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Aquaporin-9 immunohistochemistry in varicocele testes as a consequence of hypoxia in the sperm production site

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Summary

Aquaporin-9 (AQP-9) regulates tissue hydration by promoting transmembrane exchanges of both water and solutes, such as lactate. The latter is a key metabolite of primary spermatocytes and of maturing haploid germ cells (h-GCs). The present investigation was aimed at immunolocalising human AQP-9 in both normal and varicocele testes. Histology and immmunocytochemistry were investigated in archival biopsies from 20 varicocele testes and in eight unaffected ones. AQP-9 immunostaining was performed using a rabbit antibody, and either *focal* or *diffuse* cell membrane labelling was recorded. Varicocele testes showed disarranged tubular compartments, with sloughing h-GCs, tissue hyperhydration, spermiogenesis failure and fibrosis. AQP-9 immunohistology of the control testes showed a *diffuse* cell membrane staining of the primary spermatocytes and h-GCs, without any positive reaction of spermatogonia and Sertoli cells. AQP-9 cell expression in the varicocele testes was *focal* or lacking in both adluminal and sloughing GCs. AQP-9 expression occurs in normal human testis, at cell membrane of primary spermatocytes and h-GCs, suggesting a possible role of AQP-9 in the water and lactate transport from Sertoli cells to GCs. AQP-9 is focal or lacking in adolescent varicocele testes, and this suggests AQP-9 to be downregulated in such testicular disorder, leading to lactate deprivation with subsequent hypospermatogenesis.

Introduction

Varicocele affects testicular function and male fertility (Santoro *et al.*, 2001; Pasqualotto *et al.*, 2005). Multifactorial pathogenesis of the lesion includes testicular venous hypertension, hypoxia and subsequent ischaemic damages (Gat *et al.*, 2005, 2006). Progressive damages in varicocele testes imply exceeding endotubular fluid (ETF) and extracellular matrix (ECM), with subsequent germ cell sloughing and hypospermatogenesis (Naughton *et al.*, 2001; Nicòtina *et al.*, 2005).

Aquaporins (AQPs) are a family of 11 cell membrane proteins that are widely expressed in mammalian tissues, to modulate selective transmembrane water flow and osmolarity. AQP-1 overexpression has been reported in microvessel endothelium and endotubular cells of varicocele testes, as a *critical* reabsorption factor (Nicòtina *et al.*, 2005). Tissue AQP-9 is known to be developmentally regulated, as in mice as in humans (Wang *et al.*, 2004; Cavazzin *et al.*, 2006). It allows transmembrane passage of both water and neutral solutes, such as glycerol and lactate (Pastor-Soler *et al.*, 2002), varying as a consequence of tissue metabolic states (Tsukaguchi *et al.*, 1999; Carbrey *et al.*, 2003; Wang *et al.*, 2004; Cavazzin *et al.*, 2006) and ECM expansion (Tsukaguchi *et al.*, 1999; Wang *et al.*, 2004).

Aquaporin-9 expression gradually decreases under hypoxic conditions, but it is up regulated in the hyperosmotic state (Arima *et al.*, 2003; Fujita *et al.*, 2003). AQP-9 mRNA has been detected in immature spermatocytes and Leydig cells of rats (Tsukaguchi *et al.*, 1998; Nihei *et al.*, 2001; Pastor-Soler *et al.*, 2001) but, to the best of our

knowledge, it has not been reported in human testis to date.

Because of the occurrence of exceeding ETF and ECM in varicocele testes, and the proved AQP-9 role in modulating transmembrane water-flow, this study was aimed to immunolocalise AQP-9 in archival human varicocele testes, when compared with normal ones.

Material and methods

Twenty archival incisional biopsies of adolescents with a mean age of 14.3 years (range 13–18) were obtained during varicocelectomy (before the legation of spermatic veins) from idiopathic grade II or III according to Horner classification varicocele testes (Horner, 1960). Varicocele was diagnosed after physical examination and echocolour Doppler ultrasonography. Normal adolescent testes were also processed, employing archival blocks from eight post-mortem samples of subjects of matched age, without testicular lesions. Two human liver biopsies were used as positive controls. Formalin-fixed and paraffin-embedded sections were processed for both histological and immunohistochemical purposes. After microwave treatment, immunostaining was carried out by a rabbit polyclonal antibody for AQP-9 (Alpha Diagnostics International, San Antonio, TX – USA), diluted 1 : 500, using the streptavidin-biotin/LSAB method. Diaminobenzidine development (DAB substrates, Vector Laboratories, Burlingame, CA, USA) and haematoxylin nuclear counterstaining were also performed. Parallel negative controls were obtained by omitting the primary antibody incubation. Only the AQP-9 positive cells were quantitated at the interstitial or endotubular site. The percentage of such cells was then estimated and scored, as previously reported (Nicòtina *et al.*, 2005), as either *focal* (up to 25% positive cells) or *diffuse* (more than 25% positive cells).

