COMPARISON OF HOST ENDOCRINE ENVIRONMENT, DONOR TISSUE AGE AND DURATION ON SPERMATOGENESIS IN ECTOPIC MURINE TESTIS GRAFTS

By

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To the faculty of Washington State University:

The members of the committee appointed to examine the thesis of HOMER CLARENCE ADAMS III find it satisfactory and recommend that it be accepted.

____________________________
Chair
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It would not be right for me to not to start off by acknowledging my Heavenly Father, Jesus Christ. Thank you for the breath I breathe and all of these fine people you have put in my life. I love you.

To my friend, my lovely, supportive, nurturing mother who has never left my side in any decision that I have made in life whether good or bad. I love you so much that no number of acknowledgement pages nor words could do your graces correct justice. You are definitely the strongest woman I have ever met, and I will forever be indebted to you for your model influence in my life.

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Hopefully, when all is said and done I will have made you all proud. I’m out.

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COMPARISON OF HOST ENDOCRINE ENVIRONMENT, DONOR TISSUE AGE AND DURATION ON SPERMATOGENESIS IN ECTOPIC MURINE TESTIS GRAFTS

Abstract

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May 2004

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Testicular grafting has been used to provide alternative means to investigate spermatogenesis in non-testis environments. This technique could lead to identification of infertility factors and sperm from cancer treated patients. Ectopic testis grafting in different species has demonstrated multiple factors may affect sperm production. The objective of this study was to identify host endocrine factors that influence sperm production in ectopic testis grafts. Intact or gonadectomized male and female nude mice were used as recipients for testis allografts from neonatal mice. In addition, the durations of the grafts, 35 or 70 days, and the age of donor tissue 0-5, 6-10, and 11-15 days post partum (dpp) were examined to find parameters for sperm production in grafts. Graft weight, testosterone levels, seminal vesicle weight and percentage of seminiferous tubules with elongated spermatids were endpoints of analysis. It was found that testosterone was present in all of the mice indicating Leydig cell function within the grafts. At 70 days post graft, the seminal vesicle weights of castrated and intact recipients were similar indicating graft testosterone production. Testis tissue from 0-5 dpp mice had the greatest weight increase among all donor age tissue ranges and a graft duration of 70d produced heavier graft weights than the duration of 35d. Also it was found that in all recipients gonadectomized recipients had higher graft weights than intact animals. When
tubules containing elongate spermatids were examined, it was shown that in all observed
recipient groups a graft duration of 70d produced more elongate spermatids than did the
duration of 35d. Of the 70d recipients, donor tissue grafted to castrated recipients had the
highest percentage of elongated spermatids while donor tissue grafted for ovariectomized
mice displayed a statistically similar percentage of elongates to the second highest group,
intact males. From this study, testis grafts from ovariectomized females produced sperm
at similar levels to grafts on male intact recipients. In addition, grafts on ovariectomized
females produced sperm similar to grafts on castrated mice, indicating they are suitable
testis graft hosts. The best parameters for testis graft elongate spermatid production in
gonadectomized recipients were donor tissue of 0-5 or 11-15 dpp and 70d grafting period.
This is the first study in which multiple age ranges were used as testis donors for ectopic
grafting. Implications of this work indicate the extent that donor testis differentiation and
host environment may impact sperm production in grafts.
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DEDICATION

The Utmost dedication

Where would I be without my Heavenly Father? He who has brought me from
the depths of destruction to where I am today. With that being said,
I owe all my accomplishments to this day and all those that shall be bestowed upon me
to my Lord and savior, Jesus Christ. I love you infinitely.

“I can do all things through Christ who strengthens me.”

-Philippians Chapter 4 Verse 13

“The Lord is my Shepard; I shall not want. He maketh me to lie down in green pastures:
He leadeth me beside the still waters. He restoreth my soul: He leadeth me in the paths of
righteousness for His name’s sake. Ye, though I walk through the valley of the shadow of
death, I will fear no evil; for Thou art with me; Thy rod and Thy staff they comfort me.
Thou preparst a table before me in the presence of mine enemies: Thou aniontest my
head with oil; my cup runneth over. Surely goodness and mercy shall follow me all the
days of my life: and I will dwell in the house of the Lord forever.”

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CHAPTER I

REVIEW OF LITERATURE

1. SPERMATOGENESIS

2. HYPOTHALAMUS-PITUITARY-GONADAL AXIS

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Spermatogenesis

Spermatogenesis has been an area of extreme interest in past centuries and one of increasing importance in the present. Researchers have been attempting to find new ways to manipulate sperm to mature more efficiently and regulate their production in foreign environments through techniques such as strategic placement of testicular cells, observation of hormone level effects and alterations to the actual sperm production process as a whole. Ultimately, the goal with these experiments and any scientific research is to answer the questions of why and more importantly how. In order to understand spermatogenesis, it is vital to look at the various aspects involved in the production of sperm and examine how they fit together. Previous research has involved research animals, such as mice and other rodents, as models to observe spermatogenesis in a large and cost effective way. By utilizing these animals for further explanation of this process, the testis and sperm production can be better understood. The starting point for exploring this research is located within the male gonad where spermatogenesis occurs.

Sperm, the male gamete, is the endpoint of the process of spermatogenesis. Initiation of spermatogenesis and the differentiation of sperm cells occur in the testis. The testis is made up of two compartments: the seminiferous tubules and the interstitial space between the tubules. The seminiferous tubules are long cylindrical tubes where sperm production occurs. These tubules, which in the mouse there are approximately twelve, are tightly compacted within a muscular lining called the tunica dartos of the testis. This lining is involved in temperature regulation of the testis since sperm production in
mammals occurs at temperatures below the basal body temperature. At birth, a newborn male mouse testis contains a number of cells throughout the organ, including Leydig cells but within the seminiferous tubules only two cells are present: Sertoli cells and gonocytes [1, 2].

The Leydig cells of the testis are located in the interstitial space between seminiferous tubules. These cells are capable of secreting the majority of testosterone found in the serum of a male. They are able to do this because Leydig cells contain receptors for leutinizing hormone (LH), produced by the pituitary gland, which then stimulates them to produce and secrete testosterone. Leydig cells don’t store testosterone but actively secrete all that they produce which will then diffuse into the bloodstream or into the seminiferous tubules directly or indirectly by attaching to the carrier protein albumin. Intratesticular levels of testosterone in mice have been found to normally range from 25-100 ng/ml and assist in Sertoli cell proliferation during fetal and early neonatal life [3]. Testosterone has also been found to have multiple functions. First, testosterone is used in combination with the follicle-stimulating hormone (FSH) from the pituitary to assist in paracrine factor production by Sertoli cells. Secondly, testosterone works to act upon the pituitary as a negative feedback on the release of LH. Third, testosterone initially crosses the blood-brain barrier to disable the functionality of the pre-ovulatory center of the hypothalamus in males so that LH will be secreted tonically and not cyclically. Fourth, testosterone primes androgen target tissues for androgen-mediated growth. Testosterone is influential and necessary for spermatogenesis to occur in most animals [4].
Sertoli cells are epithelial cells that have a number of interesting and unique properties. First, Sertoli cells are the only known cells in the male to contain a receptor for FSH [5]. This is important since this hormone is released in most cases simultaneously with leutinizing hormone from the pituitary. FSH stimulates Sertoli cells to proliferate within the seminiferous tubule during neonatal life [6] and, with the addition of testosterone, secrete paracrine factors that help germ cells develop in the tubule. In addition, the Sertoli cell also secretes androgen binding protein, ABP that transports androgens, and inhibin which acts as negative feedback on the pituitary for FSH secretion [7].

Sertoli cells lie upon the basement membrane of the seminiferous tubule and extend to the lumen, which causes their environmental effect on germ cells to encompass the spermatogenic process from start to finish. Sertoli cells along the basement membrane and the peritubular myoid cells create a selectively permeable barrier called the blood testis barrier that prevents back flow of lumen fluids to lymph and will allow only certain factors to cross, such as testosterone [8]. This barrier consists of two major parts: (1) a barrier of myoid cells that surround the Sertoli cells and (2) the tight cell junctions between adjacent Sertoli cells. In order to protect the germ cells in their development, the Sertoli cells provide the most exclusive compartment of the protective blood-testis barrier. This barrier prevents self-immunization by the immune system against the germ cells that the testis is producing. It is also known that the breakdown of the blood testis barrier results in infertility in the male.

