SPERMATOGONIAL STEM CELL TRANSFER TO A MULE

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A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Science in Horse Science
Equine Physiology

Middle Tennessee State University
December 2015

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ABSTRACT

Spermatogonial stem cell transfer has been studied in various species and suggested potential for success. Bull, stallion, donkey, mule, boar, dog and cat testicles were examined using various injection techniques to determine distribution patterns of injected material most favorable for stem cell transfer. Spermatogonial stem cells were recovered from freshly harvested stallion testicles using enzymatic digestion and filtration and placed in suspension for transplantation. An 18 mo old sexually intact male mule was anesthetized and the stem cell suspension was injected into both testicles in the area below the head of the epididymis. Semen collected from the mule four months post-transplantation contained no spermatozoa. The stem cell transfer was unsuccessful in this case, however further research is needed to determine recipient age of injection to optimize donor cell development.

Keywords.

Spermatogonial stem cell transfer, germ cell transfer, spermatogenesis, mules

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CHAPTER 1. LITERATURE REVIEW

Introduction

Stallion fertility has been an ongoing focus in the equine industry and researched extensively since it is the source of improving a breed. It is effected by a variety of factors such as age (Stewart et al., 1998), nutrition, soundness (Love, 2011), and genetics (Brito et al., 2008). Fertility is a key aspect in the horse industry as it can improve a producer's profits through progressing the quality of a breed (Sieme and Distl, 2012). It is extremely important to understand the formation of sperm. Any attempt to improve fertility relies heavily on this knowledge and because any stage of sperm cell development can affect the level of fertility that stallion may have, it is important that the entire process is understood. A male's capacity to reproduce also increases the value of an individual animal since it could impact the producer's profitability. New technologies are always being developed to improve various species and provide more opportunities to preserve male genetics in order to continue using them beyond the life of the individual animal. These technologies include artificial insemination, cryopreservation, and cloning. Spermatogonial stem cell transfer is a technology that is currently undergoing thorough investigation in order to achieve even greater improvements to preserve male genetics. Research in pigs, dogs, rats, mice, and cattle has suggested potential for development of primary sperm cells post-transfer (Oatley et al., 2004). Mules have been found to be close in testis morphology and functionality when compared to horses and donkeys (Neves et al., 2005). These similarities indicate that mules may be successful recipients for transplantation of spermatogonial stem cells of a horse or donkey and complete

development of spermatogenesis. Mules lack endogenous germ cells, therefore the recipient testicles would provide the donor cells sufficient space that could maximize spermatogonial stem cell development.

Spermatogonial stem cells and spermatogenesis

The development of spermatogonia, the male germ stem cells, determines the fertility and functionality of the testes. These spermatogonia are found on the basement membrane of the seminiferous tubules between Sertoli cells. The Sertoli cells produce glial cell-derived neurotrophic factor, which is responsible for the spermatogonial stem cell renewal (Olive and Cuzin, 2004). The key role of spermatogonia in spermatogenesis is producing self-renewing stem cells, which initiates the production of differentiated daughter cells to offer a constant supply of spermatozoa (Guan et al., 2009). These spermatogonial stem cells make up a small population of cells resulting from the gonadocytes of newborn testes. Spermatogonial stem cells are unique in that they are the only stem cells in adult mammals that directly provide genetic material to succeeding generations (Hill and Dobrinski, 2006). Spermatogenesis is essential to the continuation of all species. Due to its great importance, it is understood to be highly efficient and resistant to harm (Ogawa et al., 1997). Spermatogenesis is an intricate process of cell development and has been extensively investigated to allow researchers to perform genetic modifications. As a result of this research, spermatogonial stem cells can be studied at nearly any stage of development. Although the time for complete sperm

development and morphology of the sperm varies between species, the process is similar across species lines.

Spermatogonial stem cells have three different types: A-spermatogonia (A1 to A4), I-spermatogonia, and B-spermatogonia (Phillips et al., 2010). A-spermatogonia are the cells responsible for providing continual source of primary spermatogonial stem cells for spermatogenesis to continue without disruption for many years. The functions of spermatogenesis are as follows: providing a male with continuous supply of male gametes, supplying genetic diversity, produce billions of sperm every day to optimize reproduction, and providing an immunologically privileged site where germ cells may thrive and self-renew. Spermatogenesis occurs in the seminiferous tubules in the testis and is separated into three stages. The first phase is known as the proliferative or mitotic phase. In this phase, spermatogonia go through mitotic divisions to develop into spermatocytes (spermatocytogenesis). This first stage results in two new cells. The second stage of development is the meiotic phase, where the developed spermatocytes undergo two divisions. The first meiotic division creates two secondary spermatocytes, which are each divided again to produce two haploid spermatids. These divisions create four secondary spermatocytes from each individual spermatocyte. Cross over and DNA replication during the meiotic stage guarantees genetic diversity. The third stage is known as spermiogenesis, or the development of mature sperm cells. There are no cell divisions occurring during this phase. The round spermatids differentiate into spermatozoa with a fully developed acrosome, head, midpiece, and tail (Chocu et al., 2012). Sperm motility requires development of flagellum, which is also developed in this final stage of

spermatogenesis. Spermiation will occur after the final development, which is the release of spermatozoa from the Sertoli cells into the lumen of seminiferous tubules. Each of these phases develops along a variety of timelines according to species, depending on sperm morphology and size (Campos-Junior et al., 2013). Mammals have a flattened and oval nucleus with a compacted and highly keratinized chromatin. While the process of spermatogenesis has been extensively investigated, the molecular mechanisms which are the basis of these cells' maintenance and diverse functions are not well known. Many new technologies utilizing spermatogonia are being developed and these primary stem cells have potential to be used in many techniques to preserve genetics in rodents, livestock, and humans.

Spermatogonial stem cell transplantation

Spermatogonial stem cell transfer (SSCT) was first reported in the mouse (Brinster and Zimmerman, 1994). The technology of SSCT permits further investigation of spermatogenesis, development of transgenic animals, and provide an alternative for preservation of male genetics (Joerg et al., 2003). Artificial insemination is not always an option for long-term preservation of genetics and may not be successful in every individual animal. The utilization of SSCT to advance reproduction would provide more opportunities to improve fertility in potentially every mammalian species than cryopreservation or cloning alone. Cryopreservation may be harmful to cells over long periods of time leading to decreased fertility and cloning may cause subfertility or infertility in the cloned animals. However, SSCT may be used in combination with

cloning, cryopreservation, and artificial insemination (Schlatt, 2002). Artificial insemination could still be used as a form of breeding with the SSCT recipient male, although SSCT allows the absence of the donor for semen collection for artificial insemination. This is similar to a natural service breeding but rather the recipient would execute the breeding rather than the donor. This is important for those donor animals that will be unable to breed after the spermatogonial stem cells are recovered and transplanted into a recipient. The use of a SSCT recipient as a substitute breeder is another advantage provided by SSCT to preserve an individual's fertility. However, optimizing success of stem cell transplantation requires many steps. A close phylogenetic relationship between the donor and recipient individuals would be advantageous to a successful SSCT, as well as the recipient having depleted endogenous germ cells (Honaramooz et al., 2002). This would allow the donor cells increased access to the basal compartment of the seminiferous tubules, providing more room for colonization and result in higher success of developed donor-derived sperm. Successful SSCT opens up the opportunity for the preservation of male fertility and an alternative to the development of transgenic animals (Honaramooz and Yang, 2011). Germ cell transplantation provides opportunities for every species to preserve male genetics and continue to improve production and breed quality. The genetics of unique or valuable livestock that may unexpectedly need to be castrated or die early would benefit from this technology since their genetics could potentially continue to be passed on through the recipient animal after the donor can no longer reproduce (Honaramooz and Yang, 2011). The process for spermatogonial stem

cell transfer consists of three main parts: preparation of the recipient, injection of the donor cells, and analysis of the sperm produced by the recipient.

