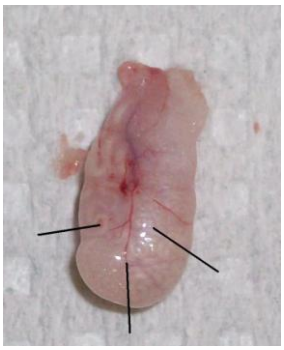
Sprague Dawley (n=31), Fischer 344 (n=4) and β-galactosidase transgenic (n=3) male rats were utilized for epididymal sperm collection. They were obtained and housed in accordance with animal care guidelines of IACUC, Kansas State University. The room was maintained at 71 to 73°F and between 30 to 70% humidity. They were maintained on an altered light/dark cycle that included 12 hours of dark from 1200 (noon) to 2400 (midnight), alternated with 12 hours of light. They were pair housed in 143 square inch solid bottom cages containing corn cob bedding unless they exceeded the recommendations set forth by The Guide for the Care and Use of Laboratory Animals. This criteria states that each animal must have greater than or equal to 70 square inches for rats weighing greater than 500 grams and the animals must be able to perform normal posture behaviors without touching the sides (Institute of Laboratory Animal Research *et al.*, 1996). Animals were moved to single housing if they exceeded these guidelines. They were fed maintenance diets (Lab Diet 5001 or 5008) with water *ad libitum* with supplemental Love Mash diet (Bio-Serv) 3 days per week. The males ranged in age from 60 days to 359 days at the time of collection.

Sperm Collection

Male rats were euthanized via carbon dioxide inhalation followed with induction of bilateral pneumothorax. Immediately following confirmation of death, the distal end of the scrotum was removed with scissors and dissection of the tunics allowed for exteriorization of the testes and *cauda epididymidis*. The *cauda epididymidis* was dissected away from the testicle and the adherent fat was removed. The tissue was rinsed in warm Dulbecco's phosphate buffered saline (DPBS) and scissors were used to cut three slits into the tissue (Figure 4.1). The epididymis was then placed in the selected cryopreservation medium (Nakatsukasa Medium I or II, Yamashiro Medium ± dbcAMP) contained in a non-tissue culture treated 12-well plate warmed to 37°C on a slide warming tray. This was repeated on the opposite side and the sample

was incubated for 10 minutes to allow the sperm to swim out of the tissues. The tissue was removed and the sample was ready for evaluation.

Figure 4.1 Location of epididymal slits



Sperm Concentration

An aliquot of the sperm sample was diluted 1:100 using 2 successive dilutions in distilled water (50 µL sample in 450 µL). A hemocytometer was prepared by placing the coverslip over the counting surface prior to loading the suspension. The sperm suspension was introduced into the V-shaped well to cover the counting area and was allowed to sit for at least 2 minutes to allow the sperm cells to settle. The hemocytometer was placed on the microscope stage and the number of cells in the 25 squares of the central counting grid was tabulated at 100x (10x phase objective with 10x eyepiece) with phase contrast. The process was repeated in the second counting chamber and an average of the two 10 mm^3 counting areas was obtained. Concentration was then determined by multiplying the number of cells by the dilution factor (100) and multiplying that total by 10,000 (obtained by calculating that the counting area contains 10 cubic millimeters and there are 1000 cu mm in each milliliter) to determine the amount of sperm cells per milliliter of sample.

Sperm motility assessment

A 15 µL sample of the sperm solution was placed on a microscope slide that was warmed to 37°C and covered with a 22 x 22 x 1 mm cover slip in a wet mount preparation. This was then examined at 100x (10x phase objective with 10x eyepiece) with phase contrast to subjectively estimate sperm motility percentages in ranges of ±5%. A Nikon digital camera (D90) attached to the microscope was utilized to obtain video footage of 10 to 20 fields at a frame rate of 24

frames per second with the number of sperm counted varying with concentration of the sample. Each field represented 0.54 mm^2 for evaluation of a total of 5.4 to 10.8 mm^2 from each sperm sample. Later evaluation of the videos enabled objective counting of motile sperm versus total sperm in each field and an overall average of total sperm motility was obtained.

Statistical Analysis

Post-hoc statistical analysis was performed using GraphPad Prism version 6.00 for Windows. Summary data is provided as mean ± standard deviation. Significance was set at $p < 0.05$.

Results

Raw data for all epididymal sperm collections is found in Table B.1. The mean concentration of all sperm collected in this manner during the course of this project, n=41, was 55.3 ± 53.0 million/mL (range 1.5 to 275 million/mL), with an average sperm motility of 88 ± 10% (range 40 to 95%). The mean age of the males was 146 ± 78 days (range 60 to 359 days). Table 4.1 summarizes the mean of each group divided by strains and media collected into. Comparison of Sprague Dawley sperm motility in different media was performed with a Kruskal-Wallis test and showed no significant difference with a p-value of 0.28. Table 4.2 provides a comparison by strain. Comparing the sperm motility of the remaining strains in various media via a Kruskal-Wallis test showed no significance with a p-value of 0.28. Table 4.3 provides a comparison based upon the medium the sperm sample was collected into. Comparing the motility of sperm across all strains to the media it was collected in utilizing a Kruskal-Wallis test showed no significance with a p-value of 0.55. Calculated sperm motility was obtained on nine Sprague Dawley samples and is shown in Table 4.4. Comparison of calculated sperm motility against estimated sperm motility showed no significant difference between the values obtained with a p-value of 0.07 via a Wilcoxon matched-paired signed rank test.

There were five males where each epididymis was collected into different media. These values provide a direct comparison between the two media without individual animal variance. This information is provided in Table 4.5. Comparing the values of the paired samples within different media was performed with a Wilcoxon matched-paired signed rank test and resulted in no significant difference with a p-value of 0.13.

Table 4.1 Summary of epididymal sperm collection

Strain	Medium	Number of Males Collected	Age (days)	Concentration (million/mL)	Total Sperm Count (million)	Sperm Motility (est)
SD	Nakatsukasa	18	103 ± 26	46.8 ± 42.3	169 ± 135.5	90 ± 8%
SD	Yamashiro without dbcAMP	7	110 ± 12	55.6 ± 43.6	72.1 ± 28.9	86 ± 7%
SD	Yamashiro with dbcAMP	5	120 ± 19	85.9 ± 98.0	187.6 ± 313.4	82 ± 21%
F344	Nakatsukasa	3	284 ± 9	34.7 ± 24.4	104 ± 73.2	88 ± 12%
F344	Yamashiro without dbcAMP	1	286	12	24	95%
B-gal	Nakatsukasa	2	304 ± 13	39 ± 0.7	137 ± 26.2	90 ± 0%
B-gal	Yamashiro without dbcAMP	1	359	27	40.5	90%
Dark agouti	Yamashiro without dbcAMP	1	247	98	98	95%
Transposagen transgenic	Nakatsukasa	2	144 ± 0	99 ± 64	297 ± 190.9	95 ± 0%

Values provided as mean ± standard deviation. Rat strains: SD- Sprague Dawley, F344- Fischer 344, B-gal- β-galactosidase transgenic. Kruskal-Wallis test of Sprague Dawley sperm motility in each media showed non-significant difference (p=0.28).

Table 4.2 Summary of epididymal sperm collection organized by rat strain.

Strain	Media	Number of males collected	Age (days)	Concentration (million/mL)	Total Sperm Count (million)	Sperm Motility (est)
SD	Nakatsukasa, Yamashiro with and without dbcAMP	31	108 ± 23	56.4 ± 56.8	150.7 ± 169.9	88 ± 11%
F344	Nakatsukasa, Yamashiro without dbcAMP	4	284 ± 7	29.0 ± 22.9	84.0 ± 71.9	90 ± 10%
B-gal	Nakatsukasa, Yamashiro without dbcAMP	3	322 ± 33	35.0 ± 6.9	104 ± 58.7	90 ± 0%
Dark agouti	Yamashiro without dbcAMP	1	247	98	98	95%
Transposagen transgenic	Nakatsukasa	2	144 ± 0	99 ± 64	297 ± 190.9	95 ± 0%

Values provided as mean ± standard deviation. Rat strains: SD- Sprague Dawley, F344- Fischer 344, B-gal- β-galactosidase transgenic. Kruskal-Wallis test comparing sperm motility of each strain across all media showed no significant difference (p=0.28).

Table 4.3 Summary of epididymal sperm collection organized by media type.

Strain	Medium	Number of males collected	Age (days)	Concentration (million/mL)	Total Sperm Count (million)	Sperm Motility (est)
SD, F344, B-gal, Transposagen transgenic	Nakatsukasa	25	144 ± 80	49.0 ± 41.6	168.9 ± 130.3	90 ± 8%
SD, F344, B-gal, Dark agouti	Yamashiro without dbcAMP	10	166 ± 95	52.6 ± 42.0	66.8 ± 31.2	89 ± 7%
SD	Yamashiro with dbcAMP	6	120 ± 19	85.9 ± 98.0	187.6 ± 313.4	82 ± 21%

Values provided as mean ± standard deviation. Rat strains: SD- Sprague Dawley, F344- Fischer 344, B-gal- β-galactosidase transgenic. Kruskal-Wallis test comparing sperm motility of all strains across different media showed no significant difference (p=0.55).

Table 4.4 Comparison of estimated sperm motility with calculated sperm motility.

Strain	Medium	Sperm Motility (est.)	Sperm Motility (calculated)	Difference
SD	Nakatsukasa	95%	96.46%	+1.46%
SD	Nakatsukasa	95%	96.16%	+1.16%
SD	Nakatsukasa	90%	87.34%	-1.66%
SD	Nakatsukasa	90%	88.48%	-1.52%
SD	Nakatsukasa	90%	89.17%	-0.83%
SD	Nakatsukasa	90%	89.43%	-0.57%
SD	Nakatsukasa	90%	88.38%	-1.62%
SD	Yamashiro without dbcAMP	95%	91.73%	-3.27%
SD	Yamashiro with dbcAMP	95%	89.45%	-5.55%

Rat strains: SD- Sprague Dawley, F344- Fischer 344, B-gal- β-galactosidase transgenic. Wilcoxon matched pairs signed rank test showed no significant difference between estimated and calculated sperm motility values (p=0.07).

Table 4.5 Inter-rat comparison of different media.

Animal ID	Strain	Medium	Concentration (million/mL)	Total Sperm Count (million)	Sperm Motility (est.)
F344 B	F344	Nakatsukasa	12	36	95%
		Yamashiro without dbcAMP	12	24	95%
SD Y	SD	Nakatsukasa	23	69	90%
		Yamashiro without dbcAMP	15	45	85%
SD AB	SD	Yamashiro without dbcAMP	5.5	15.4	90%
		Yamashiro with dbcAMP	5.5	15.4	80%
SD F	SD	Yamashiro without dbcAMP	28	56	80%
		Yamashiro with dbcAMP	28	56	40%
SD K	SD	Yamashiro without dbcAMP	63	63	95%
		Yamashiro with dbcAMP	63	63	90%

Rat strains: SD- Sprague Dawley, F344- Fischer 344. Wilcoxon matched pairs signed rank test showed no significant difference between sperm motility values obtained in different media (p=0.13).

Discussion

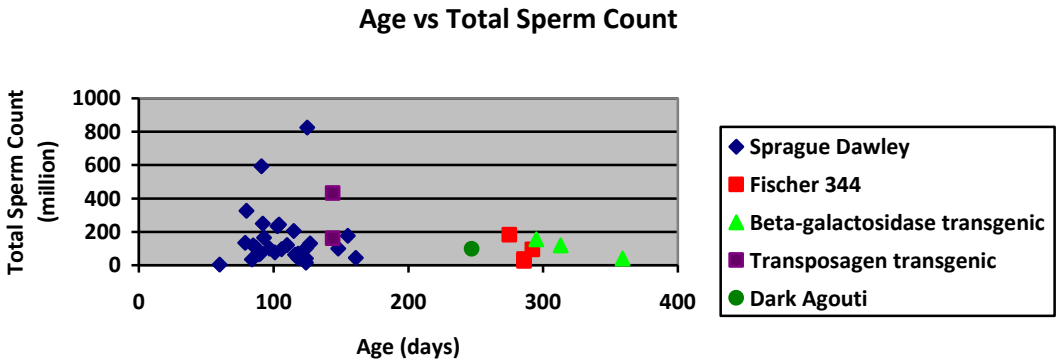
Epididymal collection is an effective means of obtaining rat sperm for insemination. There is individual animal difference in concentration as well as room for operator error depending on the effectiveness of dissection and cutting slits into the tissue. This is a procedure that can also be performed after the natural death of an animal to preserve the genetic material if it is a valuable specimen.

Scatter plots comparing data (age vs. total sperm count, age vs. sperm motility, total sperm count vs. sperm motility) reveal no obvious linear relationship between the data (Figure 4.2).

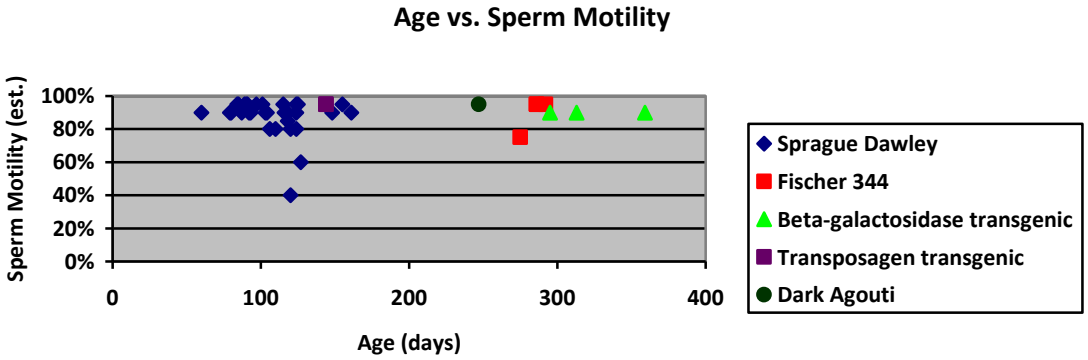
When comparing the estimated and calculated methods for determining sperm motility at collection, samples collected in Nakatsukasa medium had the least variation. The estimated value was within 6% of the calculated on all samples and within 2% on those collected in Nakatsukasa medium. No significant difference was noted between estimated and calculated sperm motility counts. With practice, estimated sperm motility provides an accurate representation of the actual motility of the sample.

The samples obtained from the same rat divided between two media allowed for direct comparison between the media. There is a general loss of motility noted in Yamashiro medium when dbcAMP is added to the solution (90% to 80%, 80% to 40%, and 95% to 90%), but this effect was not significant.

