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# Apoptosis-Related Protein Expression During Pre- and Post-Natal Testicular Development After Administration of Glucocorticoid *in utero* in the Sheep

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**Contents**
Pre-natal glucocorticoids are used in women at risk of preterm delivery to induce foetal lung maturation. However, glucocorticoids can produce negative outcomes for other tissues such as the reproductive system. We therefore tested the effects of pre-natal betamethasone on testicular morphology and apoptotic protein immune expression during pre- and post-natal development. Pregnant ewes (n = 42) bearing singleton male foetuses were randomly allocated to receive intramuscular injections of saline or betamethasone (0. 5 mg/kg) at 104, 111 and 118 days of gestation (DG). Testes were collected at 121 and 132 DG, and at 45 and 90 post-natal days (PD) and subjected to morphometric analysis (volume densities of sex cords and interstitial tissues; sex cord diameter). Immunohistochemistry (% stained area) was used to assess active caspase-3, Bax, Bcl-2 and cell-cycle proteins (PCNA). Compared with control values, betamethasone treatment decreased sex cord diameter at 121 DG, 45 and 90 PD, and sex cord volume at 90 PD. Active caspase-3 was decreased by betamethasone at 121 DG and 90 PD, but Bax was increased in all betamethasone groups. Bcl-2 and PCNA decreased in the betamethasone groups at 121 DG and 45 PD, but increased at 132 DG and 90 PD. We conclude that high levels of pre-natally administered glucocorticoid reduce foetal testicular development, perhaps via changes in the balance between pro- and anti-apoptotic proteins and cell-cycle proteins. These outcomes could compromise the future spermatogenic potential of male offspring.

## Introduction

During pregnancy, the development of the foetus is affected by maternal signals that should theoretically equip it to survive in post-natal life, but adverse events can lead to modification of organs and tissues that can disrupt health in adulthood (Rhind et al. 2001). This is the 'Barker Hypothesis', and it is especially relevant to systems that are sensitive to the environment *in utero* and, thereafter, have a fixed functional capacity (Barker 2007). There is strong experimental data for the 'programming' of predisposition to adult diseases (Abbott et al. 2002, 2008) and of changes in the balance of control systems such as the hypothalamic–pituitary–adrenal and hypothalamic–pituitary–gonadal axes (Phillips 2001; Recabarren et al. 2008). Thus, maternal stress-response hormones, such as the adrenal glucocorticoids (Sapolsky et al. 2000; Sasagawa et al. 2001), can interfere with embryogenesis and affect the development of homoeostatic systems (Bertram and Hanson 2002), leading to long-term effects in the offspring (Ward and Weisz 1980). This explains the reduction of birth weight in rats by exogenous glucocorticoid *in utero* (Stojanoski et al. 2006).

On the other hand, pre-natal glucocorticoid administration can be highly beneficial and is commonly used in pregnant women at risk of preterm delivery to enhance survival of the offspring (Bishop 1981) by improving foetal lung maturation (Liggins and Howie 1972; Ikegami et al. 1997). However, *in utero* glucocorticoids in animals and humans can also reduce birth weight (Ikegami et al. 1997) and increase predisposition to cardiovascular and metabolic diseases (Tangalakis et al. 1992; Nyirenda et al. 1998; Fowden et al. 2006). In addition, glucocorticoids regulate the balance between mitosis and apoptosis and can therefore affect the total cell number in developing tissues and organs (King and Cidlowski 1998).

Pre-natal maternal stress can also contribute to sexual dysfunction and infertility (Phillips and Lakshmi 1989; Moberg 1991), perhaps mediated by a decrease in blood testosterone in male foetuses (Ward 1994) induced by high concentrations of glucocorticoids (Sapolsky et al. 2000; Yazawa et al. 2001; Piffer et al. 2009), with potential consequences for future spermatogenesis. In the sheep, the foetal, post-natal and pre-pubertal testis comprise mostly interstitial tissue including the *endocrinocytus interstitialis* cells or Leydig cells (International Committee on Veterinary Histological Nomenclature 1994; Leydig 1850), and the solid sex cords that are precursors of seminiferous tubules in the mature testis (Kerr et al. 2006). The sex cords comprise *epitheliocytus sustentans* or Sertoli cells (Sertoli 1865; International Committee on Veterinary Histological Nomenclature 1994) plus germ cells in early embryonic, foetal and post-natal period. The cords undergo rapid enlargement and notably the development of a lumen, during the period leading to puberty (Kerr et al. 2006).

Apoptosis has been implicated in the selective deletion of cells during the development, morphogenesis and differentiation of many organs, including the gonads (Boulogne et al. 1999). Sperm production from the testis is regulated by a balance between germ cell division and germ cell loss (Russell et al. 2002). There could be nine to eleven spermatogonial cell divisions plus 2 divisions of spermatocytes, and excess cells are normally removed by death at multiple sites within the testis, permitting survival of only 25% of the possible number of pre-leptotene spermatocytes (Yamamoto et al. 2001).

