Changes in spermatogenesis and endocrine function in the ram testis due to irradiation and active immunization against luteinizing hormone-releasing hormone\textsuperscript{1,2}

J. M. Oatley\textsuperscript{*3}, A. Tibary†, D. M. de Avila*, J. E. Wheaton‡, D. J. McLean*, and J. J. Reeves*

*Department of Animal Sciences, †College of Veterinary Medicine, and Center for Reproductive Biology, Washington State University, Pullman 99164; and ‡Department of Animal Sciences, University of Minnesota, St. Paul 55108

ABSTRACT: Spermatogonial stem cell transplantation is a technique that has potential in livestock to enhance genetic gain and generate transgenic offspring through the male germ line. A means for depletion of endogenous germ cells in a recipient’s seminiferous tubules is necessary for this technology to be applied. The objectives of this study were to evaluate several methods for depletion of endogenous germ cells in the testes of adult rams and to evaluate ultrasound-guided injections into the rete testes as a means for infusing a suspension into the seminiferous tubules. Sixteen adult rams were randomly divided into 4 treatment groups (n = 4 per group). Treatments consisted of active immunization against LHRH (IMM), localized testicular irradiation (IR), LHRH immunization + irradiation (IMM+IR), and untreated control. Serial bleedings were conducted pretreatment and monthly after treatment for 4 mo, at which time all rams were castrated. Rams receiving IMM and IMM+IR treatments had higher (\(P < 0.05\)) average percentages of seminiferous tubule cross sections with depleted germ cells compared with controls. Serum testosterone was decreased (\(P < 0.05\)) in IMM and IMM+IR rams 1 mo after treatment and throughout the remainder of the study compared with controls and IR rams, which were not different from each other. Serum inhibin concentration was unchanged in all rams following treatment indicating that Sertoli cell function was unaltered. A greater (\(P < 0.05\)) average percentage of the total testicular area could be filled with the trypan blue solution by rete testis injection in IMM and IMM+IR rams. These data demonstrate the depletion of endogenous germ cells in adult ram testes without alteration of Sertoli cell viability and function that have potential as methods for preparing recipient animals for germ cell transplantation.

Key Words: Depletion, Germ Cell, Ram

Introduction

The continual process of spermatogenesis relies on spermatogonial stem cells. In rodents and goats, transplantation of these cells from the testis of a donor male into the seminiferous tubules of an azoospermic recipient has been shown to result in donor-derived spermatogenesis and generation of offspring with the donor genotype (Brinster and Avarbock, 1994; Brinster and Zimmerman, 1994; Honamarooz et al., 2003a). Spermatogonial stem cell transplantation technology has potential utility in livestock as an alternative means to enhance genetic gain and generate transgenic offspring.

Two main concepts must be developed in large animals for this technique to be efficiently applied. First, a means for preparing recipient animals for transplant is necessary. In goats, recipients were sexually immature and only one of several transfers was successful. A system that destroys or depletes endogenous germ
cells in recipient testes before transfer would allow for efficient colonization. In rodents, this has been accomplished using chemotoxic drugs or whole testis irradiation (Ogawa et al., 1997). In large animals, such as bulls, treatment with chemotoxic drugs is not practical.

Secondly, a means for infusing donor spermatogonial stem cells into recipient seminiferous tubules is obligatory. Microinjection into the efferent ductules, rete testis, or seminiferous tubules results in stem cell colonization in rodents (Ogawa et al., 1997). Due to the testicular architecture of most domestic animals, injection into the efferent ductules or seminiferous tubules is not plausible. Ultrasound guided injections into the rete testis of primates, pigs, and goats have shown to be a promising approach to overcoming this obstacle (Honaramooz et al., 2002a,b). The objectives of this study were to evaluate several methods for depletion of endogenous germ cells in the testes of adult rams and evaluate ultrasound-guided injections into the rete testes as a means for infusing a suspension into the seminiferous tubules.