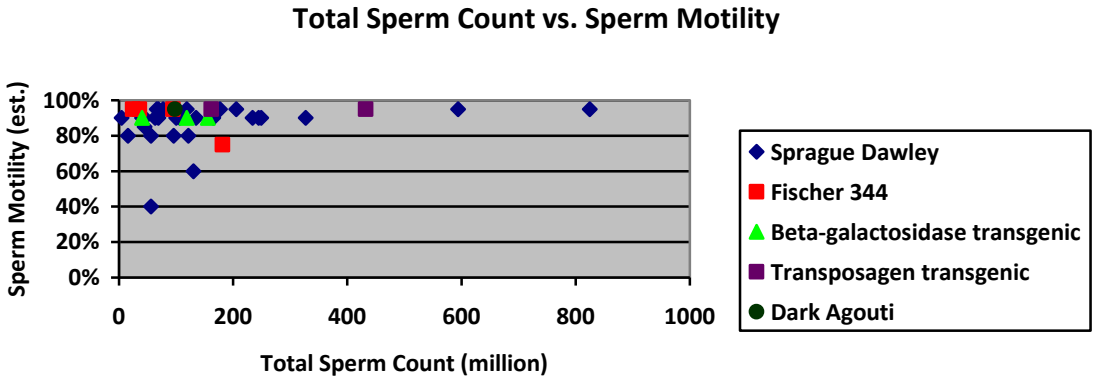
Figure 4.2 Scatter plot comparisons of epididymal sperm collection data.



No correlation noted between age and concentration (r=-0.1132, p=0.48).



No correlation noted between age and sperm motility (r=-0.02907, p=0.86)



No correlation noted between concentration and sperm motility (r=0.1158, p=0.47).

Chapter 5 - Pharmacoejaculation

One goal of this research project was to enable reduction of animals used in research. Although successful cryopreservation of sperm would achieve this, there is still the consideration that euthanasia of the male is necessary to collect an epididymal sperm sample. This is not ideal as it prevents future natural reproduction by that male and would increase animal use to obtain multiple samples. Investigation into alternatives led to evaluation of pharmacoejaculation (see chapter 2, Literature Review for more information).

Materials and Methods

See Appendix A for a full list of materials and equipment used with source and catalog number information.

Animals

Male Sprague Dawley (n=2), Dark Agouti x Sprague Dawley heterozygote (n=3), Fischer 344 (n=1) and β-galactosidase transgenic (n=1) rats were housed and fed as previously described.

Pharmacoejaculation

Male rats were transported to the laboratory and a body weight was obtained. They were placed under general anesthesia via inhaled isoflurane and placed in dorsal recumbency. A solution of p-chloroamphetamine hydrochloride (PCA) was prepared by dissolving the powder in sterile physiologic saline at a concentration of 5 mg/mL. This solution should be prepared just prior to use. This solution was administered intraperitoneally at a dose of 5 mg/kg. The penis was extruded and cleaned with chlorhexadine (Hibiclens). Ejaculate sample was collected on a glass slide for evaluation of concentration and timing of coagulant expression. The procedure was completed 30 minutes post injection, the animal was allowed to recover from anesthesia and was returned to his housing cage.

Results

Seven rats of various strains were utilized for this experiment. The first two rats began ejaculation within 10 minutes of injection and the duration was less than 5 minutes. The samples

were allowed to air dry on glass slides and examined at 100x (10x phase objective with 10x eyepiece) with phase contrast. Morphologically normal sperm were present, though the total volume of ejaculate was difficult to estimate due to collection on slides. The remaining 5 animals failed to ejaculate over the course of the 30 minutes time period. One Sprague Dawley received an additional 2.2 mg/kg (approximately a half dose) of PCA 10 minutes following the first dose to try to induce ejaculation. This was not successful. Data for all trials is presented in Table 5.1.

One animal (127 day old Sprague Dawley) exhibited hematuria over the 12 hours post-procedure and was euthanized. Gross necropsy revealed a dilated venous system, enlarged atria and a normal bladder lining suggesting the hematuria was originating in the kidneys.

Table 5.1 Pharmacoejaculation trials.

Strain	Age (days)	Weight (grams)	Amount of PCA administered (5mg/mL)	Results
B-gal	271	340g	1.7mg (0.34mL)	Ejaculation start at 11min
F344	237	394g	1.97mg (0.39mL)	Ejaculation start at 8min
DA x SD	250	570g	2.85mg (0.57mL)	No ejaculation
DA x SD	252	570g	2.85mg (0.57mL)	No ejaculation
DA x SD	256	825g	4.15mg (0.83mL)	No ejaculation
SD	127	566g	2.85mg (0.57mL) + 1.25mg (0.25mL)[a]	No ejaculation
SD[b]	127	545g	2.75mg (0.55mL)	No ejaculation

Rat strains: SD- Sprague Dawley, F344- Fischer 344, B-gal- β-galactosidase transgenic, DA x SD- Dark Agouti x Sprague Dawley heterozygote. [a]Administered an additional 2.2 mg/kg (approx. half dose) 10 minutes after first dose to try and induce ejaculation. [b]Animal exhibited hematuria post procedure, was euthanized.

Discussion

The volume of ejaculate obtained with this method was very low and did not allow for a sample that could be adequately evaluated, added to cryopreservation solution and successfully frozen. It is speculated that the volume would not yield a sample large enough to enable

insemination via the intrauterine route. There was no obvious difference in age for the animals that were induced to ejaculate and those that were non-responsive to the drug. The two strains that ejaculated were a Fischer 344 and the β-galactosidase transgenic, which is based on the Fischer 344 strain. It is possible that this inbred strain is more susceptible to the effects of PCA.

With a low incidence of ejaculation, small volume of ejaculate obtained and hematuria exhibited by one animal, it was determined that this method is not a viable alternative for epididymal collection. Utilization of this method would require further investigation and altering the methods used.

Chapter 6 - Intrauterine Insemination with Fresh Sperm Samples

Methods and Materials

See Appendix A for a full list of materials and equipment used with source and catalog number information.

Animals

Sprague-Dawley, Fischer 344 and β-galactosidase transgenic male (n=12) and female (n=19) rats were utilized for epididymal sperm collection and intrauterine insemination. They were obtained and housed in accordance with animal care guidelines of IACUC, Kansas State University. The room was maintained at 71 to 73°F and between 30 to 70% humidity. They were maintained on an altered light/dark cycle that included 12 hours of dark from 1200 (noon) to 2400 (midnight), alternated with 12 hours of light. They were pair housed in 143 square inch solid bottom cages containing corn cob bedding unless they exceeded the recommendations set forth by The Guide for the Care and Use of Laboratory Animals. This criteria states that each animal must have greater than or equal to 70 square inches for rats weighing greater than 500 grams and the animals must be able to perform normal posture behaviors without touching the sides (Institute of Laboratory Animal Research *et al.*, 1996). Animals were moved to single housing if they exceeded these guidelines. They were fed maintenance diets (Lab Diet 5001 or 5008) with water *ad libitum* with supplemental Love Mash diet (Bio-Serv) 3 days per week. The males ranged in age from 60 days to 359 days at the time of collection. The females ranged in age from 58 days to 313 days at the time of insemination.

Vaginal lavage

All females older than 8 weeks of age underwent daily vaginal lavage throughout the study to monitor their estrous cycles. At 0800 to 0900, 3 to 4 hours prior to the lights turning off, each female was manually restrained and a small volume (<1mL) of sterile PBS was introduced into her vagina via a smooth tipped glass eye dropper. The fluid was aspirated after introduction and placed in a well of a non-tissue culture treated 24-well plate. The animal was then returned to its cage and the eyedropper was rinsed three times with sterile distilled water. The process was repeated for each female prior to examination of the cell suspensions. The plate was returned to the laboratory and was examined at 100x (10x phase objective with 10x

eyepiece) with phase contrast to visualize the cells present (Figure 6.1). Cells were categorized as white cells, nucleated cells and cornified cells and recorded. Proestrus was identified when the primary cell type was nucleated cells. Estrus was classified as primarily cornified cells. Metestrus and diestrus consisted of primarily white cells. Pseudopregnancy was declared when a female had at least five consecutive days of primarily white cells in her lavage sample.

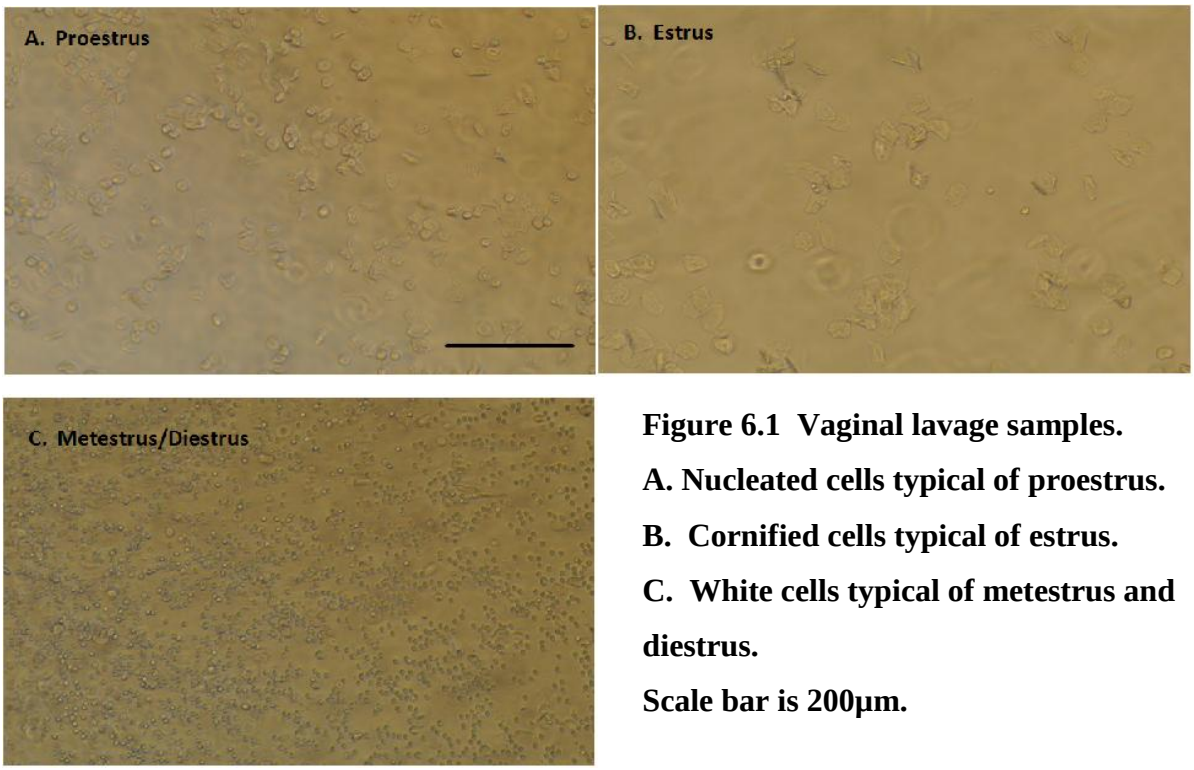


Figure 6.1 Vaginal lavage samples.
A. Nucleated cells typical of proestrus.
B. Cornified cells typical of estrus.
C. White cells typical of metestrus and diestrus.
Scale bar is 200µm.

Induction of pseudopregnancy

When a female was determined to be in proestrus based upon her vaginal lavage, she was paired with a vasectomized male. These males had previously undergone a vasectomy procedure and were test mated to confirm sterility. The pairing occurred at 0900 and they were housed in suspended wire bottom cages with a resting platform as per IACUC regulations until 1500. The cages were 143 square inches with ½ x ½ inch square wire grid floors. The resting platform provided was a 4½ x 7 x ¼ inch piece of plastic. This allowed for 3 hours for breeding prior to lights off and 3 hours into the dark phase of the light cycle. At this time, a red-lens flashlight was used to visualize copulatory plugs that had fallen through the wire bottom of the cage.

Presence of these plugs indicated breeding and induction of pseudopregnancy was assumed. The male was returned to his cage and the female was transported to the laboratory for the insemination procedure. If no plugs were present, both animals were returned to their housing cages and daily vaginal lavage was resumed on the female the following morning.

Sperm Collection

Epididymal sperm was collected from a male of the same strain as the pseudopregnant female following the methods outlined previously. It was maintained at 37°C on a slide warmer while the female was prepared for surgery. dbcAMP was added to the sperm sample at a concentration of 100 µM and incubated for at least 30 minutes prior to insemination for the Yamashrio medium with dbcAMP insemination group. The estimated number of motile sperm transferred into each side of the uterus was calculated by multiplying the concentration by the sperm motility and adjusting with the appropriate units. This number was then multiplied by the amount instilled (50 µL each side).

Intrauterine insemination

Upon arrival in the laboratory, within one hour of confirmation of breeding at 1500 to 1700 hours (3 to 5 hours after the lights turned off), a body weight was obtained on the female. She then underwent induction of general anesthesia via inhalation of 5% isoflurane in an anesthesia chamber. Isoflurane at 2 to 3% is used to maintain anesthesia throughout the procedure. The dorsal caudal half of the rat (from the last rib to the pelvis) was clipped and surgically prepared with three alternating providone iodine and 70% alcohol scrubs. Eye lubricant (LubriFresh PM) was applied to both globes and a calculated dose of 0.05 mg/kg buprenorphine was administered subcutaneously as well as 75,000 units Combi-Pen-48 (penicillin G benzathine and penicillin G procaine). After ensuring a surgical plane of anesthesia, a 1 cm dorso-lateral skin incision was made above the left ovary and extended into the peritoneal cavity (Figure 6.2). The left ovary and oviduct with the associated fat pad was exteriorized and a 22 gauge needle was used to create an opening in the uterine horn at the oviductal end. Approximately 50 µL of spermatozoa in media was instilled with a micropipette made by pulling the tip of a Pasteur pipette (Figure 6.3) and the ovary was carefully replaced into the abdomen. The body wall is closed with internal absorbable suture (PDS II 4-0) in a simple interrupted pattern and the skin closed with external staples (9 mm autoclips). The process was

repeated on the right side. Subcutaneous fluids (0.9% sodium chloride) were administered and the female was placed in a cage with a portion resting on a supplemental heat source. Recovery was monitored and when the female was able to right herself and move away from the supplemental heat source she was returned to her cage in the animal housing room. Fertilization was assumed to occur within 1 to 2 hours of sperm instillation (Howe and Black, 1963). External staples were removed at 7 days post-surgery.

Figure 6.2 Dorsal view of rat illustrating orientation of skin and body wall incisions with regard to level of last rib

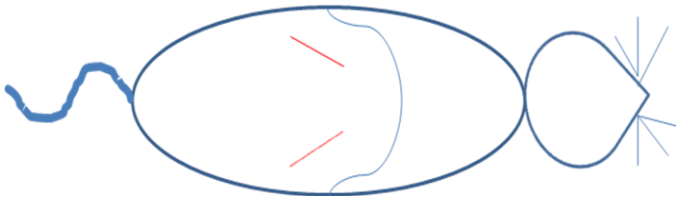
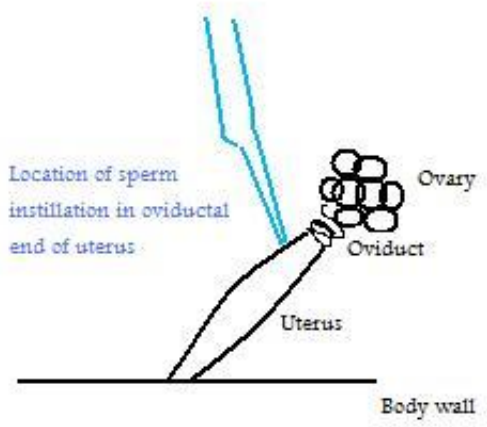


Figure 6.3 Sperm is instilled into oviductal end of uterus with micropipette.