In the adult, stress-induced endogenous glucocorticoids and exogenous glucocorticoids can also initiate apoptosis in Leydig cells thus reducing steroidogenesis and increasing the rate of apoptosis in germ cells (Yazawa et al. 2000; Drake et al. 2009). In Leydig cells,

corticosterone appears to initiate the apoptotic process by promoting activation of the Fas system, cleavage of procaspase-3 to active caspase-3, reducing the mitochondrial membrane potential, releasing cytochrome c by Bax and increasing generation of reactive oxygen species (Mackay 2000). Bax is a multi-domain pro-apoptotic member of the Bcl-2 family, and, in the adult testis, cells within the basal compartment are killed by a Bax-dependent mechanism. The death of more mature spermatocytes in the luminal compartment may also involve a Bax-independent pathway (Russell et al. 2002). Pro- and anti-apoptotic proteins work as pairs, and their relative levels determine whether cell survival or cell death takes place. Overexpression of Bcl-2 (anti-apoptotic) appears to block cell death at a critical stage and results in disruption of normal spermatogenesis and infertility (Furuchi et al. 1996; Rodriguez et al. 1997), producing a phenotype very similar to Bax-deficient mice.

We have previously shown that, in sheep, pre-natal exposure to synthetic glucocorticoids reduces Leydig cell proliferation and increases the expression of glucocorticoid receptor in foetal testicular tissue (Pedrana et al. 2008). Given the role that glucocorticoids play in regulating apoptotic signalling pathways (Sasagawa et al. 2001; Herr et al. 2007), we hypothesized that apoptosis links our observations. We therefore investigated the effect of foetal exposure to clinically relevant doses of maternally administered betamethasone on testicular morphology and on expression of apoptotic regulatory proteins (active caspase-3, Bcl-2 and Bax) and cell-cycle proteins (PCNA) in pre- and post-natal testis development in the sheep.

## Materials and Methods

### Animals and pre-natal treatments

All experimental procedures were approved by the Animal Experimentation Ethics Committee of the Department of Agriculture of Western Australia. Pregnant Merino ewes (n = 42) bearing singleton male foetuses of known gestational age (day of mating = day 0) were allocated randomly to receive maternal 5–6 ml injections of saline (control group, n = 21) or 0.5 mg/kg of betamethasone (Celestone Cronodose®, Shering Plough, Baulkham Hills, NSW, Australia, betamethasone group, n = 21) at 104, 111 and 118 days of gestation (DG). All animals were injected intramuscularly with 150 mg medroxyprogesterone acetate (Depo Provera; Upjohn, Rydalmere, NSW, Australia) at 100 DG to reduce pregnancy losses because of subsequent glucocorticoid treatment.

### Tissue collection

Pregnant ewes were killed by captive bolt at 121 (betamethasone n = 5, saline n = 5) and 132 DG (betamethasone n = 5, saline n = 6). Foetuses were immediately delivered by caesarean section and killed by decapitation. Male lambs delivered spontaneously at 150 DG were kept with their mothers on pasture supplemented with cereal grain and lupin seed until sacrifice at 45 post-natal days (PD) (betamethasone n = 5, saline n = 6) and 90 PD (betamethasone n = 5, saline n = 5). Lambs were weighed and then anaesthetized with ketamine (15 mg/kg) and xylazine (0.1 mg/kg, Troy Laboratories, Smithfield, NSW, Australia) and killed by decapitation. Foetal and post-natal testes were dissected, weighed, cut longitudinally and immersed in Bouin's fixative solution (75% picric acid saturated, 20% formaldehyde 40 and 5% glacial acetic acid) for 20–24 h at room temperature (20°C). Testis samples were dehydrated by immersion in increasing concentrations of ethanol (70, 95 and 100%), the passed through chloroform and embedded in paraffin wax. Sections (5 μm) were cut by microtome (Leica Reichert Jung Biocut 2030, Wetzlar, Alemania) and mounted for stereological and immunohistochemistry analyses.

### Stereological analyses

Stereological studies were performed on haematoxylin–eosin sections, and digital images were acquired with specific capture software (DinoCapture 2.0 software, AnMo Electronics Corporation, Taiwan) and digital camera (Dino-Eyepiece, AM-423X, AnMo Electronics Corporation, Taiwan) connected to a microscope (Premiere Professional Binocular, Model MRP-5000, Manassas, USA) at 400 × magnification.