Materials and Methods

Experimental Rams and Treatments

The Washington State University Animal Care and Use Committee approved all animal procedures. Sixteen adult rams ranging in breed from Suffolk, Colombia, and Karakul were divided randomly into four treatment groups (n = 4 per group): 1) LHRH immunized (IMM), 2) irradiated (IR), 3) LHRH immunized and irradiated (IMM+IR), and 4) control. The timeline for experimentation is depicted in Figure 1.

Eight rams (IMM and IMM+IR) received active immunization against LHRH by administration of a fusion protein cocktail (Quesnell et al., 2000). The treatment regimen consisted of a primary immunization using a modified Freund's complete adjuvant followed by two booster injections in Freund's incomplete adjuvant 1 mo apart each. Approximately 2 mo before castration, immunized rams began to receive a weekly treatment of exogenous gonadotropin in the form of 3,000 IU of Perganol (Serono, Rockland, MA) in sterile PBS by s.c. injection to compensate for the effects of the vaccine. The dose and frequency of administration was chosen based on the half-life of the hormone.

Eight rams were subjected to 12 grays of irradiation localized to the testes, which was the dose of radiation absorbed by the animal. One gray deposits 1 joule of energy into a kilogram of tissue and has been shown to cause sterility in humans. Four of the rams had been immunized against LHRH several months before, and four rams received only irradiation. Four rams also were used as untreated controls.

Serial Blood Sampling and Serum Analysis

To evaluate changes in the pituitary-gonadal axis due to treatment, serial blood samples were collected 1 mo before any treatment and monthly for 4 mo after treatment. The bleeding regimen consisted of jugular venipuncture every 15 min for 4 h. Serum was then collected by centrifugation at 3,000 $\times$ g for 20 min. All serum samples were assayed for both LH and FSH concentrations using double-antibody RIA (Acosta et al., 1983). Serum also was evaluated for circulating concentrations of $\alpha$-inhibin using a sandwich ELISA configured against the $\alpha$-subunit of inhibin (Bailey et al., 2002) and testosterone using a commercial RIA kit (DSL-400, Diagnostic Systems Laboratory, Inc., Webster, TX). The inhibin $\alpha$-subunit is the main form of the hormone produced by Sertoli cells in rams (McNeil et al., 2002); thus, measuring this hormone concentration provides a means for evaluating Sertoli cell function. Due to the pulsatile nature of testosterone, four consecutive samples from each 4-h bleeding were analyzed, and the averages of these were used as a representation of each 4-h bleeding. Inhibin concentration was analyzed in one sample from each bleeding.

Testicular Diameter, Castration, and Histological Analysis

Changes in testicular size were evaluated by measuring the testicular diameter of each ram using ultrasonography 1 mo before treatment and monthly thereafter for 4 mo. Testis diameter was not different based on breed of the animal before treatment (data not shown). Approximately 4 mo after treatment, which constituted two spermatogenic cycles, all rams were castrated using standard procedures and random sections of testicular parenchyma were collected in Bouin’s solution from one...
testis of each animal. Samples were fixed for 4 h at 4°C followed by dehydration in ethanol and xylene, and embedded in paraffin. Cross sections from each sample were cut at 8 μm and affixed to glass slides, followed by deparaffinization, rehydration, and staining with hematoxylin and eosin. Processed samples were evaluated using light microscopy at 100× magnification and digital images were captured with a cf Cool Snap digital camera (Media Cybernetics, Silver Spring, MD). Evaluation of histology samples included assessment of the average percentage of regressed seminiferous tubules and tubules containing active spermatogenesis. Average percentages were calculated for each sample by dividing the number of round tubules with depleted germ cells in three microscopic fields by the total number of round tubules in the same three fields.