Confirmation of pregnancy

At 10 to 14 days gestation (day 0 = day of insemination), transabdominal ultrasound was utilized to confirm pregnancy. The female was placed under brief general anesthesia via inhaled isoflurane at 4%. A 7 to 12 MHz linear probe with Terason t3000 veterinary ultrasound software version 4.3.1 was used to perform transabdominal ultrasound examination. Detection of pregnancy was confirmed by visualizing one or more fetuses with heartbeats. The female was then recovered from anesthesia and monitored for parturition at 21 to 23 days of gestation.

Refer to "Video 6.1 Ultrasound" to view an example of a positive ultrasound at gestational day 14.

Statistical Analysis

Post-hoc statistical analysis was performed using GraphPad Prism version 6.00 for Windows. Summary data is provided as mean ± standard deviation. Significance was set at $p < 0.05$.

Results

An example of a daily lavage record can be found in Table B.3. Throughout the study, the Sprague Dawley females were approximately 56 to 200 days old during the daily lavage collection period. Within this strain, approximately 80% maintained a 4 day estrous cycle, while the remaining 20% had 5 day estrous cycles. In the inbred Fischer 344 rats, as well as the transgenic β-galactosidase rats that are based on the Fischer 344 strain, all of the animals exhibited a 5 day estrous cycle. These rats were also older, ranging from approximately 180 to 300 days of age during the lavage period.

As pseudopregnancy in rats can be induced with cervico-vaginal stimulation, there were four females in this study that became pseudopregnant during the vaginal lavage process. This represents 6% of the 64 rats lavaged over the course of the project. Other instances of pseudopregnancy occurred when the vasectomized male bred the female but no copulatory plugs were visualized. The duration of pseudopregnancy was consistent across all strains of rats utilized and averaged 13 days over the 50 instances in this study. This included females pair-housed with vasectomized males to help train them to breed and ensure sterility.

Intrauterine insemination was performed in each strain of rats. In the Sprague Dawley strain, the goal was to obtain a minimum of 3 litters and 5 live pups from each of three cryopreservation media. See table B.2 for complete insemination data. No linear relationship can be detected between the number of motile sperm inseminated and number of pups born (Figure 6.4).

The number of pups born to Sprague Dawley rats was compared to the media used for insemination with the Kruskal-Wallis test. No significant difference was noted with a p-value of 0.30. A Mann Whitney test was used to compare the number of Sprague Dawley pups born after insemination with Yamashiro media both with and without the addition of dbcAMP. A p-value

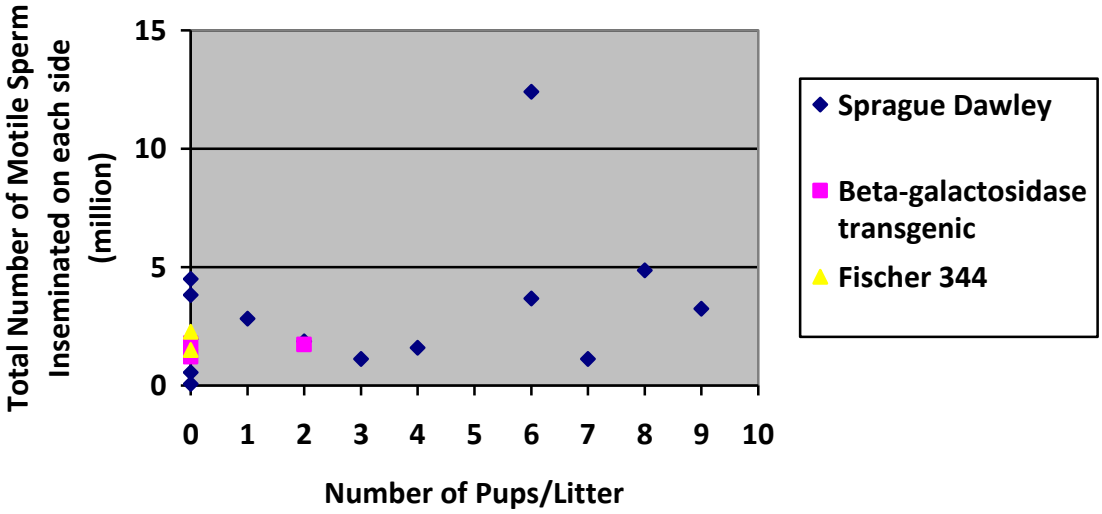
of 0.17 indicated no significant difference was found. The number of pups born utilizing the Nakatsukasa and Yamashiro media without dbcAMP in Sprague Dawley rats was compared to those born to Fischer 344 and beta-galactosidase transgenic rats. A Mann Whitney test showed a p-value of 0.049, indicating a significant difference based upon the different strains (Table B.2, summary in Table 6.1).

Table 6.1 Summary of IUI surgeries.

Strain	Medium	Age of Female (days)	Age of Male (days)	Sperm Concentration (million/mL)	Sperm Motility (est.)	Est. Number of Motile Sperm Transferred (each side)	Pregnancy Rate	Total Number of Pups Born	Pups Weaned
SD	Nakatsukasa	87 ± 23	87 ± 22	36.3 ± 27.4	93 ± 3%	1.7 million ± 1.3 million	3/4	15	10♂, 4♀
SD	Yamashiro without dbcAMP	110 ± 9	109 ± 8	80.6 ± 39.5	84 ± 8%	3.4 million ± 1.6 million	3/4	21	5♂, 15♀
SD	Yamashiro with dbcAMP	118 ± 37	120 ± 19	90.0 ± 94.7	80 ± 20%	3.9 million ± 4.4 million	3/6	10	3♂, 7♀
B-gal	Nakatsukasa	261 ± 48	289 ± 8	39.0 ± 0.7	90 ± 0%	1.8 million ± 35,000	1/2	2	1♂, 1♀
B-gal	Yamashiro without dbcAMP	313	359*	27	90%	1.22 million	0/1	0	0
F344	Nakatsukasa	284 ± 12	284 ± 12	46.0 ± 20.5	85 ± 14%	1.9 million ± 540,000	0/2	0	0

Mean values ± standard deviation. Rat Strains: SD- Sprague Dawley, B-gal- β-galactosidease transgenic, F344- Fischer 344. *An abscess was found associated with the right epididymis, sperm was collected from only the left epididymis. Estimated number of motile sperm transferred in 50 µL of solution in each uterine horn. Full data found in Table B.2.

Figure 6.4 Comparison of motile sperm inseminated on each side and litter size.



No correlation noted between total number of motile sperm inseminated on each side to the number of pups born (r=0.2823, p=0.24).

Table 6.2 IUI data from publications

Strain	Medium	Sperm Motility	Est. Number of Motile Sperm Transferred (each side)	Pregnancy rate	Litter Size
Sprague Dawley ♂, Wistar ♀[a]	Nakatsukasa	75 ± 7.7%	750,000 to 2.25 million	13/13	11.2 ± 2.2
Wistar[b]	Yamashiro with dbcAMP	80 ± 5.8%	800,000 to 1.2 million	3/6	17

[a]From (Nakatsukasa *et al.*, 2001). [b]From (Yamashiro *et al.*, 2010b). Mean values ± SEM. Estimated number of motile sperm transferred in 50 µL of solution in each uterine horn.

Discussion

When comparing Sprague Dawley rats, it was possible to produce live pups using all three media types, though it took several more trials with the solution containing dbcAMP (Figure 6.1). Thus, the pregnancy rate using this medium observed here was similar to that obtained by the group that developed the medium (50%) (Yamashiro *et al.*, 2010b). There is the possibility that dbcAMP has a negative effect on sperm motility or incidence of conception when placed in the uterine environment.

The IUI pregnancy rate utilizing Nakatsukasa medium in Sprague Dawley rats observed here (75%) was less than that reported by the group that developed this medium (100%) (Table 6.2) (Nakatsukasa *et al.*, 2001). The first insemination surgery performed in the present series was a younger female (58 days), which may have contributed to difficulty in conception, though Nakatsukasa utilized females aged 56 to 126 days and reported no issues related to age (Nakatsukasa *et al.*, 2003; Nakatsukasa *et al.*, 2001). In the present series of experiments, there was also a low number of surgeries performed (4 in our study versus 13). While the success rate here cannot reach 100% as they reported, it is possible that with a larger sample size the rate of pregnancy may increase.

The inseminations performed on Fischer 344 and β-galactosidase transgenic rats, were done using much older rats than the Sprague Dawley rats. The β-galactosidase transgenic rats were derived from the Fischer 344 strain. A significant difference in the number of rat pups produced between the Sprague Dawley (average of 5 ± 3 pups per litter) and the Fischer 344 rats (two pups in one litter) was found. Reproductive productivity decreases with age, as shown in a longitudinal study performed in Long-Evans rats (Matt *et al.*, 1987), and may have a negative impact on this procedure. Harlan reports that the Fischer 344 strain has a lower average litter size (7.5) than that of the Sprague Dawley (11.0) (Harlan Laboratories, 2012). Nakatsukasa did not report fresh insemination data in their publication that investigated IUI in various strains of rats to provide a comparison (Nakatsukasa *et al.*, 2003). Further investigation into differences in rat strain and age may be beneficial to fully evaluate restrictions on the optimal animal to use in recovery of a strain using intrauterine insemination.

Future experiments would include reducing the concentration of dbcAMP added or removing it from the media entirely. The lack of relationship between the number of motile sperm inseminated on each side and litter size may be due to low sample size as well as the somewhat low variability of the samples used. Overall, larger numbers of the procedure would allow further comparison between media and productivity with fresh sperm samples.

Chapter 7 - Cryopreservation of Sperm

Replication of Published Freezing Protocols

Methods and Materials

See Appendix A for a full list of materials and equipment used with source and catalog number information.

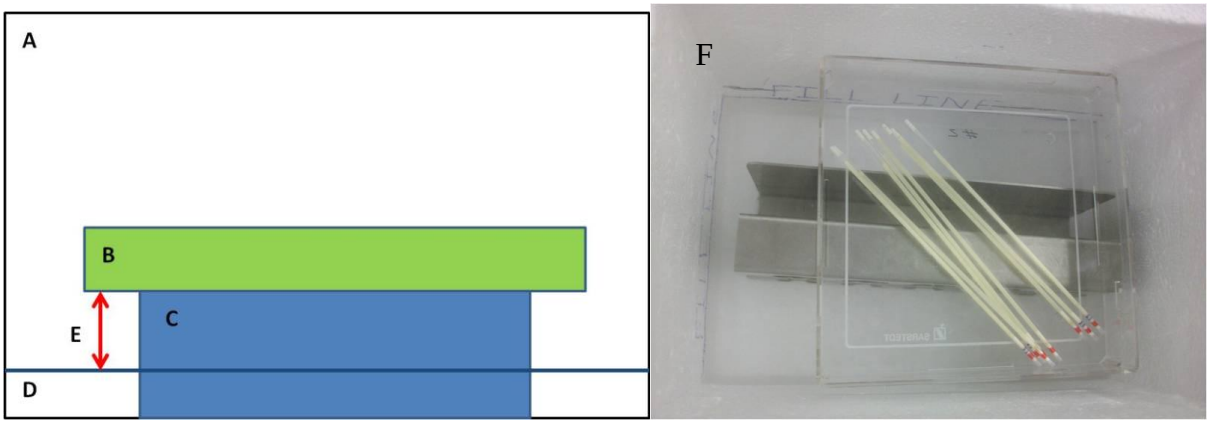
Animals and Sperm Collection

Animals were housed as indicated in previous sections. Epididymal sperm collection was performed as previously detailed utizling dark agouti (n=1) and Sprague Dawley (n=2) male rats.

Nakatsukasa Freezing

Sperm samples collected into Nakatsukasa medium I were cooled to 15°C utilizing a centrifuge (accuSpin 3R) preset to that temperature. The non-tissue culture treated 12 well plate containing the sperm sample was placed in the centrifuge for 30 minutes then transferred to the 4°C refrigerator for an additional 30 minutes. After this, equal volumes of pre-cooled 4°C Nakatsukasa medium II (with Equex STM added at 1.4%) was added and the sample was gently agitated by swirling the plate in a circular motion for 10 seconds. The sperm sample was then loaded into 0.25 mL semen straws at room temperature and heat sealed with needle holders that were heated in a hot bead sterilizer pre-heated to 240°F. The needle holders were placed in the sterilizer for 30 seconds, removed and allowed to cool for 20 seconds, then clamped on the end of the semen straw and held for 5 seconds. The straws were placed on ice cubes in an insulated container, transported to another laboratory and placed in a vapor phase chamber constructed from a Styrofoam cooler. A measured amount of liquid nitrogen was placed in the bottom of the chamber and the semen straws were placed on a platform within the chamber. The height of the platform and the level of liquid nitrogen were adjusted to ensure the straws were 2 cm above the height of the liquid nitrogen in the vapor phase (Figure 7.1). The lid was replaced on the chamber and the sperm was frozen for 10 minutes. The straws were then transferred to a storage box (Cryobox) and submerged into liquid nitrogen (Locator 6 plus) for storage.

Figure 7.1 Box used for vapor phase cooling of sperm samples.



A. Styrofoam box with outer dimensions of 11 x 8½ x 8 inches and inner dimensions of 8½ x 6 x 5½ inches. B. Plastic tray to hold sperm, 5 x 5 inches with holes in corners. C. Platform to hold tray at desired level above liquid nitrogen. D. Liquid nitrogen level. E. Desired height above vapor phase (2 cm). F. Photograph of vapor phase box.

Nakatsukasa Thawing

After being stored a minimum of three days, the semen straws were removed from liquid nitrogen and placed in ice cubes in an insulated container for transport back to the laboratory. They were then placed in a 37°C water bath for 10 seconds. The straws were cut and the sample was allowed to flow into 1.7 mL graduated tubes containing 0.5 mL modified rat one-cell embryo culture medium (mR1ECM) with 0.4% (w/v) bovine serum albumin (BSA) that was pre-warmed to 37°C. Components of mR1ECM are reported to be 76.7 mM sodium chloride, 3.2 mM potassium chloride, 2.0 mM calcium chloride, 0.5 mM magnesium chloride, 25.0 mM sodium bicarbonate, 10.0 mM sodium lactate, 0.5 mM sodium pyruvate, 7.5 mM glucose, 1.0 mg/mL PVA, 2% (v/v) minimal essential medium amino acid solution, 0.1 mM glutamine and 1% (v/v) minimal essential medium nonessential amino acid solution (Han and Niwa, 2003).

Yamashiro Freezing

Sperm samples collected into Yamashiro medium I (±dbcAMP) were cooled to 4°C by placing the 12 well plate containing the sample into a 4°C refrigerator for 90 minutes. After this equal volumes of Yamashiro medium II (with Equex STM added at 1.5%), pre-cooled to 4°C, was added and the sample gently agitated by swirling the plate in a circular for 10 seconds. The sample was allowed to equilibrate to 4°C for 30 minutes by returning it to the refrigerator. The sperm sample was then loaded into 0.25 mL semen straws at room temperature and heat sealed

with needle holders that were heated in a hot bead sterilizer pre-heated to 240°F. The needle holders were placed in the sterilizer for 30 seconds, removed and allowed to cool for 20 seconds, then clamped on the end of the semen straw and held for 5 seconds. The straws were placed on ice cubes in an insulated container, transported to another laboratory and placed in a vapor phase chamber constructed from a Styrofoam cooler. A measured amount of liquid nitrogen was placed in the bottom of the chamber and the semen straws were placed on a platform within the chamber. The height of the platform and the level of liquid nitrogen were adjusted to ensure the straws were 2 cm above the height of the liquid nitrogen in the vapor phase (Figure 7.1). The lid was replaced on the chamber and the sperm was frozen for 10 minutes. The straws were then transferred to a storage box (Cryobox) and submerged into liquid nitrogen (Locator 6 plus) for storage.

