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Pubertal Development of Intersertoli Cell Junctions in the Testis of Corriedale Ram Lambs

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[1]Departments of Anatomy and Histology and of [2]Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Uppsala, Sweden; and Depts. of Histology & Embriology[3] and of Pathology[4], Faculty of Veterinary Medicine, Uruguay.

Bielli A., T. Gastel, A. Castrillejo, A. Moraña and H. Rodriguez-Martinez: Pubertal development of intersertoli cell junctions in the testis of corriedale ram lambs. Acta vet. scand. 1995, 36, 543-551. – The ultrastructure of the tight junctional complex of pubertal ram lamb Sertoli cells was studied in immersion-fixed samples and related to clinical data and a light microscopical classification of the degree of spermatogenesis attained in the corresponding seminiferous tubule. Although the process followed the general mammalian developmental trend for tight junction complex formation, 2 unusual ultrastructural features were detected: the presence of an active Golgi complex during the early stage of tight junction formation and the transitory presence of ribosomes on both faces of the ectoplasmic cisternae bordering the developing junctions. The significance of these findings is discussed.

puberty; blood-testis barrier; ultrastructure.

Introduction

Morphological and physiological studies have revealed the existence of blood-testis barriers that are responsible for differences in composition between the fluid in the lumen of the seminiferous tubule and that of testicular lymph or blood plasma. These barriers are made up mainly of 3 elements, disposed step-wise: testicular capillary endothelium, the peritubular myoid cells and the Sertoli cells together with their special tight junctional complexes between basolateral processes (*Setchell* 1993). These junctions effectively prevent large molecules from moving through the intercellular space and reaching the germ cells situated in the adluminal compartment of the seminiferous epithelium, thus enabling Sertoli cells to maintain a specialized tubular microenvironment, essential for a successful spermatogenesis (*Byers et al.* 1993, *Jégou* 1994, *Griswold* 1995). The age of appearance and the process of ultrastructural development of these tight junctional complexes has been described in a number of animals, as the rat, mouse, hamster, guinea pig, rabbit, pig, bull, dog, mink, human (for review see *Gondos & Berndston* 1993). Although different aspects of the postnatal and pubertal lamb testis histology have been studied (*Courot* 1971, *Monet-Kuntz et al.* 1984; *Castrillejo et al.* 1995), no ultrastructural description of the interSertoli cell tight junctional complex formation is yet available for the ovine species.

Puberty, the process of entry into reproductive life (*Lindsay et al.* 1993), is a long and complex process. Among the environmental factors affecting age at puberty in mammals the most important one is nutritional status. This has been demonstrated in the ovine species for a variety of nutritional and grazing conditions (*Alkass* 1982, *Lindsay et al.* 1993). Exposure to Uru-

Table 1: Clinical and microscopical data corresponding to the ram lambs studied.

Lamb nr.	LW (kg)	SC (cm)	TW (g)	PPS (score)	DSE (score)	TJC-MAT (degree)
1	22.0	14.5	32.5	0	0	immature
2	24.5	16.0	68.0	0	1	intermediate
3	26.5	17.0	92.5	3	2	intermediate
4	28.0	20.0	133.5	4	3	mature
5	26.0	20.0	118.0	4	3	mature
6	28.0	22.0	146.9	4	3	mature
7	32.5	27.5	309.0	4	3	mature

LW: live weight, SC: scrotal circumference, TW: testicular weight, PPS: degree of penis-preputium separation, DSE: degree of development of the seminiferous epithelium, TJC-MAT: degree of maturity of the tight junctional complex.

guayan extensive grazing conditions results in a delay in the onset of puberty compared with more benign rearing systems (*Castrillejo et al.* 1995). One main goal of male pubertal development is to become capable of producing sperm. As mentioned before, the presence of interSertoli tight junctional complexes is essential for this to occur. The aim of the present study was to describe the Sertoli-Sertoli cells tight junction formation in pubertal Corriedale ram lambs kept under extensive grazing conditions.

