FREEMARTINISM IN A SHEEP

Freemartinisme bij een schaap

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ABSTRACT

In this case report an anatomical, histologic, hormonal and cytogenetic study of an intersexual sheep was carried out. At the age of 15 months, no udder could be detected in the animal, which had been classified as a female of a heterosexual twin at birth. At the age of 22 months the sheep was diagnosed as an intersex due to the presence of both male and female characteristics, viz. two testicles and a vulva. The hCG stimulation test proved that active Leydig cells were present in the male gonads. Histologic analysis after necropsy showed inflammation and atrophy of the testicular parenchyma. However, at the cytogenetic level, sexual chromosome mosaicism 54, XX / 54, XY was found after lymphocyte culture. These analyses made it clear that the intersex described here was a male pseudohermaphrodite. This condition occurs in less than 0.03% of the total sheep population. The hCG stimulation test can be used to distinguish "pseudo-male" from "pseudo-female" freemartins at a young age and without the need for necropsy.

SAMENVATTING

In deze casuïstiek worden anatomische, histologische, hormonale en cytogenetische kenmerken van een interseksueel schaap besproken. Op de leeftijd van 15 maanden kon bij dit dier geen uier aangetroffen worden, beter: had op de leeftijd van 15 maanden geen uier, hoewel het bij de geboorte als ooilam van een heteroseksuele tweeling werd geklasseerd. Op de leeftijd van 22 maanden werd het dier als interseks gediagnosticeerd door de aanwezigheid van zowel mannelijke als vrouwelijke eigenschappen, respectievelijk twee testikels en een vulva. De hCG stimulatietest wees op de aanwezigheid van actieve Leydigcellen in de mannelijke gonaden. Na autopsie werden bij histologisch onderzoek ontsteking en atrofie van het testiculair parenchym vastgesteld. Na cytogenetisch onderzoek van een lymfocytencultuur bleek er een mosaïcisme te zijn van de geslachtschromosomen (54, XX / 54, XY). Uit voorgaande onderzoeken kan besloten worden dat de beschreven interseks een mannelijke pseudo-hermafrodiet was. Dergelijke gevallen komen voor in minder dan 0,03 % van de ganse schapenpopulatie. De hCG stimulatietest kan gebruikt worden om een onderscheid te maken tussen "pseudo-mannelijke" en "pseudo-vrouwelijke" kwenen op jonge leeftijd zonder dat het uitvoeren van een autopsie noodzakelijk is.

INTRODUCTION

The genetic sex of an individual is determined at the moment of fertilization, and is not influenced by environmental factors. In mammals, the male is always heterogeneous XY, and the female homogeneous XX. Intersexes possess genital organs with both male and female characteristics, and can be subdivided into "real hermaphrodites", which have both male and female gonads, and "pseudo-male" or "pseudo-female" hermaphrodites, which have either male or female gonads combined with accessory genital organs of the other gender (Cribiu and Chaffaux, 1990). Intersexuality can be attributed to freemartinism, the term describing a sterile female born as a co-twin to a male in any species. Freemartinism is a result of anastomosis of the chorioallantoic circulation of twin male and female fetuses in utero (Lillie, 1917). The anastomosis subsequently allows the exchange of leukocytes between the fetuses and the masculinization of the female fetus. The exchange of leukocytes between male and female fetuses results in both XX and XY chromosomes being found at cytogenetic analysis.

The masculinization of the female fetus is probably caused by hormone-like action through H-Y antigens and the effect of Müllerian inhibiting substance. Whatever the cause, in almost all cases the female develops into a sterile intersex, with both XX and XY sex chromosomes, severe ovarian hypoplasia and nearly complete absence of the tubal genital organs. The external genitals of the female are normally not affected (Kennedy, 1985; McEntee, 1990). In contrast, very few morphological changes are found in the co-twin male although reduced fertility due to the influence of estrogen is possible.

With the exception of bovine freemartinism, intersexuality is rarely reported in ruminants. In cattle, intersexuality as the result of the freemartinism syndrome occurs in more than 90% of female fetuses of heterosexual multiple pregnancies (David *et al.*, 1976), while in sheep only 1% of heterosexual twins are diagnosed as intersex (Kennedy, 1985). Freemartinism is likely to occur in 0.03 % of the whole sheep population (Smith *et al.*, 1998). The male or female character of the gonads is usually reflected by the morphology of the external features, e.g. the size of the glans clitoris and the descent of the gonads (Bruere and Macnab, 1968).