Testicular tissues and compartments were analysed with image analysis software (ImageJ 1.43 m, Wayne Rasband, National Institutes of Health, USA; http:// rsb. info. nih. gov/ij) in 50 fields with a quantitative automatic tool. Sex cord areas and diameters and interstitial tissue areas were measured using the ImageJ straight and freehand tracing tool, respectively. Volume densities (Vv) were determined using the image area occupied by sex cords or interstitial tissue and the equation Vv = (An/At) 100 where An is the area overlying the tissue component of interest (sex cords or interstitial tissue), and At is the total image area. Absolute volumes (Av) were estimated using the following equation Av = Vv x testes volume, where Vv is the volume density (of sex cords or interstitial tissue), and testicular tissue density was considered to be 1.0.

### Immunohistochemistry

A streptavidin-biotin immunoperoxidase method was performed as previously described (Ortega et al. 2010). Briefly, deparaffinization and heat-induced epitope retrieval (HIER) were performed by incubating the sections in 0.01 M citrate buffer (pH 6.0) for 5 minutes in microwave as commonly use in pre-treatment in immunohistochemistry. The HIER breaks the protein cross-links formed by formaldehyde fixation and thereby uncover hidden antigenic sites. The technique involved the application of heat for varying lengths of time to formalin-fixed, paraffin-embedded tissue sections in an aqueous solution (commonly referred to as the retrieval solution). Tissue-specific endogenous peroxidases were inactivated with 3% hydrogen peroxide. Testis slides

were then washed in phosphate-buffered saline (PBS) pH 7.4, and non-specific binding was blocked with normal serum. The slides were then treated with primary antibodies acquired from Abcam (Cambridge, MA, USA): anti-active caspase-3 rabbit polyclonal IgG diluted 1 : 100 (ab4051); anti-Bax rabbit monoclonal IgG diluted 1 : 250 (ab32503); anti-Bcl2 rabbit polyclonal IgG diluted 1 : 100 (ab7973); anti-PCNA mouse monoclonal IgG2a diluted 1 : 1000 (ab29). Sections were incubated overnight for 18 h at 4°C in a humidified chamber. The slides were then incubated for 30 min at room temperature with secondary antibody (ab94698, Mouse- and Rabbit-Specific HRP Plus IHC kit IgG anti-rabbit anti-mouse) and for a further 30 min at room temperature with streptavidin–horseradish peroxidase complex (ab94698, HRP-Streptavidin–horseradish peroxidase). Diaminobenzidine tetrahydrochloride chromogen was used as a substrate (ab94698, Diaminobenzidine, DAB) with 3% hydrogen peroxide. Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted. Specificity was verified by substitution of the respective antibody with non-immune serum (diluted 1 : 500).

### Immunohistochemistry image analyses

Immunoexpression of caspase-3, Bax, Bcl-2 and PCNA was measured from digital images that had been retrieved as described above for stereological studies. The percentage of stained area in the testis parenchyma was calculated with ImageJ image analysis software in 50 fields with a quantitative automatic tool. A macro was created in ImageJ to establish a threshold for the detection of brown staining. Specific brown intensities (or positive areas) were selected for each antibody. This automated analysis for colour threshold and segmentation analysis was set for each antibody from an RGB image. The percentage of stained area was evaluated through colour segmentation analysis in which all the objects of a specific brown threshold colour are extracted. With RGB images, RGB pixels were converted to brightness values and only the percentage of pixels in the images that had been highlighted in red using image threshold were included in the measurement. These values were verified and normalized with several controls carried across runs using the same region for calibration. The methodological details of image analysis as a valid method for quantifying have been described previously (Ruifrok and Johnston 2001; Ortega et al. 2010).

### Statistic analyses

All the morphometric and immunohistochemical data were expressed as means (± SEM). Statistical analyses were carried out by ANOVA with PROC MIXED of SAS (v. 9. 1; SAS Institute Inc., Cary, NC, USA). The statistical model included the effect of treatment (betamethasone or control), days of sampling (gestational and post-natal) and the interaction between the two effects. *Post hoc* differences between groups were assessed with Tukey's tests. In all cases, the level of statistical significance was defined at $p < 0.05$.

## Results

### Stereological results

Pre- and post-natal testis parenchyma until 45 PD comprised interstitial tissue and solid sex cords in both betamethasone-treated and control animals. Lumen was observed at 90 PD in controls, but not in betamethasone-treated animals (Fig. 1). In control animals, sex cord diameter increased steadily, from 34 ± 0.3 to 50 ± 0.3 μm, with development from 121 DG to 90 PD (Fig. 2). In betamethasone-treated animals, sex cord diameter did not increase between 121 DG and 132 DG or between 45 and 90 PD. During pre-natal development at 121 DG, sex cord diameters were smaller in betamethasone-treated animals ($p = 0.045$) than in control animals. Similar effects were observed during post-natal development, with a lower sex cord diameter in betamethasone-treated animals at days 45 ($p < 0.0001$) and 90 ($p < 0.0001$) PD compared with controls. On the other hand, sex cord diameter was larger on 90 PD compared with pre-natal and 45 PD ($p < 0.0001$) in control animals (Fig. 2d).

