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Effect of germ cell depletion on levels of specific mRNA transcripts in mouse Sertoli cells and Leydig cells

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Abstract

It has been shown that testicular germ cell development is critically dependent upon somatic cell activity but, conversely, the extent to which germ cells normally regulate somatic cell function is less clear. This study was designed, therefore, to examine the effect of germ cell depletion on Sertoli cell and Leydig cell transcript levels. Mice were treated with busulphan to deplete the germ cell population and levels of mRNA transcripts encoding 26 Sertoli cell-specific proteins and 6 Leydig cell proteins were measured by real-time PCR up to 50 days after treatment. Spermatogonia were lost from the testis between 5 and 10 days after treatment while spermatocytes were depleted after 10 days and spermatids after 20 days. By 30 days after treatment most tubules were devoid of germ cells. Circulating FSH and intratesticular testosterone were not significantly affected by treatment. Of the 26 Sertoli cell markers tested, 13 showed no change in transcript levels after busulphan treatment, 2 showed decreased levels, 9 showed increased levels and 2 showed a biphasic response. In 60% of cases changes in transcript levels occurred after loss of the spermatids. Levels of mRNA transcripts encoding Leydig cell specific products related to steroidogenesis were unaffected by treatment. Results indicate 1) that germ cells play a major and widespread role in regulation of Sertoli cell activity 2) most changes in transcript levels are associated with the loss of spermatids and 3) Leydig cell steroidogenesis is largely unaffected by germ cell ablation.

Introduction

Germ cell proliferation, meiosis and differentiation during spermatogenesis is critically dependent on the actions of follicle-stimulating hormone (FSH) and androgen mediated through the Sertoli cells. Loss of androgens and, to a lesser extent, FSH disrupts spermatogenesis (Lyon & Hawkes 1970, Kumar *et al.* 1997, De Gendt *et al.* 2004) while depletion and loss of function of the Sertoli cells leads to massive degeneration of the haploid germ cells and eventually to almost complete loss of germ cells (Russell *et al.* 2001). Overall the Sertoli cells act to maintain spermatogenesis through provision of a structural support, generation of a unique environment in which the germ cells develop, movement of the germ cells as they progress through spermatogenesis and through secretion of factors which aid germ cell development and differentiation (Mruk & Cheng 2004). Spermatogenesis is highly organised and orchestrated by the Sertoli cells and appears, in most mammals, as a wave within the tubule. While the role of the Sertoli cell in the process of spermatogenesis is apparent, the extent to which germ cells regulate Sertoli cell activity is less clear. Previous studies have shown that germ cell depletion can alter expression of Sertoli cell genes (Jonsson *et al.* 1999, Maguire *et al.* 1993) and secretion of specific Sertoli

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cell proteins (McKinnell & Sharpe 1997, Guitton *et al.* 2000). In addition, co-culture experiments have shown that factors secreted by the germ cells can influence Sertoli cell activity (Boitani & Ritzen 1981, Le Magueresse & Jégou 1986, Syed *et al.* 1999, Vidal *et al.* 2001, Zabludoff *et al.* 2001, Delfino *et al.* 2003). Cryptorchidism has also been shown to affect Sertoli cell activity (Johnston *et al.* 2004, O'Shaughnessy *et al.* 2007) although this may be a direct effect of increased temperature on the Sertoli cells (Bergh & Soder 2007). Overall, there has not been an extensive survey of either the role of germ cells in regulating Sertoli cell gene expression *in vivo* or the extent to which overall Sertoli cell activity is affected. In this study, therefore, we have treated outbred mice with busulphan and measured changes in the level of 26 different mRNA species expressed specifically in the Sertoli cells as germ cell depletion progresses.

Androgen secretion by the testis is dependent upon the Leydig cells which are regulated by luteinising hormone (LH). There is also good evidence, however, that the Sertoli cells influence Leydig cell activity and that ablation of the Sertoli cell population will lead to loss of the Leydig cells (Russell *et al.* 2001). We have, therefore, also measured Leydig cell activity and function in germ cell-depleted mice to determine whether the germ cells can directly or indirectly affect the steroidogenic function of the testis.

Materials and Methods

Animals

Adult (15 weeks), outbred MF1 mice were purchased from Harlan UK (Bicester, UK). Mice were given a single injection (IP) of busulphan (30mg/Kg) in DMSO/H₂O (50/50 v/v) and were killed 5, 10, 15, 20, 30 and 50d later. At each time point 3 or 4 control animals and 5 treated animals were killed to allow for any effects of ageing of the mice. No significant differences between the control animals was seen and data from the control animals was pooled for analysis.

One testis from each animal was frozen in liquid N₂ while the other testis was weighed and cut in half. One half was frozen for subsequent measurement of intratesticular testosterone while the other half was fixed in Bouin's. Trunk blood was collected from animals and serum used to measure circulating FSH.

Measurement of mRNA levels

Real-time PCR was used to quantify the content of specific mRNA species in testes at different times following busulphan treatment. To allow specific mRNA levels to be expressed per testis and to control for the efficiency of RNA extraction, RNA degradation and the reverse transcription step an external standard (luciferase (Promega UK, Southampton, UK)) was used (O'Shaughnessy *et al.* 2002, Baker & O'Shaughnessy 2001, Johnston *et al.* 2004). Testis RNA was extracted using Trizol (Life Technologies, Paisley, UK) and luciferase mRNA (5ng) was added to each testis at the start of the RNA extraction procedure. Residual genomic DNA was removed from extracted RNA by DNase treatment (DNA-free, Ambion Inc, supplied by AMS biotechnology, UK). The RNA was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript II, Life Technologies, Paisley, UK) as described previously (O'Shaughnessy & Murphy 1993, O'Shaughnessy *et al.* 1994).

Measurement by real-time PCR used the SYBR method in a 96-well plate format. Reactions contained 5µl 2 × SYBR mastermix (Stratagene, Amsterdam, Netherlands), primer (100nM) and template in a total volume of 10µl. The thermal profile used for amplification was 95°C for 8min followed by 40 cycles of 95°C for 20 secs, 63°C for 20sec and 72°C for 30sec. At the end of the amplification phase a melting curve analysis was carried out on the products

formed and gel electrophoresis was carried out on representative samples to confirm product size. The quantity of each measured cDNA was expressed relative to the internal standard in the same sample which allows direct comparison of expression levels per testis between different samples (Johnston *et al.* 2004).