The seminiferous tubule is separated into two compartments of specific location for developing germ cells by these Sertoli-Sertoli tight junctions. Differentiating germ
cells called spermatogonia and preleptotene spermatocytes are associated with the basal compartment and all other advanced germ cells are associated with the adluminal compartment of the tubule. Tight junctions help in the progression of germ cells to prevent auto-immunity that the body could self exert upon the seminiferous tubules, block harmful toxins that could be administered in proximity to the gonad [5], as well as allow for controlled migration of the cells through the tubule to possibly keep certain factors associated with development of a germ cell at one stage in spermatogenesis from those specifically associated with those at a another stage [7].

Another important function of the Sertoli cell is to secrete seminiferous tubule fluid that is involved in the release of sperm from the Sertoli cells, which is called spermiation. At spermiation, Sertoli cells phagocytose those degenerative cells that did not complete germ cell differentiation and the cytoplasmic droplet that is leftover after spermiogenesis. The seminiferous tubule fluid is also known to contain other important factors such as testosterone that is essential in later processes of spermiogenesis [4], ABP that binds testosterone, inhibin and other enzyme inhibitors [7].

The correct testis environment allows for sperm production where germ cells can differentiate and proliferate. The initial cells from which the spermatogonial cells will emerge from are the primordial germ cells (PGCs). These cells migrate from the allantois to the genital ridge around embryonic day 10.5 in mice. PGCs then become gonocytes as the testicular environment is initially formed. Gonocytes are an undifferentiated cell that are arrested in the G0 phase of the cell cycle until after birth when they will give rise to germ cell producing spermatogonia. The starting cells for sperm production are the diploid spermatogonial stem cells that undergo mitosis to replicate themselves and
initiate spermatogonial differentiation. It is approximated that there is only 1 spermatogonial stem cell to every 5000 testis cells in a mouse testis for a total of 35,000 stem cells [9]. Spermatogonial stem cells are localized in certain niches associated with Sertoli cells along the basement membrane. The number of stem cells and niche positions has also been found to increase with age and testis growth [10]. These cells have been known to differ from other germ cells in the testis in that they have the ability to colonize a new testis following transplantation, are more resistant to toxins and they contain certain $\alpha_6$ and $\beta_1$ integrin surface cell markers unique to stem cells within the body [11].

Spermatogenesis initiates a proliferative phase and germ cells develop from type A spermatogonia that maintain intercellular bridges that connect cells. Intercellular bridges are thought to promote synchronous development of cell clones and other germ cell types by sharing of gene products. The only spermatogonial cells that do not have intercellular bridges are the single or stem cells ($A_s$). The generally accepted stem cell theory has progression of the mitotic phase of spermatogonial cells with $A_s$, $A_{pr}$, $A_{al1}$, $A_{al2}$, $A_{al3}$, $A_{al4}$, $A_{al5}$, $A_1$, $A_2$, $A_3$, $A_4$, In and then B spermatogonia. Of these cell types, $A_2$, is the indicative cell stage that spermatogenesis has started. Spermatogonial cells progress through the mitotic phase with cell divisions to double the number of germ cells at every step, except at the $A_{al}$ to $A_1$ stage of cell differentiation where only a morphological change occurs. Morphological differences in chromatin are often used to detect different spermatogonia. At completion of the mitotic phase, cells have reached the B cell stage and there have been 512 cells generated from one spermatogonial stem cell [8, 9].

There have been debates on whether it is symmetrical or asymmetrical stem cell differentiation and proliferation that occurs in the mouse and other species. In the case of
symmetrical division, the A₃ would divide into two cells: one as a committed cell that differentiates and the other a stem cell that proliferates. Or, in the case of asymmetrical division, there is a type of stem cell specificity that allows for only proliferation of some stem cells and differentiation of others. In addition, there has been an added possibility of a renewal stem cell theory consisting of a population of stem cells (A₀) that lay dormant until they are needed to proliferate for repopulating the testis. Due to this activity or lack of, they are perceived to have a slow cell cycle. The mitotic phase of this theory consists of spermatogonial cells that progress from A₀, A₁, A₂, A₃, A₄, In to B spermatogonia. The A₁, A₂, A₃ and A₄ cells are not considered different from each other. Another difference of the A₀ scheme in contrast to other theories, is that at the A₄ to In cell stage, the mitotic division can either produce In spermatogonia or retort back to A₁ spermatogonia to renew the population [9].

The differentiation of spermatogonia can be stopped either by nutritional defects such as vitamin A deficiency that holds cells at the A₄ cell stage, deletion of a gene such as Glial cell line-derived neurotrophic factor (GDNF) that causes early depletion of spermatogonial stem cells, or when there is an abundance of spermatogonial cells present associated with a Sertoli cell. This acknowledgement of cells in an area of a tubule is referred to as the density dependant regulation. The higher the number of A₂ to A₄ spermatogonia in a region of a tubule the more likely apoptosis of cells will occur to lower spermatogonia to a normal level. Likewise, this regulation of germ cells may not occur if there is an absence or shortage of spermatogonial cells associated with a Sertoli cell [9].
In the meiotic phase, type B cells divide to form preleptotene spermatocytes. The chromosomes are condensed and prepared for the two meiotic divisions. One type B cell progresses up to the diplotene stage of the cell cycle where the first meiotic division occurs to produce two diploid secondary spermatocytes. Then these two cells execute the second meiotic division to produce a total of four haploid spermatids. At the end, this process results in quadrupling the number of germ cells. A total of 4096 haploid germ cells can be produced from a single spermatogonial stem cell in the rat [8]. In this manner, spermatogonia keep stem cell numbers constant to produce large amounts of sperm and enables males to produce sperm throughout their adult life [12].

As germ cells progress between the Sertoli cells towards the lumen, the opening or center of the seminiferous tubule, the Sertoli cells regulate developmental stages of germ cells so a characteristic hierarchy of germ cells is found in the same group. By this manner, the stages together form the spermatogenic cycle that has been broken into stages that range in mice from I through XII. These stages are characteristic of the hierarchy of germ cells associated with Sertoli cells. In mice it takes four and a half cycles of seminiferous epithelium (~35 days) for spermatogonia to complete the spermatogenesis process to the elongated spermatid stage for release. Within these stages of spermatogenesis different phases occur such as the replicative phase of primary spermatocytes, the meiotic phases, the acrosomal phase for the head development of the sperm, the nuclear condensation and elongation phase for the tail and the cytoplasmic elimination and the release of the spermatid. In addition to this progression of germ cells, spermatogenesis occurs at all different stages of the cycle in waves of sequential order throughout the tubules. The Sertoli cell accommodates the cells that are at their respective
stages and progresses on to the next stage when they have completed that particular developmental process [9, 13, 14].

In the spermiogenic phase, there are many different aspects involved in the development of spermatids to spermatozoa. The formation of the flagella is initiated with the initial movement of centrioles to the cell surface, where one forms the axoneme. This structure contains microtubules and is usually implanted opposite of acrosome cap on the spermatid. As the acrosome cap forms and the nuclear region of the spermatid becomes more oriented with the cap, the cell produces a junction between the head and flagella called the neck and becomes more elongated, thereby forming elongated spermatids. In this procedure there is also nuclear condensation and elimination of cytoplasm that contribute to the formation of elongate spermatids [8].

After spermiation occurs, elongate spermatids travel out the efferent ducts of the seminiferous tubules. These ducts lead out to the epididymis where the sperm acquire the ability to be motile. They enter the caput or head of the epididymis and then migrate towards the cauda or tail of the epididymis where they are considered to have completed the maturation process. Sperm are stored here until ejaculation, in which they can be utilized through the normal mating fertilization process or other assisted reproductive methods.