Yamashiro Thawing

After being stored a minimum of three days in liquid nitrogen, the semen straws were removed from liquid nitrogen and placed in ice cubes in an insulated container for transport back to the laboratory. They were then placed in a 37°C water bath for 10 seconds. The straws were cut and the sample was allowed to flow into 1.7 mL graduated tubes and incubated at 37°C for a minimum of 5 minutes.

Sperm motility assessment

A 15 µL sample of the sperm solution was placed on a microscope slide that was warmed to 37°C and covered with a 22 x 22 x 1 mm cover slip in a wet mount preparation. This was then examined at 100x (10x phase objective with 10x eyepiece) with phase contrast to evaluate the presence or absence of sperm motility. The entire area under the cover slip was examined which totaled 484 mm^2 from each sperm sample. The total number of sperm counted varied with the concentration of the sample.

Results

Two samples were frozen utilizing the Nakatsukasa protocol and one sample utilized the Yamashiro protocol. After being stored for a minimum of 3 days, the samples were thawed following the specified procedures. The entire area under the cover slip was examined and no sperm motility was noted in any samples (Table 7.1). The samples were allowed to incubate at

37°C with sperm motility checked at 1, 3 and 5 hours post-thaw. No change in sperm motility was noted with extended incubation.

Table 7.1 Replication of freezing protocols.

Strain	Medium	Age (days)	Concentration (million/mL)	Sperm motility at collection (est.)	Duration of freezing (days)	Post-thaw sperm motility, 5min	Post-thaw sperm motility, 1hr incubation	Post-thaw sperm motility, 3hr incubation	Post-thaw sperm motility, 5hr incubation
Dark agouti	Yamashrio without dbcAMP	247	98	95%	14	0%	0%	0%	0%
SD	Nakatsukasa	161	59	95%	8	0%	0%	0%	0%
SD	Nakatsukasa	155	30	90%	4	0%	0%	0%	0%

Rat Strains: SD- Sprague Dawley

Discussion

Our efforts to replicate the published protocols failed to achieve the results reported by each group (Yamashiro *et al.*, 2010b; Nakatsukasa *et al.*, 2001). There are a variety of reasons this may have occurred. One variable to the media preparation is the egg yolk. The nutrient profile of egg yolk may vary with the diet of the chicken as well as the type of chicken the egg is collected from. It should also be considered that there may be vital details to the freezing process that are not recognized and were inadvertently omitted from the published protocol. Some areas of improvement to further test the published protocols involve increasing the number of experiments performed, loading and sealing the semen straws in a cold room maintained at 4°C to prevent warming of the sample, and transferring the sample directly from the liquid nitrogen to the warm water bath during thawing. It may also be beneficial to use a more permeable platform in the liquid nitrogen vapor phase stage to increase exposure of the sperm straws.

Discussion with Dr. Yamashiro has not revealed any answers, though he encouraged work with continued modification of the procedure until successful. After contacting a co-author from the Nakatsukasa paper, it was revealed that their laboratory continued to modify the procedure to improve sperm motility. This caused further trouble-shooting of the procedure and ultimately alteration of the initial plans for this project. Before proceeding, Dr. Stanley Leibo, at

the University of New Orleans, was contacted and provided valuable advice about how to improve the technique.

Trouble-shooting and Alterations to Published Freezing Protocols

The following alterations were made to the freezing protocols detailed above in an effort to improve sperm motility post-thaw. If no sperm motility was noted after a single trial of the alteration, further changes were made. See Appendix A for a full list of materials and equipment used with source and catalog number information.

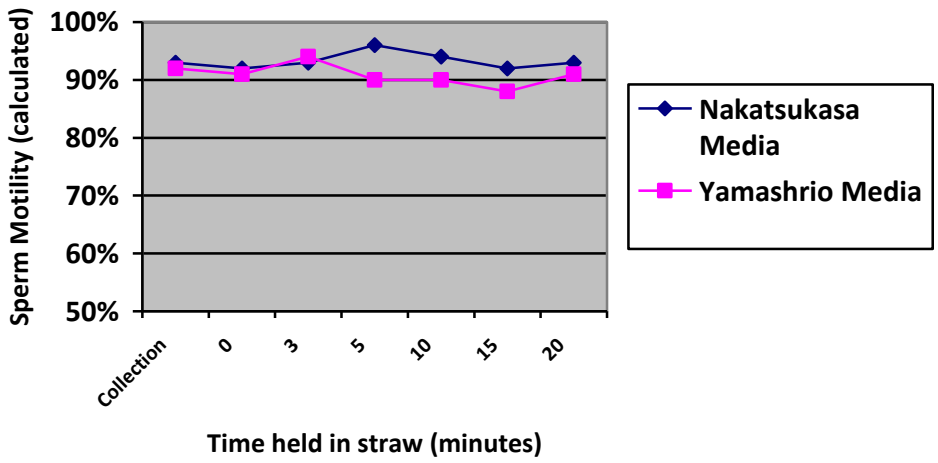
Testing Straw Diameter Effects

To evaluate whether the diameter of the semen straws was having a negative effect on sperm motility, samples were loaded into the straws and sperm motility was examined. This trial also tested the feasibility of using Critoseal to occlude the straws rather than heat sealing. A fresh sperm sample was collected from the epididymis of a male Sprague Dawley rat utilizing Nakatsukasa medium and Yamashiro medium without dbcAMP added. This sample was loaded into 0.25 mL semen straws, with an outer diameter of 2 mm and an inner diameter of 1.5 mm, and held for varying lengths of time at 37°C on a slide warmer. A straw was selected and the sample expelled onto a slide covered with a 22 x 22 x 1 mm cover slip. This was repeated at time variables of immediately, 3, 5, 10, 15 and 20 minutes after loading.

The sperm motility was evaluated at 100x (10x phase objective with 10x eyepiece) with phase contrast and compared to the sperm motility obtained from the sample prior to loading straws. A Nikon digital camera (D90) attached to the microscope was utilized to obtain video footage of at a frame rate of 24 frames per second. An area of 0.54 mm^2 was evaluated for each timed sample. Later evaluation of the videos enabled counting motile sperm versus total sperm in each field and an overall average of sperm motility was obtained.

No dramatic change in sperm motility was noted (Figure 7.2) and it was concluded that the diameter of the straws had no negative impact on sperm motility. Critoseal provided adequate occlusion for the straws and was a quicker, more reliable procedure than utilizing heat sealing.

Figure 7.2 Change in sperm motility over time due to holding in 0.25 mL semen straw.



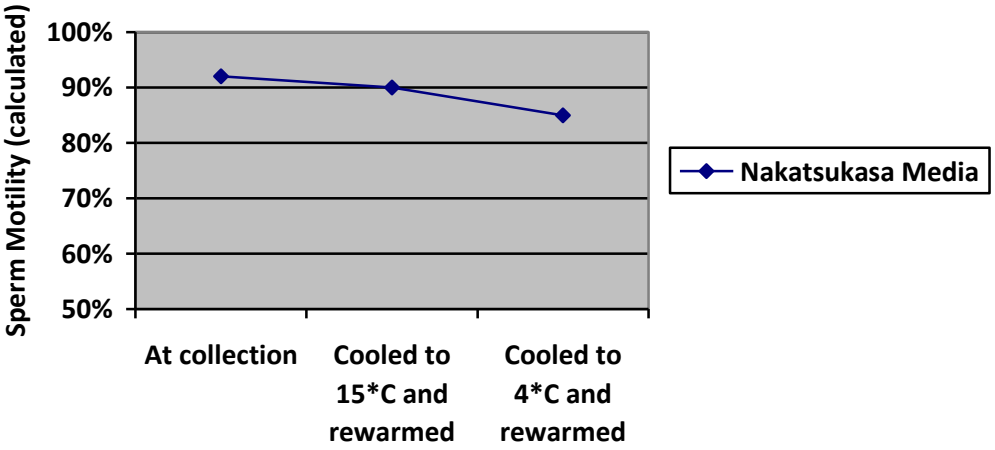
Testing Sperm Motility Loss at Each Stage of Cooling

To evaluate sperm motility loss during the duration of the cooling phase, an epdidymal sperm sample was collected from a Sprague Dawley rat in Nakatsukasa medium. The 12 well non-tissue culture tray containing the sample was placed in a centrifuge pre-cooled to 15°C and held for 30 minutes. As the tray was transferred to the refrigerator for cooling to 4°C, a sample was removed and placed on the 37°C slide warmer for 5 minutes. This sample was placed on a slide and covered with a 22 x 22 x 1mm cover slip.

The sperm motility was evaluated at 100x (10x phase objective with 10x eyepiece) with phase contrast and compared to the sperm motility obtained from the sample at collection. A Nikon digital camera (D90) attached to the microscope was utilized to obtain video footage of at a frame rate of 24 frames per second. An area of 0.54 mm^2 was evaluated for each sample. Later evaluation of the videos enabled counting motile sperm versus total sperm in each field and an overall average of sperm motility was obtained. After the sample was held at 4°C for 30 minutes, another sample was obtained and processed in a similar manner.

There was a 7.6% total loss of sperm motility from collection to the 4°C cooling phase. This is minimal compared to the decrease in overall sperm motility to <1% noted with the freezing process (Figure 7.3). It was concluded that the process of cooling to 15°C and 4°C did not create immediate effects to sperm motility. It is possible that delayed effects occurred that were not evaluated in this trial.

Figure 7.3 Change in sperm motility during cooling stages.



Utilizing 0°C for Cooling Rather than 4°C

As mentioned above, Dr. Leibo suggested a number of variables that might impact sperm cryopreservation and sperm motility. One item discussed was the reliability of the refrigerator for maintaining 4°C during the duration of sperm cooling. It was noted that each time the door was opened the temperature might fluctuate and differences may exist based upon the location the sperm was placed within the refrigerator. To remove this variability from the cooling process, it was proposed to utilize an ice block to provide 0°C conditions for the sperm straws rather than 4°C for the appropriate stages of each cooling process.

To evaluate this theory, epididymal sperm was collected from Sprague-Dawley rats in both Nakatsukasa and Yamashiro media without dbcAMP. Following collection and evaluation of sperm motility, the samples were loaded into straws and sealed with Critoseal. These straws were placed on a prepared ice block (a plastic container that was filled with distilled water and frozen at -20°C, see Figure 7.4) atop wet paper towels and covered with additional wet towels as well as closing the lid on the box containing the block. A straw was removed from these conditions at various time intervals from 1 to 120 minutes and warmed to 37°C on a slide warmer for 5 minutes prior to making a wet mount. This sample was placed on a slide and covered with a 22 x 22 x 1 mm cover slip.

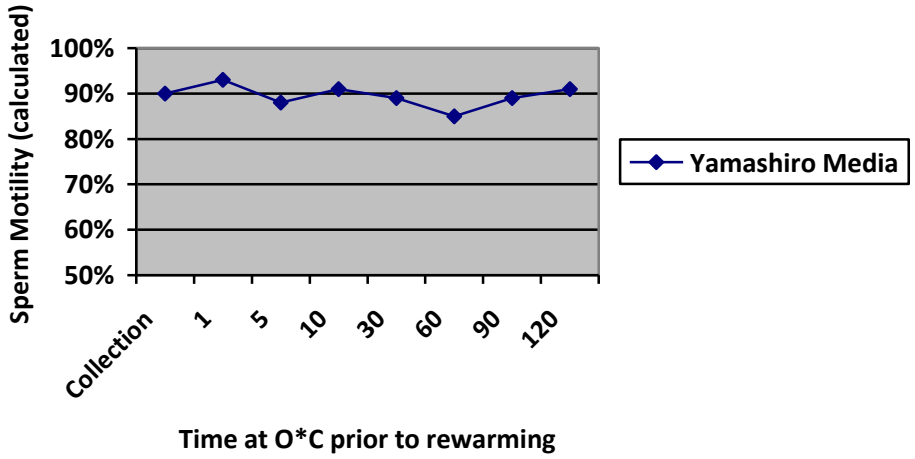
The sperm motility was evaluated at 100x (10x phase objective with 10x eyepiece) with phase contrast and compared to the sperm motility obtained from the sample at collection. A Nikon digital camera (D90) attached to the microscope was utilized to obtain video footage of at

a frame rate of 24 frames per second. An area of 0.54 mm^2 was evaluated for each sample. Later evaluation of the videos enabled counting motile sperm versus total sperm in each field and an overall average of sperm motility was obtained for the sample in Yamashiro. The Nakatsukasa medium utilized for this experiment was not centrifuged for the required amount of time and contained many large droplets and debris from the egg yolk. While subjective observation of sperm motility was possible, objective count of the sperm cells to determine a total count or sperm motility percentage was not possible.

Very minute change in sperm motility was noted over the course of the 2 hours (Figure 7.5). It was concluded that 0°C conditions provided by an ice block would be more consistent for the cooling process and has no immediate detrimental effect on sperm motility. Possible delayed effects of this cooling on the sperm motility were not evaluated.

Figure 7.4 Ice block used to provide 0°C conditions.

Figure 7.5 Change in sperm motility over time due to 0°C cooling.



Modified Nakatsukasa Freezing Protocol

After contacting multiple investigators, including Dr. Bart Carter at UT Southwestern Medical Center, Dr. Jorge Sztein at NIH, Dr. Larry Mobraaten at Jackson Laboratory, and Dr. John Bischof at University of Minnesota, for input to improving our freezing protocol to obtain post-thaw sperm motility, I was put into contact with Dr. Akiko Takizawa, a research scientist at Medical College of Wisconsin, who worked with the investigators that developed the Nakatsukasa protocol. A modified freezing protocol that was implemented with minor adjustments based upon the above trials. These adjustments included changing the height above the vapor phase of liquid nitrogen to 4 cm and maintaining it at this temperature for 15 minutes. It was also recommended to revise the media to contain Equex STM at the time of collection rather than adding it at the end of the cooling process prior to freezing and loading the sample into straws prior to initiating the cooling process (Takizawa, 2012). Based upon my conversation with Dr. Leibo, I substituted the use of an ice block to cool the sperm to 0°C rather than 4°C and sealed the straws with Critoseal instead of heat sealing.

Materials and Methods

See Appendix A for a full list of materials and equipment used with source and catalog number information.

Nakatsukasa Medium

A solution of 8% (w/v) lactose, 23% (v/v) chicken egg yolk, with 1000 units/mL of Penicillin G and 1 mg/mL streptomycin sulfate (Penicillin-Streptomycin liquid) was mixed in cell culture water. The pH is adjusted to 7.4 with 10% tris aminomethane solution. This is centrifuged at 1250 to 1750 x *g* for 15 minutes at room temperature and the upper layer of the solution was decanted. Equex STM was added at 0.7% (v/v) and mixed well on a stirring plate. This solution was divided into aliquots and stored in -20°C for up to 4 months. Aliquots should be as close to the volume used in one session to avoid storage and refreezing after thawing. Excess may be stored in 4°C and used within 2 to 4 days (based upon recommended storage time for raw egg yolks put forth by food safety organizations, such as the American Egg Board).