Primers were designed using PrimerExpress software (Applied Biosystems, Warrington, UK) using parameters described previously (O'Shaughnessy *et al.* 2007). The primers used are shown in Table 1.

Measurement of hormone levels

Levels of FSH in the serum were measured using a commercial radioimmunoassay (RIA) with rat standards (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). A dilution curve of mouse serum was parallel with the standard curve generated by the RIA. To measure intratesticular testosterone levels steroids were extracted from frozen hemi-testes in ethanol and measured by RIA as previously described (O'Shaughnessy & Sheffield, 1990).

Histology

Testes were fixed overnight in Bouin's and stored in 70% ethanol. Testes were embedded in Technovit 7100 resin, cut into sections and stained with Harris' hematoxylin and eosin.

Statistics

Effects of drug treatment were analysed initially by single factor analysis of variance followed by post-hoc analysis using Fisher's test.

Results

Testis morphology

Busulphan treatment had no apparent effect on testis morphology up to day 5 (Fig 1A & B). By day 10, however, spermatogonia had been depleted and by day 15 the number of spermatocytes had been reduced (Fig 1C & D). Twenty days after busulphan treatment some tubules contained only elongated spermatids and spermatozoa although other tubules still contained round spermatids (Fig 1E). By 30 days nearly all tubules were devoid of germ cells although some spermatozoa were still present in a few tubules (Fig 1F). Fifty days after treatment most tubules remained devoid of germ cells although early regeneration was apparent in some tubules (Fig 1G). Progressive loss of germ cell populations was reflected in declining testis weight (Fig 1H)

Hormone profiles

Circulating levels of FSH did not change after busulphan treatment (Fig 2). There was large variation in intratesticular levels of testosterone between animals but no significant change in response to busulphan treatment (Fig 2).

Germ cell genes

Expression levels of three mRNA species encoding markers of different germ cell populations were measured following busulphan treatment to monitor loss of each population. The three markers examined were *Stra8*, *Spo11* and *Tnp1* which show predominant expression in spermatogonia, spermatocytes and spermatids respectively (Oulad-Abdelghani *et al.* 1996; Shannon *et al.* 1999; Yelick *et al.* 1989). Levels of *Stra8* mRNA were significantly reduced on day 5 and were barely detectable by day 10 (Fig 3). The expression recovered slightly by 30 days but remained significantly less than control up

to day 50 (Fig 3). Expression of *Spo11* was normal up to day 10 but was significantly and markedly reduced on day 15 with little recovery up to day 50 (Fig 3). There was no change in *Tnp1* expression up to 20 days but between 20 and 30 days there was a marked decline in expression which was maintained up to 50 days.

Leydig cell specific genes

To determine whether Leydig cell function is affected by germ cell depletion levels of 5 Leydig cell specific mRNA species (*Lhr*, *Star*, *Hsd3b6*, *Cyp17a1* and *Cyp11a1*) were measured following busulphan treatment. No significant changes in transcript levels encoding the LH-receptor, StAR protein or steroidogenic enzymes were seen following busulphan treatment (Fig 4). There was, however, a significant decrease in mRNA encoding PDGF-A 20 days after treatment.

Sertoli cell specific genes

To examine the effects of germ cell ablation on Sertoli cell activity the levels of 26 Sertoli cell specific mRNA species were measured (Fig 5). There were no changes in the expression levels of 13 mRNAs (*Rhox5*, *Espn*, *Fshr*, *Tjp1*, *Aqp8*, *Fyn*, *Dhh*, *Ccnd2*, *Wt1*, *Gata1*, *Sox9*, *Msi1* and *Inhba*). The remaining 13 mRNA species showed significantly altered levels after treatment. Of these, 9 showed increased expression in response to busulphan (*Cst9*, *Shbg*, *Wnt5a*, *Clu*, *Illa*, *Cldn11*, *Cys12*, *4930486L24Rik* (Testin) and *Amh*) while 2 showed decreased expression (*Spata2* and *Sympk*) and 2 showed a mixed response (*Trf* and *Inhbb*). Most mRNA species showed a late response to germ cell ablation (after 15 days) although 5 responded within 5 days (*Cst9*, *Shbg*, *Inhbb*, *Wnt5a* and *Clu*) and one within 15 days (*Spata2*) (Fig 5).

Other testicular genes

Levels of mRNA encoding three products with unknown (BDEF36) or mixed somatic expression (GATA4 and NR0B1) were also measured after busulphan treatment. Levels of transcripts encoding the β -defensin BDEF36 and the transcription factor GATA4 increased significantly 30 days after treatment with busulphan and remained high up to day 50 (Fig 6). In contrast, *Nr0b1* (*Dax1*) transcript levels were significantly reduced 50 days after treatment (Fig 6).

Discussion

Busulphan induces apoptosis in spermatogonia within one week of treatment followed by a second wave of apoptosis in meiotic spermatocytes after 2 weeks (Choi *et al.* 2004). The expression pattern of germ cell markers was consistent with early loss of spermatogonia through apoptosis followed by loss of spermatocytes after 2 weeks and subsequent loss of spermatids between 20 and 30 days as existing spermatids mature and fail to be replaced. Histological changes in the testis after busulphan were also consistent with the changes in marker transcript levels although there tended to be a delay between loss of marker expression and loss of a particular cell population. Overall the histological and marker data indicates that spermatogonia entered apoptosis within 5 days of treatment followed by loss of spermatocytes after day 10 and spermatids after day 15. By day 30 most tubules contained only Sertoli cells and by day 50 germ cell repopulation was apparent in some tubules. It should be noted that since busulphan is a cytotoxic drug there is a possibility that it will also have direct effects on the somatic cells of the testis. It might be expected that any such effects would be rapid and, within any one cell type, have a relatively non-specific effect on transcript levels. Within the confines of the experimental design, however, no effects of this nature were seen apart from some early increases in specific Sertoli cell transcript levels (discussed below).