**Hypothalamus-Pituitary-Gonadal Axis**

Hormone secretion has essential roles in the activation of many cells and processes in the body. Two of the major organs that are involved in hormone regulation are the hypothalamus and the pituitary. These organs are located in close proximity of
each other in the lower portion of the brain and through a coordinated effort regulate many body functions. The hormones that are released by the hypothalamus are transported directly to the pituitary by way of the blood portal system. Part of this blood flow system that travels out of the anterior pituitary is directed past or in close conjunction with the hypothalamus so the hormones that are being secreted can exert negative feedback on the hypothalamus. For example β endorphin and the pituitary hormones LH and FSH, negatively feed back to regulate the release of the hypothalamic hormone gonadotropin releasing hormone, GnRH [7, 15].

The hypothalamus produces a number of hormones that act upon the pituitary through portal blood flow. These hormones are an assortment of releasing hormones that stimulate specific cells within the pituitary to secrete their respective hormones. The pituitary gland is located in the sella turcica, which is a small bony process directly underneath the brain. The pituitary is further subdivided into three specific lobes: anterior, posterior and intermediate lobes. The anterior lobe is the area primarily associated with secreting factors that regulate spermatogenesis and within this area there is a number of gonadal hormone secreting cells called gonadotropes. These cells secrete the polypeptide protein gonadal hormones of FSH and LH.

GnRH, also known as luteinizing hormone releasing hormone (LHRH), is a 10 amino acid peptide with a 2-4 minute half life synthesized in the arcuate nucleus of the hypothalamus. It is circulated through the blood system to the central nervous system and is known to act upon the anterior pituitary. GnRH is secreted at the median eminence and then travels to the pituitary through portal circulation to cause the release of gonadal hormones. The pulse frequency for GnRH is influential on which of the gonadal
hormones is preferentially secreted. It has been found that a slow frequency pulse of GnRH favors FSH secretion whereas a faster frequency pulse favors LH secretion [15]. Of the population of cells in this lobe of the pituitary, 10-15% are basophilic gonadotropes that secrete FSH and LH upon GnRH stimulation. GnRH acts on its G-protein-coupled receptors on gonadotrophs to cause an abundant increase in the production of the α subunit of LH and FSH. The α subunit is interchangeable for the dimeric proteins of FSH and LH, as well as other hormones. It is the second subunit, the β subunit, for FSH and LH which are rarely found in serum or urine that have specific activity on receptors of respective cells in the body. Both the α and β subunits are cysteine rich and have disulfide bonds and carbohydrate moieties that contribute to their individual characteristics. Although not completely understood, GnRH input on the gonadotropes of the anterior pituitary governs the dimerization of the α and β subunits for LH and FSH. Due to its chemical characteristics, FSH has a half-life of 3-4 hours and LH has a half-life of 20 minutes [15]. By this means, the trio of the hypothalamus, pituitary and gonads create an axis of continuous interaction that controls gametogenesis.

FSH is one of the gonadal hormones that once secreted from the pituitary travels to the testis and binds to its G protein-coupled, seven transmembrane domain FSH receptor (FSHR) on the Sertoli cell to cause proliferation of Sertoli cells and support germ cell differentiation. Once stimulated by FSH, Sertoli cells mature, develop tight junctions and secrete a number of hormones such as estrogen, ABP, and inhibin. Specifically, inhibin β exerts a negative feedback on the pituitary on the release of FSH [15]. Even though the effects of FSH are thought to be more quantitative and important in initiation but not maintenance of spermatogenesis, a block in spermatogenesis leads to an increase
in FSH levels. Also, the deficiency in FSH production has been linked to oligospermia and/or azoospermia whether testosterone was being produced or not [15]. Specifically, FSH is known to be responsible for the progression of spermatogenesis up to the secondary spermatocyte stage, and then testosterone appears to take over regulation of spermatogenesis. This was shown in hypogonadal (hpg) mice, which are strain of mice with a naturally occurring germline deletion in the GnRH gene. The deletion causes the inability of LH and FSH production from the anterior pituitary. These mice have been shown to be capable of completing meiosis and initiating but not completing spermiogenesis [16].

LH is the other gonadal hormone secreted from the gonadotropes of the pituitary gland. LH is known to bind to its G protein-coupled, seven transmembrane domain LH receptor (LHr) on Leydig cells within the testis to cause testosterone production and secretion. Like most hormone treatment to cells, the administration of FSH and LH to cells will initially cause the increase of hormone secretion to a point, and then a decrease in the amount of receptors on the surface occurs to regulate hormonal stimulation. The production of testosterone and its metabolites along with estradiol, negatively regulate FSH and LH production at both the pituitary and hypothalamus [20]. This hormone action of LH does make the testis the major source of testosterone production in the male with the adrenal gland providing minor additional testosterone. As mentioned before, testosterone is important in the latter portion of spermatogenesis and is also a complement to FSH in the stimulation of Sertoli cells in the testis.

The removal of the testis causes a decrease in the secretion of numerous factors and alteration of hormone levels for the interaction between the hypothalamus-pituitary-
gonadal axis. The absence of Leydig cells would cause a significant drop in testosterone [17], which would allow for LH levels to increase to unregulated amounts. The absence of Sertoli cells would similarly drop the levels of inhibin [6], which would raise FSH levels due to a lack of negative regulatory hormones [18]. So all of these things taken into consideration would then cause the axis to lack a type of homeostasis. This knowledge has been utilized to take advantage of making animal models that will secrete these gonadal hormones at abnormally high levels [19]. Given such an environment, detrimental as well as beneficial effects could be identified on a variety of cells.

**Grafting**

The ability of preserving the production of germ lines has been difficult in the past, but with new techniques such as germ cell transplantation and testicular tissue grafting, the possibility has become more of a reality. The potential capabilities of these methods can allow for many things including: retaining the ability of cancer patients to produce offspring, the preservation of endangered species, identification of potential male contraceptives, production of gametes from neonatal lethal transgenic animals and androgen replacement therapy [2, 20].

Germ cell transplantation is a method of enzymatically digesting a testis, which contains the spermatogenic cells, to form a single cell suspension. Then a small injection can be made into the efferent ducts of the seminiferous tubules through which the cells can be deposited into a recipient testis. The object of this procedure is to allow the donor cells to migrate to the basement membrane and colonize within the recipient testis. This process has been successful in previous studies with mice, rats and hamsters among other
species but is difficult in non-rodent and higher order species. One disadvantage of this process is that in cancer patients you could be possibly retransmitting tumor cells into their testis following chemotherapy when reinjecting the germ cell suspension [1, 2]. This is partially why grafting of testicular tissue has appeared as another option for germ line preservation.

Ectopic grafting of testicular tissue is a method in which either a whole or portions of testicular tissue from a donor animal is placed in a foreign area of a recipient animal, usually an immunodeficient host. The tissue once obtained from the donor animal includes the obvious removal of attachment to the epididymis and other accessory sex glands. Also, the tunica albugenia is removed so that the interstitial cells, including the Leydig cells, can have access to the recipient blood supply. Upon removal of these attachments and containing tissues, the interstitial space is disturbed as well as the exit of seminiferous tubule fluid is severed, allowing for the potentially hazardous accumulation of fluid. This can ultimately result in premature sloughing off of spermatogenic cells from the Sertoli cells into the lumen of seminiferous tubules [1]. Testis tissue is placed subcutaneously under incisions that include a small nick in the muscular layer to assure access to the blood supply and tissue. The main focus and idea in placement of the tissue is that it is in an area where it can easily access blood from the recipient animal. In addition, an area is also preferred, that will have relatively low disturbance while incubating on the animal. These grafts can be placed in areas so that visual detection of growth as well as abnormalities can be readily identified.

These procedures have been executed utilizing immunodeficient nude male mice (nu+/nu+) as recipients. This is a naturally occurring mutant mouse strain that lacks a
thymus and hence does not have immune system T-cells that could possibly reject donor tissue. From previous grafting studies and knowledge of endocrinology, the removal of the gonads of these mice seemingly makes for the ideal environment for foreign testis tissue to thrive.

