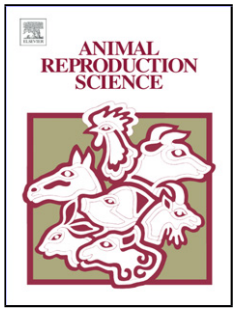

Title: Sensitivity of spermatogonia to irradiation varies with age in pre-pubertal ram lambs

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Sensitivity of spermatogonia to irradiation varies with age in pre-pubertal ram lambs

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Abridged title: Irradiation effect on spermatogonia varies with lamb age

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ABSTRACT

Although germ cells from donor rams transplanted into irradiated recipient testes have produced donor derived offspring, efficiency is low. Further optimization of recipient irradiation protocols will add precision to the depletion of recipient spermatogonia prior to germ cell transplant. Three irradiation doses (9,12,15 Gy) were administered to ram lambs aged 14 weeks (Group 1) and 20 weeks (Group 2), then testicular biopsies were collected 1, 2 and 3 months after irradiation. At 1 month after irradiation of Group 1, only the largest dose (15 Gy) reduced spermatogonia numbers below 10% of non-irradiated controls, whereas in Group 2 lambs, each irradiation dose reduced spermatogonia below 10% of controls. In both Groups, fewer differentiated germ cells were present in seminiferous tubules compared to controls. At 2 months after irradiation, spermatogonia numbers in both Groups increased more than sixfold to be similar to controls, whereas fewer differentiated germ cells were present in the tubules of both Groups. At 3 months in Group 1, each irradiation dose reduced spermatogonia numbers to < 30% of controls and fewer tubules contained differentiated germ cells. Lesser expression of spermatogonial genes, VASA and UCHL-1, was observed in the 15Gy group. In Group 2, only 12 Gy treated tubules contained fewer spermatogonia. Knowledge of these subtle differences between age groups in the effect of irradiation doses on spermatogonia or differentiated germ cell numbers and the duration of recovery of spermatogonia numbers after irradiation will aid the timing of germ cell transplants into prepubertal recipient lambs.

Keywords

Spermatogonia; Irradiation; Puberty; Stem cells, Sheep, Testis, Transplant

1. Introduction

The germ cell transplant technique in rodents has proved to be a valuable technique to study spermatogonia and the stem cell niche (Oatley and Brinster, 2006; Brinster, 2007). Its translation into domestic species has introduced a novel option for breeding improvement programs and an alternate

method to produce transgenic animals (Hill and Dobrinski, 2006). Successful germ cell transplantation with donor derived spermatogenesis and offspring was first described in mice followed a decade later by the adaptation of the technique for agricultural animals (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Honaramooz et al., 2002; Honaramooz et al., 2003). Although colonization of unprepared recipient testes by germ cell transplantation occurs, prior removal of endogenous germ cells should allow for increased colonization of the donor spermatogonial stem cells (Shinohara et al., 2001; Brinster et al., 2003; Honaramooz et al., 2003).

Key to adapting the germ cell transplant from rodents to domestic animals is solving issues related to scaling up the procedure to physically much larger animals with much greater testis volumes. Younger animals of smaller size have, therefore, been preferred as recipients for germ cell transplant particularly when testis irradiation is used to deplete endogenous germ cell populations. Several studies have used irradiation in rodents and various domestic species to estimate the optimal time for germ cell transplantation (Izadyar et al., 2000; Creemers et al., 2002; Schlatt et al., 2002; Izadyar et al., 2003; Honaramooz et al., 2005; Oatley et al., 2005; Kim et al., 2006; Trefil et al., 2006; Zhang et al., 2006). Most studies have proposed, based on the timing of endogenous recovery, that the optimal time to perform germ cell transplants in livestock animals is between 1 and 2 months post irradiation (Honaramooz et al., 2005; Herrid et al., 2006; Herrid et al., 2011). The majority of studies have used large irradiation doses (>5Gy) which are generally needed to deplete spermatogonia stem cells. Large irradiation doses must be balanced against the potential to cause long-term sterility. Doses of 9 Gy in rats and 12 Gy in mice (Meistrich et al., 1978; Pinon-Lataillade et al., 1991) have resulted in permanent sterility.

The current study in prepubertal rams expands upon earlier transplant studies using pubertal rams irradiated with 9 Gy or 15 Gy followed by germ cell transplant 6 weeks later (Herrid et al., 2009). The effect of irradiation on seminiferous tubules was not studied specifically although reductions in testicular size (scrotal circumference) in the 3 months following irradiation were indicative of reduced cellularity within seminiferous tubules and intertubular compartments. To quantify the impact of irradiation on seminiferous tubules the current study analysed testicular biopsies taken from pubertal ram lambs for 3 months following irradiation.

2. Materials and methods

2.1. Study design

Two age groups of animals were randomly selected (*n* = 12 per group). Group1 contained animals aged 14 weeks (± 2 weeks) with mean body weights of 22.6 kg and mean scrotal circumference of 13.4 cm. Group 2 were older and aged 20 weeks (± 2 weeks) with mean body weights of 27.5 kg and mean scrotal circumference of 21.5 cm. Animals from each group were randomly allocated to receive irradiation doses of 0, 9, 12 or 15 Gy. Animals were handled and treated according to the guidelines of the CSIRO animal ethics committee and located at CSIRO, Armidale, NSW, Australia.