Treatment with busulphan had no effect on intratesticular levels of testosterone confirming previous studies which have shown no effect of germ cell ablation on testosterone levels (Gomes *et al.* 1973, Morris *et al.* 1987, De Franca *et al.* 1994). Consistent with the failure to alter testicular androgen levels, busulphan treatment had no effect on levels of mRNA transcripts encoding proteins involved in steroidogenesis. Use of an external standard control for the real-time PCR studies meant that transcript levels were normalised to the whole testis and were, therefore, unaffected by changes in testis volume or cellular composition induced by busulphan. In addition, total Leydig cell number is unaffected by busulphan treatment in the adult mouse (O'Shaughnessy *et al.* 2003) and no corrections to the measured transcript levels per testis were required (O'Shaughnessy *et al.* 2007). The constant transcript levels per testis after busulphan treatment indicates, therefore, that there is no change in level per Leydig cell. This failure of germ cell ablation to affect the steroidogenic function of the Leydig cells in the adult animal contrasts with the reported effect of germ cell ablation in the fetal or prepubertal rat (Boujrad *et al.* 1995a,b). Under these circumstances Leydig cell number is reduced in the adult animal but testosterone production per cell is increased (Boujrad *et al.* 1995a,b). This would suggest that germ cells are required at the pre-pubertal stage for normal development of Leydig cell number and function but that the Leydig cells become independent of germ cell regulation once the adult cohort is formed. Alternatively, it has been shown that cryptorchidism appears to have different effects on Leydig cell function in rats and mice (de Kretser *et al.* 1979, Jegou *et al.* 1983, Mendis-Handagama *et al.* 1990a,b, Murphy & O'Shaughnessy 1991) and it is possible that there is a species difference in the Leydig cell response to germ cell depletion.

In contrast to the steroidogenic apparatus, levels of mRNA encoding PDGFA were significantly reduced coinciding with ablation of the spermatid population. This growth factor is required for normal Leydig cell development around puberty and is predominantly expressed in the Sertoli cells in the immature testis but in the adult animal it is localised in the Leydig cells (Gnessi *et al.* 2000, Fecteau *et al.* 2006). Altered expression of *Pdgfa* after busulphan suggests, therefore, that germ cell ablation can affect specific Leydig cell functions and this is likely to occur through changes in Sertoli cell activity.

The failure of germ cell ablation to affect circulating FSH levels was somewhat surprising since busulphan caused transient but significant changes in Inhibin β B –subunit mRNA levels and previous studies have shown that busulphan will increase circulating FSH levels in the rat between 6 and 10 weeks after injection (Gomes *et al.* 1973, Morris *et al.* 1987). The lack of a similar phenomenon in the mouse may be indicative of a species difference but a contributing factor in this study may also be that an outbred strain of mouse was used. This has the advantage that inbred strain-specific effects are avoided but at the expense of an overall increase in animal to animal variability which may have masked subtle changes in hormone levels.

Despite failure to affect androgen or FSH levels, germ cell ablation had a marked and widespread effect on the Sertoli cells. This study examined 26 mRNA species which have been shown, within the testis, to be predominantly or exclusively expressed in the Sertoli cells (Table 1). Of the genes studied over 50% showed altered expression following germ cell ablation and since hormone levels were unaffected this is likely to be a direct response to the loss of germ cells. In addition, since busulphan treatment does not affect Sertoli cell number (O'Shaughnessy *et al.* 2003) changes in transcript levels per testis will be a reflection of changes per Sertoli cell. While extrapolation from this set of genes should be done with caution, the results indicate that a large number of Sertoli cell genes may be directly regulated by the germ cell component. Most of the genes affected by busulphan showed a late response (after 15 days) which indicates that Sertoli cell activity is particularly sensitive to regulation by the spermatid population. This is consistent with earlier *in vivo*

studies which showed that spermatids are primarily responsible for changes in Sertoli cell function (McKinnell & Sharpe 1997, Maguire *et al.* 1993, Jegou *et al.* 1993). In addition, more recent *in vitro* studies using co-culture methods have shown specific effects of post-meiotic germ cells on Sertoli cell function (Vidal *et al.* 2001, Delfino *et al.* 2003). Sertoli cell activity also appears to be regulated by other germ cell populations and, in particular, the meiotic germ cells (Syed *et al.* 1999, AlAttar *et al.* 1997, Rey *et al.* 1994, Grandjean *et al.* 1997) although spermatogonia may also be involved (Fujino *et al.* 2006). This would be consistent with the earlier changes seen in mRNA species such as *Shbg* and *Cst9* and the loss of *Spata2* around day 15. As discussed above, it is also possible that early effects of busulphan could be due to direct effects of the drug on Sertoli cell activity but this appears unlikely since only a small number of genes are affected and in each case activity is increased after treatment.

Two recent studies have shown that there is high expression of β -defensins in the testis and male reproductive tract (Patil *et al.* 2005, Yenugu *et al.* 2006). In both studies β -defensin 36 was shown to be abundantly expressed in the testis and we have included it as a representative β -defensin in this study. While β -defensins are generally expressed in epithelia the specific localisation of *def36* expression in the testis is uncertain and *def36* has not been considered with the other two groups. Results from this study show clearly that germ cell ablation will significantly increase *def36* mRNA levels. The β -defensins act as broad spectrum antimicrobials which help protect the male reproductive tract against infection (Selsted & Ouellette 2005). It might, therefore, be expected that ablation of the germ cell population would act to increase levels of β -defensins as a protective response. During development the transcription factor GATA4 and the nuclear receptor NR0B1 (DAX1) are involved in sex determination and differentiation of the fetal Leydig cells (La Voie 2003, Park *et al.* 2005, Bielinska *et al.* 2007). In adult animals their function is less clear although both may be involved in maintenance and regulation of steroidogenesis (La Voie 2003, Niakan & McCabe 2005). The two factors are expressed in both Sertoli cells and Leydig cells in the adult animal (Tamai *et al.* 1996, Ketola *et al.* 1999) and the late changes in transcript levels after busulphan treatment indicates that normal expression of these factors is regulated by the germ cells. It is not clear whether this regulation occurs in both cell types or is restricted to only one.