It has been shown that when murine allografts are analyzed histologically, that there is often a range of spermatogenesis that occurs in the seminiferous tubules from complete atrophy to full spermatogenesis which provides the option of retrieval of spermatozoa for assisted reproduction methods [20]. For this reason, it seems apparent that these grafts should be limited in their duration on the recipient animal or that there should be some type of method in removal of the pressure exerted by the tubule fluid. Some of the regions for this testicular tissue of donor animals to be placed on the recipient animals have included the anterior chamber of the eye [18], scrotum [21], dorsal back region [2], behind the ear [6, 22, 23] and under the kidney capsule [24].

Turner [18] grafted testis tissue to the anterior chamber of the eye and observed spermatogenesis in the tissue. This approach was thought to be successful due to temperature probes that showed a similarity of temperature in the eye with that of the scrotum. To confirm his hypothesis, Turner tested six other compartments of the rat’s anatomy for potential grafting locations. Newborn donor testis tissue was used for the grafts and recipients ranged from 44 to 205 days of age with a median of approximately 120 days of age. Grafts remained on recipients for periods as long as 15 months, during which testosterone was readily produced from the grafts. This study also incorporated the use of recipients that were: intact male and female rats, castrated, spayed, and pregnant. The additional grafting locations of the rat utilized were: subcutaneous pockets, the
surface of the intestine, parietal peritoneum, the tunica vaginalis (these with scarification of tissue), muscle, liver and kidney (these without scarification of tissue). Placement in the eye chamber was done with the tunica intact so as to transfer the same amount of testis tissue per cornea and to prevent infiltration of connective tissue into the graft. This initial study proved that among the additional locations of grafts, intraocular placement of tissue had the highest percentage of transplants recovered and that there was roughly 90 percent or above recovery of transplants regardless of the hormonal status of the recipient animal. It was noted that there was a range of productive tubules to degenerative tubules, indicating that different spermatogenic processes occur simultaneously in different tubules of the same graft. Sperm heads that were identified in histological analysis could have been late spermiogenic stages or mature spermatozoa and were present by day 37 of observation. The highest percentage of sperm heads was recovered from castrated animals while the lowest percentage was noted from intact females. Pre-gonadectomy, administration of exogenous pituitary hormones or pregnancy urine extracts did not increase the appearance of sperm heads in grafts. Overall, this study showed that castrated animals were the best in growth of grafts and production of sperm heads with spayed rats producing a similar outcome to that of intact males. Pregnant and intact females were not suitable hosts for spermatogenesis in grafted tissue [18].

In 1950, Williams [22] utilized tissue grafts in chambers located on the ear of rabbits. The identification of secretions and changes in interstitial cells and tubules over extended periods of time was explored. Glass chambers in the region of the ears of rabbits that had removable cover slips for easy access to grafted tissue were developed. Four rabbits approximately six months old were used as recipients for sperm producing
testicular grafts of unknown age. Sperm heads that were visible during the initial grafting phase disappeared after the first month. All grafts, though, survived the fourteen-month duration and vascularity of grafts was more extensive in grafts that contained interstitial cells than those that did not. Interstitial and Sertoli cells had gone through some changes and spermatogenic cells were present in some tubules but did not progress beyond secondary spermatocytes after the first few months [22].

Chan and associates in 1969 [23] explored the possibility of placing testes behind the ear of normal and pseudohermaphroditic male rats. These scientists reasoned that by placing undescended testicular tissue in an area that closely simulated testicular environmental temperature that they could determine whether sterility results from cryptorchidism alone. It was found that testis tissue from 4 to 5 day old rat donors transplanted to 105-120 day old normal and castrated pseudohermaphroditic rats survived to produce all stages of spermatogenesis while similar donor age testis from pseudohermaphroditic rats to 105-120 day old normal and pseudohermaphroditic rats only produced germ cells up to the spermatid stage. This finding, though conveying a limitation in spermatogenesis in pseudohermaphroditic rats, resulted in grafted pseudohermaphroditic tissue spermatogenesis to progress further than its normal endpoint of primary spermatocytes [23].

Ectopic testis grafting was also done with subcutaneously placed tissue on the dorsal and scrotal regions of castrated rats [21]. Rats of ages seven to nine days were used as testis tissue donors to graft approximately 100mg of testis tissue per recipient. The objective of this study was to examine the level of spermatogenesis of untreated, treated and cyropreserved (glycerol or saline utilized as a cryopreservant) testis tissue.
Subcutaneous grafts were observed at intervals up to 28 days whether untreated (control) or cyropreserved. Control grafts did not increase in size comparison of those frozen in glycerol and only progressed up to the level of secondary spermatocytes. Subcutaneous saline frozen grafts remained on recipients for 8, 15, 39, 84 and 94 days. These grafts proved to be more successful than glycerol-frozen samples and had the furthest progression in spermatogenesis at day 39. Recipient rats also received an exogenous hormone supplement 10 days prior to removal of grafts. This treatment only proved to be beneficial to seminal vesicle weights and not spermatogonial differentiation within the seminiferous tubules. Scrotal grafts, however, produced some grafts that contained seminiferous tubules with full spermatogenesis from untreated and frozen grafts. The grafted rats that contained these successful tubules were also associated with full size seminal vesicles indicating the importance of testosterone production in spermatogenesis [21].

Ectopic grafts have also been placed under the kidney capsule of adult male intact and castrated rats to study Leydig cell development [24]. In this study, newborn rat testes were used for grafting tissue and after two weeks, Leydig cell numbers and nuclei size increased in grafts. Seminal vesicle weights were also utilized as a bioassay for testosterone production from the grafts. In addition, seminiferous tubules contained spermatocytes and had larger luminal diameters in comparison to normal two-week old rat testis which may have been indicative of seminiferous tubule fluid accumulation [24].

Johnson and associates [6] were another group of scientists that used grafting procedures to investigate potential differences in grafting success between sexes. These grafts were placed behind the ear utilizing Fisher rat testis at day 0 post partum and were
grafted on pituitary-intact castrated and ovariectomized rats and hypophysectomized castrated and ovariectomized rats. Grafs were shown to be mostly dependant upon gonadotropin secretion for the growth of testicular tissue. This study also is one of the few that addresses a possibility of sex playing a role in testicular graft success. Here, it was found that four testis grafts produced significantly higher amounts of parenchymal tissue and Sertoli cells per testis than did two grafts regardless of the sex of the recipient. Also it was found that males did tend to produce heavier total mass per testis than females although it was not significantly different [6].

Through grafting, viable sperm have been retrieved from mice, goat and pig grafts which was confirmed by ICSI procedures that stimulated resumption of meiosis in a mouse oocyte [2]. Pig testis tissue that was grafted on nude mice showed complete spermatogenesis earlier than in a normal pig testis and all spermatogenic stages of germ cells present in the grafted tissue [2]. Seminiferous tubule diameter increased faster as well, which as mentioned before could be detrimental to germ cell development. Asynchronous development was observed in the pig, which wasn’t observed in mouse allografts. This supports the ability of murine gonadotropins to support spermatogenesis of higher order species and that exogenous administration of hormones is not needed [2].

In one study in which hamster and monkey testis were grafted to mice, the testicular samples from adult animals whether regressed or not, displayed a lower incidence of spermatogenic recovery when compared to immature donors [20]. In addition, marmoset, photo-regressed adult hamster and murine testis grafts were not measurable for differences in weight and visually appeared smaller than when originally grafted onto nude mice. However, the immature hamster testis grafts increased 10 fold
from their weight when initially placed subcutaneously on the backs of recipient mice. This study asserts the issue that immature testicular tissue appears to be more beneficial in the effort to obtain sperm from grafts, which could be due to its ability to survive periods of ischemia or its receptibility to angiogenesis of the host animal [20].