Materials and methods

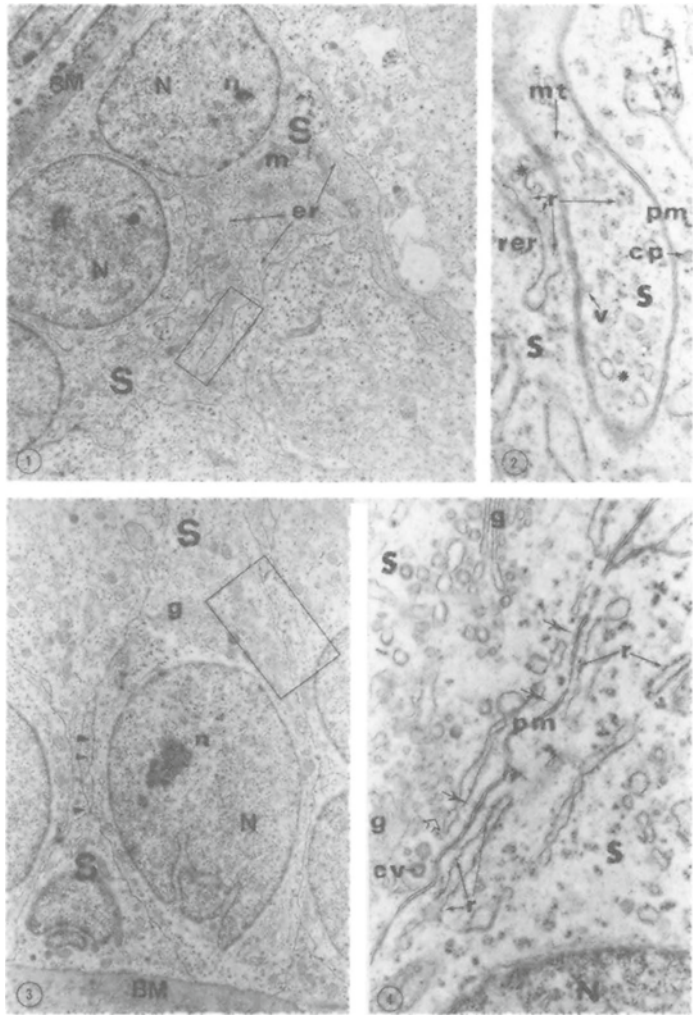
Testicular tissue was obtained from seven ram lambs *(Ovis aries)* belonging to 2 lambing groups and aged 145-175 (lamb 1) and 180-210 (lambs 2-7) days (the lambing period lasts 30 days). The ram lambs were kept with their dams under extensive grazing conditions as described in detail in *Castrillejo et al.* (1995). They were identified with collar tags and thereafter, following a night of fasting, individually weighed and clinically examined. The penis was manually exteriorized and the degree of separation of the preputial-penile mucosae registered as: 0= no separation, 1= free urethral process while gland still attached, 2= free glans penis and 3= free penis (adult appearance), Scrotal circumference was measured with a flexible tape at the widest scrotal diameter and the scrotal contents palpated. Immediately after castration, testes were weighed and examined for morphological appearance before tissue sampling.

Small (1 mm thick) tissue samples were excised from each testis at their proximal, medial (adepididymal) and distal areas and routinely processed for light and electron microscopy (*Castrillejo et al.* 1995). Semi-thin (1μm) sections were examined on a light microscope (Zeiss, Germany) to establish the degree of development of the seminiferous epithelium. The highest degree of germ cell differentiation measured in any of the tubules present in each sample was scored as: 0= only spermatogonia, 1= spermatocytes, 2= round spermatids and 3= elongated spermatids/testicular spermatozoa (i.e. complete spermatogenesis). Ultra-thin (60 nm) sections for transmission electron microscopy were cut from selected areas, mounted on uncoated copper grids, counterstained with uranyl acetate and lead citrate, and examined in a Phillips EM 201^R transmission electron microscope (Eindhoven, The Nederlands) at 60 kV.