Previous studies have examined the role of germ cells in the regulation of a small number of the mRNA species studied in this report at the mRNA level or as secreted proteins. During normal development there is a marked, prepubertal decline in AMH secretion by the Sertoli cells which is likely to be caused by increased androgen action on the Sertoli cells and by germ cell entry into meiosis (AlAttar *et al.* 1997, Rey *et al.* 2003). Since there was no significant change in intratesticular androgen levels in this study, the rise in *Amh* after busulphan treatment is consistent with regulation by the germ cells although the effect of busulphan was only seen after loss of the spermatid population. Similarly, it has been reported that levels of the Sertoli cell secretory product testin are inversely proportional to germ cell numbers (Cheng *et al.* 1989, Guitton *et al.* 2000) which is consistent with results reported here. A number of earlier studies have shown that Inhibin B levels are regulated by germ cells and data from the rat suggests that loss of post-meiotic germ cells is associated with a decline in Inhibin B (Guitton *et al.* 2000, Allenby *et al.* 1991). In contrast, Clifton *et al.* (2002) have reported that meiotic germ cells act to inhibit Sertoli cell *Inhbb* mRNA levels in culture. Interestingly, it has been shown that Inhibin B production appears to be germ cell stage-dependent with a possible inhibitory effect of IL1 α at the nadir of production (Okuma *et al.* 2006). The changes in *Inhbb* mRNA levels seen after busulphan in this study may, therefore, be related to disruption of the normal stage-dependent regulation of Sertoli cell activity although the alteration in *Il1a* transcript levels after germ cell depletion may also play a role. Sertoli cell Activin A production has also been shown to be

germ cell stage-dependent (Okuma *et al.* 2006) but *Inhba* transcript levels per testis did not change significantly after busulphan. This would suggest that there can be a complex effect of overall germ cell depletion on Sertoli cell transcripts which normally are under stage-dependent regulation. This may be because the overall effect of germ cell ablation will be a balance between the stimulatory and inhibitory effects of stage regulation aggregated across the whole testis.

Results from this study indicate, overall, that germ cells play a major (mostly inhibitory) role in regulating Sertoli cell activity and that this regulation is primarily through the post-meiotic cells. The effects of germ cell ablation were widespread, affecting 50% of the mRNA species tested suggesting that the germ cells may have a greater overall effect on Sertoli cell activity than endocrine factors which tend to be more specific (Johnston *et al.* 2004; Denolet *et al.* 2006). It is likely that the overall effect of germ cell action is to fine-tune Sertoli cell activity during the different stages of spermatogenesis in order to maximise spermatogenic output.

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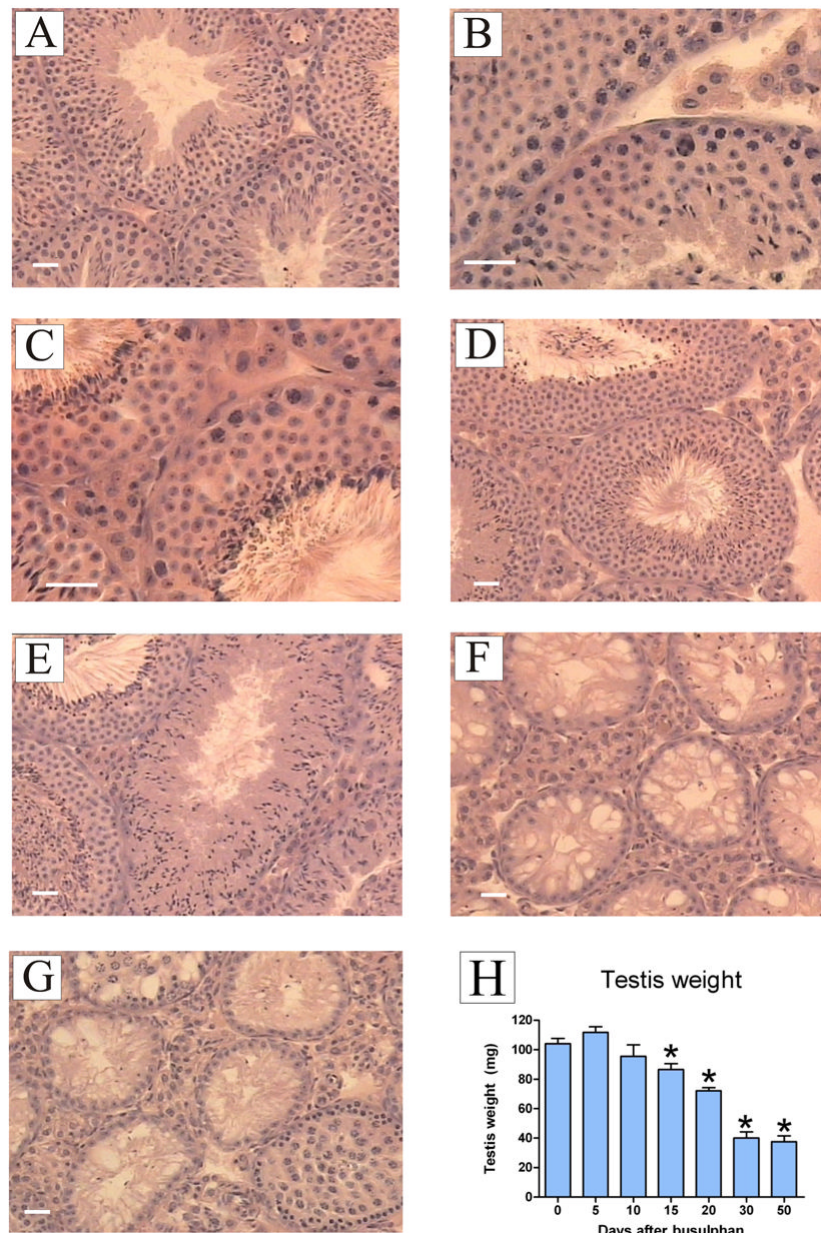


Fig 1. Testicular histology and testis weight following busulphan treatment. Adult mice were given a single injection of busulphan and killed up to 50 days later. Tissue sections show morphology in control testes (A) and 5 days (B), 10 days (C), 15 days (D), 20 days (E), 30 days (F) and 50 days (G) after busulphan treatment. There was depletion of spermatogonia 10 days after busulphan treatment while spermatocytes were reduced by day 15 and by day 20 some tubules contained only elongated spermatids and spermatozoa. By 30 days tubules were largely devoid of germ cells and by 50 days early regeneration was apparent in some tubules. Testis weight over the course of the experiment is shown in (H). The bar represents 30 μ m.

