Theriogenology 84 (2015) 763–772

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Characterization of the equine blood–testis barrier during tubular development in normal and cryptorchid stallions

K. Rode [a], H. Sieme [b], P. Richterich [c], R. Brehm [a,*]

[a] Department of Anatomy, University of Veterinary Medicine Hannover, Hannover, Germany
[b] Unit for Reproductive Medicine, Clinic for Horses, University of Veterinary Medicine Hannover, Hannover, Germany
[c] Tierärztliche Klinik für Pferde auf Boyenstein, Beckum, Germany

ARTICLE INFO

Article history:
Received 12 November 2014
Received in revised form 4 May 2015
Accepted 9 May 2015

Keywords:
Blood–testis barrier
Sertoli cell
Tight junction
Gap junction
Cryptorchidism
Horse

ABSTRACT

The formation of the blood–testis barrier (BTB) is defined as occurring with the first appearance of spermatocytes at around puberty and is vital for normal spermatogenesis. This barrier between two adjacent Sertoli cells (SCs) consists of a cell junctional protein complex, which includes tight junctions (TJs), adherens junctions, and gap junctions. In many mammalian species, BTB composition has already been investigated, whereas little is known about the equine BTB. In the present study, immunohistochemistry and qualitative Western Blot analysis were used to assess the expression and distribution patterns of the junctional proteins claudin-11 (TJ), zonula occludens-1 (TJ associated), N-cadherin (adherens junctions), and connexin 43 (gap junctions) in equine testes during tubular development and in testes of stallions exhibiting unilateral cryptorchidism. Therefore, testes of 21 warmblood stallions (aged 12 months–11 years) were obtained during routine surgical castration. In the normal adult equine testis, the junctional proteins are localized at the basolateral region of the seminiferous tubules forming a circumferential seal corresponding to the known BTB localization. N-cadherin is additionally expressed along the lateral SC surface. In immature seminiferous cords still lacking a lumen, a diffuse distribution pattern of the junctional proteins throughout the SC cytoplasm is visible. As lumen formation advances, the immunolocalization shifts progressively toward the basolateral SC membranes. Additionally, apoptotic germ cells were detected and quantified in prepubertal stallions using terminal deoxynucleotidyl transferase dUTP nick end labeling assay and correlated with junctional protein localization. In the retained testis of cryptorchid stallions, which exhibit an aberrant testicular morphology, a deviating expression of the junctional proteins is visible. The present data show for the first time that (1) the equine SC junctional complex contains claudin-11, zonula occludens-1, N-Cadherin, and connexin 43, as already described for men or mice, and that (2) different distribution patterns of these proteins exist during testicular development in the context of lumen formation (lumen scores: 1–7) and in retained testes of unilateral cryptorchid stallions.

1. Introduction

In mammalian testes, the blood–testis barrier (BTB) is being formed between adjacent Sertoli cells (SCs) during puberty by a complex of different junction types. The BTB divides the seminiferous epithelium into a basal compartment containing spermatogonia and primary spermatocytes, and an adluminal compartment containing more developed germ cells (GCs). This is essential for intact spermatogenesis [1].

The BTB consists of coexisting tight junctions (TJs) with, for example, claudins and occludin, cadherin-based adherens junctions, and connexin-based gap junctions (GJs), which are in close structural and functional contact to each other (reviewed by Pelletier and Byers [2]).

* Corresponding author. Tel.: +49511 856 7215; fax: +49511 856 7683.
E-mail address: Ralph.Brehm@tiho-hannover.de (R. Brehm).

0093-691X/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2015.05.009

Table 1
Classification of equine seminiferous tubules during tubular development.