In 1997, Ogawa et al. studied spermatogonial stem cell transplantation in mice. In a mouse testis each seminiferous tubule enters the rete testis, from which emerge 3 to 6 collecting ducts which carry spermatozoa into the head of the epididymis, where the final stages of sperm maturation takes place. The recipient mice were chemically sterilized using Busulfan to eradicate spermatogenesis so any developed sperm after transplantation could be easily identified as the donor cells. Different methods of injecting the cells were compared for transplantation including seminiferous tubule injection, efferent duct injection, and rete injection. There was no difference in the degree of spermatogenesis between these methods of injection. Colonization of the seminiferous tubules occurred in all three methods performed, thus leading to spermatogenesis. Since no method demonstrated significant increases in the degree of spermatogenesis, the suggested method would be to inject the cell suspension directly into the rete testis since it is less invasive. It has been acknowledged that, although this study was successful in mice, it may be much more difficult to perform in larger species (Ogawa et al., 1997). This is because larger animals require higher doses of anesthetics, larger equipment, and donate more tissue than a smaller, rodent animal does, which requires more solutions and suspension. The larger volume-to-surface ratio and increased convolution makes injections into the seminiferous tubules very complicated (Honaramooz et al., 2002).

Germ cell transplantation from related boars, unrelated boars, and mice spermatogonia to immunologically tolerant immature pigs was studied at the University

of Pennsylvania (Honaramooz et al., 2002). The recipient pig testes were examined four weeks after transplantation and showed evidence of the related and unrelated groups' donor cells in up to 50% of the seminiferous tubules. The results indicated a very large increase from the 10-40% efficiency of spermatogonial stem cells, compared to the results found in the 1997 study utilizing mice as the subjects. Efficiency was also proven to depend upon the ratio of donor to recipient sperm cells present in the seminiferous tubules of the subject (Honaramooz et al., 2002). The researchers also determined that donors and recipients do not need to be related and can be immunologically tolerant for spermatogenesis to occur after transplantation of spermatogonial stem cells (Honaramooz et al., 2002). While the recipients of the mouse spermatogonia remained infertile, the findings from the examination completed four weeks post-transplantation determined some of the cells remained in the recipient testicles for further development. Thus, donors and recipients do not need to be genetically identical or immunosupressed for successful transplantation. Researchers acknowledged that this was the first successful germ cell transplantation in livestock animals (Honaramooz et al., 2002). The researchers suggested that future studies focus on examining the possibility of complete spermatogenesis in donor and recipient models, whether it is a boar or another mammal. This research initiated further investigation of this technology within various livestock species across the world.

At the University of Zurich in 2003, azoospermic Klinefelter bulls were used in a germ cell transplantation study, with nonmosaic Klinefelter bulls used as the recipients (Joerg et al., 2003). Non-mosaic bulls were used because they have an extra chromosome

and does not have endogenous spermatogenesis, allowing more space for development of the donor spermatogonial stem cells. There was approximately 30.3% donor cells present in a biopsy sample taken four weeks post-transplantation. Donor cells were detected in ejaculate samples up to four months post-transplantation. Izadyar et al (2003) drew similar conclusions using Holstein-Friesian calves. Donor-derived cell development and spermatogenesis was found, but researchers determined this was due to the lack of optimization of the recipient environment. These researchers established that spermatogonial stem cell transplantation requires three important factors: an increased number of donor cells in the suspension for injection; the recipient testes must have as few endogenous spermatogenic cells as possible; and an efficient transplantation procedure so as to ensure correct injection of the suspension for development (Izadyar et al., 2003). The differentiation of the donor-derived and the recipient's spermatogonia is essential to determine the degree of success of transplantation.

In a similar study, Herrid et al. (2006) used *Bos taurus* as donor males and *Bos indicus* as the recipients. The calves were castrated 2-30 weeks after the transplant, which was considered to be sufficient time to suggest that spermatogenesis may have been able to occur. They found 50% of the recipients still contained at least one of the two dyes they used for tracking the transplant (Herrid et al., 2006). The researchers of this study suggest more research to be completed on larger animals, rather than only in mice or other rodents. They also believe the next studies should allow sufficient time for spermatogenesis to complete, if possible (Herrid et al., 2006). However, this research was successful in indicating that spermatogenesis of donor cells in a recipient may, in fact,

have potential for development, even in a different subspecies of animal. These results demonstrate that it may be possible to transplant spermatogonial cells between animals of the same genus, and that they also may be of different species or subspecies. This study did not examine maturity of the donor cells, yet it was assumed that the donor cells that colonized would develop over a longer period of time than allowed for this project (Herrid et al., 2006). The presence of the donor-derived germ cells in the recipient testicles indicated that this procedure was possible between different species and the individuals do not need to be genetically identical (Herrid et al., 2006). Comparable results to the Izadyar et al. (2003) study indicated that recipient animals should have depleted endogenous spermatogenic cells to improve spermatogenesis of the donor-derived cells (Herrid et al., 2006). However, a study performed by Hannes et al. (2003) concluded the rejection of these donor cells was rather quick and was not due to immunological rejection, but simply that the cells could not develop after being transplanted (Hannes et al, 2003). Although further research studies have suggested potential for development of donor cells in immunologically tolerant recipients.

Spermatogonial stem cell transplantation research at Cornell University succeeded in finding almost 20% donor-derived sperm in recipient dogs (Kim et al., 2008). Full spermatogenesis was documented in one individual animal with development of mature sperm in the epididymis. After comparing various methods to estimate the amount of donor-derived developed sperm through marking donor cells or recipient cells, this study concluded that the percentages of donor-derived sperm ranged from 10-35% (Kim et al., 2009). These researchers were also the first in successfully finding SSCT can be

performed in dogs without any modifications on the recipient's immune system (Kim et al., 2009). This evidence leads to potential SSCT in other species when it is preferred to have no immunological modifications in the recipient male, such as humans.

Potential spermatogonial stem cell transplantation in human infertility

Stem cells of mice and humans appear to have similar cellular and molecular characteristics (Conrad et al., 2008). Human stem cells differentiate into various somatic cells of all three germ layers when grown under the proper environment. This differentiation suggests that human stem cells have the appropriate properties to allow for stem cell retrieval for growth and transplantation, similar to the properties of embryonic stem cells, in order to preserve genetics of an individual. (Conrad et al., 2008).

The infertility rate in men following chemotherapy or overexposure to gonadotoxins is high (Vlajkovic et al., 2012). The current technology to preserve the genetics of adolescent boys preparing to undergo anticancer therapy is to bank three semen samples with 48 hours between each collection (Vlajkovic et al., 2012). These patients have the option of cryopreservation of their semen prior to starting any cancer treatments so as to preserve their capability to produce offspring following a cure. This technique requires all samples to be free of any malignant cells, otherwise the healthy cells will become subfertile or unusable (Vlajkovic et al., 2012). An SSCT would provide a safer method with a higher success rate for preservation of genetics for these patients.

A study examining the potential for development of human stem cells in mouse testes used men with obstructive azoospermia, or poor development of spermatogenesis

(Nagano et al., 2002). One month after transplantation of the six donor spermatogonia into mouse testes, human germ cells were observed to be present on the basement membrane of the tubule of the mouse. It was concluded that proliferation of spermatogonia only occurred for one month after transplantation of cells, yet undifferentiated spermatogonia may be observed for up to six months in the mouse testes (Nagano et al., 2002). The results of this study support the theory that human germ cells may be transferred to a recipient of a different species.

