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Spermatogonia survival in young ram lambs following irradiation, Busulfan or thermal treatment

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ABSTRACT

Many alternatives to surgical castration have been explored to induce short or long term infertility in male animals. Comparatively few have been carefully evaluated in very young production animals. A comparison of four treatments known to deplete testicular cells in rodents (heating, cooling, chemotherapy, radiation) was undertaken using ram lambs.

Neither testicular cooling (0 °C) nor heating (45 °C) affected testis weights, tubule diameters or germ cell numbers. Low dose chemotherapy (Busulfan 4 mg/kg) treatment caused dramatic falls in white blood cells and platelets numbers which recovered within 3 weeks. Spermatogonia numbers were not significantly reduced (27% change).

The impact of irradiation doses (0–15 Gy) delivered with high precision to the testis by a 6 MV photon beam was assessed by serial biopsies. Sertoli cell numbers were depleted by 90% at 3 weeks in 15 Gy treated testes. Spermatogonia were depleted 8 weeks after irradiation with 9 Gy, 12 Gy and 15 Gy. By 13 weeks, only in the 15 Gy treated testes were spermatogonia and Sertoli cell numbers lower. At 13 weeks testis atrophy resulted in 3/6 lambs irradiated with 12 Gy or 15 Gy. Irradiation of very young lambs clearly compromised testis function, thermal treatment was ineffective and busulfan treatment resulted in minimal effects.

1. Introduction

Treatments that interfere with the number or function of germ cells or Sertoli cells facilitate studies on male infertility and prepare recipient testes prior to germ cell transplant (Brinster and Zimmermann, 1994; Brinster, 2002). Depletion of male germs cells to improve colonization of recipient testes by germ cell transplantation has been evaluated in small and large animals (Ogawa et al., 1999; Shinohara et al., 2001; Brinster et al., 2003; Honaramooz et al., 2003). Treatments may also cause non-surgical sterilization such as in agricultural or domestic animals via injection of sclerosing agents into the testis or epididymis (Plant et al., 1979; Mercy et al., 1985; Jana et al., 2005; Kutzler, 2015). Treatments such as chemotherapy, irradiation, heat, cold, ischemia, torsion and cryptorchism have been well characterized in mice and rats, but less so in domestic or agricultural species (Jegou et al., 1984; Bucci and Meistrich, 1987; Young et al., 1988; Brinster et al., 2003; Honaramooz et al., 2005; Zhang et al., 2007).

The effects of prolonged periods of temperature changes on the fertility of adult males is well known (Setchell, 1998). Models using scrotal insulation to evaluate the effects of increased testicular temperature cause an increase in abnormal spermatozoa and reduction in spermatozoa number (Wildeus and Entwistle, 1983; Rocha et al., 2015). In rats, testis weights declined to 70% of controls after one week with numbers of spermatocytes and spermatids most affected (Jegou et al., 1984). Hypothermic treatments (10 °C for 30 min.) in mice increased apoptosis in spermatogonia (Blanco-Rodriguez and Martinez-Garcia, 1997), but not in rat testes (cooled to 0 °C for 60 min.) (Zhang et al., 2004), while in juvenile piglets, short term heating (39 °C) of the testes reduced gonocytes numbers by 50% (Frankenhuis et al., 1981).

In mice, a chemotherapeutic drug, Busulfan (1,4-butanediol

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dimethanesulfonate) is selectively toxic to spermatogonial stem cells at a dose of 30 mg/kg (Bucci and Meistrich, 1987; Wang et al., 2010). However, Busulfan has a narrow safety margin and causes severe myelosuppression with precipitous falls in numbers of platelets and white blood cells (Bucci and Meistrich, 1987). For example, in dogs, Busulfan doses up to 15 mg/kg are tolerated, whereas higher doses have resulted in almost 100% mortality (Deeg et al., 1999). With wide differences in tolerance between species to Busulfan the current study will add to the limited information available for young rams.

Testis irradiation to prepare recipients for germ cell transplantation has shown high irradiation doses (> 5 Gy) are required to deplete spermatogonia stem cells (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). Doses of 9 Gy in the rat and 12 Gy in the mouse (Meistrich et al., 1978; Pinon-Lataillade et al., 1991) have resulted in permanent sterility. Irradiation is more effective when delivered as split doses over multiple days although a single dose, which is more practical in adult farm animals can induce a temporary reduction in fertility (Honaramooz et al., 2005; Oatley et al., 2005; Kim et al., 2006). With less information available on irradiation in very young ram lambs, we hypothesized that the predominance of gonocytes and of actively dividing Sertoli cells in very young lambs would result in higher sensitivity to a range of irradiation doses. There is little information on the impact of heat, cold, chemotherapeutic drugs (eg Busulfan) or irradiation on testis function of young, prepubertal ram lambs and therefore this study investigated the effects of these treatments.