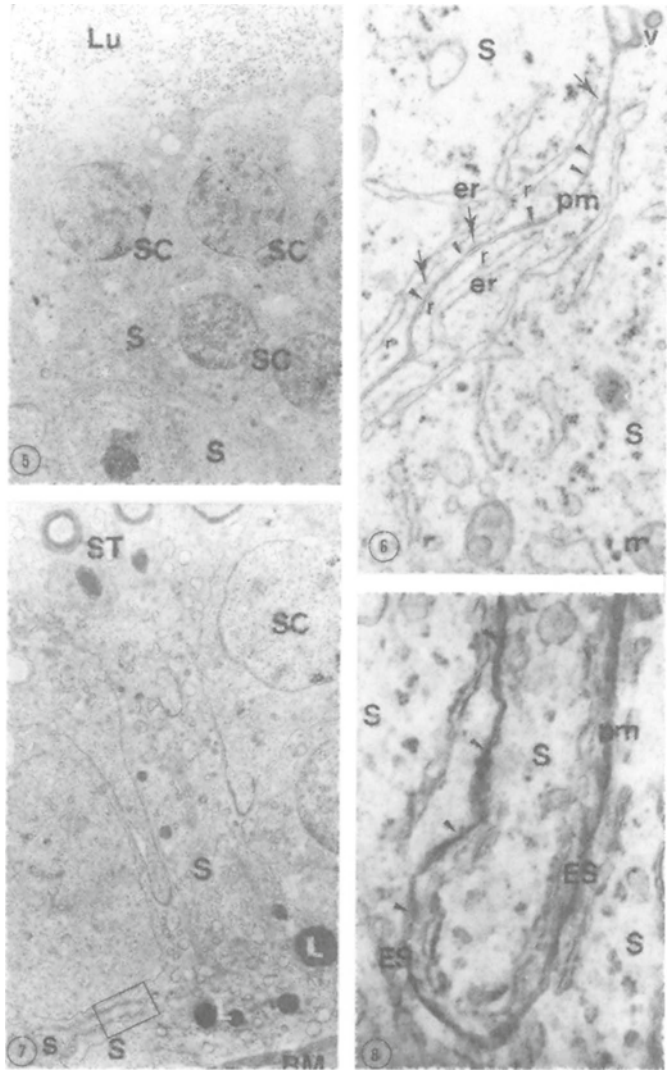
Results

Clinical data and the light microscopy classification of the degree of spermatogenesis for

each ram lamb are shown in Table 1. The electron microscopy showed that the testicular samples were properly fixed. In the youngest animal (score 0, Figs. 1-4) the testicular parenchyma harboured only seminiferous cords, consisting of Sertoli cells and spermatogonia, and with no lumen present whatsoever. Sertoli cells were cylindrical, with a few short ramifications. Nucleoli had not yet become vacuolated (Figs. 1 and 3). Although no lipid vacuoles or lysosomes were visible, several sections of Golgi complexes around the nucleus and near the lateral plasma membrane were frequently seen (Figs. 1, 3-4). The cytoplasm contained abundant mitochondria with tubular cristae, poliribosomes and both rough and smooth endoplasmic reticulum (Figs. 3 and 4). Lateral plasma membranes of these immature Sertoli cells displayed desmosome-like structures (Fig. 3) but tight junctions were not visible. In the short lateral Sertoli-Sertoli cell projections, microtubules, endoplasmic cisternae (rough and smooth) and vesicles were gathered near the plasma membranes. Two grossly different pictures were found: in one of them (Fig. 2), many vesicles and a few cisternae were present. Some clear vesicles were seen in the Sertoli cell plasmalemmae. Also, coated pits were evident budding off from plasma membranes or located in the nearby cytoplasm. In the second kind of image (Fig. 4), endoplasmic reticulum cisternae were more abundant, and were positioned in parallell with the plasma membranes. Two grossly different pictures were found: in one of them (Fig. 2), many vesicles and a few cisternae were present. Some clear vesicles were ween in the Sertoli cell plasmalemmae. Also, coated pits were evident budding off from plasma membranes or located in the nearby cytoplasm. In the second kind of image (Fig. 4), endoplasmic reticulum cisternae were more abundant, and were positioned in parallell with the plasma membranes. Points of contact were observed between the rim membranes of opposing subsurface cisternae. The membranes appeared to be fused at these points. Cisternae bore ribosomes on their outer membrane face both towards the cell body as well as towards the plasma membranes. Sparse, thin filament bundles were located in the cytoplasm between the endoplasmic cisternae and the plasma membranes. Golgi complexes were often seen near the cisternae. Numerous vesicles (originating from the Golgi complexes?) appeared to be associated to the endoplasmic cisternae, while coated vesicles seemed to fuse with Golgi complexes and *trans*-Golgi networks. The ultrastructural features described above implied an immature degree of tight junctional complex development (Table 1). In 2 other ram lambs (spermatogenesis scores 1-2) the structures corresponding to the seminiferous cords showed lumina, so that they could be called seminiferous tubules. The most developed germ cells within each seminiferous tubule were either spermatocytes (Fig. 5) or round spermatids. In a few tubules, degenerating long spermatids were present (data not shown). Sertoli cells had more profuse ramifications and some lipid droplets appeared in their cytoplasm. Sertoli-Sertoli cell tight junctions had already an adult-like character (Fig. 6) and were extensively limiting the baso-lateral areas of these cells. Parallell endoplasmic cisternae lined both sides of the tight junctions, as a part of an ectoplasmic specialization. The outer face of these cisternae bore occasional ribosomes. Some sparse, thin filaments were visible between the endoplasmic cisternae and the plasma membranes. Electron-dense material, present in the form of adherens-like junctions, lined both cytoplasmic faces of plasma membranes. Occluding junctions were interspersed with the former ones, showing a close morphological resemblance to a typical mammalian tight-junctional complex. The only deviating aspect of their morphology