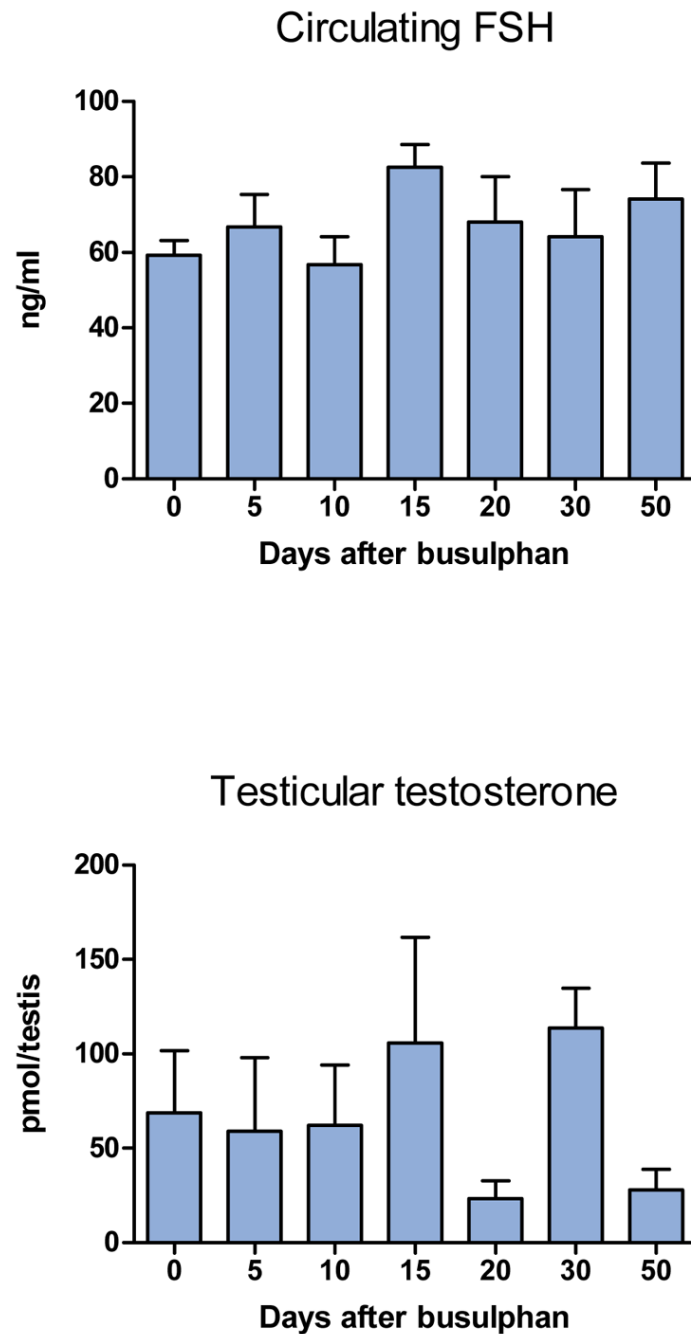
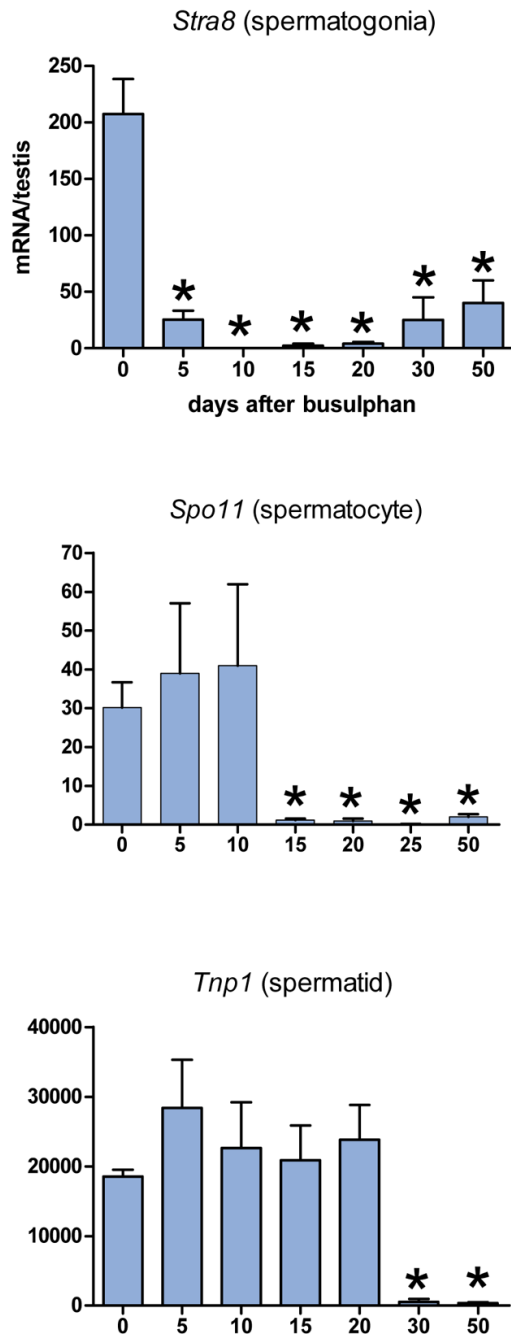


Fig 2. Levels of serum FSH (A) and intratesticular testosterone (B) following busulphan treatment. Serum and tissue was collected at different times after a single injection of busulphan and hormone levels measured as described in Materials & Methods. The results are expressed as mean \pm SEM for four or five animals in each busulphan-treated group and 18 animals in the control group. There was no significant ($P<0.05$) effect of busulphan on levels of either hormone.

**Fig 3.**

Effect of busulphan treatment on levels of 3 mRNA transcripts encoding markers of germ cell differentiation. Expression was measured by real-time PCR, and results are expressed relative to the external control luciferase. Data shows expression of the spermatogonial marker *Stra8*, the spermatocyte marker *Spo11* and the spermatid marker *Tnp1*. The results are expressed as mean \pm SEM for four or five animals in each busulphan-treated group and 18 animals in the control group. Groups marked with an asterisk (*) are significantly ($P < 0.05$) different to control values.