2.2. Selection of animals

The two age groups were selected to compare the effects of irradiation in young Merino rams during early and late puberty. During early puberty (Group 1 animals aged 14 weeks with a scrotal circumference range of 13 to 15cm) the most advanced germ cell types within the seminiferous tubules are spermatogonia and/or spermatocytes (Skinner et al., 1968). This early puberty group was

compared to a late puberty group, (Group 2 aged 20 weeks with a scrotal circumference range of 18 to 25cm) which corresponds to seminiferous tubules containing active spermatogenesis with spermatozoa at the most advanced germ cell stage.

2.3. Testicular irradiation

The irradiation doses of 9, 12 or 15 Gy were delivered by a 6 MV photon beam produced by a linear accelerator at a dose rate of 2.5 to 3 Gy/min. Each lamb was anesthetized using a 0.1 ml/kg i.v. of a combination of tiletamine and zolazepam (Zoletil 100mg/ml; Virbac Corporation). The diameter of the testes was measured, and delivery of irradiation calculated accordingly to deliver a consistent dosage throughout the full depth of the testis to within +/- 6.5%, at a dose rate of 2.5 to 3 Gy/min. Control animals were not placed inside the linear accelerator and did not receive an irradiation dose.

2.4. Records and sample collection

At 1, 2 and 3 months after irradiation, the left testis was biopsied. The biopsies were performed under general anaesthesia (2% to 3% Isoflurane vapour in oxygen + NO_2). The biopsies were fixed in Bouin's solution for 3 to 6 hours, followed by transfer into 70% alcohol.

At 3 months after irradiation both testes were removed under general anaesthesia. Testis and epididymis weights were recorded. Testis fluid was collected by an impression smear taken from the freshly cut surface of the testis, while fluid from the head and tail of the epididymis was collected and transferred to a glass slide. All slides were examined using a light microscope for the presence of spermatozoa. The presence/absence, motility and morphology of spermatozoa in the testis and epididymal fluids were recorded.

Testis tissue was collected and fixed in Bouin's solution overnight and transferred into 70% Ethanol prior to paraffin embedding and processing. Sections were cut at 5 µm then left unstained for immunohistochemistry or stained with haematoxylin and eosin (H&E).

2.5. Histology and immunohistochemistry

Immunohistochemistry was conducted essentially as described previously using antibody dilutions of 1:400 for PGP 9.5/UCHL-1 (Dako, Denmark;(Herrid et al., 2009). Spermatogonia were identified using the PGP 9.5/UCHL-1 antibody to count positive cells counted in 100 tubule cross sections. Sertoli cells (non-staining) were counted from the same slides. Antigen retrieval was conducted by boiling sections in 0.01M citrate buffer (pH 6.0) for 10 min on high power in a microwave oven. After cooling and rinsing, the sections were quenched in 1.5% peroxide for 10 min then rinsed in TRIS-buffered saline + 0.05% tween-20 (TBST). The primary polyclonal rabbit antibody to PGP 9.5/UCHL-1 was applied to sections at a dilution of 1:400 in TBS + 0.5% BSA for 30 min at room temperature. Sections were rinsed with TBST, then Envision + Dual Link System peroxidase (anti-rabbit and anti-mouse complex, Dako, Denmark) was applied for 30 in. Sections were rinsed in TBST and colour developed using peroxidase DAB solution (Dako, Denmark) for 3 to 5 min. Sections were rinsed then counterstained with Harris's hematoxylin (4:1) for 20 seconds, de-hydrated and mounted.

The H&E testis sections were used to record tubule diameter and examine seminiferous tubules by examining 100 tubule cross-sections for the presence of differentiated germ cells (spermatocytes, round and elongated spermatids and spermatozoa). Spermatogenic progression score (most advanced germ cell type present) was assessed using 25 tubule cross sections to assign tubules to a category based on the most advanced germ cell type present within that tubule (Huang et al., 2008). The spermatogenic progression score categories were: 0 = Sertoli cell only, 1 = spermatogonia, 2 =

spermatocytes, 3 = round spermatids, 4 = elongated spermatids, 5 = spermatozoa. An average score was then calculated and used as a repopulation index to indicate onset and progress of recovery of spermatogenesis following irradiation.

2.6. Gene Expression for VASA and UCHL-1 – RNA extraction and RT-PCR analysis

The VASA gene is expressed in all ovine germ cells, including gonocytes, while UCHL-1 is expressed by a sub-population of ovine gonocytes and by all ovine spermatogonia (Rodriguez-Sosa et al., 2006; Borjigin et al., 2012; Yue et al., 2015).

Total RNA was extracted from irradiated and control sheep testicular tissue using TRIzol Reagent (Invitrogen) following manufacturer's instructions, with slight modifications. As slightly larger sample sizes were used (100 to 300 mg), samples were firstly cut up into smaller sections using a scalpel and homogenized, using glass beads in 1.5 ml of TRIzol Reagent. After homogenization, 750 µl (half of the sample) was removed and transferred into a new Eppendorf tube and another 250 µl of TRIzol reagent was added to each tube. The subsequent steps were conducted as directed by the manufacturer. After RNA extraction, contaminating DNA was removed from the RNA preparations using TURBO DNA-free Kit (Ambion) and confirmed PCR for GFRα1. All samples were run on an agarose gel and RNA concentration was estimated by spectrophotometry (Nanodrop). For first strand cDNA synthesis, the SuperScript III First-Strand System for RT-PCR (Invitrogen) was used according to the manufacturer's instructions. There was 2 µg of each RNA sample used in the RT reactions and the resulting cDNA samples were diluted to a concentration of 10 ng/µl.