Testis anatomy

Spermatogonial stem cell transplantation varies between species based on anatomical features of the testicles of the focus animal, time for spermatogenesis to occur, as well as the morphological and functional features of the sperm and testicles. Girish et al. (2001) examined the anatomy of dog testicles and determined the mediastinum testis (rete testis) that contained tubular and intertubular parts. These tubular components were fibrous and consisted of a few smooth muscle cells (Girish et al., 2001). A similar study examined the anatomy of the rete testis in the feline. Researchers established there are three main parts to a cat rete testis: the interlobular part, the mediastinal part, and the tunical part. Each part was composed of tubular structures or channels for transit of spermatogonia (Viotto et al., 1991).Hemeida et al. (1978) examined testicular anatomy in the boar, goat, ram, bull, and stallion. The researchers primarily investigated the presence of ductuli efferentes and blind-ending ductules in each different species. The results of the study indicated that there were ample numbers

of blind-ending ductules in the stallion testicles examined, and therefore may have a direct effect on spermiostasis and potentially infertility (Hemeida et al., 1978).

Effects on fertility

There are a variety of factors that can affect the fertility of a stallion. Pregnancy rates alone are not enough to prove the level of fertility, especially when only considering the number of pregnant mares a stallion successfully breeds per season. Pregnancy rates do not account for the number of times each mare was bred before being impregnated, therefore it is important to examine both overall pregnancy rates and those mares that became pregnant on the first cycle (Love, 2011). The level of motility, as well as the velocity of the sperm, and even the morphology of each individual sperm, is important to determine a male's reproductive capability (Campos-Junior et al., 2013). Stallions with a greater percent of sperm motility, a higher mean progressive sperm velocity, and lower percent of coiled sperm tails had a higher number of pregnant mares per season (Pesch et al., 2005). This number of impregnanted mares did not take into account whether or not these pregnant mares were first cycle pregnancies or not. There is also evidence that other morphological abnormalities in the sperm lead to lower fertility, including detached or abnormal sperm heads, deformed sperm bodies, and higher percentage of proximal droplets (Love, 2011). Research has indicated that increased quality of semen that is used for insemination achieves a higher pregnancy rate than poor quality semen. Therefore it is crucial that semen, which will be used for artificial insemination following

cryopreservation, be processed correctly to ensure the highest potential fertility (Heckenbichler et al., 2011).

A well known example of sub-fertility that poses an issue in the horse industry is the lower rate of fertility caused when using frozen or cooled semen for artificial insemination. Sub-fertility of frozen or cooled semen is commonly due to changes in temperature, poor preparation of the semen, or human error in the lab (Monteiro et al., 2011). Seminal plasma stabilizes mature sperm, yet is very sensitive to cryopreservation, so it is important to understand the specific temperature at which to keep the semen and how long it may be kept frozen before insemination (Monteiro et al., 2011). For example, semen which is not slowly cooled and then kept at the specific temperature of 4 to 6º C will become sub-fertile, if not infertile (Heckenbichler et al., 2011). This form of sub-fertility may occur with improper storage material or if the straws are removed from the freezing or cooling equipment. Research is being done to attempt to resolve these issues and make this procedure more successful and effective. For example, Barrier-Battut et al. (2013) completed a study to examine the possibility of removing the seminal plasma to improve the stability of the membrane, which is predicted to be the main problem in poor fertility of cooled semen (Barrier-Battut et al., 2013). Although this study was not successful in proving that removing seminal plasma would increase fertility of cooled or frozen semen, it did find the importance of seminal plasma as a stabilizer of the sperm (Barrier-Battut et al., 2013). Additionally, not all stallion semen is viable when frozen or cooled. Therefore, some individuals' semen may have a lower fertility rate after freezing or cooling (Heckenbichler et al., 2011). Various studies suggest that "germ cell

transplantation has an advantage over the only currently available approach [to male genetic preservation], cryopreservation of sperm" (Hill and Dobrinski, 2006). For instance, the genetics of prepubescent males may also have potential for preservation with SSCT through isolation of the primary spermatogonial stem cells from the prepubescent donor (Schlatt, 2002b). From human error in freezing temperature, to the necessary transportation of the semen, cryopreservation alone is not necessarily the most consistent or successful way to preserve stallion genetics. There are many opportunities for error throughout the freezing process of semen and there is also the potential for limiting the preservation time for each semen sample.

An influential factor on stallion fertility is age (Stewart and Roser, 1998). Sperm motility or semen quality and thus, fertility, is directly affected by aging. Idiopathic testicular degeneration is an example of sub-fertility in stallions that occurs due to age. This condition worsens as the horse gets older and causes changes in the size and the uniformity of the testicles, as well as diminished sperm production and general quality of the semen (Brito et al., 2010). Age also affects the change in immunoreactive inhibin concentrations in the plasma and within the testicle, which has been directly correlated with fertility rates in stallions (Stewart and Roser, 1998). It has been theorized that as the horse ages the concentrations of both intratesticular and plasma immunoreactive inhibin decrease, causing a decline in fertility (Stewart and Roser, 1998). The intratesticular immunoreactive inhibin levels were decreased in subfertile stallions, and this decline may be used for an indicator of diminishing fertility in individual males. The paracrine role of inhibin should be further researched to determine its effect on testosterone levels and

Leydig cells to verify the cause of the decrease in fertility (Stewart and Roser, 1998). Although if plasma follicle stimulating hormone (FSH) levels are too high, fertility may also be diminished due to improper interactions with affected hormones, such as inhibin and testosterone (Stewart and Roser, 1998). An increase in inhibin is related to pituitary function, but may also be due to testicular dysfunction. Both levels of inhibin and plasma FSH play crucial roles in stallion fertility and may fluctuate with various external factors (Roser, 1997). This study also provides evidence that spermatogonial stem cells retrieved for donors should be from recipients of younger, or of prepubescent age, to avoid diminished sperm quality or fertility which may come with age.

Improving fertility

Preserving and improving stallion fertility is an important focal point for breeders in the horse industry. Loss of fertility in stallions may be due to aging, or unavoidable castration or euthanasia for a variety of reasons. Improving stallion fertility is a main focus of equine reproductive research since it is central to progressing breeds and advancing equine genetics.

Cryopreservation and the fertility of epididymal sperm of stallions were analyzed to determine viability (Monteiro et al., 2011). It was discovered that sperm retrieved from the epididymal cauda immediately after castration or after no more than 24 hours of refrigeration have the same fertility rate as sperm that has been ejaculated (Monteiro et al., 2011). The key for the sperm storage function in the epididymal cauda is the accrual of spermatozoa to promote ejaculation, as well as increasing the sperm count in each

ejaculation (Monteiro et al., 2011). The epididymal fluids quiet the sperm cells while they are stored in the cauda. At ejaculation, the seminal plasma, or a semen extender, dilutes the epididymal fluid and initiates sperm motility (Rota et al., 2004). The process of direct retrieval of sperm from the testicle, which permits removal of sperm directly from the epididymal cauda, allows for an easier, more controlled retrieval of semen samples that increases a producer's ability to preserve a stallion's genetics. Nevertheless, this direct recovery of sperm cells will not increase a stallion's ability to reproduce if the quality of the stallion's sperm is already low.