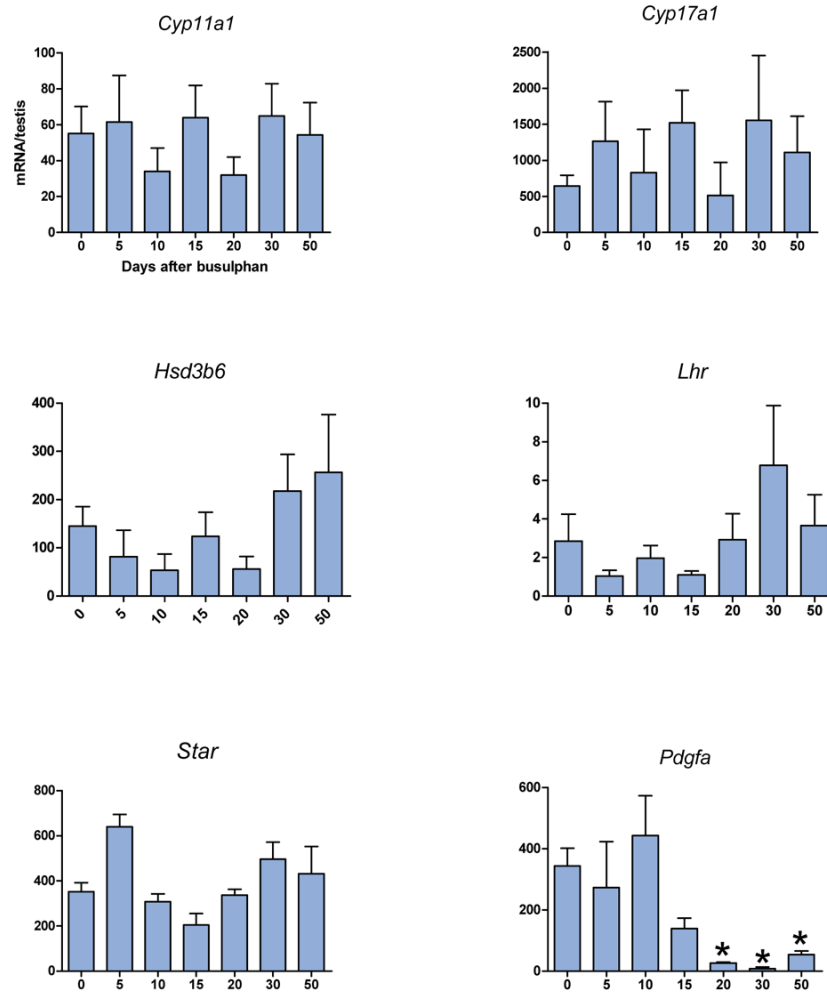
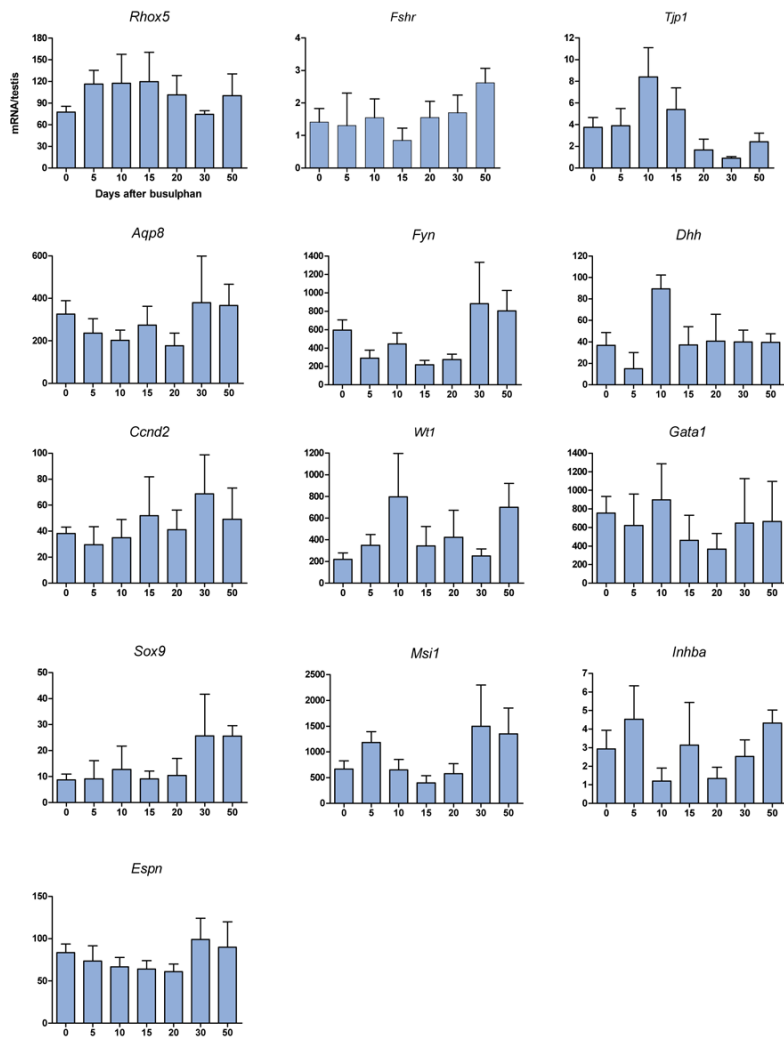


Fig 4. Effect of busulphan treatment on levels of mRNA transcripts encoding Leydig cell-specific products. Expression was measured by real-time PCR, and results are expressed relative to the external control luciferase. The results are expressed as mean \pm SEM for four or five animals in each busulphan-treated group and 18 animals in the control group. Groups marked with an asterisk (*) are significantly ($P < 0.05$) different to control values.