Master mixes for each primer set contained 500 nM each of the forward and reverse primers, 25 µl SYBR green (Applied Biosystems) and 15.5 µl water for each reaction. Forward primer sequence for VASA was AGAAAGTAGTGATACTCAAGGACCA and reverse was TGACAGAGATTAGCTTCTTCAAAAGT. For UCHL-1 the forward primer was CCCCTGAAGACAGAGCAAAG and reverse primer was CCGACATTGGCCTTCCTG. The master mixes were aliquoted into 96 well PCR plates and 2.5 µl (25 ng) of cDNA was added to each well. Each sample was then divided into 4 x 5 µl replicates in 384 well PCR plate using a robot (Biomek 2000, Beckman). Quantitative PCR was performed using a 7900HT Sequence Detection System (Applied Biosystems). A dissociation stage was added to the RT-PCR and was assessed to ensure only one product was being amplified then results from the qPCR reactions were normalized against a reference gene (18s or GAPDH)

2.7. Statistical analysis

Data from each testicular development group were analysed using a linear mixed model analysis in Genstat software. Time and treatment (irradiation dose) were set as fixed effects and animal as a random effect. To obtain multiple comparisons between treatments within each time point, separate ANOVA's were conducted at each time point, and all pairwise comparisons were obtained using the Bonferonni procedure. These analyses were only conducted if the treatment or the treatment by time interaction was significant in the overall linear mixed model. The relative gene expression was transformed to a Log base 2 value prior to analysis to achieve better data normality (Reverter et al., 2003). Multiple comparisons between the overall treatments and time points were made using the Bonferonni correction procedure from the initial linear mixed model. Means were considered statistical significant if $P<0.05$.

3. Results

3.1. Body and testis measurements

There was no effect of irradiation treatment on growth rate. In the older animals (Group 2), wool loss and thickening of the scrotum occurred from Day 30, at all irradiation doses. There were no signs of inflammation or radiation burns to the skin. Scrotal wool started to regrow between Days 40 and 50, leaving only small areas of wool loss after 90 days (Figure 1).

Following irradiation of the older animals (Group 2) mean scrotal circumference decreased from 8 to 15 days post irradiation and by Day 40 was 3.5 cm less than controls. Mean scrotal circumference then increased to be similar to controls by 3 months post irradiation. After castration at 3 months, testicular weights were similar to controls. In Group 1, testicular weights at 3 months post irradiation were not significantly less for irradiated testes (33 g ±5, 40 g ±14, 42 g ±6 compared with controls 65 g ±13; $P = 0.137$).

3.2. Irradiation effects at 1 month post irradiation

Each irradiation dose significantly reduced the number of spermatogonia per cross section in both Group 1 (1.3 at 15 Gy; 2.6 at 12 Gy; 4.3 at 9 Gy compared with 6.7 in Controls; $P<0.05$, Table 1, Figure 2) and Group 2 (0.5 at 15 Gy; 0.7 at 12 Gy; 1.2 at 9 Gy compared with 10.2 in Controls; $P<0.05$). There was no effect of treatment on Sertoli cell numbers or tubule diameter in either group although Group 2 tubule diameters tended to be less than controls (106–109 µm for 9-15 Gy treatments compared with 137 µm in controls, $P = 0.053$). At 1 month post irradiation, tubules from Group 1 animals consisted of a single layer of cells (Sertoli cells and a few spermatogonia) and in some sections, gaps between Sertoli cells were observed.

Stage of spermatogenesis was assessed by recording presence of germ cell stages in tubule cross sections. In Groups 1 and 2, fewer irradiated tubules contained differentiated germ cells (Table 1). Further analysis of the progression of spermatogenesis in tubules was provided by spermatogenic progression scores (Figure 3). In Group 1, scores were less than controls for tubules treated with 12 Gy and 15 Gy ($P<0.05$) and in Group 2 scores were less at each irradiation dose (0.4 at 15 Gy; 0.5 at 12 Gy; 1.0 at 9 Gy compared with 3.2 in Controls; $P<0.05$).

3.3. Irradiation effects at 2 months post irradiation

In Group 1, the number of spermatogonia per cross section increased approximately six fold in irradiated tubules to be similar to controls. For both Groups, the number of spermatogonia per cross section were similar to controls (Figures 4, 5). There was also no effect of treatment on Sertoli cell numbers. Group 1 tubule diameters were less in 12 Gy- and 15 Gy-treated tubules ($P<0.05$) but were not different in Group 2. In control animals, there was a numerical decrease in spermatogonia per cross section in Group 1 and 2 between the 1- and 2-month biopsies (Group 1 controls from 6.7 to 5.8 and Group 2 controls from 10.2 to 5.4).