Although many of the issues in cryopreservation are caused by human error in transportation or not keeping the semen at a cool enough temperature (Neild et al., 2002), it is more difficult to increase fertility in a stallion that already has poor quality semen. Blanchard et al. (2012) examined the effectiveness of lower dosages of semen used for artificial insemination through concentrating the semen samples collected from stallions. This study aimed to increase pregnancy rates of stallions with lower quality semen and therefore reduced fertility (Blanchard et al., 2012). An aged stallion that had diluted semen and poor sperm morphology with proximal droplets was studied by Blanchard et al. (2012). They examined the different possibilities of semen processing before insemination to maximize fertility with extreme sub-fertile stallions, but found no differences between treatments (Blanchard et al., 2012). However this study did not determine their methods of concentrating semen samples prior to insemination improved fertility of the stallions. The researchers used the entire ejaculate of each subject with no set semen volume and did not measure the sperm functionality other than motility and

morphology, which may have contributed to the failure to improve fertility. Researchers of this study concluded that further research should consider standardizing the semen volume used and examining the sperm functionality in its entirety to determine any slight improvement after concentrating semen samples (Blanchard et al., 2012). The results indicate that very few steps can be taken in older, less fertile stallions in order to increase their reproductive ability. The researchers concluded that age and low quality semen cannot be overcome through modifications meant to increase a stallion's fertility (Blanchard et al., 2012). Although cryopreservation may be rather successful in younger, fertile stallions (Monteiro et al., 2011), the horse industry would benefit from a longer term, more effective method for preserving stallion genetics after they have exceeded their sexual high point in their reproductive life.

Spermatogonial cell transplantation would allow stallions that are to be castrated, euthanized, or simply physically incapable of reproducing, to continue to pass on their genes via a surrogate breeder. Typically these procedures utilized a two-step enzymatic digestion of the cells and tissues from the donor animal, and cooled the cells for storage before being transplanted into the recipient animal. Studies on mice were some of the first done with germ (spermatogonial) cell transplantation, and the results of a 1997 study found 10-40% efficiency within the subjects used (Ogawa et al., 1997). The most successful results found were to have round spermatids and some following stages of spermatogenesis still stained with the blue dye used to track the transplant. This study simply examined the possibility of transplanting spermatogonial cells, not necessarily the spermatogenesis following the transplant (Ogawa et al., 1997).

Functionality of the mule testis

The mule is a member of the *Equus* genus and is a hybrid of a horse and a donkey - both of the *Equidae* family and the *Equus* genus. Horses, *Equus caballus*, and donkeys, *Equus asinus*, differ in the number of acrocentric autosomal chromosomes (Neves et al., 2005). Stallions, donkeys, and mules present nearly identical features in male reproductive functions such as Leydig cell and Sertoli cell function, as well as testicular anatomy. In stallions, to bring one sperm cell to full maturity takes approximately an average of 57 days (Johnson et al., 1997). Breeding these two animals leads to sterility since spermatogenesis in mules, *Equus mulus*, infrequently develop past spermatocytes. However, mule testes are functional regarding the presence of somatic cells (Neves et al., 2005). Therefore it may be possible to transplant spermatogonial cells from a stallion to a recipient mule, since sperm morphology, sperm development, and testis function are the same. The study performed by Neves et al. (2005) indicated the foundation to the sterility of mules is not due to somatic elements. The amount of Sertoli cells and Leydig cells from the subject mules were found to be very similar to that of a fertile donkey, indicating a mule's seminiferous tubules may be able to sustain complete spermatogenesis (Neves et al., 2005). The researchers concluded that the pairing failure of homologous chromosomes during meiosis in mules is the direct cause of their sterility. Mules have 63 chromosomes, compared with the horse's 64 and a donkey's 62, both which have complete pairings (Neves et al., 2005).

In the comparisons between mule and donkey testicular functionality, the results indicated that through transplantation of spermatogonial stem cells, which have

developed past this missing step in spermatogenesis, mule testes have the potential to sustain the later phases in sperm development. Morphological analysis indicated that Leydig cells in donkeys and mules were very similar, however the Sertoli cells were similar in ultrastructure yet slightly varied with a smaller nucleolus in mule testicles (Neves et al., 2005). The results indicated that 95% of the individual mules' seminiferous tubules contained spermatogonia and spermatocytes, proving that mule seminiferous tubules are capable of sustaining complete spermatogenesis after meiosis has occurred prior to transplantation of cells (Neves et al., 2005). Sertoli cells differentiate in the gonad and function as the organizational center, so the slight variation in mule Sertoli cells may affect the failure to pair in mule acrocentric autosomal chromosomes (Neves et al., 2002). These studies are the foundation to the theory that mules would serve as successful recipients for spermatogonial stem cell transplantation. A transplantation from horses to mules would simplify the identification of donor-derived cells because the recipient cells are non-existent or depleted.

CHAPTER II. SPERMATOGONIAL STEM CELL TRANSFER FROM A STALLION TO A MULE

Introduction

Spermatogonial stem cell transplantation has been studied in various species and the target for injection has been theorized to be the rete testis within the testicle. However, the equine testicle does not contain a discrete rete testis, or mediastinum, that we could cannulate. The lack of this anatomical feature make the use of SSCT extremely difficult in the equine. This study examined the possibility for successful injection through using castrated testicles from various species that contain a discrete rete testis. The goal of this part of the study was to determine the true target for injection of spermatogonial stem cells into a recipient testicle to optimize spermatogenesis. This study is important to improve the SSCT methodology between various species in order to advance technologies to preserve male genetics. Human genetics would also benefit from the results of this study because through determining the optimum target for SSCT injection, successful transplantation would lead to continuing offspring of that donor male.

Materials and Methods

All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Middle Tennessee State University (protocol 14-007) and the approval letter is included in the appendix.

In vitro testicular injection in various species

In order to determine the optimal injection technique, testicles from the bull, mule, donkey, foal, boar, dog and cat were examined by means of dissection and contrast radiography. The optimal injection site was identified as the area of the greatest dispersion of contrast material between species. Castrated bull testicles were injected with 10 mL of fluorescein into the rete testis of the testicle and cut open transversely with a scalpel to observe the dispersion of the fluorescent dye. Based on testis size and structure 270 mgI/mL iodixanol contrast was injected through a 20 gauge needle into castrated testicles of a bull (3 to 5 mL), mule (5 mL), donkey (1 to 4 mL), foal (5 mL), boar (1 mL), dog (1 mL), and cat (0.5 mL). The testicle was then radiographed longitudinally twice, with the second radiograph taken after rotating the testicle 90° for a lateral view. The dispersion of the iodixanol was then evaluated in the two planes.

Fluorescein solution (10 mL) was injected into a bull testicle using ultrasound imaging to direct the catheter into the rete teste. The sample was cut open and visually examined through comparing the fluorescent dye dispersed throughout the testicular tissue. More fluorescein, 3 mL, was further injected directly up into the head of the epididymis and visually examined again to determine if any more dispersion had occurred. A second bull testicle was cut transversely to expose the rete testis. A catheter was inserted into the rete testis and an ultrasound was used to examine diffusion as it was injected to observe the dispersion of the solution. A catheter was inserted into another bull testicle using ultrasound imaging. Iodixanol contrast (3 mL) was injected into the

testicle and a radiograph of the testicle was taken. Another 2 mL of contrast was injected and another radiograph was taken, as well as a radiograph of the testicle rotated 90°. The final bull testicle sample used had the vaginal tunic cut away in order to expose the epididymis. 1 mL of the contrast into the head of the epididymis. The testis was radiographed to examine the dispersion of contrast media. These radiographs were taken to determine the optimum target for injection through observing the dispersion of the contrast media.

Iodixanol contrast (2 mL) was injected into a donkey testicle using ultrasound imaging. Before and after injection radiographs were made of the testicle to observe the diffusion of the contrast. More iodixanol contrast (1 mL) was injected just below the head of the epididymis and redirected for another 1 mL to be injected and radiographs were made to examine the diffusion. A second donkey testicle was injected with 1 mL of iodixanol contrast into the head of the epididymis and a radiograph was taken. Another 3 mL of iodixanol contrast was added and another radiograph was taken to compare the diffusion. These methods were compared to determine the difference in diffusion between injection targets to optimize success of an injection when performing a SSCT.