Morphometric variables for testis parenchyma differed between betamethasone-treated and control animals during both pre- and post-natal development (Fig. 2). In controls, 54% of testis parenchyma volume was occupied by sex cords at 121 DG, after which there was a decrease at 132 DG ($p < 0.0005$) and again by 90 PD ($p < 0.0001$), concomitant with an increase in interstitial tissue increased ($p < 0.0001$). By contrast, in betamethasone-treated animals, sex cord volume did not change among both pre-natal period DG and 45 PD. Moreover, sex cords volume density was smaller ($p = 0.008$) at 90 PD betamethasone-treated animals compared with control animals (Fig. 2c). Interstitial tissue absolute volumes (Av) decreased in betamethasone-treated animals at 45 ($p = 0.0034$) and 90 PD ($p < 0.0001$) compared with control groups.

### Immunohistochemistry results

Immunoexpression of caspase-3, Bax and Bcl-2 differed between pre- and post-natal developmental periods. Pro-apoptotic active caspase-3 was detected in perinuclear region of the cytoplasm and in the nuclei in Sertoli, Leydig and germ cells in both the betamethasone and control groups (Fig. 3a–b). In saline controls, immune-reactive caspase-3 staining, ranging from weak to intense, was detected during both the pre-natal and post-natal periods, with maximum expression during the pre-natal period from 121 DG. In the betamethasone groups, caspase-3 expression was lower at 121 DG ($p = 0.002$) and 90 PD ($p = 0.004$) compared with control values (Fig. 4a). Bax expression was detectable in the cytoplasm of Sertoli and Leydig cells from both betamethasone and control groups and was localized in the perinuclear region of germ cells (Fig. 3c–d). Betamethasone-treated animals tended to have more Bax expression at 121 DG ($p = 0.07$), after which the intensity increased (Fig. 4b), such that values were significantly higher than those in the controls at 132 DG ($p = 0.0005$) and 45 PD ($p < 0.0001$) and 90 PD ($p = 0.04$).

Immunoexpression of Bcl-2 was localized to the cytoplasm of Sertoli and Leydig cells, and to the nuclear