Freemartinism can be diagnosed reliably by the examination of chromosomes from cultured leucocytes. However, no correlation has been found between the percentage of male cells (XY) in repeated blood cultures from freemartin sheep and the degree of masculinization of the genitals (Bruere and Macnab, 1968; Greene *et al.*, 1977). Histologically, as in bovine freemartins, mesonephrotic and paramesonephrotic ducts are present in each of the gonads and their development is in close accordance with the degree of masculinization of the genitals. No structures comparable to an ovarian follicle have ever been found (Bruere and Macnab, 1968).

The aim of this report is to give an anatomical, histologic, hormonal and cytogenetic description of a freemartin sheep. The applicability of the hCG stimulation test to distinguish "pseudo-male" from "pseudo-female" freemartins is discussed.

MATERIAL AND METHODS

History of the animal

The sheep described in this report was born in the winter of 1998 as co-twin to a ram. It was the offspring of a Suffolk ewe and a Texel ram. At birth it was reported to be a female. At the age of 10 months it was presented for mating to a ram, but it did not breed. At 18 months of age, the sheep began to show male libido towards ewes in heat, although it urinated like a ewe. Finally, after evaluation of the external genitals, hormonal analysis and cytogenetic analysis, the animal was identified as an intersex and was euthanized for necropsy at the age of 23 months.

Anatomical and histologic analysis

The external appearance and genitals were evaluated in the live animal. Testicular consistency was evaluated by ultrasound. During necropsy, samples were taken from testicles, ampullae, vesicular glands and urethra. These tissue samples were fixed, embedded and cut in thin sections that were dried on uncoated slides. Staining was performed with haematoxylin-eosin and Van Gieson.

Hormonal analysis

Blood samples for the analysis of testosterone were taken on heparin before and after a single i.v. injection (t_0) of 4500 IU hCG (Chorulon®, Intervet, Boxmeer, the Netherlands) in three sheep: the intersex, an intact ram and an intact ewe. Blood samples were collected every 15 min in the first hour, every 30 min in the second hour and every two hours for the following 6 hours after injection. During the next three days, blood samples were taken at 12-hour intervals. The plasma testosterone and progesterone concentrations were analyzed by RIA (Henry *et al.*, 1987).

Cytogenetic examination

A blood sample of the intersex was taken prior to necropsy and lymphocyte cultures were prepared according to the method described by Galli *et al.* (1987). Both chromosome counts and karyotype examinations were carried out.

RESULTS

Anatomical and histologic analysis

At birth, the lamb was classified as a female but at 15 months, when the sheep was shorn, it was noticed that the udder was absent. Cranial to the normal location of the udder, two bulges of about 1 cm x 2 cm in diameter could be palpated. The external genitals were similar to those of a ewe, except for an enlarged clitoris glans at the ventral commissure of the vulva.

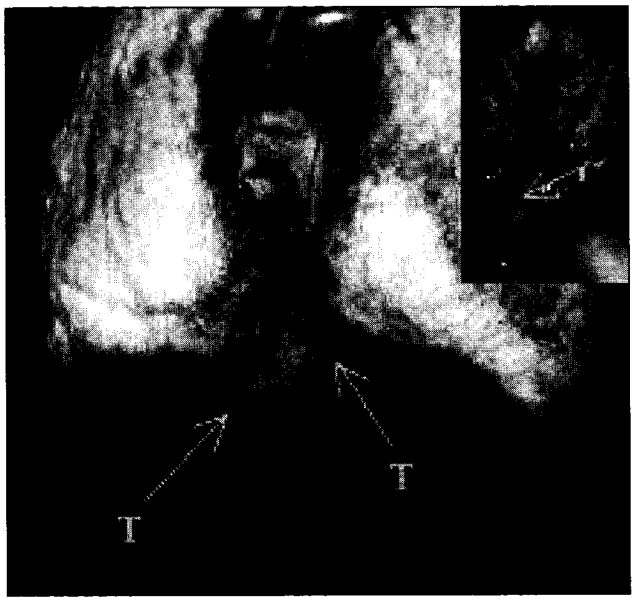

Fig. 1. Position of the testicles (T) and enlarged clitoris (C) in the ovine intersex.

Fig. 3. Histologic picture of the left testis showing a few tubuli seminiferi embedded in a lot of connective tissue (HE, x 400).