More recent research involved murine grafts on the back of nude mice that produced a complete round of spermatogenesis in the similar time frame of four weeks for normal mice. Following this duration of the study, these grafts were found to restore hormone levels of FSH and LH in castrated hosts and the elongated spermatids, through assisted reproductive methods, produced normal fertile offspring [1]. A similar study involving the ICSI procedure was done with rabbit and murine testis samples that were grafted underneath the tunica albugenia of recipient nude mice. These grafts became vascularized and produced elongated spermatids that were used to produce progeny from mice as well as rabbits [25].
Conclusion

With many issues today pertaining to the physiology of spermatogenesis and ways to increase its vitality, grafting procedures may become one of the premier avenues to utilize in increasing longevity in animals as well as human kind. The understanding of how hormonal regulatory functions affect sperm production may help in acquiring a new outlook on controlling gametogenesis and fertility processes in males and potentially females. Ultimately, developing and sustaining ectopic environments for sperm production will be beneficial for the science of today and years to come.
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CHAPTER II

COMPARISON OF HOST ENDOCRINE ENVIRONMENT, DONOR TISSUE AGE AND DURATION ON SPERMATOGENESIS IN ECTOPIC MURINE TESTIS GRAFTS

1. INTRODUCTION

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COMPARISON OF HOST ENDOCRINE ENVIRONMENT, DONOR TISSUE AGE AND DURATION ON SPERMATOGENESIS IN ECTOPIC MURINE TESTIS GRAFTS

Introduction

Spermatogenesis has been explored in many species to better understand the mechanisms that regulate sperm production within the testis. Obtaining more information about how to possibly manipulate and preserve germ cells may lead to new possibilities in fertility research and removal of deleterious genetics from commercial species. The most significant advance to study spermatogenesis has been in the area of germ cell transplantation. This technique can restore fertility in infertile male mice. However, the application for human cancer patients may be limited because it has been shown that there may be a high incidence of transmitting pathogenic cells during the spermatogonial stem cell transplantation process. This risk has led to the development of testicular grafting as another option to produce sperm from infertile donors [1, 2].

In previous testis grafting studies, there has been a preference in utilizing castrated males as recipient animals. Although this is a logical choice considering hormonal physiology, few scientists have addressed the possibility of utilizing females as well as male recipients. With the knowledge that rat testis tissue grafted to ovariectomized females have produced elongate spermatids [3], increased parenchymal tissue and Sertoli cells per testis similar to castrated males [4] female recipients should not be excluded. In addition, utilization of nude mouse model recipients which have a
compromised immune system, makes them an ideal model to use for testis grafts in that the possibility of immunological rejection of tissue is alleviated.

Testis development begins early in life in rodents and the appearance of specific germ cells during development has been clearly defined [5]. It has been shown that prior to 5 days old there are only spermatogonial stem cells and Sertoli cells in the seminiferous tubules. Then the first wave of spermatogenesis begins from 6 days old with slow progression until elongated spermatids are first observed around 30 days old [6]. In light of this information, previous studies have not made an effort to keep a consistent range of donor age testis tissue to use in grafts, but instead have mainly used neonatal tissue of mice and rats up to 20 days old [1, 3, 4, 7, 8]. These studies resulted in providing inconsistency between observed groups or were too narrow of a range to determine if there was a donor age effect on testis graft success.

The actual duration of grafts, though, has been examined fairly well in previous research. It was found that a period of grafting from 28 to 37 days was sufficient to produce sperm heads in grafts of rodent testis [8]. Sperm from such grafts have been harvested and utilized in assisted reproductive methods to produce progeny from mice and rabbits [1, 9]. Graft durations on recipients are approximately equal to the time needed to complete one round of spermatogenesis in mice (35 days) and rats (52 days) [10]. This is important to note, because with the common problem of tubule fluid accumulation in grafts and the sloughing of cells into the lumen of seminiferous tubules, the shorter time needed to generate elongate spermatids would be more beneficial for application of this technique.
The objective of this research was to identify specifically what influence a recipient nude mouse’s hormonal environment, different age ranges of donor testis and durations of grafts for spermatogenic cycles had on the productivity of spermatogenesis from murine testis grafts. We hypothesized that castrated male recipients would produce the largest grafts and highest percentage of seminiferous tubules containing elongated spermatids of all observed recipients. Also that ovariectomized females would display a similar efficiency in testicular graft production. In addition, testis tissue from 0-5 days old and grafting periods sustained for 70 days on a recipient would obtain the highest rates of testicular growth and the greatest percentage of tubules with complete spermatogenesis.

**Materials and Methods**

*Experimental Design*

Neonatal testis tissue was obtained from three different age ranges (0-5 days old, 6-10 days old and 11-15 days old) of NCr heterozygous nude mice (nu-/nu+); (Taconic Laboratories, Germantown, NY). Tissue was then placed on the backs of immuno-compromised nude mice (nu+/nu+); (Taconic Laboratories, Germantown, NY). Recipient mice consisted of four groups: intact males, intact females, castrated males and ovariectomized female nude mice of at least 6 weeks of age. Each donor mouse group consisted of a total of five mice per donor age range of testis tissue. Each recipient mouse received four testicular grafts placed subcutaneously, two per side of the dorsal midline. A whole testis was counted as one graft from the 0-5 day old range whereas tissue older
than 6 days old was cut in half equating to two grafts for the experiment. Grafts were left on recipient mice for either 35 or 70 days at which point they were removed for histological and gene expression analysis.

*Acquiring and Placement of Donor Testis*

To retrieve donor testis from neonatal mice, heterozygous pups from nude breeder pairs were utilized. The Animal Care and Use Committee at Washington State University approved all experimental procedures (protocol #3163). Breeding pairs were monitored daily and age of the pups was designated 0 days old the first afternoon in which they were observed in the cage, after which sequential days progressed up to 15 days old. Donor mice were sacrificed and a small incision was made in the genital ridge in close proximity to the femur and os coxae that exposed the small fat pad associated with the testis. The gonad was removed and then placed in ice cold 1X Hanks Buffer Solution. All testes were detunicated by delicately pulling off the tissue with forceps to expose interstitial tissue to the recipient blood supply. For samples older than 6 days old, the testis was cut in half to graft similar amounts of testis tissue onto the recipient animal. Tissue samples did not exceed a period of 90 minutes in Hanks Buffer Solution before being grafted. For the placement of gonads on recipient animals, an intraperitoneal injection of 0.1 mg/kg ketamine and 0.05 mg/kg xylazine (Fort Dodge Animal Health, Fort Dodge, IA) was given. Small incisions were made, two per side of the dorsal midline that included small cuts to the muscle layer to ensure a blood supply to the tissue. Testis
tissue was then placed under the skin and the skin sutured closed. Animals were allowed to recover from the procedure under a heated lamp to prevent hypothermia.

**Gonadectomy of Recipients**

For castration of males, an incision was made on the abdomen through both the skin and peritoneal cavity. The fat pads associated with the testis were pulled out to allow for the removal of the gonad. The testicular artery was tied off to prevent excessive internal bleeding at the time of removal of the testes. The gonads were then removed, fat pads replaced and the peritoneal cavity as well as the outer skin layer were individually sutured.

In the case of ovariectomies of females, an incision was made along the back closer to the rib cage of the animal. Connective tissue between the peritoneal cavity and skin was separated to allow the incision to be maneuvered from one side to the other of the mouse. Upon encountering an area approximately halfway down the side of the female, the peritoneum was cut to expose the ovary. Forceps were used to remove the ovarian bursa so that the ovary could be extruded. Exposed tissue was placed back into the internal fossa and the other ovary of the animal was removed by the same procedure. All animals were gonadecotomized at the time of receiving testicular grafts.

**Removal of Tissue**

At the end of the grafting period, mice were sacrificed by administration of carbon dioxide, and cervical dislocation. Grafts were then removed from the backs of the mice by removing the outer layer of skin and gently teasing away testicular tissue that
was then either put in Bouin’s fixative for histology analysis or Trizol reagent for mRNA analysis. Seminal vesicles were removed and weighed from male recipients as a bioassay for testosterone production.

**Histological Preparation and Analysis**

Testis grafts were placed in Bouin’s fixative for 4-6 hours at 4° C. After this time period samples were washed two to three times with 75% ethanol (EtOH) until prepared for blocking. The grafts were then dehydrated twice for at least 4 hours sequentially in 95% and 100% EtOH and cleared in xylene. Then, samples were infiltered in Kendall’s Paraplast Plus Tissue Embedding Medium at 65° C. The following day tissue samples were placed in blocks by using histology cartridges and placed at 4° C. Blocks were sectioned at 8 μm (5 sections/strip) and placed in a warm water bath of 40° C and affixed to glass slides treated with Mayer’s Fixative albumin (egg white, glycerol and salicylic acid). Slides were then placed on a 37° C heating block for 12 hours. Slides were cleared in xylene, and rehydrated in 100%, 95%, 70%, 50% and 30% EtOH followed by tap water. Slides were then stained in hematoxylin for 3 minutes, and then dehydrated back up to xylenes from 70% EtOH. Cover slips were placed on the slides with a 1:1 Permount to xylene solution and allowed to dry before analysis.