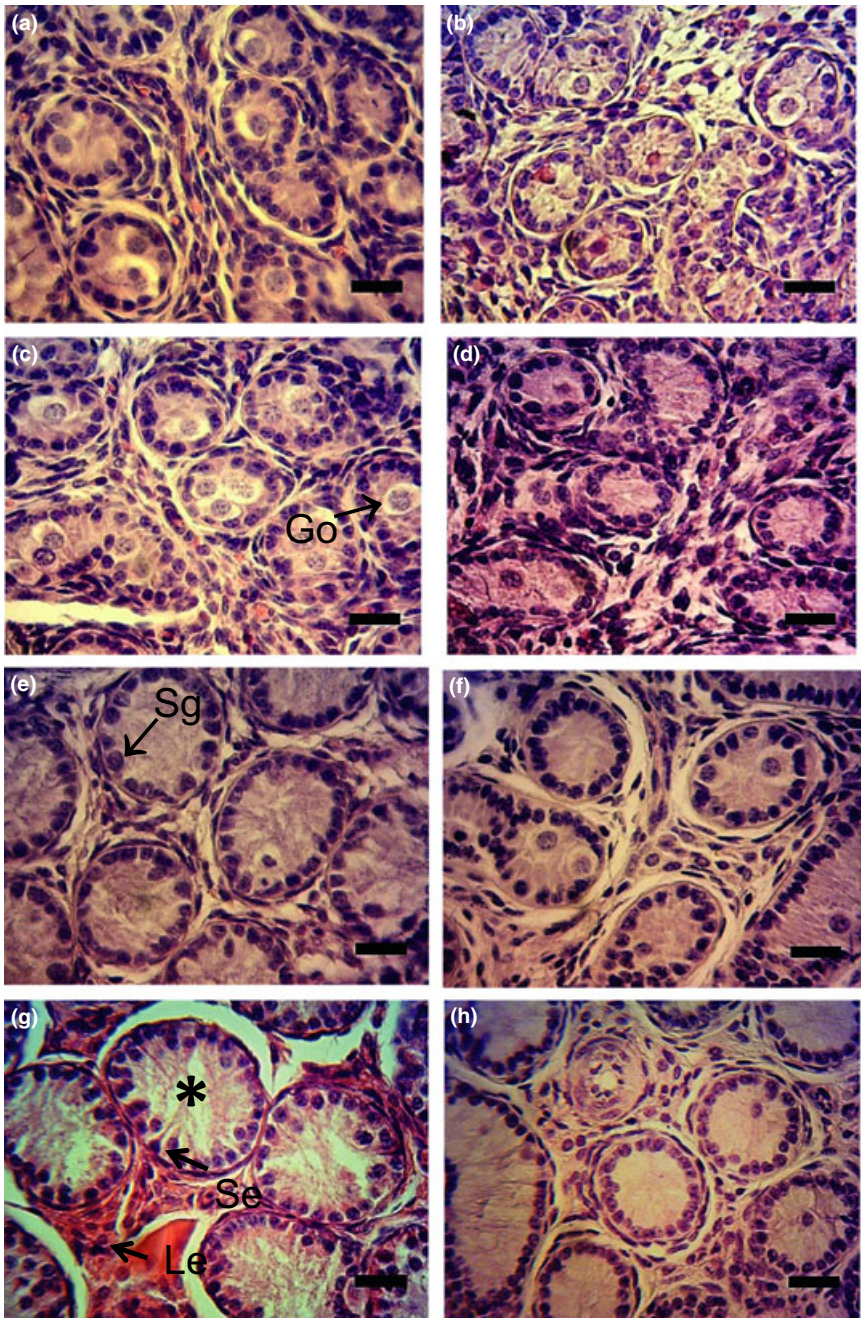


Fig. 1. Optical microscopy images of ovine testicular tissue after *in utero* administration of saline (control) or betamethasone at 104, 111 and 118 days of gestation (DG). (a) Control testis at 121 days of gestation; (b) Betamethasone-treated at 121 days of gestation; (c) Control testis at 132 days of gestation; (d) Betamethasone-treated at 132 days of gestation; (e) Control testis at 45 days after birth; (f) Betamethasone-treated at 45 days after birth; note gonocytes (Go) in centrally localized in the sex cords; (g) Control testis at 90 days after birth; (h) Betamethasone-treated at 90 days after birth; note * lumen in sex cords indicating appearance of seminiferous tubules, Leydig cells (Le) with eosinophilic cytoplasm, Sertoli cells (Se) acquiring adult shape. Magnification 400 × . Scale bar: 10 μm

and perinuclear regions of gonocytes (Fig. 3e–f). Betamethasone-treated animals showed a decrease in Bcl-2 expression at 121 DG ($p = 0.001$) and at 45 PD ($p = 0.005$), and two high peak (Fig. 4c) at 132 DG ($p < 0.0001$) and 90 PD ($p = 0.0006$). This pattern of increasing and then decreasing Bcl-2 expression was similar to the expression of proliferating cell nuclear antigen (PCNA). Gonocytes were centrally located in the sex cords and Sertoli cells at periphery, and both cell types expressed PCNA in both betamethasone and control animals (Fig. 3g–h). Pre- and post-natal PCNA expression was greater at 132 days of gestation than at other times, in both betamethasone and control animals, but differed between treatments at other times. In betamethasone-treated animals, PCNA staining was darker at 132 DG ($p < 0.0001$) and tended to be darker at 90 PD ($p = 0.07$) compared with control animals. Testicular PCNA expression was less intense in betamethasone-treated animals at 121 DG ($p = 0.006$) and 45 PD ($p = 0.009$) compared with controls (Fig. 4d).

## Discussion

Administration of glucocorticoid *in utero* influences testicular development in the sheep foetus, and the effects carry over to post-natal development. There was a reduction in sex cord diameter and volume densities that persisted long into post-natal pre-pubertal development, with a delay in opening of the sex cords as they develop into functional seminiferous tubules typically around day 90 after birth in the male sheep (Schanbacher et al. 1974). These histological changes were accompanied by changes in the balance between apoptosis-inhibiting and apoptosis-inducing proteins as the

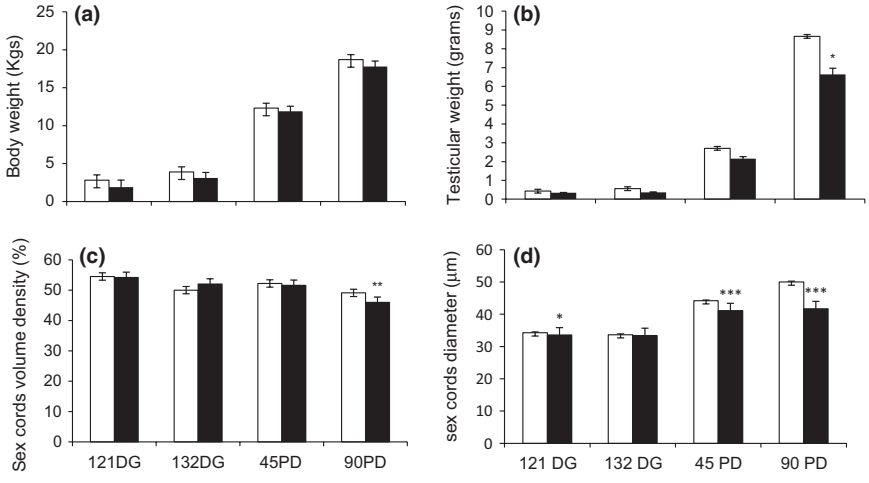


Fig. 2. Body weight (a), left testis weight (b), sex cord volume density (c) and sex cord diameter (d) in betamethasone (black bars) and control (white bars) lambs at 121 and 132 days of gestation (DG) and at 45 and 90 post-natal days (PD). Values are mean ± SEM. Differences among treatments within sampling times are indicated with asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