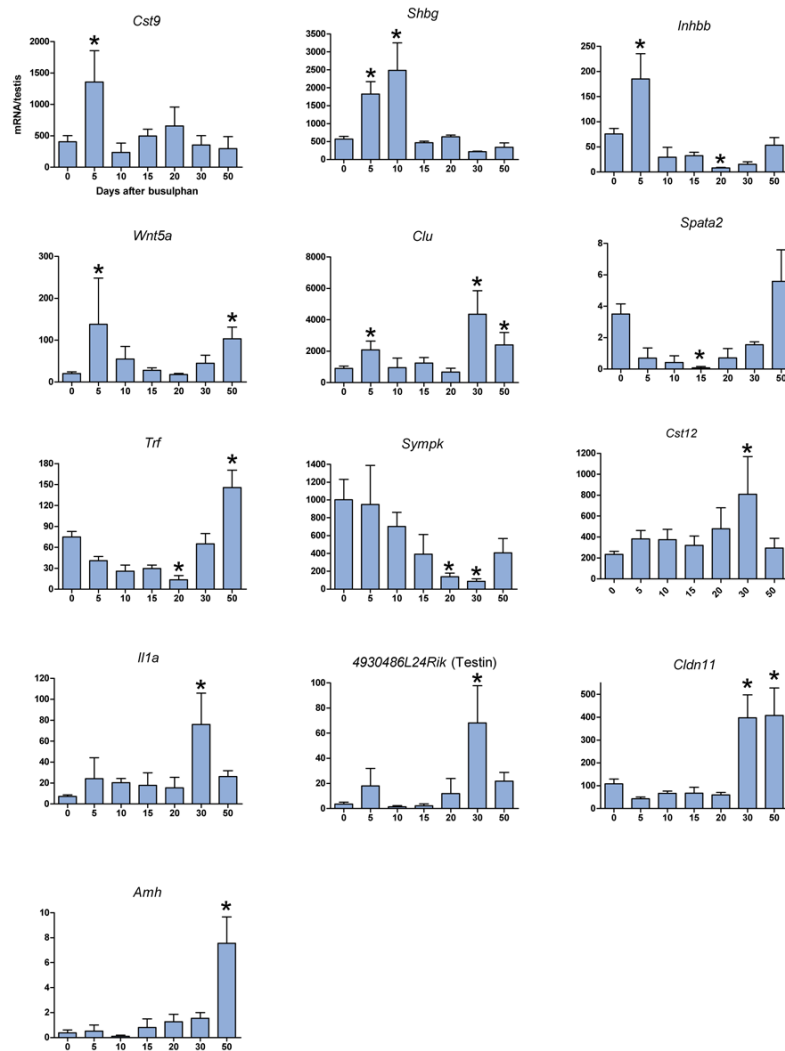
**Fig 5.**

Fig 5A Effect of busulphan treatment on levels of mRNA transcripts encoding markers of Sertoli cell-specific products. Expression was measured by real-time PCR, and results are expressed relative to the external control luciferase. The results are expressed as mean \pm SEM for four or five animals in each busulphan-treated group and 18 animals in the control group. Transcripts showing no change in levels after busulphan treatment have been grouped here.

Fig 5B Effect of busulphan treatment on levels of mRNA transcripts encoding markers of Sertoli cell-specific products. Expression was measured by real-time PCR, and results are expressed relative to the external control luciferase. The results are expressed as mean \pm SEM for four or five animals in each busulphan-treated group and 18 animals in the control group. Transcripts showing a significant difference to control values ($P < 0.05$, marked *) have been grouped here and are ordered according to the time at which an effect of busulphan is first seen.

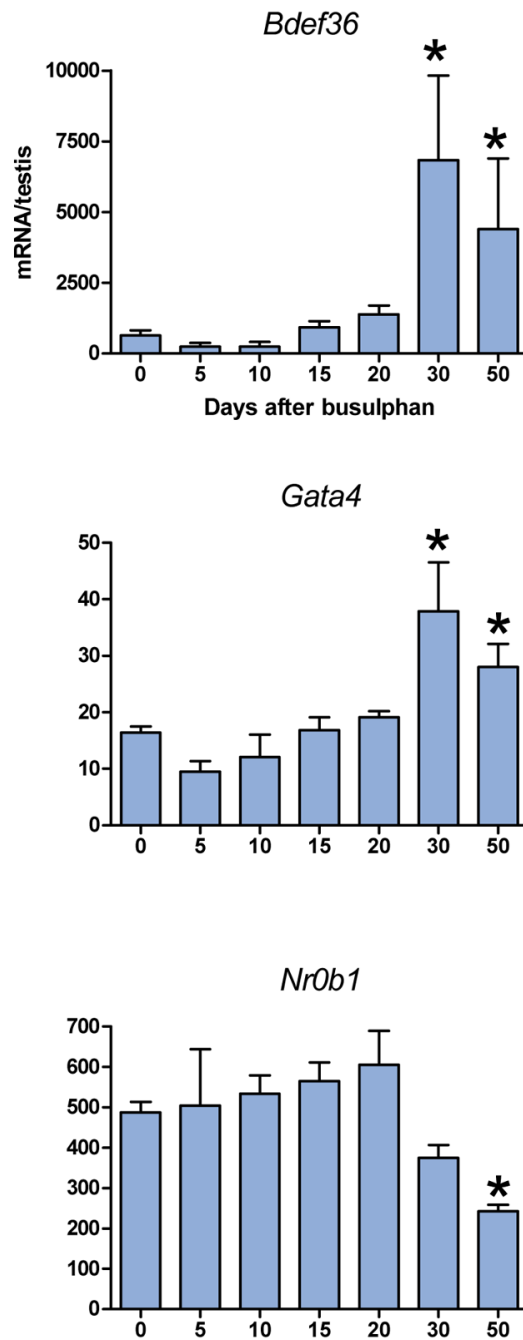


Fig 6. Effect of busulphan treatment on levels of mRNA transcripts encoding BDEF36, GATA-1 and NR0B1 (DAX1). Expression was measured by real-time PCR, and results are expressed relative to the external control luciferase. The results are expressed as mean \pm SEM for four or five animals in each busulphan-treated group and 18 animals in the control group. Groups marked with an asterix (*) are significantly ($P < 0.05$) different to control values.