Ultrasound-Guided Rete Testis Injection

Just before castration the testis not used for histology was infused with a 0.4% trypan blue/PBS solution using ultrasound-guided injection into the rete testis. Briefly, rams were positioned in dorsal recumbence in a surgery cradle and given a local anesthetic injection of lidocaine at the base of the scrotum. A small incision was then made through the scrotum. The testis was viewed by ultrasonography (Aloka 500-7.5 MHz, Aloka, Inc., Tokyo, Japan) and a 20-gauge, 7.62-cm anesthesia needle was guided into the rete testis. Three milliliters of trypan blue/PBS solution was then infused into the rete testis over a 5-min period by positive pressure from a peristaltic pump (Harvard Apparatus, Holliston, MA). Immediately after injection, the testis was removed, split down the midline to expose the rete testis and surrounding testicular parenchyma, extensively washed with saline to remove trypan solution not infused into seminiferous tubules, and digital images were captured. Analysis of the percentage of area filled with trypan blue was conducted by evaluating the digital images with a software program (Scion image, Scion Corp., Frederick, MD).

Statistical Analyses

All data were analyzed using the Proc GLM function of SAS systems software (SAS Inst., Inc., Cary, NC). Differences between means for treatment values of serum hormone concentrations and testis diameters were compared to control values using repeated measures analysis with contrast transformation. This type of analysis utilized a multivariate ANOVA to determine differences between treatment and control means. Data for percentage of regressed tubules and percentage of area filled by rete testis injection were analyzed for differences between means using Duncan’s multiple range test for significance. Means were considered significantly different at $P < 0.05$.

Results

Effect of Treatment on Testicular Somatic Cell Endocrine Function

Serial blood samples were collected from all rams to evaluate changes in the pituitary testis axis in response to treatment. Serum testosterone concentration was measured to assess Leydig cell functionality. Sertoli cell functionality was evaluated by measuring serum inhibin concentration. The average concentrations of LH and FSH in all rams were not significantly different among groups before treatment (Figure 2). At 2 mo after treatment and throughout the remainder of the
Germ cell depletion in rams study, serum LH concentration in immunized animals (IMM and IMM+IR) decreased below the detection limit of the assay, but was not statistically different from control rams (Figure 2A). The lower detection limit value (0.3 ng/mL) was thus used to statistically compare the samples to control values. In contrast, serum FSH concentrations in immunized rams 1 mo after treatment and continuing throughout the remainder of the study decreased by approximately 56% of preimmunization levels and were less (P < 0.05) than controls (Figure 2B). In IR rams, serum LH concentrations were greater (P < 0.05) than controls at 1 mo after treatment but not different at 2, 3, or 4 mo (Figure 2A).

Serum testosterone concentration in all rams was not different before treatment (Figure 3A). In control and IR rams, serum testosterone concentrations dramatically increased (P < 0.05) as the animals entered the breeding season at the end of the study. In both LHRH-immunized groups, serum testosterone concentrations decreased to below the detection limit of the assay 1 mo after the final booster and throughout the remainder of the study and were less (P < 0.05) than those of the control group (Figure 3A). Serum testosterone concentration in the IR group and control group were not different throughout the entire treatment period (Figure 3A).

Serum inhibin concentrations were greater (P < 0.05) in all the immunized (IMM and IMM+IR) and IR rams than in controls before treatment (Figure 3B), but they were not different from each other. The average concentration of serum inhibin actually rose in IMM+IR rams and remained greater (P < 0.05) than controls throughout the treatment period. The average inhibin concentration in IMM rams remained relatively constant throughout the treatment period and was not different than controls by the end of the study. In IR rams, the average serum inhibin concentration increased throughout the treatment period and was greater (P < 0.05) than in controls throughout but not different than IMM+IR rams until the 4-mo blood sampling. Treatment of IMM and IMM+IR animals with Perganol had no effect on serum concentrations of any of the hormones evaluated.