At vaginoscopic examination, the intersex had a 7-cm-deep vagina with a urethral opening leading to the bladder, but more cranially the vagina narrowed abruptly and no cervix could be seen. Testis-like gonads could be palpated subcutaneously at the external inguinal ring.

At 18 months of age, the left testicle began to descend and this process was completed at the age of 22 months (Fig. 1). At inspection, this adult intersex appeared to be male with a small ram head, two bases for horn formation and heavy forequarters. The descended left testicle was very large (16 cm x 11 cm), while the right testicle remained inguinal and hypoplastic (4.5 cm x 1.5 cm). Ultrasound examination demonstrated an abscess with several septa in the left testicle. The whole spermatic cord could be palpated, including the cremaster muscle, the testicular blood vessels and the ductus deferens.

Fig. 2. Internal genitals of the intersex: R = hypoplastic right testicle (6 x 1.5 cm), L = inflammated left testicle (16 x 11 cm), E = epididymis, D = ductus deferens, A = ampulla ductus deferentis, B = bladder, G = glans clitoris. Other male anatomical structures as prostate, bulbourethral glands, a penis and the retractor penis, ischiocavernosus and bulbospongiosus muscles were absent in this intersex. The depth of the vagina is 7 cm.

At necropsy, most of the male reproductive tract could be identified: 2 testicles, 2 epididymides, 2 ampullae ductus deferentis and 2 vesicular glands (Fig. 2). Other male anatomic structures were absent. Histologically, extensive fibrosis and infiltration of inflammatory cells were detected in both testicles, making the differentiation of the different cell types difficult. The nuclei of the Sertoli cells and the spermatogonia were round and located basally in the tubuli seminiferi, while the Leydig cells could be recognized by their large nucleolus. In the hypoplastic right testicle, inactive seminiferous tubules with Sertoli cells and a few spermatogonia were present (Fig. 3). In the functional part of the inflamed left testicle, atrophic seminiferous tubules without spermatogonia were detected. In the fibrotic tissue of the left testicle, no sign of spermatogenesis was noticed.

In the vagina, a normal urethral opening including a suburethral diverticle and an enlarged glans clitoris containing erectile tissue, were found. No traces of cervix, uterus, uterine tubes or ovaries could be detected (Fig. 2).

Hormonal analysis

The progesterone concentration of the intersex was much lower than in a ewe at the end of the luteal phase of the cycle (Fig. 4). Before the administration of hCG, no difference in testosterone concentration could be found between the intersex, the ram and the ewe. The testosterone concentration in the ram incre-

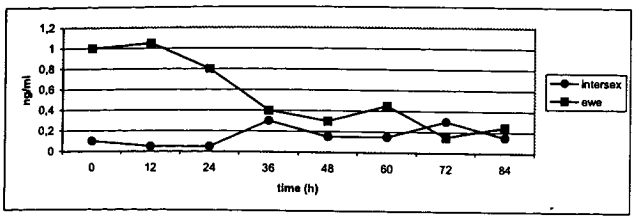

Fig 4. Progesterone concentrations in the intersex and in a ewe at the end of the luteal phase (t_0 = moment of injection of 4500 IU hCG).

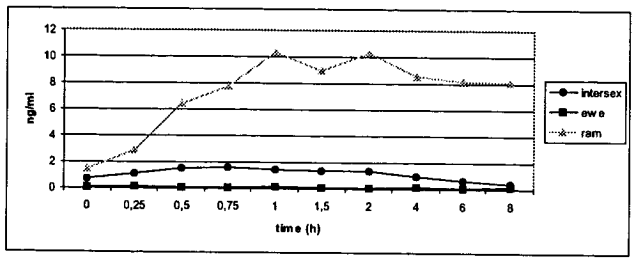

Fig 5. Testosterone concentrations after IV-administration (t_0) of 4500 IU hCG.

ased substantially to >8 ng/ml within 45 min after injection of hCG (t_0) and remained above this level for a period of at least 7 hours. In the intersex the testosterone concentration increased within 45 min after hCG injection to 1.9 ng/ml and dropped below 1.5 ng/ml 2 hours after hCG injection. In the ewe no increase (>0.2 ng/ml) in testosterone was notified (Fig. 5).