Progression of spermatogenesis was based on the furthest differentiation of germ cells present in individual cross-sections of tubules. Tubules containing pre-meiotic, meiotic germ cells, or elongated spermatids were counted. The most advanced germ cell present in a tubule labeled that tubule in one of the three categories. Slides were examined under 200X power using brightfield microscopy.
**Gene expression Analysis**

Testis grafts were also examined for similar gene expression to that of control male testis. At the time of removal from the recipient, a minimum of one testis graft was put into 500μl of ice-cold Trizol reagent (Sigma-Aldrich Co., St. Louis, MO). Samples were immediately homogenized using a Polytron electric homogenizer and then stored at –20° C. For the extraction of mRNA, samples were thawed and then 100μl of chloroform was added to the Trizol/mRNA mixture. The contents were shaken vigorously for one minute and allowed to sit at room temperature for 3 minutes. Samples were then centrifuged for 15 minutes at 10,000 x g at 4° C. The upper aqueous layer was removed and transferred to a new centrifuge tube, 250μl of isopropanol was added, contents gently inverted several times and stored overnight at –20° C to allow for RNA precipitation. Samples were then centrifuged for 10 minutes at 10,000 x g at 4° C to pellet RNA. The supernatant layer was removed, discarded, and 500μl of 75% EtOH was added to wash the RNA pellet. The centrifuge tube was then spun for 5 minutes at 5,000 x g at 4° C. The aqueous layer was again removed and the pellet was left out for 12-15 minutes to allow all of the ethanol to evaporate. Pellets were then rehydrated with 20μl of Ambion RNase free water and stored at –80° C. Concentration and purity of mRNA was determined with a spectrophotometer at 260/280nm and also samples were electrophoresed on a 1.0% agarose gel with Gel Star staining (Cambrex Bio Science Rockland, Inc., Rockland, ME) for visual images of the 18S and 28S mRNA bands (Coolsnap Pro, MediaCybernetics, Carlsbad, CA).

The mRNA was converted to cDNA by combining 2μg of total RNA and 1μl of 0.5 μg/μl oligo dT primer (Invitrogen, Carlsbad, CA) with RNase free water for a total
volume of 12μl. These tubes were then incubated at 70° C for 10 minutes. cDNA was synthesized by adding a mixture of 5X Reaction buffer (Invitrogen, Carlsbad, CA), 0.1M dithiothreitol (DTT) (Invitrogen, Carlsbad, CA), 10mM dNTPs (Invitrogen, Carlsbad, CA), and 200U/μl Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen, Carlsbad, CA). Samples were incubated at 37° C for 60 minutes followed by 95° for 5 minutes. cDNA was then tested for integrity by polymerized chain reaction (PCR) analysis using primers for β-actin. For this procedure a reaction volume of 25μl consisted of 10X Buffer (Promega, Madison, WI), 25mM MgCl₂ (Promega, Madison, WI), 10mM dNTP (Invitrogen, Carlsbad, CA), 20pM 3’ β actin primer, 20pM 5’ β actin primer, 5U/μl Taq DNA polymerase (Promega, Madison, WI), and double distilled water. PCR reactions were at 95° C for 5 minutes and 30 cycles of 95° for 30 seconds, 60° for 30 seconds, and then 72° C for 30 seconds followed by one cycle at 72° C for 7 minutes. A 10μl aliquot of PCR reaction plus 2μl of loading buffer was electrophoresed on a 0.9% agarose gel with Gel Star staining (Cambrex Bio Science Rockland, Inc., Rockland, ME) to assess production of β-actin amplimers by PCR (Coolsnap Pro, MediaCybernetics, Carlsbad, CA). After confirmation, the stock cDNA was then used to test for the expression of 17-β hydroxysteroid dehydrogenase (17-β HSD) and follicle-stimulating hormone receptor (FSHr) with the same PCR protocol used for β-actin only differing in using the respective primers. Primer sequences were as follows; 5’ – CGTCCAAAGCAGGAGTGATT-3’, 5’- TGGGTTCTGTTTTTCCTGTCC-3’ (17-β HSD), 5’- CATCACTGGGAACACCACAG-3’, 5’- AGTTGCATGGCATGTGTGAT-3’ (FSHr).
**Testosterone Radioimmunoassay**

Blood samples were taken from each mouse and stored at –20 degrees C. Samples were then thawed and 100μl of serum was added to DSL-4000 testosterone assay kits (Diagnostic System Laboratories, Webster, TX) to measure steroid (ng/ml) levels in circulation. Standards for the testosterone assay were 0.10, 0.50, 2.50, 10.00, and 25.00 (ng/ml).

**Statistical Analysis**

Statistical analysis was done for graft weights, testosterone, elongate tubule percentage, and premeiotic tubule percentage using the Proc-GLM procedure of SAS utilizing a completely randomized design three-way treatment structure with alpha equivalent to 0.05.

**Results**

Four groups of mice were used as recipients for testis tissue which were castrated males, ovariectomized females, intact males and intact females. In addition, three age groups of 0-5 days old, 6-10 days old and 11-15 days old were used for donor tissue and two graft durations of 35 and 70 days were observed. The ability of recipient animals to support spermatogenesis and for donor testis tissue and duration to initiate spermatogenesis was evaluated based on graft growth potential and degree of spermatogenesis within grafted tissue.
Donor testis graft weights

Effect of graft duration on graft weight

Graft weight was analyzed for respective growth potential according to the two durations in the experiment of 35 and 70 days. Overall, the duration of 70 days produced a mean graft weight of 15.42mg ± 2.82 which was significantly higher than the 35 day duration mean graft weight of 12.06mg ± 0.84. The data was then pooled to examine the effect of duration on graft weights between recipient animal hormonal environments. The heaviest average graft weight between recipient animals and among durations was that from castrated males for 70 days. These castrated males also produced grafts that were significantly heavier than any other recipient group at 70 days (Fig. 1). For the 35-day duration, both male and female gonadectomized recipients had the heaviest graft weights which were also significantly heavier than that of intact female recipients (P<0.05) (Fig. 2).

Effect of donor age on graft weight

To measure the growth of testicular grafts placed on the backs of recipient nude mice, the weights of grafts at time of removal were analyzed. Overall, testicular tissue grafts from 0-5 day old pups produced a mean graft weight of 17.20mg ± 1.14 which was significantly higher than the mean graft weights of 6-10 day old and 11-15 day old tissue which were 11.43mg ± 1.14 and 12.60mg ± 1.16 respectively. The data was then pooled to examine the effect of age range donor tissue on graft weight between recipient animal
Figure 1. Measurement of testicular graft weights between recipients for the effect of a duration of 70 days. Analysis was done independent of donor tissue age. Columns with different letters are significantly different (p<0.05). Ovx = ovariectomized.
Figure 2. Measurement of testicular graft weights between recipients for the effect of a duration of 35 days. Analysis was done independent of donor tissue age. Columns with different letters are significantly different (p<0.05). Ovx = ovariectomized.
hormonal environments. In the 0-5 day old age range gonadectomized animals had significantly heavier (P<0.05) graft weights than those of intact animals (Fig. 3). The donor group at the age of 6-10 days old produced overall graft weights that were the lowest for donor age group and intact males contained grafts that were statistically similar to gonadectomized animals (Fig. 4). At the donor tissue age range of 11-15 days old, castrated males produced significantly heavier graft weights than any other recipient (P<0.05) (Fig. 5). In every age range intact females produced the lightest graft weights (Fig. 3, Fig. 4, Fig. 5). In all 70 day duration donor tissue range recipients, there was at least one observation of a tubule that displayed complete spermatogenesis, and as a whole, tissues displayed varying levels of efficiency in spermatogenesis (Fig. 6, 7, 8).