Iodixanol contrast (5 mL) was injected into a foal testicle in the central vein and radiographs were taken before and after injection, and at a 90° rotation.

Iodixanol contrast (5 mL) was injected into a mule testicle using ultrasound imaging to direct the catheter into the rete teste and radiographed. Iodixanol contrast (5 mL) was injected in a second mule testicle in four different locations in the region below the epididymis. Radiographic images were collected to examine diffusion.

Iodixanol contrast (1 mL) was injected into a dog testicle using ultrasound imaging and the testicle was radiographed. Another 1 mL of the contrast was added and another radiograph was taken to examine the success of dispersion.

Iodixanol contrast (0.5 mL) were injected into two cat testicle samples using ultrasound imaging to guide the catheter. Radiographic images were collected before and after the injections to examine diffusion.

Iodixanol contrast (1 mL) was injected into two pig testicles using ultrasound guidance. Radiographs were made before and after the injections.

Preparation of the spermatogonial stem cells

A healthy 2 year old Tennessee Walking Horse stallion was anesthetized with 1.1 mg/kg xylazine followed by 2.2 mg/kg ketamine. Castration was performed by the Henderson method (Kilcoyne, 2013). No anesthetic was injected into the testes. The testicles were immediately put on ice for two hours pending preparation of the samples. The tunica albuginea was first removed from the testicles and the testicular tissue was minced with a scalpel. These pieces were further minced to maximize surface area. The seminiferous tubules were transferred to a conical tube which contained 5 mL of collagenase 4/DNAse solution and incubated at 37° C in the rolling hybridization oven for 15 minutes until the tubules resemble string. The digest was triturated and incubated for 90 minutes until the tubules separated and made a fluid liquid.

The seminiferous tubule fragments were dispersed by repeated flushing with a 5 mL pipette. The resulting fragments were collected in a conical centrifuge tube and

centrifuged at 200 xg for 5 minutes at 18 ^{o} C. The remaining tubule fragments were washed with Phosphate Buffered Saline (PBS) and centrifuged at 200 xg for 5 minutes at 18 ^{o} C. This was repeated two more times in order to acquire a pellet with increased spermatogonial stem cells with the large amount of tissue being centrifuged.

The seminiferous tubule fragments were incubated in 10 mL of dispase solution in conical tube at 37 ^{o} C for 20 minutes in the rotating hybridization oven. The remaining tubule cells were dispersed using a 5 mL pipette and 10 mL of fetal bovine serum was added to stop any further digestion.

After digestion, the mixture was passed through a 100 µm filter, followed by a 70 µm pore sized filter. The cells were pelletized via centrifugation at 450 xg for 5 minutes at 18 ^{o} C. The enzyme solution was decanted and the cells were washed three times with PBS. The cells were plated overnight on laminin-coated dishes to allow separation of the cells.

The next day, the recipient, an 18 mo old prepubertal mule, was placed under general anesthesia - using xylazine at 1.1 mg/kg followed by ketamine at 2.2 mg/kg i.v. and maintained with an infusion of 1 L of 5% guaifenesin, with 1000mg ketamine and 500 mg xylazine administered to effect. Then 15 mL of the spermatogonial stem cell suspension was injected into each of the testicles with ultrasound guidance into the area of the rete testis.

At 4 mo post SSCT, the mule reached puberty as evidenced by his sexual arousal when exposed to a mare in estrus. A semen collection was performed using a Missouri style artificial vagina, and the seminal fluid was examined microscopically before and

after concentration by centrifugation. The collection was repeated at 6 mo post SSCT and again at 7 mo.

Results

Castration sample injections

The examination of the testes injection methodology between different species exhibited the anatomical variation between equine and other animals. It was previously known that horse testicles vary in the rete testis is less discrete in proportion to the testicle, as well as having a web of many, small seminiferous tubules.

The fluorescein was seen throughout the bull testicles and into the head of the epididymis, as seen in figure 1. Directly injecting the suspension into the testicle just beneath the head of the epididymis after being incised indicated similar diffusion to injecting the suspension directly into the rete testis, as observed on the ultrasound (Figure 2). There was relatively good diffusion in the third testis as well. When 1 mL of the iodixanol was injected for the final test, the contrast did not disperse much of the solution through the testicle.

The next pair of testicles examined were those of a donkey. The level of dispersion was low. There was slightly more diffusion after another 1 mL was injected just below the head of the epididymis and the catheter was redirected to inject another 1 mL of contrast (Figure 3). There was no dispersion into the testicle in the second test, rather the contrast stayed in the head of the epididymis. There was no diffusion into the testicle when injected into the epididymis (Figure 4).

The foal testicle injection resulted in a web of contrast diffusion into the efferent ducts. The testicle was squeezed and exhibited further diffusion, as seen in figure 5. Further dispersion in the region of the epididymis was observed after dissection.

There was very good dispersion into the epididymis with both mule testicles. Dispersion into the epididymis was observed, as seen in figure 6.

One dog testicle had very good dispersal of the solution. An additional 1 mL of the contrast was injected and even better dispersal was observed in figure 7.

Two cat testicles were examined and had similarly good diffusion of solution throughout the testicle. Contrast was in the region of the epididymis on the X-ray images in figure 8.

The final testicles examined were pig samples. Both testicles had comparable dispersion as that of the cat and dog testicles. Contrast media is seen throughout the testicle extending into the epididymis in figure 9.

Spermatogonial stem cell transfer to a mule

The first collection sample was 40 mL of fluid, however the sample was very clear and primarily accessory fluid. No spermatozoa was seen anywhere in the sample observed under the microscope. A second collection was performed an hour later and the same procedure repeated. The same results were found when another 40 cc sample underwent examination and centrifugation.

The second collection day performed a month later resulted in approximately 40 mL more turbid fluid. Although no other changes were observed when a sample was

viewed on a slide on a microscope and spun down with a centrifuge. There was only accessory fluid and no evidence of any mature sperm.

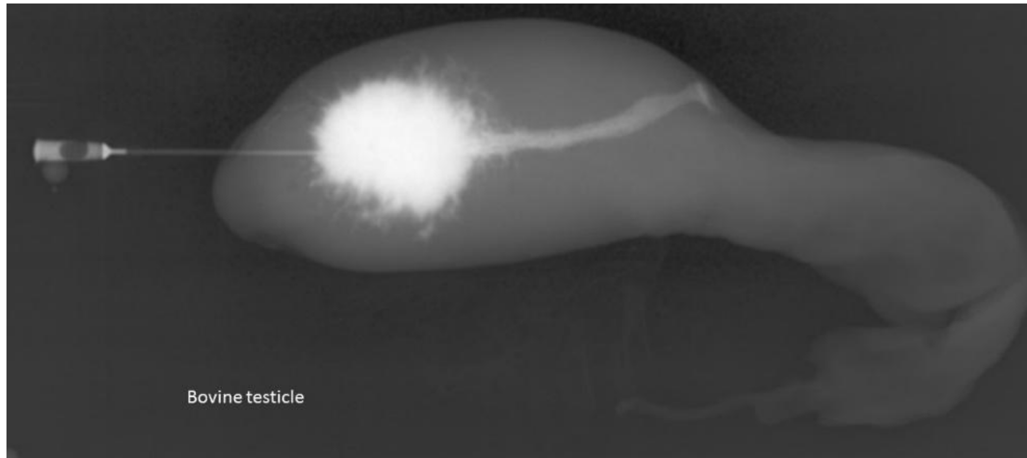

Figure 1. Bovine testicle injected with 3 mL of iodixanol contrast. Mediastinum testis is visible.