Figures 1-4: TEM of Sertoli cells in seminiferous cords from a prepubertal ram lamb. Figure 1 shows cylindrical, epithelium-like Sertoli cells (S), with short cytoplasmic prolongations. Note the abundance of mitochondria (m) and endoplasmic reticulum (er). No tight junctional complexes are present (N = nucleus; n = nucleolus; BM = basal membrane; ×8000). Figure 2 (×35,000) shows a higher magnification of the area marked in 1, depicting a Sertoli-Sertoli invagination (S) with the early stage in tight junction formation. Some endoplasmic cisternae (rer) and vesicles (*) run parallel to the plasmalemmae (pm). Note the presence of ribosomes on both sides of the endoplasmic cisternae (r); v = clear vesicle; cp = coated pit; mt = microtubule. Figure 3 shows a Sertoli cell (S) with a typical nucleus (N), immature nucleolus (n) and the supranuclear Golgi complexes (g) nearby the area of formation of a tight junction (inset); arrowheads, desmosomes (×10,000). Figure 4 (×40,000) depicts a magnification of the area marked in 3, with a more advanced degree of inter-Sertoli tight junctional complex. The er cisternae and vesicles (close to the Golgi complex: g) line the adjacent plasma membranes (pm). Note the presence of occluding junctions (arrows). Note the presence of ribosomes on both sides of the reticular cisternae (r); cv = coated vesicle; open arrow = vesicle at the endoplasmic cisterna; N = nucleus.



Figures 5-8: Electron micrographs of seminiferous tubules from ram lambs. Figure 5 (×4,000) shows a partial view of the ad-luminal epithelium, that had only reached the spermatocyte stage (SC); Lu = lumen; S = Sertoli cell. Figure 6 (×35,000) shows an intermediate stage of tight junction development, from the epithelium in 5, with the typical structure of the ectoplasmic specialization in 2 adjacent Sertoli cells (S). Sparse bundles of actin-like filaments (arrows) are located between the endoplasmic cisterns (er) and the plasma membrane (pm); arrowheads = occluding junctions; v = vesicles. Note that still some ribosomes (r) are still present on the outer face of the er cisterns; m = mitochondria. Figure 7 (×6,000) shows the seminiferous epithelium in a mature seminiferous tubule, that had reached complete spermatogenesis. Typical lipid droplets (L) are present in the basal Sertoli cytoplasm (S); BM = basal membrane; SC = spermatocyte; ST = spermatid. Figure 8 (×45,000) shows a higher magnification of the area marked in 7, with a completely developed interSertoli tight junction; pm, = plasma membrane; S = Sertoli cell; ES = ectoplasmic specialization; arrowheads = occluding junction.