*Testosterone levels of recipient animals*

Blood samples retrieved at the time of graft removal were examined to detect testosterone hormone levels in the recipients. Data was pooled for analysis and displayed intact males to have the highest testosterone levels (ng/ml) which were statistically similar to castrated males. Females produced lower testosterone than that of males as expected, but ovariectomized female testosterone levels were statistically similar to castrated males (P<0.05) (Fig. 9).
Figure 3. Measurement of testicular graft weights between recipients for donor age tissue of 0-5 dpp. Analysis was done independent of duration. Columns with different letters are significantly different (p<0.05). Ovx = ovariectomized.
Figure 4. Measurement of testicular graft weights between recipients for donor age tissue of 6-10 dpp. Analysis was done independent of duration. Columns with different letters are significantly different (p<0.05). Ovx = ovariectomized.
Figure 5. Measurement of testicular graft weights between recipients for donor age tissue of 11-15 dpp. Analysis was done independent of duration. Columns with different letters are significantly different (p<0.05). Ovx = ovariectomized.
Figure 6. Photomicrographs of cross sections of hemotoxylin stained testicular grafts. Testis grafts were from 0-5 dpp donors and were left on the recipient for a duration of 70 days. Fig. A) Intact male recipient; B) Castrated male recipient; C) Day 3 control testis; D) Intact female recipient; E) Ovariectomized female recipient; F) Day 82 control testis. = 100 µm.
Figure 7. Photomicrographs of cross sections of hemotoxylin stained testicular grafts. Testis grafts were from 6-10 dpp donors and were left on the recipient for a duration of 70 days. Fig. A) Intact male recipient; B) Castrated male recipient; C) Day 7 control testis; D) Intact female recipient; E) Ovariectomized female recipient; F) Day 82 control testis. $= 100 \mu m$. 
Figure 8. Photomicrographs of cross sections of hemotoxylin stained testicular grafts. Testis grafts were from 11-15 dpp donors and were left on the recipient for a duration of 70 days. Fig. A) Intact male recipient; B) Castrated male recipient; C) Day 13 control testis; D) Intact female recipient; E) Ovariectomized female recipient; F) Day 82 control testis. = 100 μm.
Figure 9. Measurement of serum testosterone in recipient animals. Analysis was done independent of duration and donor tissue age. Columns with different letters are significantly different (p<0.05). Ovx = ovariectomized.
Graft Spermatogenesis Analysis

Analysis of Tubules Containing Elongate Spermatids

The definitive measurement of the success of the graft was the visual presence of elongate spermatids within the grafts. Cross sections of seminiferous tubules were evaluated individually based upon the extent of spermatogenesis that occurred. In this analysis, since there was no interaction between donor tissue age, data was pooled to examine differences in recipients among the duration of grafting periods. Of the three categories for the presence of pre-meiotic, meiotic and elongate spermatids within tubules, castrated males of 70 days produced the highest percentage of elongate spermatids. This percentage was significantly higher than any other group except for intact males of 70 days (P<0.05). Ovariectomized females at 70 days also had tubules that produced elongate spermatids that were statistically similar in number to that of intact males at 70 days. In each respective recipient hormonal environment 70-day durations produced a higher percentage of tubules containing elongate spermatids than did durations of 35 days (Fig. 10).

Analysis of Tubules Containing only Premeiotic Cells

Overall there was no interaction between donor tissue age or duration, so data was pooled to examine differences in recipients among their respective durations. In each type
Figure 10. Analysis of recipient animal and duration effect on the presence of elongated spermatids within seminiferous tubules of grafted tissue. Statistics were done independent of donor tissue age. Columns with different letters are significantly different (p<0.05). Ovx = ovariectomized.
Figure 11. Analysis of recipient animal and duration effect on the lack of spermatogenesis occurring within seminiferous tubules of grafted tissue. Statistics were done independent of donor tissue age. Columns with different letters are significantly different (p<0.05). Ovx = ovariectomized.
Figure 12. Photomicrographs of cross sections of hemotoxylin stained testicular grafts at 100X (A, B, D, E) and 200X (C and F) magnification for premeiotic cells. Grafts were from 6-10 dpp donor testis tissue and were left on the recipient for a duration of 70 days. Fig. A) Intact male recipient; B) Castrated male recipient; C) Day 7 control testis; D) Intact female recipient; E) Ovariectomized female recipient; F) Day 82 control testis. \( \text{bar} = 100 \mu m \).
Figure 13. Photomicrographs of cross sections of testis tissue for observation of interstitial space in testis grafts. A) Normal appearing interstitial space {Intact male recipient (70 day duration) 11-15 donor age}; B) Muscle invasion of interstitial space {Intact female recipient (70 day duration) 6-10 donor age}; C) Expanded interstitial space {Intact female recipient (70 day duration) 0-5 donor age}; D) Control male testis at 82 days of age with normal interstitial space.

= 100 μm
Figure 14. 0.9% Agarose gel for detection of testis gene expression of germ cells within donor testis tissue of 0-5 dpp for a 70 day duration. Lane 1: 100-base pair DNA ladder; Lanes 2-5: Detection of Follicle Stimulating Hormone receptor (FSHr) of each observed recipient; Lane 6: skipped; Lanes 7-10: Detection of 17-β Hydroxysteroid Dehydrogenase (HSD) of each observed recipient; Lane 11: skipped; Lanes 12-15: Detection of β-actin (BAct) of each observed recipient; Lane 16: skipped; Lane 17: FSHr (negative control); Lane 18: HSD (negative control); Lane 19: BAct (negative control).
of recipient there was a tendency of increased tubules with only premeiotic germ cells in
70-day graft durations versus 35-day graft durations. The only recipient group that
differed from this trend was that of castrated males such that graft durations of 70 days
displayed a significantly lower amount of premeiotic tubules than those of 35-day
durations (P<0.05). Grafts from castrated recipients at 70 day duration grafts also
displayed the lowest overall number of premeiotic tubules observed while ovariectomized
female recipients of 70 day grafts displayed the highest number of premeiotic tubules
among all observed recipients (Fig. 11). It was commonly observed that tubules unable to
conduct complete spermatogenesis were either closely associated with few tubules (Fig.
12) or subjected to muscle invasion or abnormal interstitial expansion (Fig. 13).

Gene Expression Analysis

To confirm that active spermatogenesis was occurring within the grafts, gene
expression of specific gene products indicating somatic cell function was done. One
sample from each recipient treatment group of 70 day duration and 0-5 day old donor
tissue was taken and analyzed for the presence of genes of expressed by somatic cells.
The expression of FSHr was present in all graft recipients examined and 17-
β Hydroxysteroid Dehydrogenase was present in all but the intact female graft recipients
(Fig. 14).

Discussion

This study conveys a number of approaches in the area of germ cell preservation.
Mice, regardless of sex or presence of gonads, were shown to be able to support various
levels of spermatogenesis in donor grafted testicular tissue. Testis tissue obtained from numerous neonatal animals was able to sustain the ability to produce sperm following transfer to an extra-testicular location on an adult recipient. This is important in that it provides more information in the durability of spermatogonial stem cells in trauma-like situations to be able to resume spermatogenesis for the production of germ cells capable of fertilizing oocytes.