Yamashiro Medium

Modified Kreb's ringer bicarbonate (mKRB) solution was first prepared by mixing 94.6 mM sodium chloride, 4.78 mM potassium chloride, 1.71 mM calcium chloride dehydrate, 1.19 mM magnesium sulfate heptahydrate, 1.19 mM potassium phosphate monobasic, 25.07 mM sodium bicarbonate, 32.37 mM sodium DL-lactate, 0.5 mM sodium pyruvate, and 5.56 mM D(+)glucose in sterile cell culture water. To this, 0.1 M D(+)raffinose pentahydrate, 750 units/mL penicillin G and 0.75 mg/mL streptomycin sulfate (Penicillin-Streptomycin liquid), 20% (v/v) egg yolk, 0.04% (w/v) sodium dodecyl sulfate and 1.85 mM adenosine triphosphate (ATP) are added. The solution was centrifuged twice at 7000 x *g* for 30 minutes at room temperature and filtered via filter syringe and 0.45µm filter paper. The pH was then adjusted to 7.3 with 3 M sodium hydroxide. Equex STM was added at 0.7% (v/v) and mixed well on a stirring plate. Aliquots should be as close to the volume used in one session to avoid storage and refreezing after thawing. Excess may be stored in 4°C and used within 2 to 4 days (based upon recommended storage time for raw egg yolks put forth by food safety organizations, such as the American Egg Board).

Animals and Sperm collection

Animals were housed as indicated in previous sections. Only Sprague Dawley male rats (n=3) were utilized for this portion of the work. Epididymal sperm collection was performed as

previously detailed with the exception of the media utilized. The epididymides were placed into this Equex containing medium and sperm were allowed to swim out of the tissue.

Nakatsukasa Freezing

Rather than processing the sample through the cooling process in a tray, the sperm sample was loaded into 0.25 mL semen straws and sealed with Critoseal. They were then placed in a pre-cooled 15°C centrifuge for 30 minutes and transferred to a 0°C ice block (Figure 7.4) for 30 minutes. The straws were transported to another laboratory on the ice block and placed in a vapor phase chamber constructed from a Styrofoam cooler (Figure 7.1). A measured amount of liquid nitrogen was placed in the bottom of the chamber and the semen straws were placed on a platform held 4 cm (rather than 2 cm) above this level. The lid was replaced on the chamber and it was held at this temperature for 15 minutes (rather than 10 minutes). The straws were then transferred to a storage box and submerged into liquid nitrogen for storage.

Yamashiro Freezing

Similar changes were made to the Yamashiro protocol. The sperm sample was loaded into 0.25 mL semen straws and sealed with Critoseal. They were placed on a 0°C ice block (Figure 7.4) for 90 minutes. The straws were transported to another laboratory on the ice block and placed in a vapor phase chamber constructed from a Styrofoam cooler (Figure 7.1). A measured amount of liquid nitrogen was placed in the bottom of the chamber and the semen straws were placed on a platform held 4 cm (rather than 2 cm) above this level. The lid was replaced on the chamber and it was held at this temperature for 15 minutes (rather than 10 minutes). The straws were then transferred to a storage box and submerged into liquid nitrogen for storage.

Thawing Sperm

Rather than having individual thawing protocols for each media type, all semen straws were thawed in a similar manner. After being stored a minimum of three days, the semen straws were removed from liquid nitrogen and placed in ice cubes in an insulated container for transport back to the laboratory. They were then placed in a 37°C water bath for 10 seconds. The straws were cut and the sample was allowed to flow into 1.7 mL graduated tubes containing 0.5 mL mR1ECM with 0.4% BSA that was pre-warmed to 37°C.

Results

Three samples, two collected in Nakatsukasa medium and one collected in Yamashiro medium without dbcAMP, were frozen utilizing this procedure. Post-thaw sperm motility was evaluated as previously described. The estimated sperm motility of both samples was <1% and manual counting confirmed 0.85% and 0.53% sperm motility in Nakatsukasa medium and 0.58% sperm motility in the Yamashrio sample (Table 7.2). Refer to "Video 7.1 Sperm motility after thaw" for an example of a sample with <1% sperm motility.

Table 7.2 Post-thaw sperm motility with modified Nakatsukasa freezing protocol.

Strain	Medium	Age (days)	Concentration (million/mL)	Sperm motility at collection (calculated)	Duration of freezing (days)	Post-thaw sperm motility
SD	Nakatsukasa	91	198	96.46%	12	0.85%
SD	Nakatsukasa	97	33.5	96.16%	5	0.53%
SD	Yamashiro without dbcAMP	90	46	91.73%	13	0.58%

Rat strains: SD- Sprague Dawley

Discussion

Upon finally having success obtaining sperm motility post-thaw, plans were made to continue to alter the freezing process to further increase this value. There were multiple changes in this protocol that likely contributed to obtaining motile sperm. By loading the sperm straws prior to cooling and minimizing the time spent manipulating them; the temperature of the sample within the straw was able to be kept more constant. The addition of Equex at the time of collection allows it to assist in the stabilization of the lipids in the plasma membrane as the sample proceeds through the cooling process, thus reducing the amount of cold shock that may occur. Though several changes were made in this process, it was speculated that altering the rate of freezing would continue to increase sperm motility. Further experiments were based upon this assumption.

Altering Cooling Steps of Modified Nakatsukasa Freezing Protocol

Similarly to previous troubleshooting, a single trial was performed with each variation. Post-thaw sperm motility was estimated and recorded as either zero, <1% or >1%. If >1% sperm motility was noted, further experiments would be designed around those conditions.

Straws were prepared as previously indicated using epididymal sperm collected from Sprague Dawley male rats (n=10) in Nakatsukasa medium. See Appendix A for a full list of materials and equipment used with source and catalog number information.

Elimination of cooling steps prior to 0°C cooling

After loading the straws, they were placed on an ice block as previously described (Figure 7.4) for either 30 or 60 minutes. The straws were then placed in the vapor phase at 4 cm above the level of liquid nitrogen for 15 minutes and submerged into liquid nitrogen for storage. Straws were thawed following the above protocol. Sperm motility for both conditions was estimated to be <1%.

Elimination of cooling steps prior to vapor phase

After loading the straws, they were held at room temperature for approximately 10 minutes as they were transported from the laboratory to the liquid nitrogen storage tank or held on the slide warmer at 37°C for 1 hour. They were then placed in the vapor phase at 4 cm above the level of the liquid nitrogen for 15 minutes then submerged into liquid nitrogen for storage. Straws were thawed following the above protocol and no sperm motility was found from either condition.

Elimination of cooling steps prior to submersion in liquid nitrogen

After loading the straws, they were immediately submersed in liquid nitrogen with no prior cooling steps. Straws were thawed following the above protocol. One of the straws was noted to have a crack in it prior to thawing, likely due to the rapid temperature change at freezing. No sperm motility was noted.

Discussion

This series of experiments illustrated the importance of step-wise cooling prior to freezing. While the first stage of cooling from 37°C to 15°C does not appear to be vital to maintaining sperm motility, it is important to cool the sample to 0°C prior to placing them in the vapor phase of liquid nitrogen or cooling to -196°C.

Chapter 8 - Intrauterine Insemination with Frozen Sperm Samples

Methods and Materials

See Appendix A for a full list of materials and equipment used with source and catalog number information.

Animals

Sprague Dawley male (n=4) and female (n=4) rats were utilized for epididymal sperm collection and intrauterine insemination. They were obtained and housed as previously described. The males ranged in age from 90 days to 155 days at the time of collection. The females ranged in age from 120 days to 180 days at the time of insemination.

Vaginal lavage

All females older than 8 weeks of age underwent daily vaginal lavage as previously described.

Induction of pseudopregnancy

When a female was determined to be in proestrus based upon her vaginal lavage, she was paired with a vasectomized male. Pseudopregnancy was determined as previously described.

Sperm Collection and Freezing

Epididymal sperm was collected from a male of the same strain as the pseudopregnant female following the methods outlined previously. It was frozen utilizing the previously described modified freezing protocol in Nakatsukasa medium or Yamashiro medium without dbcAMP.

Intrauterine insemination

The surgical procedure was performed as previously described, utilizing a sperm sample thawed in mRIECM with 0.04% BSA that was warmed to 37°C. 50µL of each diluted sperm sample was instilled into each side of the uterus. Sperm motility of the thawed samples ranged from 0 to 0.85%, resulting in a total motile sperm number instilled in each side ranging from 0 to 20,375.

Confirmation of pregnancy

At 10 to 14 days gestation, transabdominal ultrasound was performed as previously described.

Results

Inseminations were performed utilizing sperm samples obtained in both Nakatsukasa and Yamashiro media without dbcAMP that were previously frozen (Table 8.1). The mean post-thaw sperm motility was 0.48 ± 0.35% in a mean concentration of 35.5 ± 35.8 million/mL resulted in a mean estimated number of motile sperm transferred in 50 µL of sample into each uterine horn was 9491 ± 9143. No pregnancies resulted from these inseminations.

Table 8.1 Intrauterine insemination utilizing frozen sperm samples.

Strain	Medium	Age of Female	Age of Male at Time of Collection	Duration of Freezing	Sperm Concentration	Sperm Motility (post-thaw)	Est. Number of Motile Sperm Transferred (each side)	Ultrasound Confirmation	Pups Born and Weaned
SD	Nakatsukasa	120d	155d	33 days	4.5 million/mL	0%	0	Negative	0
SD	Nakatsukasa	141d	91d	13 days	10 million/mL	0.85%	4250	Negative	0
SD	Nakatsukasa	149d	105d	7 days	81.5 million/mL	0.50%	20,375	Negative	0
SD	Yamashiro without dbcAMP	180d	90d	9 days	46 million/mL	0.58%	13,340	Negative	0

Rat Strains: SD- Sprague Dawley. Estimated number of motile sperm transferred in 50 µL of solution in each uterine horn.

Table 8.2 Summary of IUI with frozen samples from this experiment and literature

Strain	Medium	Sperm Motility	Est. Number of Motile Sperm Transferred (each side)	Pregnancy rate	Litter Size
SD[a]	Nakatsukasa	0.45 ± 0.24%	8208 ± 6205	0/3	0
SD[a]	Yamashiro without dbcAMP	0.58%	13,340	0/1	0
SD ♂, Wistar ♀[b]	Nakatsukasa	9 ± 3.3%	90,000 to 270,000	9/13	4.6 ± 4.9
SD (Closed colony)[c]	Nakatsukasa	9.6 ± 1.5%	288,000 to 480,000	3/5	3.7 ± 1.2
Wistar (Closed colony)[c]	Nakatsukasa	10 ± 0%	300,000 to 500,000	3/5	4.3 ± 2.1
BN (Inbred)[c]	Nakatsukasa	2.0 ± 0.6%	60,000 to 100,000	3/5	3.0 ± 1.0
F344 (Inbred)[c]	Nakatsukasa	10.0 ± 2.5%	300,000 to 500,000	4/5	7.0 ± 1.4
LEW (Inbred)[c]	Nakatsukasa	6.7 ± 1.7%	201,000 to 335,000	1/5	4.0 ± 0.0
Long-Evans (Inbred)[c]	Nakatsukasa	5.7 ± 3.3%	171,000 to 285,000	3/5	2.0 ± 1.0
WKY (Inbred)[c]	Nakatsukasa	10.7 ± 6.7%	321,000 to 535,000	2/5	4.0 ± 1.4
Tremor (Mutant)[c]	Nakatsukasa	9.3 ± 3.3%	279,000 to 465,000	5/5	4.8 ± 1.6
Zitter (Mutant)[c]	Nakatsukasa	8.7 ± 1.8%	261,000 to 435,000	4/5	6.8 ± 2.4
Human A-transferase (Transgenic)[c]	Nakatsukasa	10.6 ± 2.0%	318,000 to 530,000	5/5	6.0 ± 2.7
GFP (Transgenic)[c]	Nakatsukasa	12.3 ± 1.5%	369,000 to 615,000	3/5	6.7 ± 2.3
Wistar[d]	Yamashiro with dbcAMP	43.3 ± 2.8%	433,000 to 649,500	0/27	0

[a]Data collected in this study, mean ± SEM. [b]Data collected in (Nakatsukasa *et al.*, 2001), mean ± SEM. [c]Data collected in (Nakatsukasa *et al.*, 2003), mean ± SEM. [d]Data collected in (Yamashiro *et al.*, 2010b). Rat Strains: SD- Sprague Dawley, BN- Brown Norway, F344- Fischer 344, LEW- Lewis, WKY- Wistar-Kyoto, GFP- green fluorescent protein. Estimated number of motile sperm transferred in 50 µL of solution in each uterine horn.

Discussion

The low concentration of motile sperm in the inseminated samples precluded pregnancy in the animals in our study. Some reasons for this may have been that the motile sperm were either unable to reach the ovulated oocyte or there were not enough sperm to form the oviductal reservoir (Suarez, 2002) to enable fertilization.

When compared with the total number of motile sperm instilled by Nakatsukasa, our values were much lower (Table 8.2). The minimum number of sperm that produced a pregnancy in their study was 60,000 motile sperm. The maximum number achieved here was just over 20,000. While the goal to increase this number is by increasing the post-thaw sperm motility, one may also attempt to increase the volume of a lesser concentrated sample or increase the concentration of sperm stored in the straws. It would also be possible to utilize the low sperm motility samples by performing insemination via IVF or ICSI.

Chapter 9 - Evaluating Sperm Motility Patterns

After reviewing information concerning the differences in motility between sperm at collection and capacitated sperm (Lindemann *et al.*, 1987), videos of sperm motility obtained from previous collections were reviewed. In particular, a set of videos obtained from the collection from a 118 day old male Sprague Dawley rat were reviewed, where one epididymis was placed into the modified Nakatsukasa medium with Equex STM added and the other was placed in modified Yamashrio medium with Equex STM added. Comparing samples from the same male eliminated differences in sperm motility based upon individual animal sperm motility patterns.

Subjective analysis of the videos showed that there was a distinct difference in motility patterns of the sperm cells. The sperm in Nakatsukasa medium displayed a more straight line motion with minimal curvature to the tails. Refer to "Video 9.1 Nakatsukasa sperm motility pattern" to view an example of this sperm motility pattern. Approximately two-thirds of the motile sperm in Yamashiro medium exhibited motion consistent with that discussed by Lindemann, with extreme curvature of the head and tail, at times bending into a fish-hook shaped position (Lindemann *et al.*, 1987). Refer to "Video 9.2 Yamashiro sperm motility pattern" to view an example of this sperm motility pattern.