**Effects of Treatment on Testicular Diameter**

The testicular diameter of all rams was measured to provide an evaluation of changes in active spermatogenesis caused by treatment. Testis diameter has been shown to be correlated with sperm output in many livestock species (Courot and Ortavant, 1981; Berndtson et al., 1987). One month before treatment, mean testis diameter was not different among groups (Figure 4). One month after treatment, IMM and IR rams had smaller (P < 0.05) testis diameters than controls. Rams receiving irradiation alone had a smaller (P < 0.05) testis diameter than controls until 3 mo after treatment, but by the time of castration, the average testis diameter of these rams had reached control sizes and were not different. All rams immunized against LHRH had smaller (P < 0.05) testis diameters than controls throughout the 4-mo period after treatment and were not different from each other. Treatment of immunized rams with Perganol had no effect on testis diameter.

**Effects of Treatment on Spermatogenesis**

Preparation of a recipient animal for germ cell transplantation requires that endogenous germ cells be depleted within the seminiferous tubules to allow donor spermatogonial stem cells to colonize. The effect of

![Figure 3. Average serum concentrations of testosterone (A) and inhibin (B) in rams subjected to different treatment regimens for depletion of endogenous germ cells. Testosterone concentration was analyzed for evaluation of Leydig cell function and viability. Inhibin concentration was determined for evaluation of Sertoli cell function and viability. In both panels, bars with an asterisk are different (P < 0.05) than controls. For both panels, Control = untreated animals, IR = animals receiving testicular irradiation, IMM = animals actively immunized against LHRH, and IMM+IR = animals receiving active immunization against LHRH and testicular irradiation. Perganol was given to all LHRH-immunized rams between the 3 and 4 mo bleeds. Data are presented as the mean ± SEM. PRE = sampling before treatment.](image)
Ultrasound-Guided Rete Testis Injection

To evaluate differences in the ability to infuse a liquid solution into the seminiferous tubules of ram testes, an ultrasound-guided injection into the rete testis was performed on one testis from each ram. As an evaluation of the utility of any of these treatments for preparing recipient animals for germ cell transplantation, the total filled area was measured as a percentage of the whole testis. After injection, dissection, and extensive washing, infused blue dye could be seen within the testicular parenchyma (Figure 7). The average area filled by the rete testis injection of the IMM and IMM+IR groups was greater ($P < 0.05$) than that of the control and IR groups (Figure 8). Approximately 53% of the testicular area could be filled with the rete testis injection in IMM and IMM+IR rams.

Discussion

In this study, we evaluated three different treatment regimens for their individual abilities to deplete germ cells in the seminiferous tubules of adult ram testes without causing harmful effects on testicular somatic cell function or viability.

Adult rams receiving localized testicular irradiation had a significant decrease in average testis diameter 1 and 2 mo after treatment compared with controls. This observation suggests a depletion of germ cells and loss of active spermatogenesis within the testes. Studies in livestock have demonstrated the correlation of testis size to active spermatogenesis (Courtot and Ortavant, 1981; Berndtson et al., 1987). Therefore, measuring testis diameter is an effective means for assessing loss of germ cells and active spermatogenesis following treatment. At 3 and 4 mo after treatment, average diameter was not different from controls, suggesting that germ cell depletion occurred after irradiation but slowly came back after 3 mo, thereby indicating that spermatogonial stem cells were not destroyed by treatment. Spermatogonial stem cell viability following testis irradiation has also been demonstrated in rodents (Meistrich et al., 1995; Hacker-Klom et al., 2000).

Histological evaluation of testes revealed a significant 19% increase in average percentage of regressed seminiferous tubule cross sections in IR rams compared with controls. Approximately 22% of seminiferous tubules in these rams were unable to reestablish spermatogenesis following two spermatogenic cycles after irradiation, suggesting that spermatogonial stem cells were destroyed in these tubules. Histological evaluation of the seminiferous epithelium in these rams occurred after their average testis diameter had regained values comparable to controls. Therefore, a higher percentage of tubules may have been regressed at earlier time points after treatment when testis diameter was significantly smaller than controls. A much higher percentage of germ cell-deficient tubules than that observed in irradiated rams 4 mo after treatment would be ideal in animals intended as recipients for germ cell transplantation. Evaluation of regressed seminiferous tubules should be conducted at time points earlier after treatment (i.e., 1 and 2 mo).