Table 1

Gene	Abbrev	Genbank	Forward primer	Reverse primer	Reference to mRNA localization*
Sertoli cell					
Anti-Mullerian hormone	Amh	nm_007445	tcctacatcggcgaagatgatagg	aggctctgtgtgccccgag	Munsterberg & Lovell-Badge (1991)
Aquaporin 8	Aqp8	nm_007474	gctggcagtcacagatgatggga	cttgagcatggcaaaagcctg	Badran & Hermo (2002)
Claudin 11	Cldn11	nm_008770	gctccaaggccctgtgggc	tgtcaacagcagcaagatggcg	Morita <i>et al</i> (1999)
Clusterin	Clu	nm_013492	ccacgccatgaagattctcctgc	ctcccaggacggcgttctga	Morales <i>et al</i> (1987)
Cyclin D2	Ccnd2	nm_009829	ggaaccctggccgacagtcacc	aatcatcagacggcgggtacatg	Tan <i>et al</i> (2005b)
Cystatin 12	Cst12	af440737	ggatgacgattttgacctaacagtctct	ttctctctctggacctctctgca	Li <i>et al</i> (2002)
Cystatin 9 (Testatin)	Cst9	nm_009979	gatattgcccttcctaggagagcc	agagaagctacgacaccagctccatggg	Kanno <i>et al</i> (1999)
Desert hedgehog	Dhh	nm_007857	ggcgacagaccctctgatg	aaggcacggcctctctgtatggg	Bitgood <i>et al</i> (1996)
Espin	Espn	nm_019585	gctctgtgtggcattacct	ggtcatgccctctggggcg	Bartles <i>et al</i> (1996)
Follicle stimulating hormone receptor	Fshr	nm_013523	ggccaggtcaatacaccctctg	tgccttgaatatagactgttgcanaatg	Kliesch <i>et al</i> (1992)
Fyn proto-oncogene	Fyn	nm_008054	gaaagcgccctgtatgaaaggtt	tgtggcgaggcctcctatagc	Maekawa <i>et al</i> (2002)
GATA binding protein 1	Gata1	nm_008089	atggtcagaaccggcctctctc	ggagcttgaatatagagggcggcaggg	Tan <i>et al</i> (2005a)
Inhibin beta-A	Inhba	bc053527	catggacagacctggagatca	tgtctctgttctgttagccttgg	Kaipia <i>et al</i> (1992)
Inhibin beta-B	Inhbb	nm_008381	gagcgcctctccgagatcatca	cgtaacctctctctgtctccct	Kaipia <i>et al</i> (1992)
Interleukin 1alpha	Il1a	nm_010554	tggcgcctgagctggcnaa	tcatgaagtgagccatagcttgcac	Jonsson <i>et al</i> (1999)
Musashi homolog 1	Msi1	nm_008629	tcactttcagaccaggcggg	gftcacagacagcccccca	Saunders <i>et al</i> (2002)
Reproductive homeobox 5	Rhox5	nm_008818	aggftgccagcattcagctg	ggcgagccctctctgatctt	Lindsey & Wilkinson (1996)
RIKEN 4930486L24R1k		ay146988	aaagacaatggcgccctcgc	ggccccactttagccaactgcc	Cheng <i>et al</i> (1989)
Sex hormone binding globulin	Shbg	nm_011367	gacattccccagcctcatgca	tgcctcggagacagaccacag	Wang <i>et al</i> (1989)
Spermatogenesis associated 2	Spata2	nm_170756	gccgftggggcctgtgctt	ttccccaaatcaaacccaagg	Graziotto <i>et al</i> (1999)
SRX-box containing gene 9	Sox9	nm_011448	cgggagctcagcaagactctg	tgtccgtttcacgacttctc	Frojdman <i>et al</i> (2000)
Symplekin	sympk	xm_485873	caagaagaaggcccaagcagcga	aggaagttgtcaagcaggggggg	Keon <i>et al</i> (1996)
Tight junction protein 1	Tjp1	nm_009386	ggcgagagacacaagatgtccgc	ctctgaaatgaggattatcttccacca	Byers <i>et al</i> (1991)
Transferin	Trf	nm_133977	caaatgcatagcttccgacc	cggcatcgtacaccaacc	Skinner & Griswold (1980)
Wilms tumor homolog	Wt1	nm_144783	gctccagctcagtgaaatggacagaa	ggccatccagatcacagccg	Mundlos <i>et al</i> (1993)
Wingless-related MMTV integration	Wnt5a	nm_009524	ctgtcttaccatcgctgtgctgg	ggccatggagacagtgccgg	O'Shaughnessy <i>et al</i> (2007)

Gene	Abbrev	Genbank	Forward primer	Reverse primer	Reference to mRNA localization *
site 5A					
<u>Leydig cell</u>					
Cytochrome P450 side chain cleavage	Cyp11a1	nm_019779	cacagagcatcaagcagcaaaa	gcattgagaaccgctgggc	O'Shaughnessy <i>et al</i> (2002)
3B-hydroxysteroid dehydrogenase type 6	Hsd3b6	nm_013821	gtccagactgggactgctgacac	aatcctctggccccaaaaccctc	O'Shaughnessy <i>et al</i> (2002)
Steroidogenic acute regulatory protein	Star	nm_011485	cgtcggagctctctgcttgcttc	tegtccccgttctctgctg	O'Shaughnessy <i>et al</i> (2002)
Cytochrome P450 17alpha-hydroxylase	Cyp17a1	nm_007809	tggcccacatctctctctgcccig	aggcgcagcctttctctg	O'Shaughnessy <i>et al</i> (2002)
Luteinising hormone receptor	Lhr	nm_013582	tcaggaaattgccgaagaagaacacag	gaagtcataatcgaatcccagccactg	O'Shaughnessy <i>et al</i> (2002)
Platelet -derived growth factor A	Pdgfa	nm_008808	gagcggctggctcgaagtcag	ctgcgaatgggcatcaggcc	Gnessi <i>et al</i> (1995)
<u>Germ cell</u>					
Transition protein 1	Trp1	nm_009407	ggcgatgagcaagtcgcaa	ccactctgataggaictttggctttfgg	Yelick <i>et al</i> (1989)
Sponulation protein meiosis-specific	Spo11	nm_012046	cggcggccctctagttctgagg	ggatcatc-cgaaggccgacagaat	Shannon <i>et al</i> (1999)
Stimulated by retinoic acid gene 8	Stra8	nm_009292	gaagtgcatggctcaccctgg	gctcgtatggcgggcccctg	Oulad-Abdelghani <i>et al</i> (1996)
<u>Others</u>					
Beta defensin 36	def36	nm_001037 247	tccccagfacccacagaacg	tfgccgfggagattccagcatt	see text
Gata binding protein 4	Gata4	nm_008092	cccttcacagcccagctctg	aggctgctctccctccctcctc	Ketola <i>et al</i> (1999)
Nuclear receptor subfamily 0b1 (Dax1)	Nr0b1	nm_007430	cggaggcggggcactgct	caatgtattcacccactgcaggc	Tamai <i>et al</i> 1996

* Reference describing localisation of mRNA transcripts in the testis