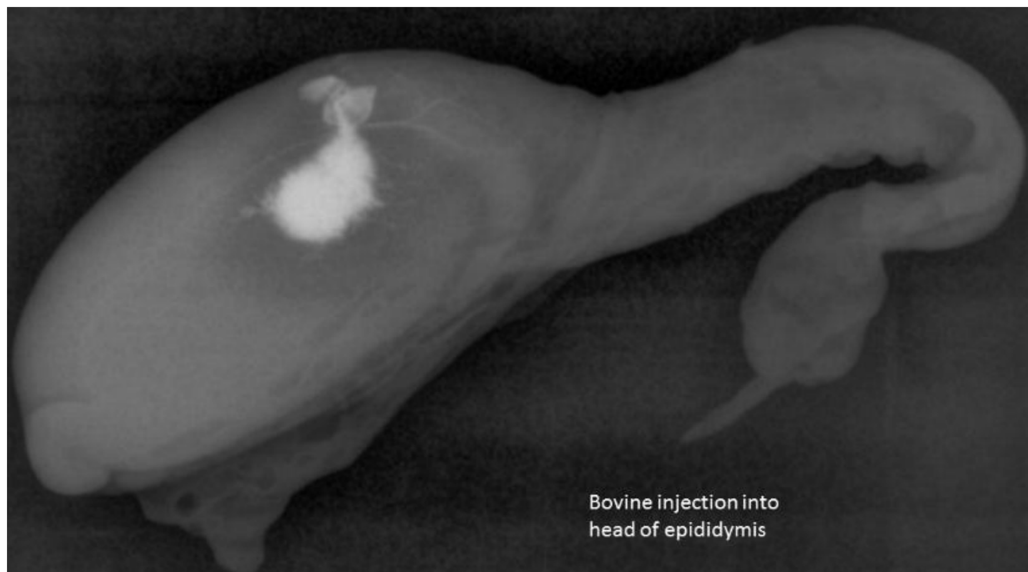

Figure 2. Bovine testicle injected with 1 mL of iodixanol contrast into the epididymis. No contrast media is seen in testicle.

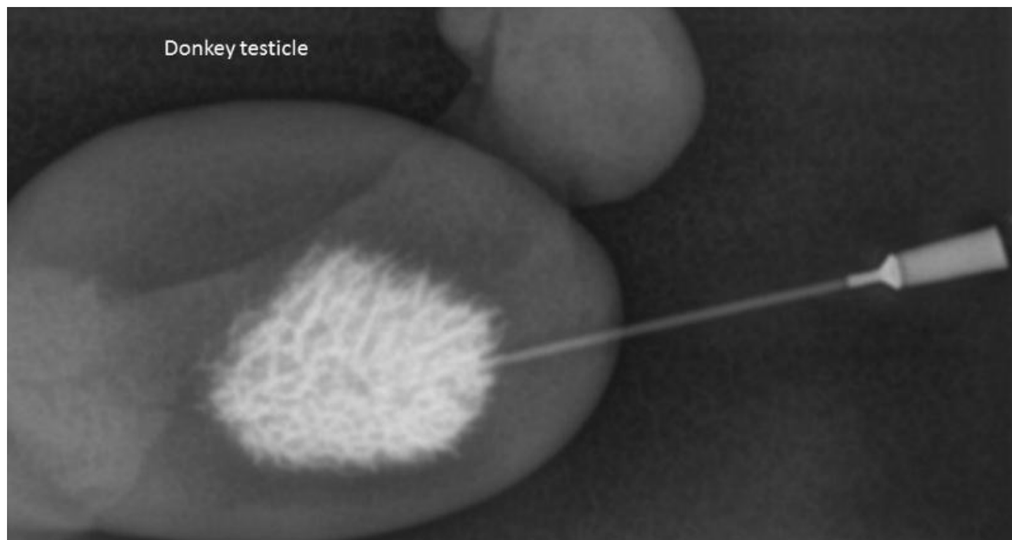

Figure 3. Donkey testicle injected with 2 mL iodixanol contrast below the area of the epididymis. Contrast can be seen in tubules.

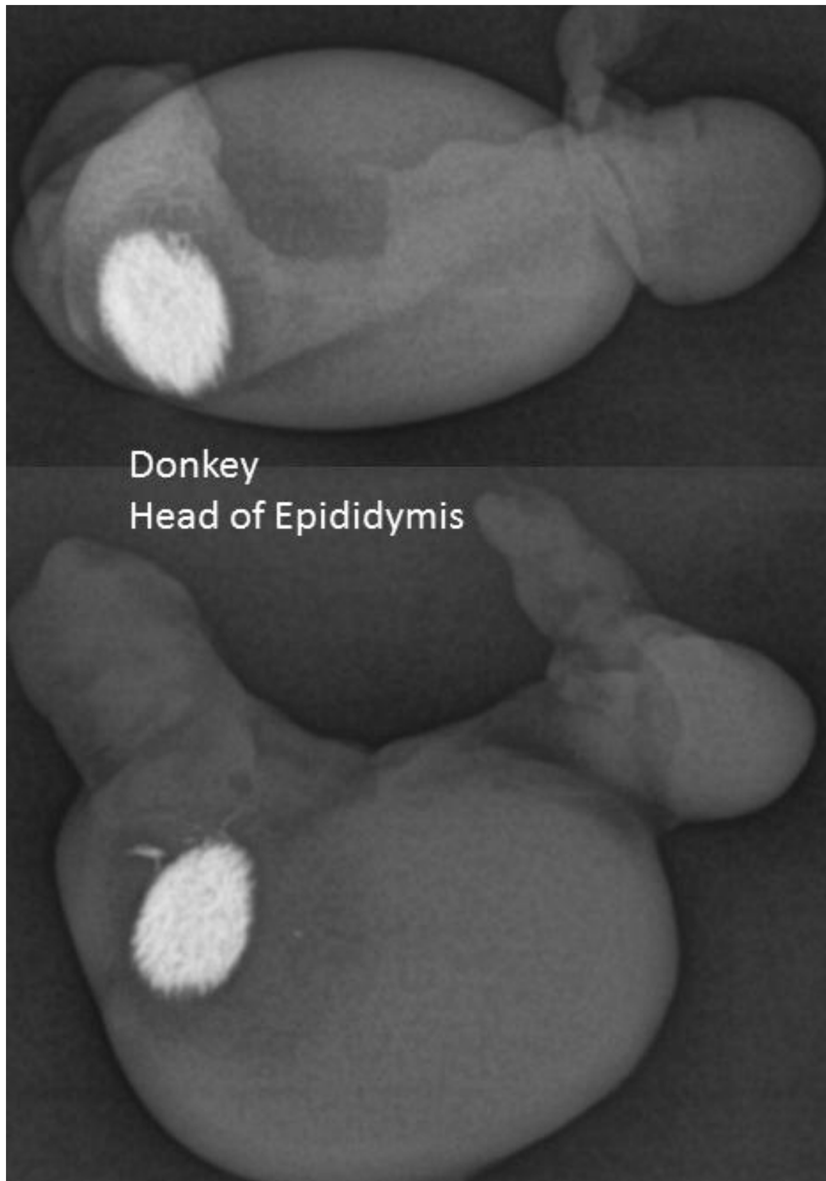

Figure 4. Donkey testicle injected with 1 mL of iodixanol contrast into the epididymis. No dispersion of contrast into testicle.