This subjective observation suggests that a portion of the sperm collected in Yamashiro medium may be undergoing capacitation prior to freezing. This may be induced by the additional calcium, bicarbonate and ATP present in the solution. It is possible that our low post-thaw sperm motility in samples frozen in Yamashiro medium is due to capacitated sperm having a shorter life span (Bailey *et al.*, 2000). This would be amplified by the addition of dbcAMP to the solution. This may also suggest the reason Yamashiro was unable to produce pregnancies using IUI with frozen-thawed sperm, despite a higher post-thaw sperm motility level (Yamashiro *et al.*, 2010b). Further objective evaluation utilizing a computer assisted sperm assessment (CASA) program that is specific for rat sperm or performing frame by frame evaluation of video would be necessary to draw any definite conclusions from this observation.

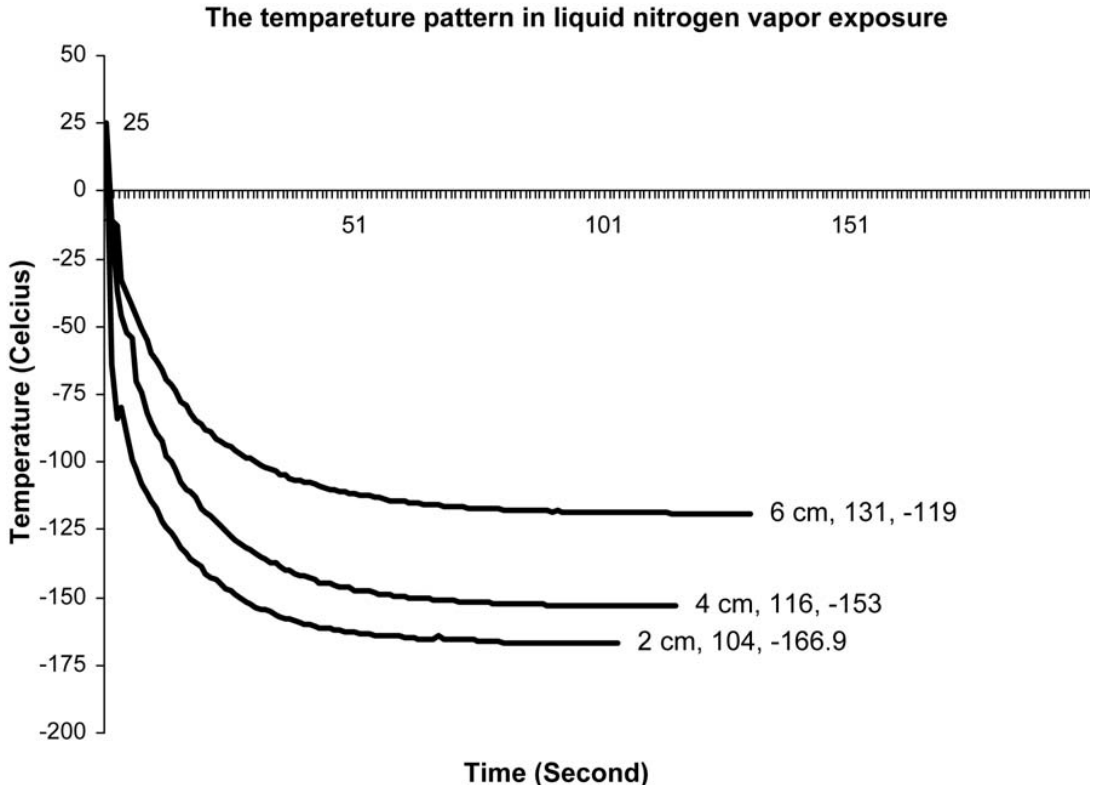
Chapter 10 - Determining the Effect of Various Freezing Rates on Epididymal Sperm

Introduction

The modified freezing protocol detailed above had multiple changes that allowed for obtaining sperm motility post-thaw in collected sperm samples. One change was increasing the distance above liquid nitrogen during the vapor phase as well as the length of time the sample was held at this temperature prior to submerging it into the liquid phase. These alterations result in a slower freezing rate which can result in more time for the solution to equilibrate across the cell membrane leading to improved stabilization.

A study conducted on carp sperm cryopreservation detailed the temperature change when samples are placed in the vapor phase of liquid nitrogen at various heights above the liquid (Irawan *et al.*, 2010). Straws used were similar to those utilized in our study, though the container that was used to produce the vapor phase was larger with an overall volume of 19,899 cm^3 (Irawan *et al.*, 2010), while the container here was only 4597 cm^3. There were also differences between the freezing solutions used as the carp sperm was placed in a solution containing salts, sugars such as fructose, glucose and sucrose in various combinations, and bicarbonate solution. The cryoprotectants used were DMSO, methanol and propylene glycol (Irawan *et al.*, 2010). These differences may have an impact on the overall cooling rate. Temperature of the sperm solution was measured within the straw using a K-type thermocouple every second. A cooling rate average was obtained for each condition and it was found that samples 2 cm above the liquid nitrogen cooled at a rate of 110.9°C/minute. At 4 cm above the liquid nitrogen the cooling rate was 92.2°C/minute and at 6 cm it was 66.1°C/minute (Irawan *et al.*, 2010). The optimal cooling rate proposed for rat sperm was 53 to 70°C/minute (Hagiwara *et al.*, 2009), which corresponds most closely to the freezing rate detected at 6 cm. A profile of the freezing temperature was created based upon their freezing data and is reproduced in Figure 10.1.

Figure 10.1 Figure from (Irawan *et al.*, 2010).



Data at the end of each line indicate the height above liquid nitrogen, the time in seconds it took to reach a plateau in temperature and the temperature, in °C, that this plateau was reached.

An experiment was also conducted in mice to evaluate differences in heights above liquid nitrogen during the freezing process as compared to the use of a programmable freezer. Heights of 1, 2, 3, and 4 cm above the liquid nitrogen were utilized and it was found that 2 cm provided an increased post-thaw sperm motility (Yildiz *et al.*, 2010).

For the experiment here, pooling sperm from several animals would reduce the impact of biological variability on the experiment. It was elected to use Nakatsukasa medium as more samples have been processed using that medium in the preliminary research and it was the only medium with successful IUI production of pups with frozen-thawed sperm in the literature.

Thus, the hypothesis was formed that allowing slower cooling by increasing the distance above the liquid nitrogen level during the vapor phase will increase the post-thaw sperm motility of cryopreserved rat epididymal sperm samples.

Materials and Methods

See Appendix A for a full list of materials and equipment used with source and catalog number information.

Animals

Sprague Dawley male rats were utilized for epididymal sperm collection. They were obtained and housed in accordance with animal care guidelines of IACUC, Kansas State University. The room was maintained at 71 to 73°F and between 30 to 70% humidity. They were maintained on an altered light/dark cycle that included 12 hours of dark from 1200 (noon) to 2400 (midnight), alternated with 12 hours of light. They were pair housed in 143 square inch solid bottom cages containing corn cob bedding unless they exceeded the recommendations set forth by The Guide for the Care and Use of Laboratory Animals. This criteria states that each animal must have greater than or equal to 70 square inches for rats weighing greater than 500 grams and the animals must be able to perform normal posture behaviors without touching the sides (Institute of Laboratory Animal Research *et al.*, 1996). Animals were moved to single housing if they exceeded these guidelines. They were fed maintenance diets (Lab Diet 5001 or 5008) with water *ad libitum* with supplemental Love Mash diet 3 days per week. The males ranged in age from 79 days to 103 days at the time of collection.

Nakatsukasa Medium Preparation

A solution of 8% (w/v) lactose, 23% (v/v) chicken egg yolk, with 1000 units/mL of Penicillin G and 1mg/mL streptomycin sulfate (Penicillin-Streptomycin liquid) was mixed in cell culture water. The pH is adjusted to 7.4 with 10% tris aminomethane solution. This is centrifuged at 1250 to 1750 x *g* for 15 minutes at room temperature and the upper layer of the solution is decanted. Equex STM is added at 0.7% (v/v) and mixed well on a stirring plate. This solution is divided into aliquots and stored in -20°C for up to 4 months. Aliquots should be as close to the volume used in one session to avoid storage and refreezing after thawing. Excess may be stored in 4°C and used within 2 to 4 days (based upon recommended storage time for raw egg yolks put forth by food safety organizations, such as the American Egg Board).

Epididymal Sperm Collection and Pre-Freezing Evaluation

Sperm Collection

Two male rats were euthanized via carbon dioxide inhalation followed with induction of bilateral pneumothorax. Immediately following confirmation of death, the distal end of the scrotum in each rat was removed with scissors and dissection of the tunics allowed for exteriorization of the testes and *cauda epididymidis*. The *cauda epididymidis* was dissected away from the testicle and the adherent fat was removed. The tissue was rinsed in warm PBS and scissors were used to cut three slits into the tissue as illustrated in Figure 4.1. The epididymis was then placed in Nakatsukasa cryopreservation medium with Equex added and warmed to 37°C on a slide warming tray. This was repeated on the opposite side and both epididymides were removed from the second animal and placed in the same medium sample. The pooled sample was incubated for 10 minutes to allow the sperm to swim out of the tissues. The epididymal tissue was removed and the sample was ready for evaluation.

Sperm Concentration

The sperm sample was diluted 1:100 using 2 successive dilutions in distilled water (50µL sample in 450µL). A hemocytometer was prepared by placing the coverslip over the counting surface prior to loading the suspension. The sperm suspension was introduced into the V-shaped well to cover the counting area and was allowed to sit for at least 2 minutes to allow the sperm cells to settle. The hemocytometer was placed on the microscope stage and the number of cells in the 25 squares of the central counting grid was tabulated at 100x (10x phase objective with 10x eyepiece) with phase contrast. The process was repeated in the second counting chamber and an average of the two 10 mm^3 counting areas was obtained. Concentration was then determined by multiplying the number of cells by the dilution factor (100) and multiplying that total by 10,000 (obtained by calculating that the counting area contains 10 cubic millimeters and there are 1000 cu mm in each milliliter) to determine the amount of sperm cells per milliliter of sample.

Sperm motility assessment

A 15 µL sample of the sperm solution was placed on a microscope slide that was warmed to 37°C and covered with a 22 x 22 x 1 mm cover slip in a wet mount preparation. This was then

examined at 100x (10x phase objective with 10x eyepiece) with phase contrast to estimate sperm motility percentages in ranges of ±5%. A Nikon digital camera (D90) attached to the microscope was utilized to obtain video footage of 20 fields at a frame rate of 24 frames per second with the number of sperm counted varying with concentration of the sample. Each field represented 0.54 mm^2 for evaluation of a total of 10.8 mm^2 from each sperm sample. Later evaluation of the videos enabled counting motile sperm versus total sperm in each field and an overall average of sperm motility was obtained.

Freezing Sperm Samples

The sperm sample was loaded into 0.25 mL semen straws and sealed with Critoseal. They were then placed in a pre-cooled 15°C centrifuge for 30 minutes and transferred to a 0°C ice block (Figure 7.4) for 30 minutes. The straws were transported to another laboratory on the ice block and placed in one of three vapor phase chambers constructed from a Styrofoam cooler (Figure 7.1). A measured amount of liquid nitrogen was placed in the bottom of the chamber and the semen straws were placed on a platform held 2, 4 or 6 cm above this level. For each trial the order of addition to the chambers was altered. For example, the first trial was placed in the order of 2, 4, and then 6 cm while the second trial was in the order of 4, 6, and then 2 cm. The lids were replaced on the chambers and samples held at this temperature for 15 minutes. The straws were then transferred to a storage box and submerged into liquid nitrogen for storage.

Thawing Sperm Samples

After being stored for four to six days, the semen straws were removed from liquid nitrogen and placed in ice for transport back to the laboratory. They were then placed in a 37°C water bath for 10 seconds. The straws were cut and the sample was allowed to flow into 1.7 mL graduated tubes containing 0.5 mL modified rat one-cell embryo culture medium (mR1ECM) with 0.4% (w/v) bovine serum albumin (BSA) that was pre-warmed to 37°C. Additional warm mR1ECM with 0.4% BSA was added to each sample to bring the total volume of the sample to 1 mL. They were incubated for 5 minutes at 37°C.

Post Thaw Sperm Motility Assessment

A 15 µL sample of the sperm solution was placed on a microscope slide that was warmed to 37°C and covered with a 22 x 22 x 1 mm cover slip in a wet mount preparation. This was then

examined at 100x (10x phase objective with 10x eyepiece) with phase contrast. The entire area under the cover slip was examined (484 mm^2) and if no motile sperm were found, the sperm motility was recorded as 0%. When motile sperm were detected, a Nikon digital camera (D90) attached to the microscope was utilized to obtain video footage of 20 fields at a frame rate of 24 frames per second with the number of sperm counted varying with concentration of the sample. Each field represented 0.54 mm^2 for evaluation of a total of 10.8 mm^2. Later evaluation of the videos enabled counting motile sperm versus total sperm in each field and an overall average of sperm motility was obtained.

LIVE/DEAD® Stain of Post Thaw Sperm Samples

The LIVE/DEAD® sperm viability kit produced by Invitrogen contains two reagents used to identify live mammalian sperm versus dead cells. Propidium iodide (PI) is a commonly used stain for identifying dead cells. The second component, SYBR 14, was developed as a membrane-permeant nucleic acid stain. Both stains target DNA and can be utilized with flow cytometry. They can be excited with visible-wavelength light and viewed with fluorescence microscopy. SYBR 14 stains all sperm cells green and PI will cause cells with damaged cell membranes to stain red. This red stain covers the green and thus causes dead sperm to fluoresce red while the cells with intact cell membranes that failed to take up PI will remain green (Ramalho-Santos *et al.*, 2007). Rat sperm cells that displayed SYBR-14 staining correlate with expected viability (Gravance *et al.*, 2001).

Each component of the LIVE/DEAD® stain was removed from -20°C storage and allowed to thaw at room temperature. The SYBR 14 stock solution (1 mM in DMSO) (LIVE/DEAD® sperm viability kit) was diluted 1:50 in HEPES-buffered saline solution with bovine serum albumin containing 10 mM HEPES buffer, 150 mM sodium chloride, and 10% BSA, with pH adjusted to 7.4 with 3 M sodium hydroxide. For each milliliter of sperm solution, 8 µL of diluted stain was added. The samples were then incubated at 37°C for 30 minutes. After this incubation 2 µL of the stock propidium iodide solution (2.4 mM in water) (LIVE/DEAD® sperm viability kit) was added to each sample. This was allowed to incubate at 37°C for 2 minutes. A wet mount preparation was made as previously discussed and the staining was evaluated at 200x (20x fluorescence objective with 10x eyepiece) with phase contrast utilizing TRITC (PI) and FITC (SYBR 14) fluorescence. The entire area under the cover slip was

examined for a total of 484 mm^2 of sample. Sperm cells exhibiting green fluorescence were counted and recorded for each sample and compared to the total number of motile sperm observed in the sample.

Statistical Analysis

A post-hoc Friedman test was performed using GraphPad Prism version 6.00 for Windows. A nonparametric test was selected due to our small sample size and this test was selected due to having matched data. Summary data is provided as mean ± standard deviation. Significance was set at $p < 0.05$.