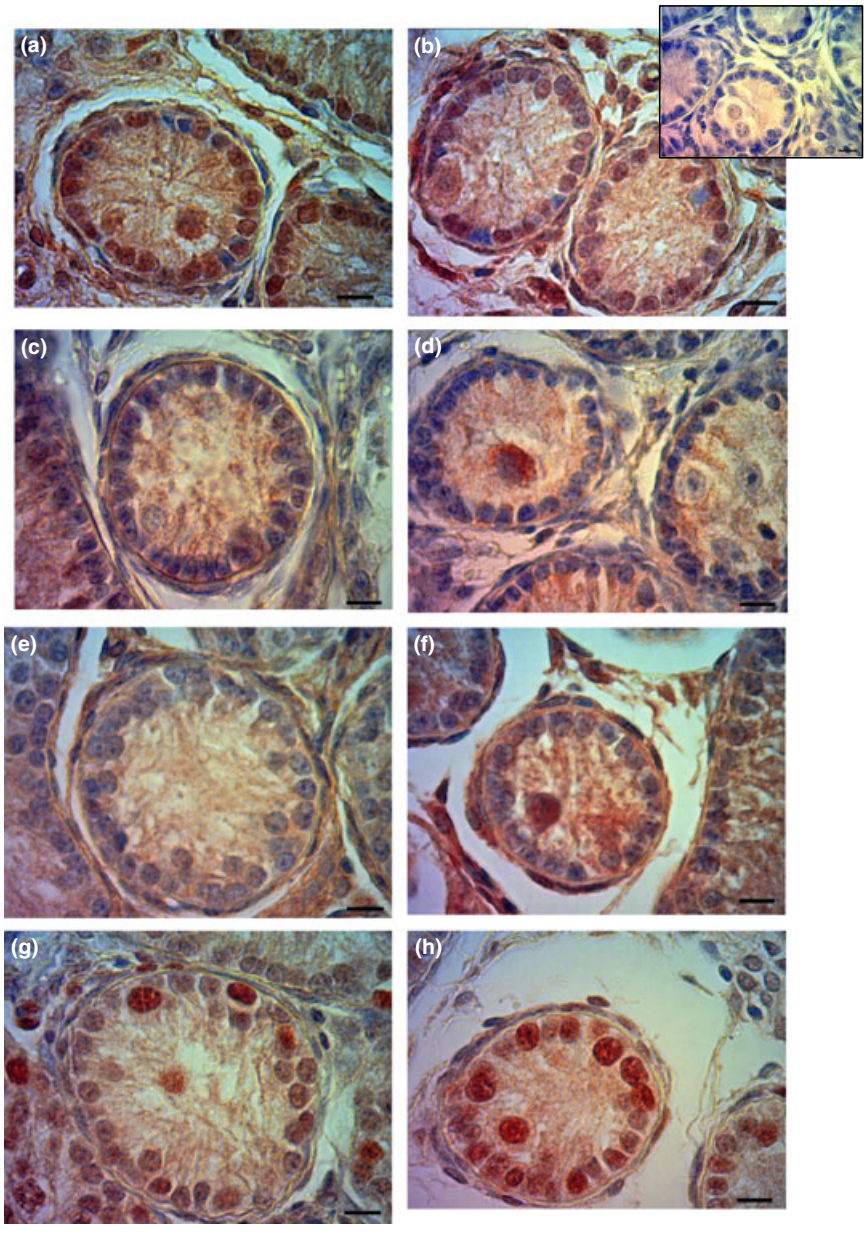


Fig. 3. Immunoexpression of factors reflecting apoptosis or cell proliferation in testicular parenchyma at 90 days after birth following *in utero* treatment with betamethasone or saline at 104, 111 and 116 days of gestation (DG). (a) Caspase-3, betamethasone-treated; (b) Caspase-3, control; (c) Bax, betamethasone-treated; (d) Bax, control; (e) Bcl-2, betamethasone-treated; (f) Bcl-2, control; (g) PCNA, betamethasone-treated; (h) PCNA, control. Magnification 600×. Scale bar: 10 µm. Top right: Negative control for immunohistochemistry