Lumen score	Morphology	Intratubular cells
1	Tubules lack a lumen (solid spermatic cords)	Sertoli cells and gonocytes randomly dispersed throughout the cord
2	Tubules contain a single vacuole	Sertoli cell nuclei moved toward the basal lamina Gonocytes are located centrally
3	Tubules contain several independent vacuoles	Sertoli cells and spermatogonia Occasional appearance of early (leptotene and zygotene) spermatocytes
4	Tubules contain several aggregating vacuoles	The most advanced germ cell type is the pachytene spermatocyte
5	Tubules have an open lumen, but the seminiferous epithelium has only the height of one cell layer	Only Sertoli cells and spermatogonia
6	Tubules have an open lumen	Germ cell population is still incomplete
7	Tubules have an open lumen	Full population of germ cells

The equine BTB has not been the object of intense investigation so far, so its molecular composition is unknown [3–8]. Only Heninger et al. [5] analyzed the formation and density of the equine BTB by hypertonic fixation but did not investigate its molecular composition. The studies from Clemmons et al. [3], Heninger et al. [5], and Heninger [8] classified the seminiferous tubules on the basis of their "functional" tubular development according to a modified lumen score (LS) system (Table 1) ranging from LS 1 (least developed) to LS 7 (mature). They also assessed the shrinkage of intratubular cells after perfusing the testes of six yearling stallions *via* the testicular artery with either 2% glutaraldehyde (450 mOsm/L) or 2% glutaraldehyde with 10% dextrose (1214 mOsm/L) [5] and showed that within LS 1 through LS 4 tubules, every intratubular cell type exhibited shrinkage. In turn, advanced GCs in the adluminal compartment of LS 6 and LS 7 tubules did not show shrinkage artifacts compared to cells in the basal compartment indicating that an intact selective barrier was probably formed, which prevents the hypertonic solution from entering the adluminal compartment. Tubules of LS 5 showed shrinkage of all cell types present, but because advanced GCs were not present in this stage, the real density of the SC barrier could not be evaluated. However, the authors suggested that the BTB is formed early in LS 5 [5].

Heninger et al. [5] also investigated apoptotic rates of GCs during the initiation of spermatogenesis in young stallions. They showed two apoptotic GC peaks throughout tubular development taking place in LS 4 and, to an even greater extent, in LS 6. These results correspond to the findings of Rodriguez et al. [9], who reported that GC apoptosis is required for the establishment of functional murine spermatogenesis, although occurring there as a single wave.

A functional BTB is a prerequisite for intact spermatogenesis [1], and the disruption of this barrier can be associated with impaired spermatogenesis and consequently with subfertility or infertility [10–17]. Thus, the knowledge of the normal development and molecular composition of the BTB is a requirement for (1) the assertion of aberrations of this barrier and (2) the development of adequate therapy strategies of subfertility or infertility associated with alterations of BTB components.

Therefore, the objectives of the present study were to assess the expression and localization patterns of the BTB proteins, claudin-11 (TJ protein), zonula occludens-1 (ZO-1, TJ-associated protein), N-cadherin (adherens junctions protein), and connexin 43 (Cx43, GJ protein), (1) within the equine seminiferous epithelium in the context of lumen formation and (2) in unilaterally cryptorchid equine testes *via* immunohistochemistry (IHC). To validate these results at protein level, qualitative Western blot (WB) analysis was performed. Another objective was to investigate whether (unilateral) cryptorchidism has any influence on junctional protein expression. To correlate the distribution of the junctional proteins, especially of claudin-11 as a TJ protein, with the appearance of apoptotic GCs and with the corresponding LS, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed.

2. Materials and methods

The study was supervised by Prof. Dr Ralph Brehm (Department of Anatomy, University of Veterinary Medicine Hannover, Germany). All testes were obtained during routine surgical castration, so an approval for animal experiments by the Lower Saxony State Office for Consumer Protection and Food Safety was not necessary.

Table 2
Allocation of the stallions into age groups.

Age group	Normal	Cryptorchidism
Prepubertal (n = 4)	12 mo (n = 2) 15 mo (n = 1)	12 mo (n = 1)
Pubertal (n = 9)	1.5 y (n = 1) 2 y (n = 3) 2.5 y (n = 2)	2 y (n = 2) 2.5 y (n = 1)
Adult (n = 8)	3 y (n = 2) 5 y (n = 2) 6 y (n = 1) 11 y (n = 1)	3 y (n = 2)