Rams that were actively immunized against LHRH had a significant decrease in testes diameter compared with controls beginning 1 mo after the final booster and remaining until the conclusion of the treatment period. Localized testicular irradiation of rams that had been previously immunized against LHRH did not have an additive effect on decreasing testicular diameter. These observations alone suggest that active immunization against LHRH results in loss of active spermatogenesis and depletion of germ cells. Previous studies have also shown the inhibitive effects of immunoneutralizing LHRH on spermatogenesis (Carelli et al., 1982; Awoniyi et al., 1989; Ladd et al., 1989). Treatment of immunized rams with Perganol in this study was unable to bypass...
Germ cell depletion in rams

Figure 5. Photomicrographs of cross sections of seminiferous tubules from rams subjected to different treatment regimens to deplete endogenous germ cells. Panel A = control ram testis with normal spermatogenesis occurring. Panel B = irradiated ram testis with tubules containing normal seminiferous epithelium and regressed tubules (arrow head). Panel C = LHRH-immunized ram testis containing germ cell depleted seminiferous tubules (arrow head). Panel D = LHRH immunized + irradiated ram testis containing germ cell depleted seminiferous tubules (arrow head). Bar = 100 μm for each panel.

Figure 6. Average percentage of regressed germ cell depleted seminiferous tubule cross sections in rams subjected to different treatments for depletion of endogenous germ cells. Control = untreated animals, IR = animals receiving testicular irradiation, IMM = animals actively immunized against LHRH, and IMM+IR = animals receiving active immunization against LHRH and testicular irradiation. Bars with different letters differ, $P < 0.05$. Data are presented as the mean ± SEM.

the immunization and stimulate an increase in testis diameter, suggesting that some of the spermatogonial stem cells were destroyed over the treatment period and could not reinitiate spermatogenesis in those seminiferous tubules.

Evaluation of the seminiferous epithelium in testes of rams immunized against LHRH showed significantly higher average percentages of tubules that were germ cell depleted. The IMM rams had a 33% average increase in regressed tubule cross sections compared to controls, whereas IMM+IR rams had an average 60% increase. Approximately 63% of the seminiferous tubule cross sections in IMM+IR were regressed. Those animals received localized testicular irradiation after the final LHRH vaccine booster. Therefore, mitotically active germ cells that were not depleted as a result of LHRH immunization were subjected to deleterious defects from irradiation and were possibly eliminated, resulting in a higher average percentage of regressed tubules than immunization alone. In both the immunized groups, some seminiferous tubules contained active spermatogenesis; however, the percentage was so low that the animals were considered infertile after electroejaculation and semen analysis (data not shown).
Evaluation of Leydig cell function in terms of testosterone production revealed no differences between IR and control rams. However, beginning 1 mo after the final booster, all rams immunized against LHRH had concentrations of serum testosterone that were significantly less than controls. This observation suggests that active immunization against LHRH effectively neutralized LH output by the anterior pituitary. This observation is in accordance with previous studies demonstrating the effects of LHRH immunization on testicular steroidogenesis in bulls (Oatley et al., 2001; Aissat et al., 2002). Even though the average serum LH concentration in both groups of immunized rams was approximately one-half of the concentration in controls, they were not statistically different at the 5% level. The low number of animals in each treatment group was most likely the reason for lack of statistical significance.

Treatment of the immunized rams with Perganol was unable to reestablish testosterone concentrations to control values. For a treatment to be effective as a
Germ cell depletion in rams 611

Figure 8. Average percentage of area filled by ultrasound-guided injection of trypan blue into the rete testis of testes from rams subjected to different treatments for depletion of endogenous germ cells. Control = untreated animals, IR = animals receiving testicular irradiation, IMM = animals actively immunized against LHRH, and IMM + IR = animals receiving active immunization against LHRH and testicular irradiation. Bars with different letters differ, P < 0.05. Data are presented as the mean ± SEM.