In Group 1, fewer tubules contained differentiated germ cells after high irradiation doses (52% in 12 Gy and 66% in 15 Gy compared with 99.3% in the controls). Accordingly, spermatogenic progression scores were less in Group 1 tubules (1.5 at 12 Gy, 1.8 at 15 Gy compared with 3.6 in the controls; $P<0.05$). Scores were greater than those at the time of the first biopsy which indicated that spermatogenesis had commenced. In Group 1, the most advanced germ cells that were observed were round spermatids in the 9 Gy-treatment group, spermatocytes in 12 Gy- and 15 Gy-treated tubules and spermatozoa in controls.

In Group 2, fewer irradiated tubules contained differentiated germ cells (23% in 15 Gy; 35% in 12 Gy; 43% in 9 Gy compared with 94% in the controls; $P<0.05$). Spermatogenic progression scores were less for Group 2 treatments ($P<0.05$).

3.4. Irradiation effects at three months post irradiation

In contrast to the 2 month evaluation there were less spermatogonia per tubule cross section in Group 1 irradiated testes (3.0 at 15 Gy; 2.9 at 12 Gy; 3.2 at 9 Gy compared with 10.0 in the controls; $P<0.05$). Fewer tubules contained either round (1.7% 15 Gy, 11% 12 Gy, 7.7 % 9 Gy compared with 77% in the controls) or elongated spermatids (2% 15 Gy, 0.3% 12 Gy, 7% 9 Gy compared with 54% in the controls). There were no spermatozoa in treated tubules (0% compred with 24% in the controls; $P < 0.05$). Spermatogenic progression scores from all treatments were less than controls ($P<0.05$), with spermatocytes the most advanced germ cell type present in treated tubules, compared to round and elongated spermatids for the controls.

In Group 2, the 12 Gy treatment had less spermatogonia per tubule cross section than controls (9.6 compared with 12.7 in controls; $P<0.05$). A lesser percentage of 15 Gy-treated tubules contained spermatozoa (16% compared with 36% for the controls; $P<0.05$) and 15-Gy treated tubules had a lesser spermatogenic progression score ($P<0.05$).

Tubule diameters increased over the 3 month period for both treated and control tubules in Group 1 (from 92 to 138 µm) and Group 2 (from 115 to 172 µm; $P<0.05$). Sertoli cell numbers per tubule cross section decreased over the 3 month period for both treated and control tubules in Group 1 (from 27 to 21) and Group 2 (from 26 to 20; $P<0.05$).

3.5. Gene expression analysis of Group 1 testes

At 3 months post irradiation relative abundance of VASA and UCHL-1 mRNA in Group 1 was less in the 15 Gy but not the 9 Gy treatment group compared to controls ($P<0.05$). The relative abundance of C-kit, PLZf and GRFα1 mRNA had a consistent though non-significant reduction. The relative abundance of mRNA of the two Sertoli cell associated genes (SCF and GATA 4) was not significantly different between irradiation treatment groups, although SCF expression tended to be greater in the 15 Gy-treated testes.

4. Discussion

This study demonstrated testicular irradiation at moderately large doses (12 or 15 Gy) reduced spermatogonia numbers by up to 95% in ram lambs. The fewest spermatogonial numbers were observed in the 5-month-old Group 2 lambs at 1 month following irradiation. In Group 2 lambs, the initial marked decrease in numbers of spermatogonia at 1 month was associated with a 3.5 cm reduction in mean scrotal circumference and a trend ($P = 0.053$) towards lesser tubule diameters. This is consistent with studies in mice where irradiation reduced spermatogonia in mice by 90% 4 weeks after irradiation with 12 Gy, in humans, where 6 Gy reduced spermatogonia numbers by 60%, but differs from rats where differentiated germ cells (round and elongated spermatids) remained 3 weeks after irradiation with 9 Gy (Rowley et al., 1974; Meistrich et al., 1978; Pinon-Lataillade et al., 1991; Zhang et al., 2006).

Differential duration of effect of irradiation was observed between the two groups of prepubertal ram lambs. While spermatogonia numbers decreased precipitously at 1 month following irradiation in

both Group 1 and 2 lambs, the inhibition of spermatogenesis was prolonged in the younger Group 1 lambs, such that 3 months after 12 or 15 Gy irradiation spermatogonia numbers were only 30% that of controls. This may indicate that larger irradiation doses were required to reduce the stem cell population and then to inhibit the regenerative capacity of the tubules (Erickson, 1976). In Group 1, expression of germ cell genes, VASA and UCHL-1 (PGP 9.5) was also less in the 15 Gy- but not in the 9 Gy-treated testes. Spermatogonia numbers from older Group 2 lambs recovered earlier after irradiation to be 87% to 93% of control values by 3 months after irradiation.

The initial large reductions in numbers of spermatogonia were not permanent as recovery of spermatogonia numbers began at 2 months after irradiation in both groups. The current study used lambs aged 14 to 20 weeks and a single delivery of irradiation which builds upon information available for young goats aged less than 10 weeks which received fractionated doses of irradiation of 9 Gy delivered over 3 consecutive days (Honaramooz et al., 2005). In this previous study, initial reductions in spermatogonia numbers were followed 6 months later by variable recovery of spermatogenesis in 29% to 60% of tubules. In adult rams, a larger proportion of tubules recovered germ cells by 4 months following irradiation with 12 Gy (van Vliet et al., 1988; Oatley et al., 2005).