Cytogenetic analysis

The cytogenetic analysis showed the presence of sexual chromosome mosaicism 54, XX / 54, XY in lymphocytes. The lymphocyte count, carried out on several metaphasic plates of different cultures with well spread, separate chromosomes, showed that half of the lymphocytes contained 6 metacentric and 48 acrocentric chromosomes and were female (54, XX), while in the other half of the lymphocytes one acrocentric X chromosome was replaced by the small submetacentric male Y chromosome (54, XY).

DISCUSSION

In this paper, a case of freemartinism (54, XX / 54, XY) in a sheep is described. The animal was classified as a "pseudo-male" hermaphrodite because the testicular gonads were combined with male and female genital organs. In contrast to cattle, where freemartinism occurs in more than 90% of heterosexual twins and the males are mostly normal and fertile (Dunn *et al.*, 1979), the male freemartins in sheep have a prevalence of ≤ 1% in heterosexual twins and are sterile (Kennedy, 1985). However, recent studies indicate that the prevalence of freemartinism in sheep is increasing (Parkinson *et al.*, 2001).

Most freemartin sheep described in the literature are of the "male type", with only seven freemartins of the "female" type having been described (Chaffaux *et al.*, 1987). The absence of ovarian tissue in the present intersex could be anticipated because the plasma progesterone concentration never exceeded the threshold level of 0.5 ng/ml for a period of 84 hours. In contrast to Parkinson *et al.* (2001), who found higher testosterone concentrations in freemartins than in ewes but no response of testosterone to GnRH or eCG, the testosterone concentration in our study was increased after administration of hCG. The hCG stimulation test showed an increase in testosterone to about 2 ng/ml in the intersex, although it was not as pronounced as in the intact ram (>8 ng/ml). This indicates the presence of Leydig cells, although less functional or less abundant than in the intact ram. This was confirmed by histologic examination, which proves that the hCG stimulation test can be used not only for the detection of cryptorchidism but also to distinguish "pseudo-male" from "pseudo-female" intersexes. Although in this intersex no hCG stimulation test was performed at a young age, we may assume that the hCG stimulation test could have been applied from puberty on. At histologic examination the distinction between Sertoli cells, Leydig cells and spermatogonia was complicated by the infiltration of inflammatory cells and fibrosis in the testicles. The production of Müllerian inhibiting substance by the Sertoli cells during the embryonic phase may have inhibited the development of the uterine tubes, uterus and cervix in the intersex, while on the other hand inflammation and extensive fibrosis in both testicles may have resulted in a decrease of the Müllerian inhibiting substance and a delayed initiation of the transabdominal migration of the testicles (Ladds, 1993). The suboptimal testosterone concentration in the intersex was sufficient to induce the differentiation of the canals of Wolff but insufficient for a normal inguino-scrotal descent of the right testicle. Differentiation of the canals of Wollf can also be induced either by exogenous testosterone produced by the adrenal glands or by tumors, both in "pseudo-male" as in "pseudo-female" hermaphrodites (Austin and Short, 1982).

The incidence of freemartinism as a result of chimaerism in heterosexual twins is low in sheep (≤1%) compared to cattle (>90%). In sheep breeds with high

fertility and a high percentage of multiple pregnancies (e.g. Romanov sheep), the infertility caused by chimaerism is very low (Cribiu and Chaffaux, 1990). This low incidence of ovine freemartinism is attributed to the infrequency (0.8 – 10% of multiple pregnancies) of significant interplacental vascular anastomoses in this species (Stromont *et al.*, 1953, Alexander and Williams, 1964). The most important factor for the production of chimaerism and freemartinism in multiple pregnancies seems to be the degree of inbreeding (Dain, 1971). Long (1980) found an incidence of ± 1% of freemartinism in sheep presented for market sale, but this rate may be overestimated because ewes with fertility problems are more likely to be sold. Besides the hypothesis of vascular anastomosis, mosaicism (54 XX / 54 XY) can also be explained on the basis of developmental abnormalities, the fusion of two embryos (Eldridge, 1985) or the fertilization of both the oocyte and the first polar body by two spermatozoa (Fechheimer and Harper, 1980).

From the available literature it can be concluded that the prevalence of freemartinism in sheep with pronounced signs of intersexuality as described in this case report is very low. The hCG stimulation test could be used to distinguish "pseudo-male" from "pseudo-female" freemartins without the need for necropsy. It remains to be determined whether the hCG stimulation test can be used in true hermaphrodites, where both types of gonadal tissue are present.

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