Results

Epididymal samples were collected from ten rats into 5 groups. The concentration and pre-freezing sperm motility was determined. Mean concentration was 37 ± 12.5 million/mL and varied from 22.5 million/mL to 54.5 million/mL. Pre-freezing sperm motility mean was 88.56 ± 0.81%, ranging from 87.34% to 89.43%. The number of sperm counted for sperm motility evaluation of each sample varied with concentration and ranged from 2365 to 4099 sperm cells at collection and 304 to 1177 sperm cells in thawed samples, with means of 3478 ± 678 and 647 ± 244, respectively. The average sperm motility of thawed samples was calculated. At 2 cm above liquid nitrogen, the mean sperm motility was 0.19 ± 0.23%, ranging from 0 to 0.53%. A 4 cm height produced mean sperm motility of 0.18 ± 0.13%, ranging from 0 to 0.36%. The 6 cm height produced mean sperm motility of 0.31 ± 0.12%, ranging from 0.17 to 0.50%. Data presented in Table 10.1 and summarized in Table 10.2.

The Friedman test for sperm motility post-thaw at 2, 4 and 6 cm resulted in a p-value of 0.23 (Figure 10.2).

Each thawed sample was stained with LIVE/DEAD® sperm stain to evaluate membrane integrity. Some samples that had no motile sperm on the slide evaluated for sperm motility contained motile sperm in the slide prepared after staining (Table 10.3). In all instances, sperm that were motile on microscopic evaluation stained green due to SYBR 14 uptake and non-motile sperm provided red staining due to PI.

Table 10.1 Concentration, sperm motility and sperm counts of individual samples.

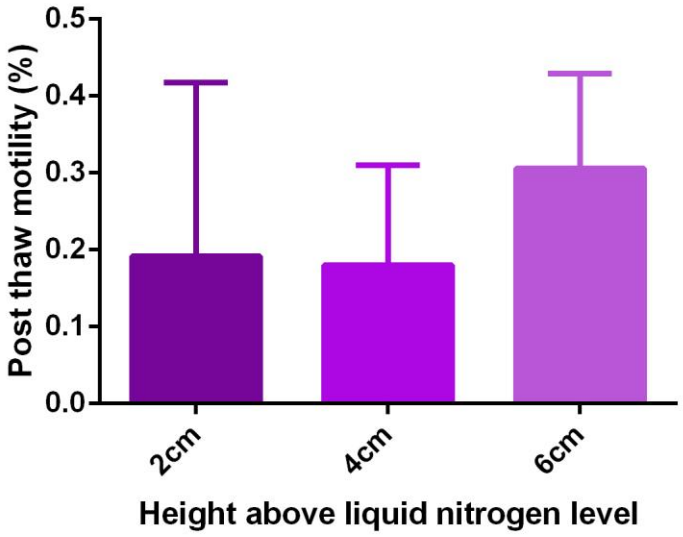
Group	Concentration at collection	Number motile sperm/Total number counted at collection	Sperm motility at collection	Number motile sperm/ Total number counted, 2 cm	Sperm motility after thaw when frozen at 2 cm	Number motile sperm/ Total number counted, 4 cm	Sperm motility after thaw when frozen at 4 cm	Number motile sperm/ Total number counted, 6 cm	Sperm motility after thaw when frozen at 6 cm
1	39 million/mL	3001 / 3436	87.34%	No motile sperm noted	0%	2 / 893	0.22%	2 / 1177	0.17%
2	41.5 million/mL	3627 / 4099	88.48%	1 / 769	0.13%	1 / 639	0.16%	2 / 797	0.25%
3	27.5 million/mL	3507 / 3933	89.17%	2 / 664	0.30%	2 / 552	0.36%	2 / 399	0.50%
4	22.5 million/mL	2115 / 2365	89.43%	No motile sperm noted	0%	No motile sperm noted	0%	1 / 304	0.33%
5	54.5 million/mL	3142 / 3555	88.38%	3 / 569	0.53%	1 / 635	0.16%	1 / 361	0.28%

Table 10.2 Summary of concentration, sperm motility and sperm count data.

	Concentration at collection	Total number counted at collection	Sperm motility at collection	Total number counted, post thaw	Sperm motility after thaw when frozen at 2cm	Sperm motility after thaw when frozen at 4cm	Sperm motility after thaw when frozen at 6cm
Mean	37 ± 12.5 million/mL	3478 ± 678	88.56 ± 0.81%	647 ± 244	0.19 ± 0.23%	0.18 ± 0.13%	0.31 ± 0.12

Mean ± standard deviation.

Figure 10.2 Mean sperm motility of post-thaw samples after being frozen at varying heights above liquid nitrogen in the vapor phase.



Error bars display standard deviation for each group. p=0.23 via Friedman test.

Table 10.3 Results of LIVE/DEAD® stain.

Group	Post-thaw after freezing at 2cm		Post-thaw after freezing at 4cm		Post-thaw after freezing at 6cm	
	Number of sperm fluorescing with SYBR 14 at 2 cm	Number of motile sperm noted during sperm motility evaluation	Number of sperm fluorescing with SYBR 14 at 4 cm	Number of motile sperm noted during sperm motility evaluation	Number of sperm fluorescing with SYBR 14 at 6 cm	Number of motile sperm noted during sperm motility evaluation
1	1	0	3	2	2	2
2	3	1	0	1	0	2
3	3	2	8	2	4	2
4	1	0	0	0	1	1
5	0	3	1	1	1	1

All sperm staining green due to SYBR 14 were motile. Area examined was 10.8mm^2. Remaining sperm on slide were non-motile and stained red due to PI stain. Mann-Whitney test comparing difference between number of sperm fluorescing with SYBR 14 and number of motile sperm during sperm motility evaluation showed no significant difference (p=0.84).

Discussion

With a p-value of 0.23, there is not a statistical difference between the post-thaw sperm motility of samples frozen at differing heights above the liquid nitrogen. A difference may be easier to appreciate and may approach significance if the value of post-thaw sperm motility exceeded 1% or the sample size was increased. The power of our current study was estimated to be between 0.2 and 0.3. If we assume an r-squared value of 0.1 to 0.2 (calculated r-squared for our current data was 0.1633), the sample size needed to obtain a power of >0.8 is 20 to 30 using a rank ANOVA power curve simulation (Figure C.1). If the temperatures detected in the carp paper are applicable to our freezing set up (Irawan *et al.*, 2010), it is possible that further increase in height above the liquid nitrogen may continue to increase post-thaw sperm motility.

The presence of motile sperm in stained samples where no motile sperm was noted on sperm motility examination is likely due to sampling differences. When the overall number of motile sperm is so low, the chances of having one motile sperm in 15 µL of sample is low as well. It is possible that the sample was mixed more thoroughly after staining which suspended the motile sperm higher in the sample which predisposed it to being selected for the wet mount. The correlation of membrane permeability and sperm motility is expected. Further evaluation of sperm integrity could give insight to fertilizing capabilities aside from sperm motility.

Chapter 11 - Conclusion

Rat sperm has proven to be one of the more difficult species to undergo successful cryopreservation. Though many protocols have been developed, the ability to retain sufficient sperm motility to produce pups via IUI has not been published outside of the work in Japan (Nakatsukasa *et al.*, 2001; Nakatsukasa *et al.*, 2003). Through my preliminary work and experiment evaluating the use of height above liquid nitrogen in vapor phase, I have been unable to produce adequate post-thaw sperm motility to utilize IUI. The samples produced may be used for ICSI or IVF, though the IUI method would have more practical applications.

Future experiments would focus on producing higher post-thaw sperm motility. The thawing process was not addressed during this project. Ways to alter this step would be to adjust the speed of the thawing process, utilizing different dilution media that has more substances to increase sperm motility, or by altering the incubation time of sperm following thawing. Another aspect would be to create a novel freezing medium that contains a permeating cryoprotectant and attempt to develop an appropriate freezing and thawing protocol. It would be desirable to eliminate the egg yolk for an ingredient that is chemically defined.

Evaluation of sperm concentration and the effects on post-thaw motility was performed at the conclusion of this experiment (Table B.4). Though no correlation was noted between the concentration of the sample at freezing and the post-thaw motility (Figure B.1), a larger sample size may be needed to fully evaluate the effects. In future work, utilizing a consistent concentration of sperm for freezing and insemination will allow more detailed comparisons of the results obtained.

Other aspects of rat sperm cryopreservation that may be altered include investigating other means of collecting sperm that do not necessitate the euthanasia of the male. This would permit collection while maintaining the male's natural reproductive capabilities and potentially more stored samples if a male could be collected multiple times.

In improving our methods for cryopreservation, increasing the amount of information we can collect on the sperm samples would be valuable. This may include using a computer assisted sperm analyzer (CASA) to obtain more consistent and rapid results for sperm motility rates as well as provide information on progressive sperm motility and velocity patterns. It would also be beneficial to examine the sperm morphology with a higher magnification, possibly even

utilizing electron microscopy, to further evaluate damage to the sperm cells caused by the cryopreservation process.

After a successful cryopreservation protocol is developed, other projects may involve investigating the effect of the age of males, the length of storage, and the impact of various strains of rats. Further developing skills in IVF and ICSI would allow for the use of the lower sperm motility samples.

Success in this area of research is important to further the welfare of research animals by allowing reduction of number used to maintain strains. It will also allow the ease of animal transfer and preservation of strains. Additional investigation is necessary as there are many aspects that may be altered to improve the technique and its reproducibility.

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Appendix A - Supplies and Equipment Used

Item Name	Company	Location	Catalog Number
Adenosine triphosphate	Sigma Aldrich	St. Louis, MO	A3377-5g
Alcohol, 100% and 70%	Decon Labs, Inc.	King of Prussia, PA	2701
Autoclip, 9 mm	MikRon Precision Inc.	Gardenia, CA	205016
Bedding, Bed-O'cobs®	Pharma Serv	Framingham, MA	AND4B
Bovine serum albumen	Sigma Aldrich	St. Louis, MO	A3912
BrightLine Hemocytometer	Hausser Scientific	Horsham, PA	1492
Buprenorphine, 0.3mg/mL	Reckitt Benckiser	Richmond, VA	N/A
Calcium chloride dihydrate	Fisher Scientific	Waltham, MA	C-79
Cell culture water	Sigma Aldrich	St. Louis, MO	W3500
Centrifuge (for cooling sperm), accuSpin 3R	Fisher Scientific	Waltham, MA	N/A
Centrifuge, Sorvall RC 6+	Thermo Scientific	Waltham, MA	46910
Chicken egg	Local farms	Manhattan, KS	N/A
Chlorhexidine Gluconate Solution, 4.0% w/v, Hibiclens	Mölnlycke Health Care	Nacross, GA	26004-05-RM
Combi-Pen-48 (Penicillin G Benzathine and Penicillin G Procaine Suspension)	Bimeda Inc.	Oakbrook Terrace, IL	1COM301
Conical tube for aliquot storage, 15 mL	BD Falcon	San Jose, CA	352097
Cover glass, 22 x 22 x 1 mm	Fisher Scientific	Waltham, MA	12-542-B
Critoseal, Leica Biosystems	Fisher Scientific	Waltham, MA	02-676-20
Cryobox	Thermo Scientific	Waltham, MA	189470
Cryopreservation system, Locator 6 plus	Thermo Scientific	Waltham, MA	CY509113
D(+) glucose	Sigma Aldrich	St. Louis, MO	G6152-500g
D(+) raffinose pentahydrate	Sigma Aldrich	St. Louis, MO	R0250-100g
Dibutyryl cyclic-AMP sodium salt	Sigma Aldrich	St. Louis, MO	D0627-25mg
Digital camera, D90	Nikon Instruments Inc.	Melville, NY	D90
Dulbecco's Phosphate Buffered Saline	Gibco/Life Technologies	Grand Island, NY	14190
Equex, STM	Nova Chemical Sales	Scituate, MA	EQ8
Filter cube, FITC	Chroma	Bellows Falls, VT	31001 409
Filter cube, TRITC	Chroma	Bellows Falls, VT	31002 409
Graduated tubes, 1.7 mL	BioExpress	Kaysville, UT	C-3718-1
Heat supply, post-operative, Circulating water blanket	Gaymar Industries, Inc.	Orchard Park, NY	TP-500
HEPES buffer	Sigma Aldrich	St. Louis, MO	H-3375
Hot bead sterilizer	Fine Science Tools	Foster City, CA	18000-45
Incubator, AutoFlow Water jacketed CO_2	Nuaire	Plymouth, MN	NU-8500
Isoflurane	Abbott	Abbott Park, IL	4/5/5260

Item Name	Company	Location	Catalog Number
Lactose	Fisher Scientific	Waltham, MA	NC9654357
Live/Dead sperm viability kit, propidium iodide	Invitrogen	Grand Island, NY	L7011 B
Live/Dead sperm viability kit, SYBR 14	Invitrogen	Grand Island, NY	L7011 A
Love Mash rat diet	Bio-Serv	Frenchtown, NJ	S3823P
LubriFresh PM eye lubricant	Major Pharmaceuticals	Livonia, MI	NDC 0904-5168-38
Magnesium sulfate heptahydrate	Sigma Aldrich	St. Louis, MO	230391-25g
Maintenance diet, LabDiet	PMI Nutrition International	St. Louis, MO	5001, 5008
Micropipette, Disposable Pasteur Pipet	Fisher Scientific	Waltham, MA	13-678-20A
Microscope slides, Frosted, 25 x 75 x 1 mm	Fisher Scientific	Waltham, MA	12-544-2
Microscope, Nikon Diaphot	Nikon Instruments Inc.	Melville, NY	N/A
Microscope, Nikon Eclipse	Nikon Instruments Inc.	Melville, NY	TE2000-S
Modified rat 1-cell embryo culture medium	Chemicon/Millipore	Billerica, MA	MR-054-D
Needle, 22 gauge x 1"	Monoject/Covidien	Mansfield, MA	8881201449
Non-tissue culture treated plate, 12 well	BD Falcon	San Jose, CA	351143
Non-tissue culture treated plate, 24 well	BD Falcon	San Jose, CA	351147
p-Chloroamphetamine hydrochloride	Sigma Aldrich	St. Louis, MO	C9635-1g
Penicillin-Streptomycin, Liquid	Gibco/Life Technologies	Grand Island, NY	15140
pH meter, Accumet Research AR15	Fisher Scientific	Waltham, MA	13-636-AR15BC
Potassium chloride	Fisher Scientific	Waltham, MA	BP366-500
Potassium phosphate	Fisher Scientific	Waltham, MA	P285-3
Providone Iodine Solution	Dynarex	Orangeburg, NY	1416
Refrigerator, 4°C	Thermo Electron Corporation	Waltham, MA	REB5004D21
Semen straws, 0.25 mL	AgTech	Manhattan, KS	F06
Slide Warmer	Fisher Scientific	Waltham, MA	12-594
Sodium bicarbonate	Fisher Scientific	Waltham, MA	S233-500
Sodium chloride	Fisher Scientific	Waltham, MA	5271-3
Sodium DL-lactate	Sigma Aldrich	St. Louis, MO	71720-5g
Sodium dodecyl sulfate	BioRad	Hercules, CA	161-0300
Sodium hydroxide	Fisher Scientific	Waltham, MA	BP359-212
Sodium pyruvate	Sigma Aldrich	St. Louis, MO	P4562-2g
Statistical analysis software, GraphPad Prism	GraphPad Software	La Jolla, CA	Ver. 6.00
Subcutaneous fluids, Veterinary 0.9% sodium chloride for injection	Abbott	Abbott Park, IL	04925-04-05
Suture, PDS II 4-0, SH 26 mm 1/2C taper, 27"	Ethicon	Cornelia, GA	Z315H
Syringe Filter, 0.45 µm	Fisher Scientific	Waltham, MA	09-719-7
Syringe, 1 mL slip tip	Becton Dickinson (BD)	San Jose, CA	309659