was the few remaining ribosomes on the outer faces of the endoplasmic cisternae. The degree of maturation of these tight junctional complexes was classified as intermediate (Table 1). In the other 4 ram lambs practically all seminiferous tubules displayed complete spermatogenesis (socre 3). Sertoli cells had a completely mature character. The nucleoli were vacuolated and basally located lipid droplets were frequent. Their cytoplasmic processes were fully developed and the longer lateral projections showed extensive tight junctions in Sertoli-Sertoli contact zones (Figs. 7-8). Higher magnifications of these tight junctions showed they were ultrastructurally indistinguishable from those appertaining to any adult mammalian testis (Fig. 8) and consequently they were classified as mature (Table 1).

Discussion

To our knowledge, the present study constitutes the first description of the process of Sertoli-Sertoli cell tight junction formation in the ovine species. The process reported in the present article is similar in general trends to that previously described for other mammals. Since no blood-testis permeability tracer studies were performed, only the structural aspects of the process can be described. The point in time at which the blood-testis barrier began to function could be determined indirectly as the time at which the seminiferous tubule lumen was formed.

In the younger animal, the cytological characteristics of the Serotli cells studied reflected the immaturity of these sustentacular cells. The abundance of polyribosomes and rough endoplasmic reticulum indicated that these Sertoli cells were performing strong synthetic activity. Moreover, mitochondria with tubular cristae were often seen. In the mitochondria with transverse non-tubular cristae present in neonatal Sertoli cells of rodents, the cristae become tubular with the onset of differentiation and the increase in steroid metabolism (*Gondos* 1993). Tubular cristae are, in general, characteristic of cells with high steroid metabolism and occur in adult Sertoli cells. All these cytological characteristics in prepuberal ram lambs correspond to a stage shortly before tight junctional complexes appearance. The incipient ectoplasmic specializations and occluding junctions that were visible in some other Sertoli cells denote a slightly more mature state.

The view of vesicles fusing with the endoplasmic cisternae of the ectoplasmic specialization and their progressive increase in length are in agreement with the classical tight junction formation process reported for other mammals. As far as we know, there is no previous description of Golgi complexes in close proximity to these membranes, as if they were undergoing an active interchange of vesicular structures with both the endoplasmic cisternae and the adjacent plasma membrane in the tight junctional complexes of the developing blood-testis barrier. It is nevertheless expectable that this phenomenon would occur. Golgi complex functions are not only linked to secretory activities but also to the recycling of membrane between organelles and from the cytoplasm to the surface for extension and renewal of the cell membrane (*Fawctt* 1994). In any case, it is reasonable to expect major changes in composition and recycling of the maturing Sertoli cell plasma membrane. Coated pits originating from the plasma membrane and apparently migrating towards the Golgi complexes could represent sites of active interchange of molecules between neighboring Sertoli cells. Coated pits have been reported in all cytoplasmic regions of mature Sertoli cells (*Russell* 1993). It is suspected that the receptor-mediated uptake of transferrin occurs via bristle-coated pits in Sertoli cells (*Morales & Clermont* 1986). These coated pits and vesicles could also provide an endocytic means of mem-

brane recycling between Golgi complexes, ectoplasmic specializations and plasma membranes. Coated vesicles seemed to fuse with some part of the Golgi complex in our material (data not shown), although we could not determine which part. They could also represent coated shuttle vesicles that move between Golgi cisternae and are devoid of clathrin. In many cells the Golgi complex has been reported to be an undulated structure whose *cis* and *trans* faces will not appear in maturing ram lamb Sertoli cells as classically described in ultrathin sections, thus making it difficult to identify the part of the Golgi complex to which these coated vesicles fuse and to further speculate on the significance of this finding. The fact that the Golgi complexes and *trans*-Golgi networks are situated close to the endoplasmic cisternae also suggests that the Golgi apparatus contributes in some way to the formation of the organized structure of cisternae parallel to both the filament layer and the tight junction cell membrane domain. We can only speculate as to the function of these structures at this stage of development of the Sertoli cells. Further studies on this aspect of the tight junctional complex formation should consider biochemical or immunocytochemical approaches.