Differential sensitivity to increasing irradiation dose was observed between Groups 1 and 2 in the present study. In the younger Group 1 lambs, each increase in radiation dose resulted in a decrease in spermatogonia numbers. Yet in Group 2, each dose resulted in a similar large reductions in spermatogonia numbers. At the time of irradiation in the 5-month-old Group 2 animals, spermatogenesis would be well advanced (Skinner et al., 1968). The presence of differentiating spermatogonia, which are more sensitive to irradiation, may be an explanation for the increased sensitivity of spermatogonia of the older lambs to radiation (Erickson, 1976; van der Meer et al., 1992).

The pattern of change in spermatogonia number over time differed between the treatment groups. Spermatogonia numbers in Group 2 initially decreased rapidly, then increased steadily over the next 2 months to be similar to or approach that of controls. Spermatogonia numbers from Group 1, however, increased at 2 months after irradiation to be similar to controls then decreased again to be significantly less than control numbers at 3 months. This pattern of an initial increase followed by a later decrease has also been reported in the pre-pubertal and adult rats (Erickson and Blend, 1976; Pinon-Lataillade et al., 1991; Kangasniemi et al., 1996). Radiation may have induced a defect in spermatogonial stem cells that limited self-renewal order to repopulate depleted seminiferous tubules (Beamer et al., 1988; Parreira et al., 1998). Defects in the Sertoli cell could also result in the failure of spermatogonia stem cells to self-renew (Zhang et al., 2007).

Regeneration of spermatogonia numbers in Group 2 over time was remarkable as spermatogonia numbers were initially reduced by 88% to 95%. The number of spermatogonia per cross section in the 15 Gy-treatment-group increased 20 fold from 0.5 at 1 month to 11.1 at 3 months post irridiation. The spermatogenic progression score in each Group 2 treatment increased slowly between 1 and 2 months then more rapidly in the final month post irridiation. Although recovery of spermatogenesis by 3 months was rapid in Group 2, less spermatogonia numbers in the 12 Gy-treated tubules and a lesser spermatogenic progression score in 15 Gy-tubules indicated recovery was not yet complete. Also, 12% of the 15 Gy-treated tubules did not contain differentiated cells which appears similar to the proportion of regressed tubules (20%) observed in adult rams treated with a 12 Gy treatment (Oatley et al., 2005). Although results of the present study indicated substantial recovery of spermatogenesis by 3 months post irradiation, it is possible that complete recovery of all tubules will not occur (Meistrich, 1986).

The information that spermatogonia numbers are least at 1 month after irradiation then increase quickly to be similar to controls after 2 months, has applications for timing of both germ cell transplant and for sterilization procedures. To improve the efficiency of 'sterilization' of ram lambs, a second irradiation dose administered between 1 and 2 months after the initial dose would occur when there were mitotically active spermatogonia which are likely to have a greater sensitivity to radiation and thus an increased probability of permanent sterility. Temporary reduction in spermatogonia numbers also creates an opportune time for germ cell transplantation to take advantage of vacant stem cell niches. It is presumed greater available numbers of niches will increase uptake of transplanted germ cells (Brinster et al., 2003). The opportunity, however, is likely to be brief in older Group 2 animals as by 2 months spermatocytes were again present, followed 1 month later by spermatozoa production even at the largest dose of 15 Gy. The rapid recovery of these animals, indicates that the testicular environment can still support spermatogenesis.

Another factor to consider is the longer term impact of irradiation damage to spermatogonial stem cell niches and to Sertoli cell function. Any effect of irradiation on Sertoli cells is important as number and function is essential to a healthy seminiferous tubule epithelium (de Rooij et al., 2002). In the present study, irradiation did not change Sertoli cell numbers, which indicated the resistance of these cells to irradiation in 3 to 5-month-old lambs. While changes to Sertoli cell numbers were not observed, Sertoli cell morphology or function may have been impaired with an outcome of less support for germ cell differentiation (Kangasniemi et al., 1996; Zhang et al., 2007).

The radiation doses delivered had minimal side effects on the health of the animals. Skin changes were seen only in the older Group 2 animals where loss of wool and thickened skin occurred. This effect was short term and recovery was apparent within 3 months of irradiation. In contrast, relatively larger irradiation doses (17.5 Gy) intentionally directed within 1 to 2 mm of the ovine skin surface causes long term damage to, and loss of, wool follicles (Sorell et al., 1990). In the present experiments, radiation delivery and damage to the skin surface was calculated precisely and predicted to be minimal. Thus wool loss was temporary in Group 2 lambs and not of practical use for fly strike prevention. In Group 1 lambs, there was no apparent wool loss and it is presumed that wool follicles were less mitotically active than in the older lambs of Group 2.

It has previously been reported that there is production of live progeny following testis germ cell transplantation of donor cells into irradiated recipient rams. Of the two recipients that produced donor derived progeny, one was irradiated at a similar developmental age as the current Group 1 animals and the other at a similar age as Group 2 animals (Herrid et al., 2009; Herrid et al., 2011). The current results from both Groups 1 and 2 support the use of larger doses of irradiation (12-15 Gy) to prepare pre-pubertal lambs as recipients for germ cell transplant and are consistent with results of previous studies in other species suggesting that germ cell transplants should be performed prior to repopulation of the tubules and between 3 and 4 weeks after irradiation (Shinohara et al., 2001; Honaramooz et al., 2005).