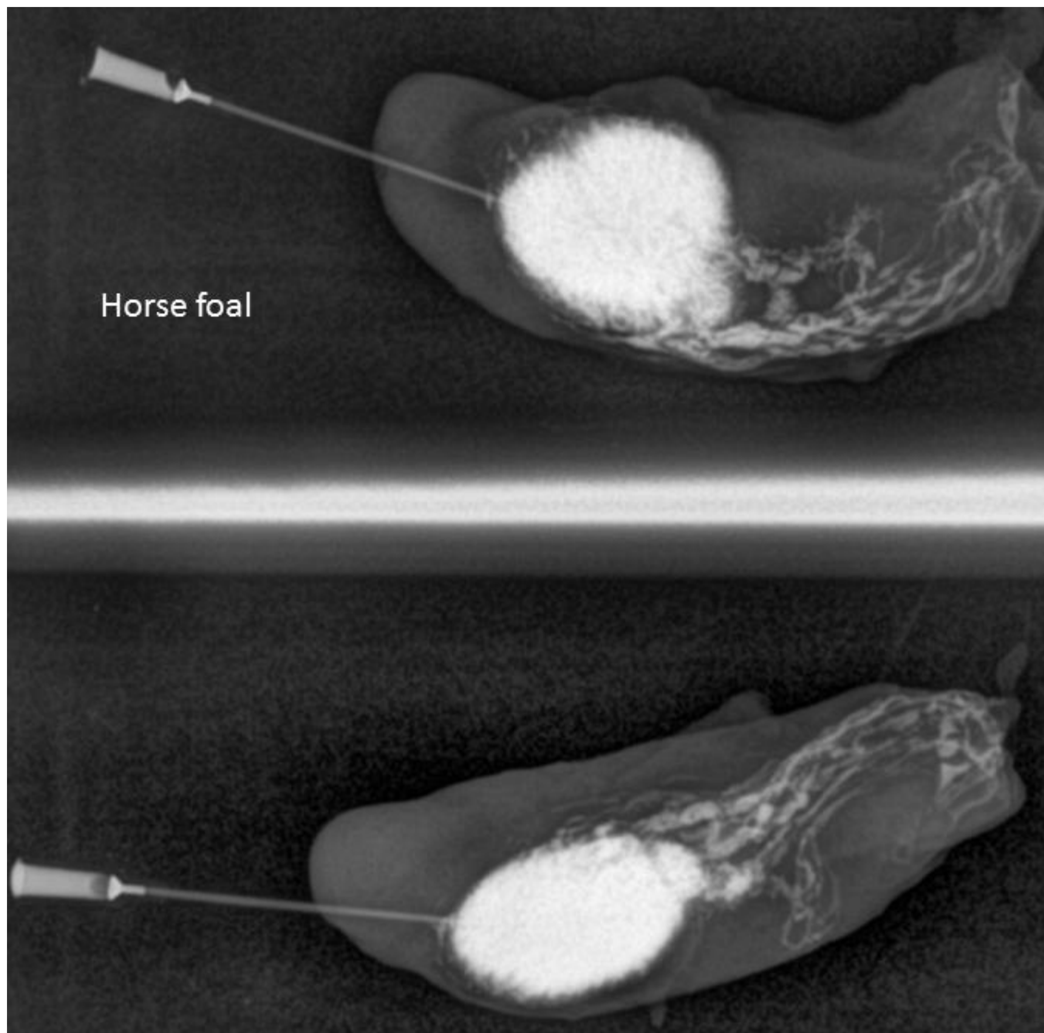

Figure 5. Horse foal testicle injected with 5 mL of iodixanol contrast into the area below the epididymis. Contrast media can be seen in vessels.

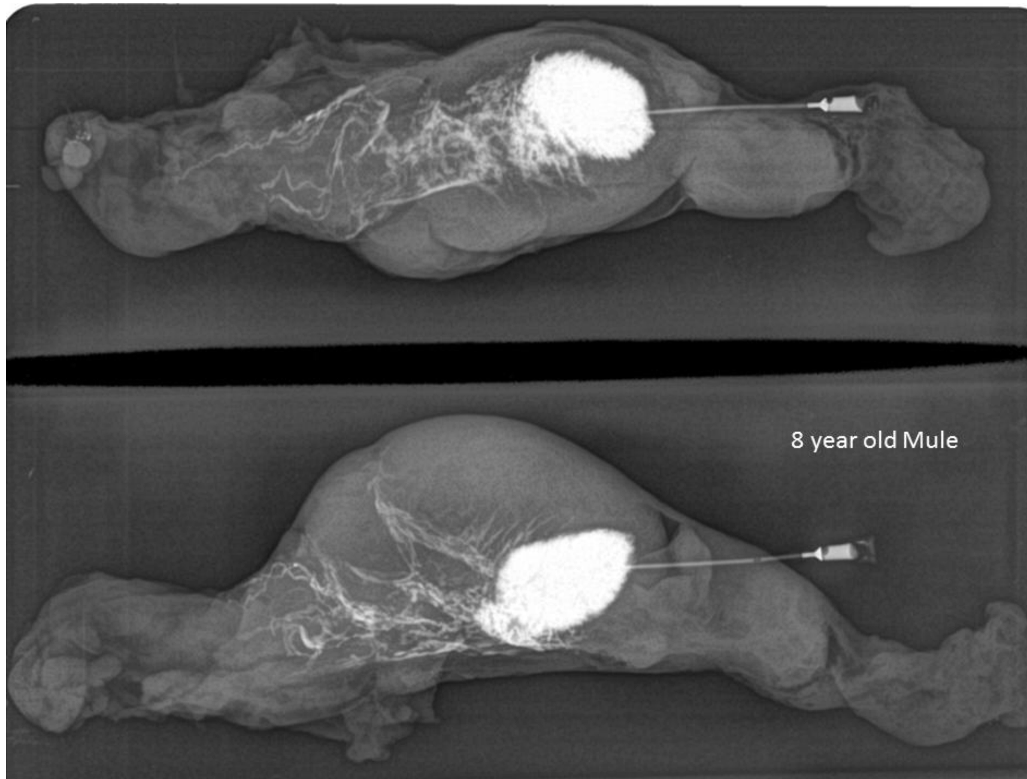

Figure 6. Two mule testicles injected with 5 mL of iodixanol contrast into the area below the epididymis. Contrast media is seen in a focal area surrounding the needle tip and in the vessels extending toward the head of the epididymis.

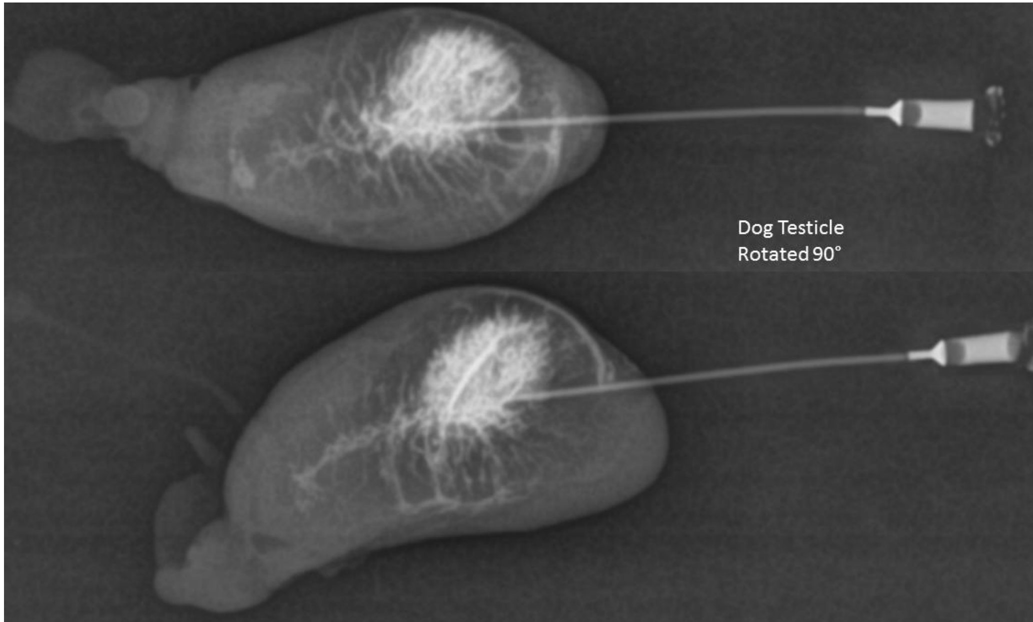

Figure 7. Dog testicle injected with 1 mL of iodixanol contrast. Viewed in 2 planes; contrast media is seen distributed in tubules.