Another novel feature of Sertoli-Sertoli tight junctional formation in ram lamb testes has been reported in this paper, i.e. the transitory presence of ribosomes on the endoplasmic cisterna surface facing the plasma membrane. These cisternae apparently arise from the nuclear envelope in rabbit pubertal Sertoli cells (*Sun & Gondos* 1986). The nuclear envelope is another organelle in which ribosomes are limited to only one face of an extensive endoplasmic system of cisternae that is lined on the other side by a filament network, the nuclear lamina of lamin intermediate filaments. The presence of ribosomes on the ER outer face probably represents a short stage in the process of ectoplasmic specialization formation. The development of the array of actin fibres linking the external face of the adult endoplasmic cisterna to the adjacent plasma membrane could perhaps account for the disappearance of the ribosomes from that face. Indications that 2 actin-binding proteins, a-actinin and fimbrin, may be present in the ectoplasmic specialization have been reported (see review by *Vogl* 1989. It would be interesting to find out whether these proteins appear in adult ovine ectoplasmic specializations, particularly in the endoplasmic cisterna outer membrane, and if so, whether their levels change as the ribosomes disappear.

Ram lambs of intermediate spermatogenetic status already showed adult-like tight junctional complex images. This should be no surprise, since the blood-testis barrier should develop before the first spermatocytes can survive (*Gilula et al.* 1976, *Hagenäs et al.* 1981, *Gondos* 1993). The character of mature tight junctions in young rams did not differ from that of the rest of the mammals studied so far (see Introduction).

Our findings indicate that the tight junctional complex in Corriedale ram lambs reared under extensive grazing conditons in Uruguay appeared at aroud 145-180 days of age. Although no other age of appearance has been given for lambs, *Courot* (1971) reported that Sertoli cells stop dividing in lambs at 40 days of age. It is well known from other species that adjacent Sertoli cells normally develop tight junctions soon after they cease dividing (*Gondos* 1993). The apparent discrepancy in timing between our results and those of *Courot* (1971) can be explained by considering that the ram lambs from our study were raised under extensive conditions that delay pubertal development (*Castrillejo et al.* 1995). Although rearing conditions were not specified in Courot's study, it is very improbable that the availability of food was also low for lambs raised at an experimen-

tal station in Europe (Nouzilly, France). The age at which tight junctions appeared in our study is in agreement with the notion that spermatogenesis in these animals is attained at 180-210 days (*Castrillejo et al.* 1995). Prepuce-penis adherences disappear during pubertal development owing to an increase in testosterone levels. The disappearance of these adherences has been used to monitor the course of puberty (*Brown et al.* 1994, *Castrillejo et al.* 1995). Our results indicate that the ram penis separated from the prepuce at approximately the same time as the interSertoli tight junctional complex formed. Corriedale rams raised extensively in Uruguay produce their first spermatozoa around the time that their scrotal circumference reaches 23 cm (*Castrillejo et al.* 1995). According to our present results, blood-testis formation (as evidenced indirectly by the appearance of the seminiferous tubule lumen and interSertoli cells occluding junctions) occurs somewhat earlier, when the scrotal circumference in the ram lambs has reached 14.5-16 cm. Although scrotal circumference is easy to measure, it must be kept in mind that factors such as breed and feed availability can cause this clinical sign to vary considerably.

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Sammanfattning

Utvecklingen under puberteten av junctions mellan sertoliceller i testiklarna hos corridale bagglamm.

Ultrastrukturen av »tight junctional complex« i Sertoli celler hos icke könsmogna bagglamn studerades i immersions fixerade prov. De ultrastrukturella fynden relaterades till kliniska data och ljusmikroskopisk klassifikation av graden av spermatogenes i intilliggande tubuli seminiferi. Fastän processen följde den generella utvecklingstrenden hos däggdjur för bildandet av »tight junctional complex«, upptäcktes två ovanliga ultrastrukturella egenheter; närvaron av ett aktivt Golgi komplex under det tidiga stadiet av uppbyggnaden av »tight junction« och den övergående närvaron av ribosomer på båda sidor av de ektoplasmatiska cisternerna som gränsar till de »junctions« som bildas. Betydelse av dessa fynd diskuteras.

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