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Legends to figures

Fig 1. In the sixth week post irradiation, wool loss and thickening of the skin was observed in irradiated animals in the older age group (left), but not in control animals of similar age (right).

Fig 2. Irradiation resulted in differential effects over time on the mean number of spermatogonia per cross section between Groups 1 and 2. In Group 1, only 12 and 15 Gy significantly reduced spermatogonia number by 1 month after irradiation, while at 3 months after irradiation, spermatogonia numbers were less in all treated tubules. In Group 2, each irradiation dose reduced spermatogonia numbers to <12% of controls by one month and by 3 months spermatogonia numbers were similar to controls in the 9 and 15 Gy treatment groups but less in 12 Gy-treated tubules.

Fig. 3. Spermatogenic progression scores (mean advanced germ cell type) for control and irradiation treatments (9, 12 and 15 Gy) at 3, 8 and 13 weeks post irradiation. Means with different superscript letters within each time point, were different (P<0.05).

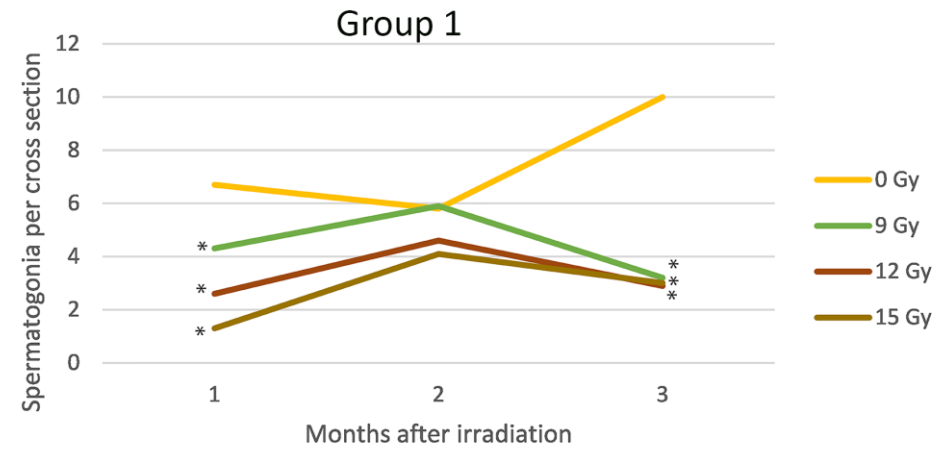
Fig 4. Group 1 seminiferous tubules containing spermatogonia highlighted by UCHL-1 antibodies. Irradiation doses are in separate rows (e.g., 0 Gy is A,B,C) and columns depict the interval since irradiation (e.g., 1 month is A,D,G). Micrographs are magnified at 400x. At 1 month after irradiation, 12- and 15-Gy treated tubules possess no differentiated germ cells, markedly fewer spermatogonia (brown) and an equivalent numbers of Sertoli cells compared to controls.

Fig 5. Group 2 seminiferous tubules containing spermatogonia highlighted by UCHL-1 antibodies. Irradiation doses are in separate rows (e.g., 0 Gy is A,B,C) and columns depict the interval since irradiation (e.g. 1 month is A,D,G).) Micrographs are magnified at 400x. At 1 month after irradiation, 9-, 12- and 15-Gy treated tubules possess no differentiated germ cells, far fewer spermatogonia (brown) and an equivalent number of Sertoli cells compared to controls.

Fig 6. Relative abundance of VASA (a) and UCHL-1 (b) mRNA in testicular tissue from control and irradiated animals (9 and 15 Gy) at 3 months post irradiation. Means with different superscript letters within each time point are different (P<0.05).