means for preparing recipient animals for germ cell transplantation, detrimental influences on testicular somatic cell viability and function must be avoided. Testicular irradiation does not seem to harm Leydig cell function in terms of ability to respond to LH and produce testosterone. The lack of response to exogenous gonadotropin in immunized rams suggests that Leydig cells in those animals were atrophied or unable to respond. A decline in Leydig cell number in the testes of adult rats following immunization against LHRH has been implicated (Duckett et al., 1997). Likewise, immunization of pigs against LHRH has been shown to result in reduced LH receptor in the testis and reduced ability of Leydig cells to produce testosterone on exposure to exogenous LH (Awoniyi et al., 1988). Another explanation for lack of a response to exogenous gonadotropin in this study may have been due to the dose of Perganol being too low. However, Oatley et al. (2001), using a different gonadotropin preparation demonstrated a re-establishment of testosterone concentration in LHRH immunized bulls given PMSG.

Even though active immunization against LHRH results in high percentages of tubules with germ cell depletion, Leydig cell function may be impaired. To serve as adequate recipient animals in germ cell transplantation, these animals may need to be supplemented with exogenous testosterone. It is also important to note that spermatogonial stem cell colonization following transplantation in mice has been shown to be enhanced in a low testosterone environment (Ogawa et al., 1998; Dobrinski et al., 2001). Therefore, active or passive immunization against LHRH may be beneficial as a means to prepare recipient animals in terms of initial donor stem cell colonization.

Viability and functionality of Sertoli cells following treatment, evaluated as the ability to produce inhibin, revealed no deleterious differences in treated animals compared with controls. Both treatment groups receiving testicular irradiation (IR and IMM + IR) had significantly higher serum inhibin concentrations compared with controls throughout the study. The IMM rams had significantly greater concentrations than controls initially but were not different by the end of the study. These data suggest that neither irradiation nor active immunization against LHRH has deleterious effects on Sertoli cell function.

In both of the LHRH immunized groups, serum inhibin concentrations did not significantly decrease following immunization. Serum FSH concentrations in these animals was decreased by approximately 56% following immunization compared with their preimmunization values. Because a primary regulator of inhibin is pituitary FSH, these data suggest that the decrease in FSH concentration following active immunization against LHRH is not low enough to significantly inhibit Sertoli cell function. In these same animals, testosterone concentration was significantly decreased compared with the preimmunization levels suggesting that LHRH immunization inhibited LH release from the pituitary. In fact, LH concentration in the serum was below the detection limit of the assay. Other studies have also shown differential effects on FSH and LH release following neutralization of LHRH, in which LH was completely inhibited, whereas FSH was not affected as severely (Culler and Negro-Vilar, 1986, 1987; Aissat et al., 2001).

Infusion of trypan blue into the rete testis of recipient rams demonstrated the ability to transfer a liquid solution into the testes of large domestic adult animals using ultrasound-guided injections. A significantly greater area could be filled with the solution in testes from rams immunized against LHRH than both control and irradiated rams. This observation suggests that treatment of rams by immunization against LHRH results in a testicular parenchyma mass with a greater percentage of seminiferous tubules devoid of germ cells that may allow for filling with a germ cell suspension. Previous studies have also shown the possibility of injecting a liquid solution into the seminiferous tubules of goats and pigs using ultrasound-guided injections into the rete testis (Honaramooz et al., 2002, 2003a,b). These studies used immature animals rather than adults and did not evaluate the effectiveness of the procedures based on the percentage of area filled.

Implications

Spermatogonial stem cell transplantation in livestock could provide a means to enhance genetic gain in domestic herds as well as generate transgenic offspring through the male germ cell line. For this technology to
be applied, a means for preparing recipient animals through depletion of endogenous germ cells within the seminiferous tubules that provides an environment allowing donor stem cells to seed must be developed. This method must be practical and cost-effective for the technology to be feasible. The treatment regimens evaluated in this study may be further refined and used in future germ cell transplantation experiments to achieve this goal.

**Literature Cited**