Figure 8. Cat testicle injected with 0.5 mL of iodixanol contrast. Contrast media is seen throughout the testicle and into the epididymis.

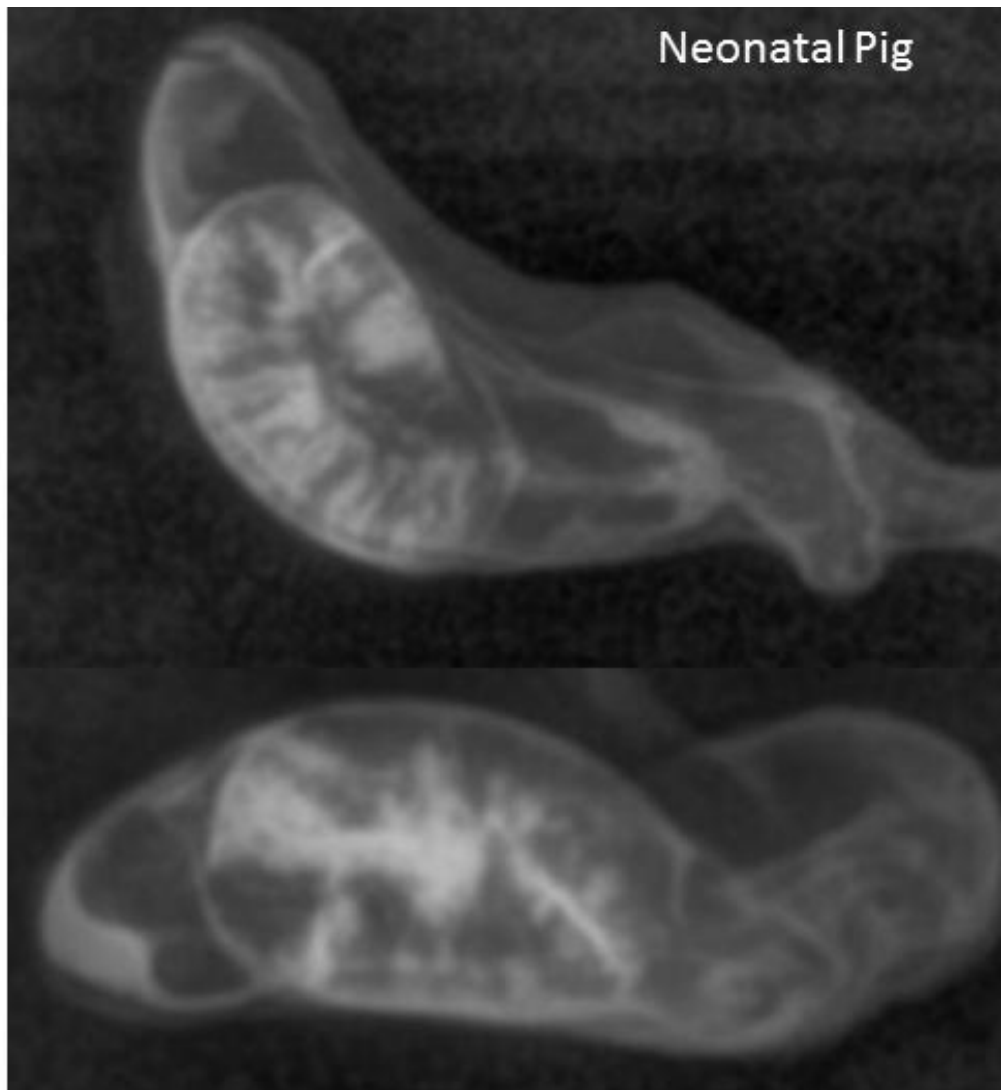

Figure 9. Neonatal pig testicles injected with 1 mL of iodixanol contrast. Contrast media is seen throughout the testicle extending into the epididymis.

Discussion

The dispersion of fluid was achieved in the different species in all cases. There was no advantage to one injection technique over the others. The best dispersion seen in an equine testicle was when the injection site was in the area of the testicle just below the epididymis. Other non-equine species have a discrete rete testis which can be found by ultrasound examination. Further research may be necessary to determine specific area within an equine testicle to improve the chances of successful injection. Ultrasound guidance is valuable when injecting the horse testicle to ensure that cells are not injected into the central vein.

The spermatogonial stem cell transfer from a Tennessee Walking Horse to a mule was unsuccessful, to date, in developing mature donor sperm in the recipient testicles. Mature sperm was found in a bovine-bison hybrid male after 10 months post-transfer. Therefore, semen collection will be repeated after 10 months post injection. If no spermatozoa are identified at that time, the procedure will be repeated on the basis that after puberty the testicle may be better suited to receive donor cells and allow them to implant and produce sperm. The cells were given four months to develop and there was no evidence of any parts of mature spermatozoa in any of the three collections performed two months apart. Deposition of the suspension into the testicle was confirmed by ultrasound guidance. Additional research is needed on male mule reproductive anatomy to support the study done by Neves and colleagues in 2005 which indicated that mule testicles contain functional somatic cells (Neves et al., 2005). The anatomy of equine testicles may also need further assessment when determining a successful injection site.

Conclusion

In this case, a mule was unable to successfully develop donor spermatogonia from a Tennessee Walking horse stallion in four months. There is evidence from a variety of species that injection of the donor cells into the rete testis of the recipient testicles is not essential for dispersion of the cells through the testicular tissue. However, additional research is needed to further determine the target for injection within an equine testicle. Research should be done to determine the time frame for successful cell development prior to expected date of maturity to determine the success of injection.

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APPENDICES

APPENDIX A: IACUC APPROVAL

5/5/2014

Investigator(s) Name: Emily Smith, Dr. John Haffner
Investigator(s) Email: emanncyclone@gmail.com, john.haffner@mtsu.edu

Protocol Title: "Stallion Spermatogonial stem cell transfer in the rete testis of a mule."
Protocol Number: 14-007

Dear Investigator,

The MTSU Institutional Animal Use and Care Committee has reviewed your research proposal identified above (including the addendum submitted 4/21/2014) and has approved your research in accordance with PHS policy. Approval is granted for three (3) years. Your study expires 5/5/2017. Please note you will need to file a Progress Report annually regarding the status of your study and submit an end-of-project report.

According to MTSU Policy, an investigator is defined as anyone who has contact with animals for research purposes. Anyone meeting this definition needs to be listed on the protocol and needs to complete the IACUC training through citiprogram. If you add investigators to an approved project, please forward an updated list of investigators to the Office of Compliance before they begin to work on the project.

Any change to the protocol must be submitted to the IACUC before implementing this change. Any unanticipated harms to subjects or adverse events must be reported to the Office of Compliance at (615) 494-8918.

Also, all research materials must be retained by the PI or faculty advisor (if the PI is a student) for at least three (3) years after study completion. Should you have any questions or need additional information, please do not hesitate to contact me.

Sincerely,

Kellie Hilker
Compliance Officer
615-494-8918
kellie.hilker@mtsu.edu