Fig. 1

Fig. 2



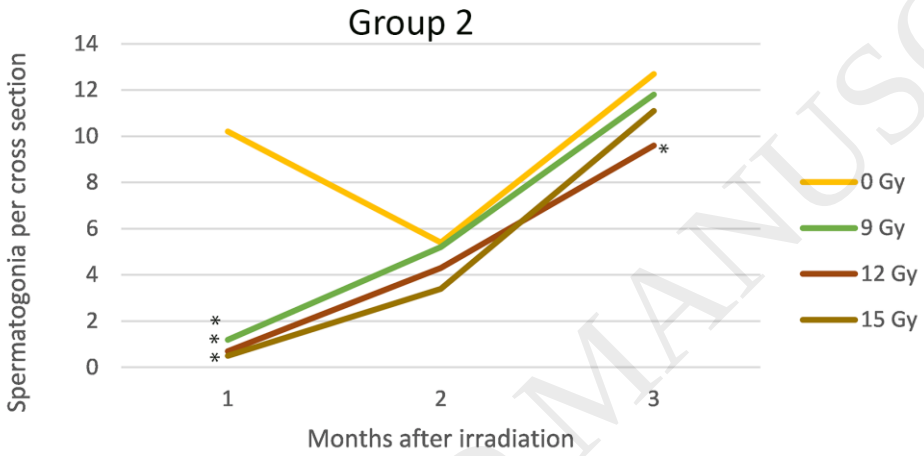


Fig 3A. Group 1

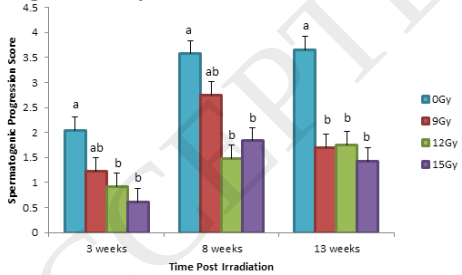


Fig 3B. Group 2

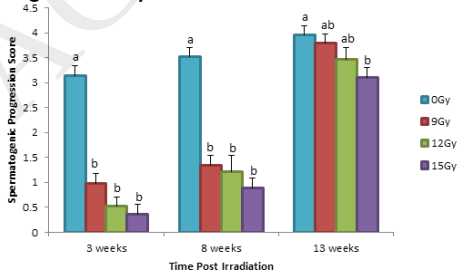


Fig. 4

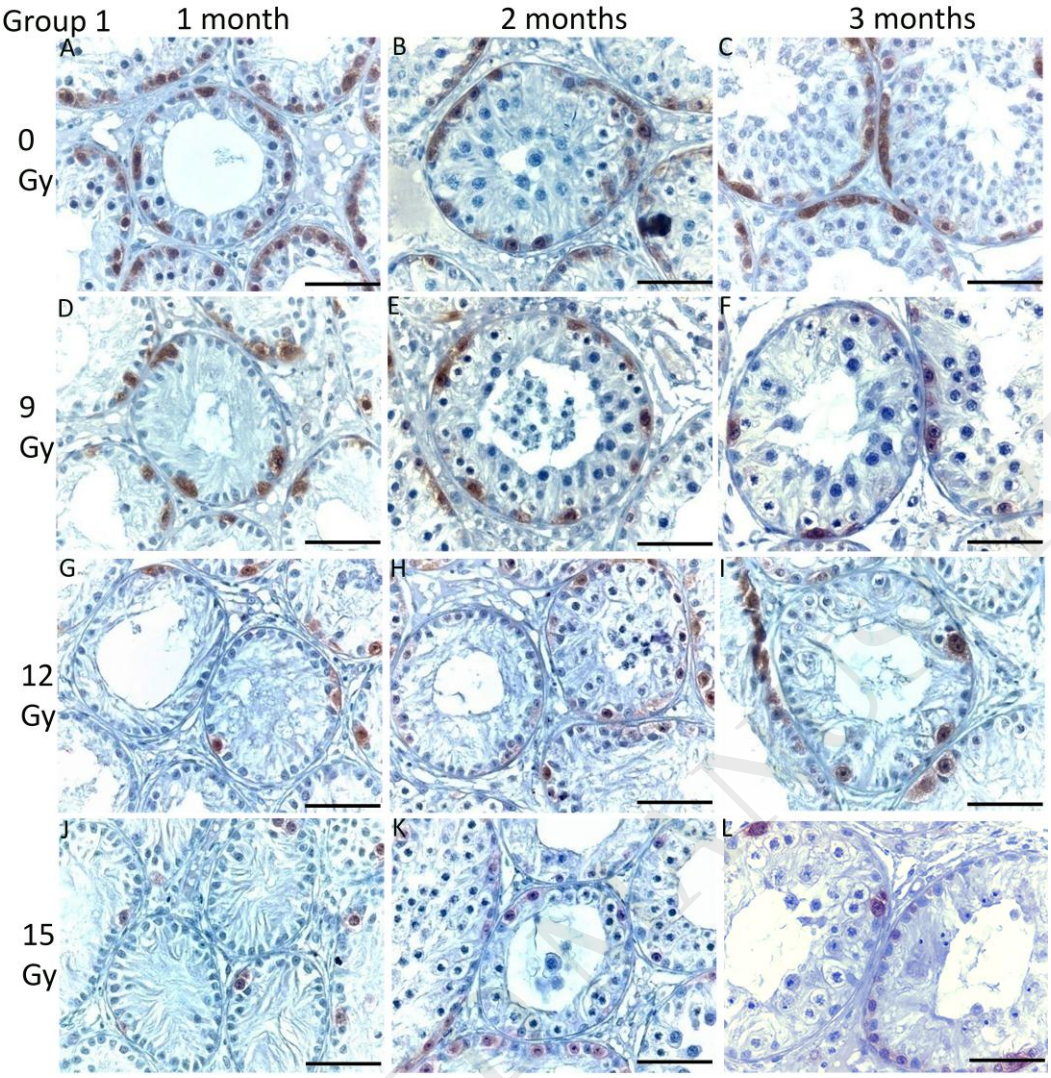


Fig 5.