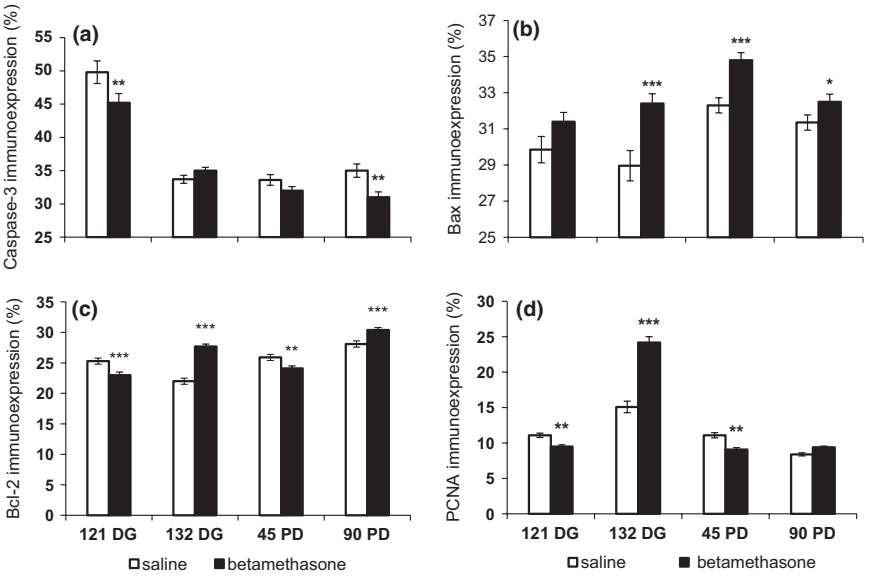


Fig. 4. Percentage immune expression of apoptotic and cell-proliferation proteins (mean ± SEM) in testicular parenchyma after *in utero* administration of saline (control) or betamethasone at 104, 111 and 118 days of gestation (DG). Tissues were sampled at 121 and 132 days of gestation (DG) and at 45 and 90 post-natal days (PD). (a) Caspase-3, (b) Bax, (c) Bcl-2; (d) PCNA. Within sampling times, asterisks indicate differences between betamethasone-treated and control groups: *p < 0. 05, **p < 0. 01, ***p < 0. 001

testis develops, suggesting that the glucocorticoids have immediate and long-term effects on the processes that modify tissue structure during the development of spermatogenic function. It is feasible that *in utero* glucocorticoid treatment affects future fertility.

Overall, the histological evidence is that pre-natal glucocorticoid treatment delays pre-pubertal testicular development, the consequences of which would be postponement of the first spermatogenic wave – normally, gonocytes begin to form spermatogonia at 65 post-natal days, and primary spermatocytes are evident at 69 post-natal days (Schanbacher et al. 1974). During foetal life, an increase in sex cord size is mainly a consequence of increase in sex cord length, not diameter (Hochereau-de Reviers et al. 1995). These descriptions match the observations in the present study. During post-natal life, increments in testicular weight are due primarily to an enlargement in the diameter of seminiferous cords that accompanies germ cell proliferation (Clermont and Huckins 1961). However, we could not detect an increase in sex cord diameter in animals treated *in utero* with betamethasone, suggesting that the effect on sex cords persists for several months and thus, in the sheep, well into post-natal life.

Tissue development involves both cell proliferation and cell death. Our data strongly suggest that a cell death pathway that involves changes in the balance of the apoptosis enzymes, caspase-3, Bax and Bcl-2, is activated within the testis by glucocorticoid administration during the last trimester of gestation. Glucocorticoid treatment reduced the expression of active caspase-3 while increasing the expression of Bax and Bcl-2, in association with the arrested growth of the sex cords. The translocation of caspase-3 to the nucleus appears to be a prerequisite for DNA degradation and subsequent germ cell apoptosis (Kim et al. 2001). This agrees with our observation of expression of caspase-3 in the perinuclear region as well as in the nucleus of the gonocytes of control foetuses. Our observations are also consistent with previous studies showing that apoptosis in germ cells was preceded by a redistribution of Bax from cytoplasmic to perinuclear regions (Yamamoto et al. 2001). In the present study, there was clear cytoplasmic perinuclear and nuclear staining for Bax in gonocytes from betamethasone-treated animals, suggesting that the redistribution of this enzyme to the nucleus can be driven by glucocorticoids.