Item Name	Company	Location	Catalog Number
Syringe, 6 mL with 21gauge x 1½" needle	Monoject/Covidien	Mansfield, MA	8881516150
Tris Hydroxymethyl Aminomethane Hydrochloride	Fisher Scientific	Waltham, MA	BP153-500
Ultrasound, t3000 with 7-12 MHz linear probe	Terason Ultrasound	Burlington, MA	t3000
Water bath, Isotemp 205	Fisher Scientific	Waltham, MA	15-462-5Q

Appendix B - Additional Data Tables

Table B.1 Raw Data of Epididymal Sperm Collection

Strain	Medium	Age (days)	Concentration (million/mL)	Total Sperm Count (million)	Sperm Motility (est)
SD	Nakatsukasa	60	1.5	4.5	90%
SD	Nakatsukasa	85	39.5	118.5	95%
SD	Nakatsukasa	87	35.5	106.5	90%
SD	Nakatsukasa	115	68.5	205.5	95%
SD	Nakatsukasa	127	43.5	130.5	60%
SD	Nakatsukasa	155	59	177	95%
SD	Nakatsukasa	161	30	45	90%
SD	Nakatsukasa	84	11.5	34.5	95%
SD	Nakatsukasa	91	198	594	95%
SD	Nakatsukasa	97	33.5	100.5	95%
SD	Nakatsukasa	104	81.5	244.5	90%
SD	Nakatsukasa	118	23	69	90%
SD	Nakatsukasa	124	34	102	95%
SD	Nakatsukasa	103	39	234	90%
SD	Nakatsukasa	92	41.5	249	90%
SD	Nakatsukasa	93	27.5	165	90%
SD	Nakatsukasa	79	22.5	135	90%
SD	Nakatsukasa	80	54.5	327	90%
SD	Yamashiro without dbcAMP	101	77.5	77.5	95%
SD	Yamashiro without dbcAMP	106	95.5	95.5	80%
SD	Yamashiro without dbcAMP	110	121.5	121.5	80%
SD	Yamashiro without dbcAMP	120	28	56	80%
SD	Yamashiro without dbcAMP	90	46	69	95%
SD	Yamashiro without dbcAMP	118	15	45	85%
SD	Yamashiro without dbcAMP	124	5.5	40.5	90%
SD	Yamashiro with dbcAMP	116	63	63	90%

Strain	Medium	Age (days)	Concentration (million/mL)	Total Sperm Count (million)	Sperm Motility (est)
SD	Yamashiro with dbcAMP	120	28	56	40%
SD	Yamashiro with dbcAMP	148	100	100	90%
SD	Yamashiro with dbcAMP	89	44	66	95%
SD	Yamashiro with dbcAMP	124	5.5	15.4	80%
SD	Yamashiro with dbcAMP	125	275	825	95%
F344	Nakatsukasa	275	60.5	181.5	75%
F344	Nakatsukasa	286	12	36	95%
F344	Nakatsukasa	292	31.5	94.5	95%
F344	Yamashiro without dbcAMP	286	12	24	95%
B-gal	Nakatsukasa	313	39.5	118.5	90%
B-gal	Nakatsukasa	295	38.5	155.5	90%
B-gal	Yamashiro without dbcAMP	359	27	40.5	90%
Dark agouti	Yamashiro without dbcAMP	247	98	98	95%
Transposagen transgenic	Nakatsukasa	144	144	432	95%
Transposagen transgenic	Nakatsukasa	144	54	162	95%

Table B.2 Intrauterine insemination utilizing fresh sperm samples

Strain	Medium	Age of Female (days)	Age of Male (days)	Sperm Concentration (million/mL)	Sperm Motility (est.)	Est. Number of Motile Sperm Transferred (each side)	Ultrasound Confirmation	Pups Born	Pups Weaned
SD	Nakatsukasa	58	60	1.5	90%	67,500	Negative	0	0
SD	Nakatsukasa	85	85	39.5	95%	1.87 million	At least one pup seen	2	1♀
SD	Nakatsukasa	89	87	35.5	90%	1.60 million	At least one pup seen	4	3♂, 1♀
SD	Nakatsukasa	115	115	68.5	95%	3.25 million	At least 5 pups seen	9	7♂, 2♀
SD	Yamashiro without dbcAMP	101	101	77.5	95%	3.68 million	At least 3 pups seen	6	4♂, 2♀
SD	Yamashiro without dbcAMP	106	106	95.5	80%	3.82 million	Negative	0	0
SD	Yamashiro without dbcAMP	110	110	121.5	80%	4.86 million	At least one pup seen	8	1♂, 7♀
SD	Yamashiro without dbcAMP	122	120	28	80%	1.12 million	At least 6 pups seen	7	6♀
SD	Yamashiro with dbcAMP	72	116	63	90%	2.83 million	At least one pup seen	1	1♀
SD	Yamashiro with dbcAMP	122	120	28	80%	1.12 million	At least 2 pups seen	3	1♂, 2♀
SD	Yamashiro with dbcAMP	78	120	28	40%	560,000	At least 2 pups seen	0	0
SD	Yamashiro with dbcAMP	148	148	100	90%	4.5 million	Negative	0	0
SD	Yamashiro with dbcAMP	127	89	44	Est. 90%, actual 89.45%	1.97 million	Negative	0	0
SD	Yamashiro with dbcAMP	163	125	275	90%	12.4 million	At least 3 pups seen	6	2♂, 4♀
Bgal	Nakatsukasa	227	283	39.5	90%	1.78 million	Negative	0	0
Bgal	Nakatsukasa	295	295	38.5	90%	1.73 million	At least one pup seen	2	1♂, 1♀
Bgal	Yamashiro without dbcAMP	313	359*	27	90%	1.22 million	Negative	0	0
F344	Nakatsukasa	275	275	60.5	75%	2.27 million	Negative	0	0
F344	Nakatsukasa	292	292	31.5	95%	1.50 million	Negative	0	0

Rat strains: SD- Sprague Dawely, F344- Fischer 344, Bgal- β-galactosidase transgenic. *An abscess was found associated with the right epididymis, sperm was collected from only the left epididymis. Estimated number of motile sperm transferred in 50 µL of solution in each uterine horn.

Table B.3 Example of vaginal lavage record.

Date	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Rat 8	Rat 9	Rat 10
22-Apr-12	CN	C	WNC	CN	C	CN	CN	WNC	WNC	WN
23-Apr-12	WNC	CN	WNC	WN	WNC	CN	CN	WNC	WNC	WNC
24-Apr-12	NC	C	WN	WNC	WNC	NC	WNC	WNC	NC	WNC
25-Apr-12	WNC	NCW	NC	NC*	WN	WN	WN	C	C	C
26-Apr-12	WNC	WNC	C	C	NC	WN	WN	NWC	WN	WNC
27-Apr-12	C	C	WNC	WNC	NC	NC	NC	WNC	WN	WNC
28-Apr-12	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
29-Apr-12	CN	NC	WNC	WNC	WNC	WN	WNC	NC	CN	NC
30-Apr-12	WN	WNC	NC*	NC	NC	WN	WN	C	WNC	CN
01-May-12	WNC	WNC	NC	NC	NC	WNC	NC	WNC	WNC	WN
02-May-12	NC*	NC	WN	WNC	WNC	CN	CN	WNC	CN	WNC
03-May-12	C	C	WNC	CNW	CW	WNC	WNC	NC	CN	NC
04-May-12	WN	CN	WN	CN	WN	WNC	WNC	C	WN	CN
05-May-12	WNC	WN	WNC	C	NC	NC	NC	WNC	WNC	WNC
06-May-12	WNC	WNC	NC	WN	WN	CN	C	WN	WN	WNC
07-May-12	NC*	NC	NC	WNC	WN	WN	WN	NC*	NC	NC
08-May-12	C	CN	WN	WN	WNC	WNC	WNC	C	WNC	CN
09-May-12	WN	WN	WN	NC*	NC	NC*	NC*	WN	WNC	WN
10-May-12	WNC	WNC	NC	CN	WNC	CN	CW	WNC	NC	WNC
11-May-12	WN	WN	CN	WN	WNC	WNC	NCW	NC	CN	NC
12-May-12	NW	NC	WNC	WNC	NC	WNC	WNC	CW	WNC	CN
13-May-12	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
14-May-12	NC*	WN	WN	WNC	WNC	CN*	WN	WN	NC*	WNC
15-May-12	WN	WN	C	NC*	WN	WNC	WN	NC*	CN	NC*
16-May-12	WN	NC*	CN	WNC	NC*	WNC*	WN	C	WNC	CN
17-May-12	NC	C	WNC	WNC	NCW	NC	W	WNC	WNC	WNC
18-May-12	C	NCW	WN	WNC	WNC	CN	WNC	WNC	WNC	WN
19-May-12	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
20-May-12	WN	WNC	CN	NC	NC	WNC	WN	C	NCW	NCW

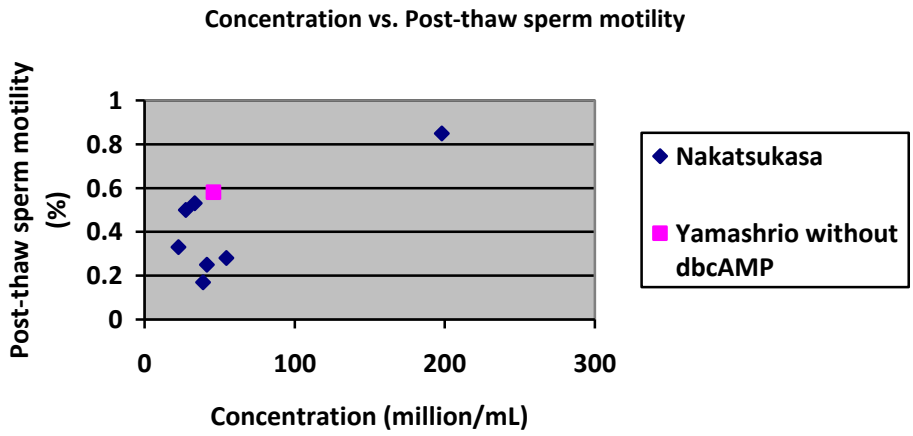
Cell types: C- cornified, W- white blood cell, N-nucleated. Grey "xx" indicates no lavage was performed on that day. Red font with "*" indicates pairing with vasectomized male. Cells are color coded according to stage of estrous cycle- darkest color indicates estrus, lighter colors indicate metestrus and diestrus.

Table B.4 Comparison of concentration and post-thaw sperm motility.

Strain	Medium	Concentration (million/mL)	Sperm motility at collection (calculated)	Post-thaw sperm motility
SD	Nakatsukasa	198	96.46%	0.85%
SD	Nakatsukasa	33.5	96.16%	0.53%
SD	Yamashiro without dbcAMP	46	91.73%	0.58%
SD	Nakatsukasa	39	87.34%	0.17%
SD	Nakatsukasa	41.5	88.48%	0.25%
SD	Nakatsukasa	27.5	89.17%	0.50%
SD	Nakatsukasa	22.5	89.43%	0.33%
SD	Nakatsukasa	54.5	88.38%	0.28%

Rat Strain: SD- Sprague Dawley. Data from liquid nitrogen vapor phase experiment taken from 6cm sample due to having data points for all groups.

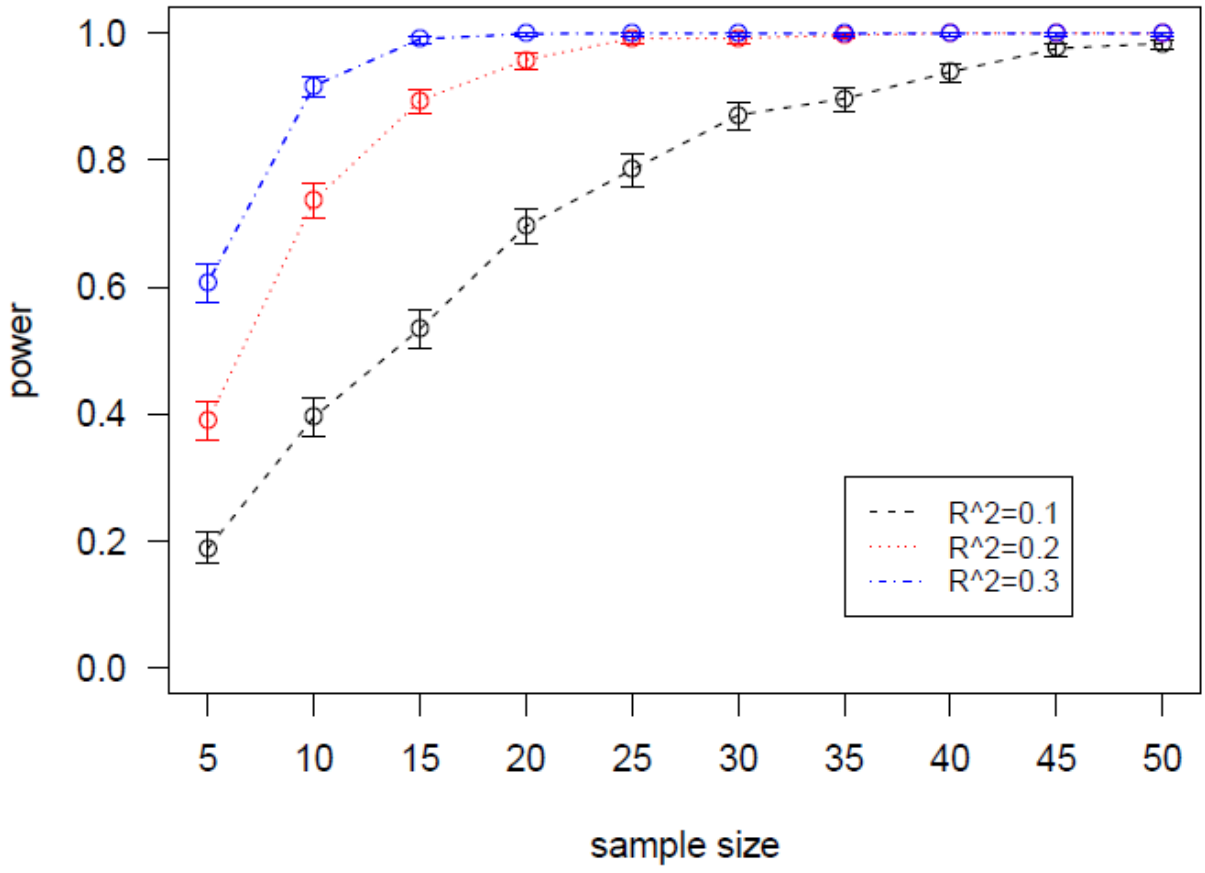
Figure B.1 Scatter plot comparison of concentration and post-thaw sperm motility.



No correlation found between concentration and post-thaw sperm motility (r=0.2619, p=0.54).

Appendix C - Sample size calculation

Figure C.1 Simulated rank ANOVA power curve for determining sample size.



Statistical simulation run and provided by Sasha Moola.