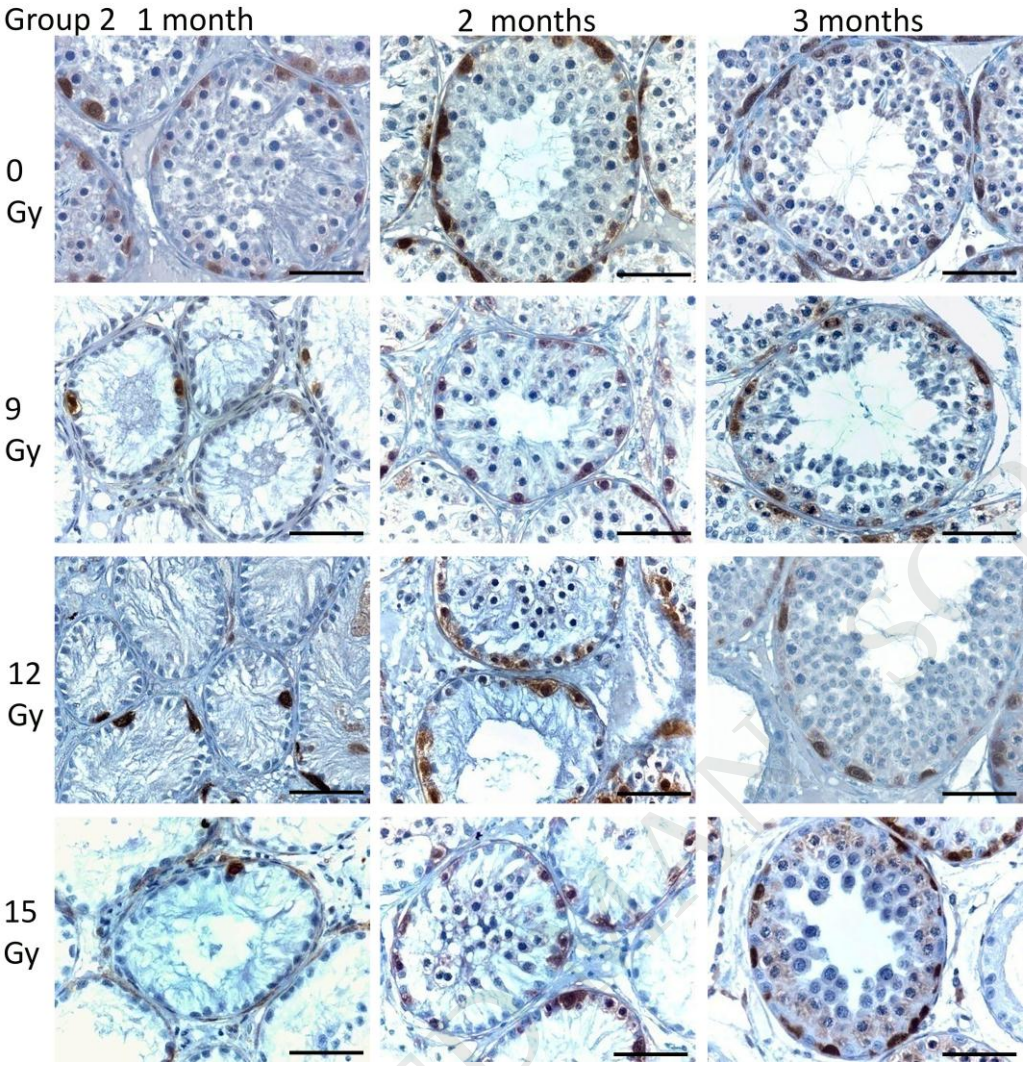
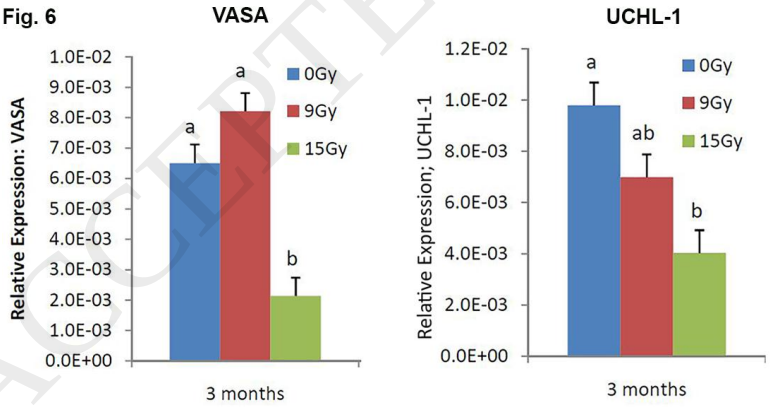


Fig. 6



Tables

Table 1

Numbers of spermatogonia within seminiferous tubule cross sections and percentage of sections containing differentiated germ cells for control (0 Gy) and irradiated treatments (9, 12, 15 Gy) at 1, 2 and 3 months after irradiation.

Duration after irradiation	**Group**	**Radiation dose**			
		0 Gy	9 Gy	12 Gy	15 Gy
		Spermatogonia per tubule cross section			
1 month	1	6.7^{a}	4.3^{ab}	2.6^{b}	1.3^{b}
	2	10.2^{a}	1.2^{b}	0.7^{b}	0.5^{b}
2 months	1	5.8^{a}	5.9^{a}	4.6^{a}	4.1^{a}
	2	5.4^{a}	5.2^{a}	4.3^{a}	3.4^{a}
3 months	1	10^{a}	3.2^{b}	2.9^{b}	3.0^{b}
	2	12.7^{a}	11.8^{ab}	9.6^{b}	11.1^{ab}
		% of tubules containing differentiated germ cells			
1 month	1	84^{a}	26^{b}	10^{b}	0^{b}
	2	80^{a}	15^{b}	4^{b}	3^{b}
2 months	1	99.7^{a}	96.7^{a}	52^{b}	66^{b}
	2	94^{a}	57^{b}	36^{b}	13^{b}
3 months	1	98.3^{a}	66^{b}	68^{b}	50^{b}
	2	100^{a}	96^{a}	96.2^{a}	88^{a}

*Numbers in the row with different superscripts differ (P<0.05)