Onset of spermatogenesis begins with an apoptotic germ cell wave that limits their efficiency during the first cycle in most mammals and ensures a balance between spermatogonia and supporting Sertoli cells (Rodriguez et al. 1997). Our data are consistent with this proposal – control testes showed an increase in the expression of active caspase-3 in 90-day-old lambs, reflecting the description of a massive apoptotic wave normally occurring in pre-pubertal testis of the rat (Jahnukainen et al. 2004). Sertoli cells can support only a limited number of germ cells, a major factor in the regulation of germ cell death (Pentikäinen et al. 1999). Once the activation of caspase-3 begins, the destiny of the cell has been decided because the process cannot be reversed (Said et al. 2004). The lack of apoptosis during the first wave of spermatogenesis in the prepuberal period has been associated with the accumulation of spermatogonia and infertility in later life (Jahnukainen et al. 2004). Therefore, the decrease in active caspase-3 in pre- and post-natal testis that we observed in the present study suggests that reproductive performance is likely to be compromised in adulthood. Furthermore, we found an increase in pro-apoptotic Bax protein during pre- and post-natal testicular development in betamethasone-treated animals. Bax deficiency in adults resulted in accumulation of pre-meiotic germ cells in mature animals, and absence of spermatocytes and mature sperm (Russell et al. 2002). Previous studies have shown that inducible Bax expression triggers a rapid caspase-dependent apoptosis, but if caspase activity is inhibited, a slower non-apoptotic death proceeds (Finucane et al. 1999). In the present study, *in utero* betamethasone administration determined an increased in Bax immunoexpression in pre- and post-natal period. Whether or not these increased enhance in Bax could be sufficient for subsequent death still remains unknown.

Concerning the anti-apoptotic protein, Bcl-2, our results showed an overexpression in lamb testis at 132 days of gestation 90 post-natal days in betamethasone animals. Members of the anti-apoptotic oncogene family, Bcl-2 and Bcl-xL, are capable of inhibiting or delaying the release of cytochrome c, while significantly preventing cell death. The increase in Bcl-2 immunoexpression in betamethasone-treated animals indicates that betamethasone should play a modulating role in blocking the mammalian cell death machinery by acting in caspase function level, during late gestation and pre-pubertal period, suggesting a future alteration of spermatogenesis. In accordance with previous studies (Russell et al. 2002), the misbalance between pro- and anti-apoptotic proteins could be sufficient to disturb the normal development of future spermatogenesis. The anti-apoptotic mechanism of Bcl-2 includes the formation of heterodimers between Bcl-2/Bcl-xL and Bax which would interfere with the availability and translocation of the Bax protein from the cytoplasm to the mitochondria. While an *in vivo* competition exists between Bax and Bcl-2, a single copy of Bax gene promoted apoptosis in the absence of Bcl-2. In contrast, overexpression of Bcl-2 still repressed apoptosis in the absence of Bax (Knudson and Korsmeyer 1997).

With respect to cell proliferation, we studied PCNA expression in the testicular parenchyma and found that *in utero* betamethasone increased it during the pre-natal period and decreased it during the post-natal period. The pre-natal increase was associated with an increase in Bcl-2 and Bax expression. In sheep, there are two periods of intense mitotic activity of Sertoli and Leydig cells, one before day 110 of gestation and the second after birth. Gonocyte proliferation increases progressively from sexual differentiation until birth (Hochereau-de Reviers et al. 1995). Consequently, the increased expression of PCNA in testis cells from glucocorticoid-treated animals at pre-natal period probably reflects an increase in the proliferation of gonocytes and at that of post-natal period reflects an increase in proliferation of spermatogonia. A consequence of this outcome would be overproduction of spermatogonia and therefore, eventually, testicular atrophy and infertility, as describe for the mouse (Jahnukainen et al. 2004). Overproduction of germ cell requires a mechanism such as apoptosis to match the germ cells with the supportive capacity of Sertoli cells (Said et al. 2004). Prevention of this physiological apoptotic process will thereby damaged Sertoli cell capacity, producing an alteration of normal spermatogenesis (Rodriguez et al. 1997). In the present study, we demonstrated that glucocorticoids interfere in the balance between apoptosis-regulating enzymes that control or trigger apoptosis (caspase-3, Bax, Bcl-2), and probably interfere with future spermatogenesis.

## Conclusion

Administration of glucocorticoid *in utero* influences testicular development in the sheep foetus, and the effects carry over to post-natal development. A reduction in the growth of the sex cords, and in the development of a lumen, persists long into post-natal pre-pubertal development. This would probably delay the development of spermatogenic function and the onset of spermatogenesis. The histological changes appear to be driven by changes in tissue-modification processes, including those that control cell proliferation and cell death. It is feasible that *in utero* glucocorticoid treatment affects future fertility.

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### Conflict of interest

None of the authors have any conflict of interest to declare.

### Author contributions

Graciela Pedrana worked in experimental design, tissue collection, testis sampling, processing, immunohistochemical assays, statistical analyses and writing of the manuscript; Maria Helen Viotti worked in processing samples, immunohistochemical assays, statistical analyses and writing of the manuscript; Elisa Souza processing histological samples and morphometry analyses, statistical analyses and writing results of morphological data; Deborah Sloboda worked in experimental design and writing of the manuscript; Graeme B. Martin worked in experimental design, writing paper and rewriting correction; Daniel Cavestany designing of statistical analyses; Hugo H Ortega defining work for immunohistochemical assays and methodology used for image analyses, analysed data, drafted and writing manuscript.

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