With the knowledge of successful grafts of testicular tissue, there were also some results potentially advantageous for testicular grafting. The age of the donor animal testis tissue was examined closely to understand the influence of spermatogenic progress of the graft by recipient animal endocrinology. The age donor tissue of 0-5 days old produced the largest graft weights among all three observed groups. Though graft weight increase can be due to an accumulation of seminiferous tubule fluid thereby relocating germ cells from the Sertoli cells into the lumen [3], it here has been shown to be correlated to increased spermatogenesis. The younger age donor testis tissue may progress better due to the fact that at days 0-5 there is minimal spermatogenesis that is occurring within the murine testis allowing for initiation of spermatogenesis to occur on the recipient’s back. The lowest observed mean graft weight at removal for donor tissue among the three groups was that of 6-10 days old, which corresponds with the beginning of meiotic activity within murine testes at about day 10 post partum. Since this age of tissue produced a lower level of spermatogenesis, this may indicate that there are essential processes that occur in this time frame that once disturbed are extremely detrimental to spermatogenesis and therefore graft production.
Another variable in previous grafting experiments has been the length of time to leave a graft on a recipient animal before time becomes harmful to sperm production. For the duration of 35 days there was no significant difference between the graft weights of the gonadectomized recipients or intact male recipients, though gonadectomized recipient animals produced heavier weights than intact animals. However, when these grafts were sustained for 70 days, technically two rounds of spermatogenesis in mice, it was found that a duration of 70 days for grafts produced heavier graft weights than 35 day graft durations with castrated males supporting the largest graft weight increase than any other recipient. This difference in graft production may explain that there is a need for the graft to become acclimated with the recipient animal to successfully conduct sperm production. The change of gonadectomized animals producing statistically similar graft weights to intact males at 35 days to separating themselves from both intact male and female recipients at 70 days, conveys the a possible importance of efficiency compensating for gonad removal with time intervals for grafts. Turner [3] alluded to this in his study with grafts in the anterior chamber of the eye of rats that there may be some benefit of time of grafting with respect to removal of gonads to increase the potency of hormones from the pituitary [3], but the decrease in testosterone levels and therefore increase in luteinizing hormone secretion occurred almost immediately in observed control mice.

Though the experimental analysis of donor age on grafting success was very informative, the results may have been influenced towards the younger donor tissue 0-5 days of age because of its closer proximity to the end of spermatogenic cycles at the 35 and 70 day durations. It is important to note, that the first spermatogenic cycle is more
accelerated with respect to future cycles and that the other donor tissue ranges were further along in development. The other donor ages were theoretically 6 to 15 days further along in initiation of the first round of spermatogenesis and therefore may have been less productive in spermatogenesis due to the fact that this testicular development needed to be compensated for by removing them at a respective time frame that would mimic that of the 0-5 day donor testis tissue.

Since testosterone is important in the conduction of spermatogenesis in the testis, seminal vesicles as well as serum hormone levels were measured. Castrated males were able to obtain statistically similar seminal vesicle weights to that of intact males after a duration of 70d (See Appendix: Table 1). As expected in the serum analysis, intact males displayed the highest amount of testosterone in circulation, but an interesting finding was that the testosterone levels of castrated male recipients and ovariectomized female recipients were statistically similar (P<0.05). This may partially explain why ovariectomized females were able to produce elongate spermatids in a similar fashion to castrated males in some instances. In addition, the extremely low testosterone level of intact females may have been the cause of their low production of elongate spermatids. Turner [3] reported this inability of intact females to produce successful grafts and hypothesized that it was due to testosterone from these grafts being produced at a sub-threshold level or direct inhibition by ovarian hormones that rendered the grafts unable to respond to testosterone [3].

Evaluation of these grafts for production of spermatids that can be used for assisted reproduction methods and study of efficiency in spermatogenesis was done by analyzing seminiferous tubules [9]. All 70 day durations in each respective group
produced more elongate spermatids than did those of 35 days durations and castrated males did the best of all recipients while ovariectomized recipients did similar to intact males. The removal of gonads seems to have allowed for exclusivity of gonadotropic hormones to the grafts and aid in the production of elongate spermatids. The production from intact males with respect to ovariectomized females may be due to the fact that testosterone from the male naturally supports the production of sperm versus the female where there may have to be a conversion of biological activity such as the pre-ovulatory center in the brain to mimic a hormonal situation to that of a male. In examination of elongate spermatids, all previous findings from different points of analysis were comprised to present logical conclusions of recipient efficiency in graft production. These results convey the male as being the preferred animal of choice to produce advanced germ cells within testicular grafts, but also prove that ovariectomized females can produce a similarly receptive environment for testis grafts. It was also expected that intact females would clearly display the lowest percentage of elongate spermatid tubules since Turner [3] found that degenerative tissue occurred in intact females in which the graft persisted for 60 days or more.

Not only were tubules that contained elongate spermatids measured but the percentage of tubules that contained only premeiotic germ cells were tabulated as well. This was an important observation to see how many tubules did not initiate or resume meiosis. This again was representative of the previous findings of this study in that castrated males had the lowest percentage of premeiotic tubules, thereby indicating that the majority of tubules in the grafts were at least conducting meiosis. Also contrary to note, is that the castrated male was the only recipient group to have a lower percentage of
tubules that contained premeiotic germ cells at 70 days versus 35 days. These recipients could differ from the female recipients due to the above mentioned differences in male and female gonadotropic hormonal environment and differ from intact males possibly because of the adjustment to the removal of the testis, therefore making it more efficient over time. Another interesting finding was that ovariectomized females with graft durations of 70 days had the highest percentage of premeiotic tubules which was marginally higher than intact females of the same graft duration. Ovariectomized females were respectively the second best in graft weights in almost all categories examined, but once again some contributors to this occurrence may be due to pressure of fluids as a contributing factor for atrophy of seminiferous tubules, or also Turner’s finding that normal females are liable to invasion of their donor tissue, even though the female pituitary is sufficient to sustain mitotic and meiotic processes if it has become fibrotic, complete germ cell differentiation becomes difficult [3]. This point was evident in that abnormal interstitial space in grafts whether expanded or invaded by muscle, caused proximal tubules to have minimal spermatogenic processes.

With the completion of this study, it was found that the graft duration of 70 days, donor age tissue of 0-5 days old and gonadectomized animals were the preferred options for grafting procedures. Also among these observed parameters, it was noted that individual observed groups of castrated males with 0-5 day old graft tissue sustained for 70 days, castrated males with 11-15 day old graft tissue sustained for 70 days and ovariectomized females with 0-5 day old graft tissue sustained for 70 days emerged with the best overall results (See Appendix: Table 2) for spermatogenesis from these grafts.
Implications

This study has provided more insight into the beneficial parameters to utilize in maximizing the productivity of testicular grafts. Castrated males have been used predominately in testicular grafting procedures, but this study shows that ovariectomized females also provide a valid option as carriers for tissue samples. In addition, the finding of specific beneficial and detrimental age ranges of murine tissue for production of elongate spermatids may suggest that there may be similar time points to consider in other species to obtain optimal spermatogenesis. Duration of the grafts was also found to be an important factor in sperm production, in that some studies were concerned about long periods of grafts on recipients. With the experiments that were sustained for two rounds of spermatogenesis producing more sperm than those sustained for only one round of spermatogenesis, recipient environment adjustment may be a critical factor in graft success. Further studies will need to be done to confirm where a peak of sperm production occurs in the mouse with respect to duration of grafts. This study provides a good building block for efficiency modifications to current grafting protocols.
Bibliography


6. De Rooij DG and Russell LD. All you wanted to know about spermatogenesis but were afraid to ask. Journal of Andrology 2000; 21:776-798.


APPENDIX

EXPERIMENTAL ORGAN AVERAGES w/ ± Errors

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<th>Sv wt</th>
<th>Ut wt</th>
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<td>143.72 ± 30.32</td>
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Table 1. Analysis of individual experimental units accounting for all observed parameters (recipient hormonal environment, donor tissue age and duration of graft). Different letters indicate significant differences (P<0.05). No statistics were done on uterus weight. F = female, M = male, (I) = intact, (Cas) = castrated, (Ovx) = ovariectomized. Number immediately following ( ) indicates the graft duration and the next numbers seperated by the hyphen are the age range of the donor tissue. Ut weight = uterus weight. Sv wt = seminal vesicle weight.
# EXPERIMENTAL PARAMETER AVERAGES w/ ± Errors

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<th>% Elongates</th>
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<th>Graft weight (mg)</th>
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<th>% Elongates</th>
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<th>Graft weight (mg)</th>
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Table 2. Analysis of individual experimental units accounting for all observed parameters (recipient hormonal environment, donor tissue age and duration of graft). Different letters indicate significant differences (P<0.05). F = female, M = male, (I) = intact, (Cas) = castrated, (Ovx) = ovariectomized. Number immediately following ( ) indicates the graft duration and the next numbers separated by the hypen are the age range of the donor tissue. Testost = testosterone.