2. Materials and methods

Animals were located at CSIRO, Armidale, NSW, Australia and handled and treated according to the guidelines of the CSIRO animal ethics committee. Very young (six week old 15–20 kg) merino ram lambs were randomly allocated to treatments with short term testicular heating or cooling (n = 9 per treatment) followed by castration 2 weeks later. Six week old lambs were chosen as testis weights remain less than 10 g, while seminiferous tubule structure has changed little since birth and tubules continue to contain only gonocytes and Sertoli cells (Skinner et al., 1968).

2.1. Testicular heating and cooling in ram lambs

Animals were anaesthetised (Isoflurane vapour in oxygen) and placed on their sternum. The testes were immersed in an insulated cup containing water at 45 °C or 0 °C for 1 h. Treatments were performed once only. Control animals were similarly anesthetized without immersion of their testes. Animals were castrated 2 weeks after treatments and the testes collected, weighed and sections fixed in Bouins fixative for histological analysis.

2.2. Busulfan treatment

Merino ram lambs (3–4 months old) were randomly allocated into control or Busulfan treated groups (4 per group). At that age, testes would be around the time of onset of spermatogenesis but prior to appearance of spermatids or release of sperm into the tubule lumen (Herrera-Alarcon et al., 2007; Boukenaoui et al., 2012).

In a preliminary study to determine the safety margins of busulfan, a dose of 8 mg/kg induced thin diarrhea with lethargy and lack of appetite beginning from 5 days post busulfan injection(Olejnik et al., 2005). Therefore a reduced dose of 4 mg/kg was used in the current experiment.

Busulfan (Sigma, St Louis, MO, USA) was dissolved in 50% DMSO in saline to a concentration of 40 mg/ml then administered intravenously at doses of 0 or 4 mg/kg. Animals were then monitored daily for signs of toxicity to Busulfan, such as lethargy, diarrhea and inappetance. Body weight, rectal temperature and blood samples were collected twice weekly.

Animals were castrated 6 weeks later then testis sections fixed in Bouins solution overnight before transfer into 70% ethanol prior to paraffin embedding and processing. Sections were cut at 5 µm then stained with haematoxylin and eosin (H&E).

2.3. Testicular irradiation

Six week old merino ram lambs, each with a scrotal circumference of < 10 cm (consistent with tubules containing gonocytes with no active spermatogenesis) were randomly allocated to receive irradiation doses of either 0, 9, 12 or 15 Gy (n = 3 per treatment).

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. Prior to irradiation, animals were anesthetized, using Zoletil 100 mg/ml (i.v., 0.1 ml/kg; Virbac Corporation). The precision with which radiation is delivered to the testis is of particular importance in large domestic animals where testicular volume is much larger than in mice or rats. The dose delivered was carefully calculated from testis measurements so that the prescribed dose was be within +/- 10% of the calculated dose. The dose delivered was validated by performing measurements in a phantom using an ionisation chamber prior to in vivo irradiation. For a typical testis of 5 cm thickness the dose was delivered to the entire organ volume to within +/- 6.5%, at a dose rate of 2.5 to 3 Gy/min.

Each week during the first 13 weeks of the experiment, animals were weighed and their scrotal circumference measured. At week 3 and week 8 after irradiation, the left testis was biopsied using an open incisional technique under general anesthesia (2–3% Isoflurane vapour in oxygen + NO_2). The incisions in the tunica albuginea and scrotal skin were closed using 2-0 vicryl and nylon respectively. Biopsies were fixed in Bouin solution for 3–6 hours, then transferred into 70% alcohol.

At week 13 after irradiation the non-biopsied right testis was removed under general anesthesia. The twice biopsied left testis remained until 8 months post irradiation, when it was surgically removed to assess longer term effects of irradiation.

After removal, 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 under a light microscope for the presence of spermatozoa. Testis tissue was collected and fixed in Bouins 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).

2.4. Histology and immunohistochemistry

Immunohistochemistry was carried out as described previously using antibody dilutions of 1:400 for PGP 9.5/UCHL-1 (Dako, Denmark) and 1:40 for VASA (Sapphire Bioscience) (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. Gonocytes were identified using the VASA antibody. Sertoli cells (non-staining) were counted from the same slides. Sections for immunohistochemistry were dewaxed in xylene for 2 × 5 min. and rehydrated through decreasing gradients of alcohol into water. Antigen retrieval was carried out by boiling sections in 0.01 M citrate buffer (pH 6.0) for 10 min. on high power in a microwave oven. Sections were allowed to cool for 40 min. then rinsed in tap water. The sections were quenched in 1.5% peroxide for 10 min. and rinsed in TRIS-buffered saline + 0.05% tween-20 (TBST) for another 10 min. The primary polyclonal rabbit antibody to PGP 9.5/UCHL-1 applied to sections at a dilution of 1:400 in TBS + 0.5% BSA for 30 min. at room temperature in a humidified chamber. Sections were rinsed with TBST, then