Results

Tubular and extra-tubular changes were objectified in the studied varicocele testes, displaying disarranged tubular compartments. Detachment and sloughing of GCs occurred there, with a varying degree of germ cell and Sertoli cell impairment, up to spermiogenesis failure, as observed in seven of 20 cases (35%). Unlike normal testes (Fig. 1a), the varicocele ones showed dilated microvessels, more or less expanded ETF and ECM and a plenty of extracellular vesicles inside the seminiferous tubules. A high proportion of Sertoli cells, spermatogonia and spermatocytes also showed variously sized cytoplasmic vacuoles, while retained cytoplasmic droplets could be observed in spermatozoa, if present. Leydig cell clusters were recognisable, but a varying degree of interstitial

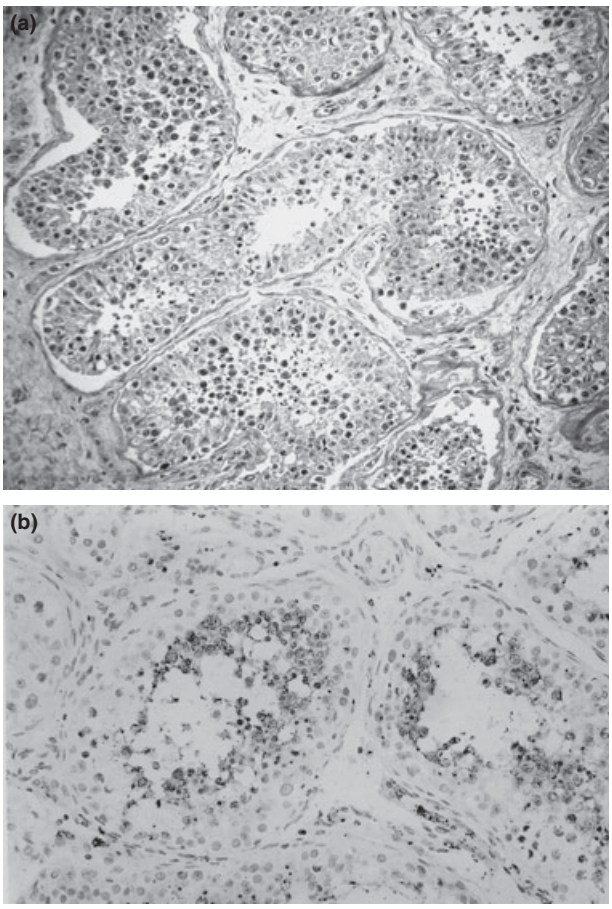

Fig. 1 (a, b) Normal testis of a 16-year-old boy. (a) Lobular arrangement of seminiferous tubules is associated with normal endotubular compartments and spermatogenesis. (b) A *diffuse* AQP-9 cell-membrane and cytoplasmatic immunostaining depicts h-GCs (original magnification 250×) (black arrows: primary spermatocytes; white arrows: secondary spermatocytes).

oedema prevailed, with notable peritubular and extratubular fibrosis (Figs 2a and 3a).

AQP-9 immunohistology of adolescent normal testes showed a *diffuse* cell membrane and cytoplasmic immunostaining of primary spermatocytes, adluminal h-GCs and sperm cells, without any positive reaction in the basal compartment of seminiferous tubules, including both spermatogonia and Sertoli cells. A *focal* immunolabelling also depicted Leydig cells (Fig. 1b).

The studied varicocele testes showed a more or less reduced endotubular AQP-9 immunostaining, among the low-density primary spermatocytes and h-GCs. AQP-9 cell positivity was *focal* or lacking in sloughing GCs, as well as in microvessel endothelial and Leydig cells (Figs 2b and 3b).

Positive controls displayed AQP-9 binding cell membrane of hepatocyte facing sinusoids, in centrolobular areas.

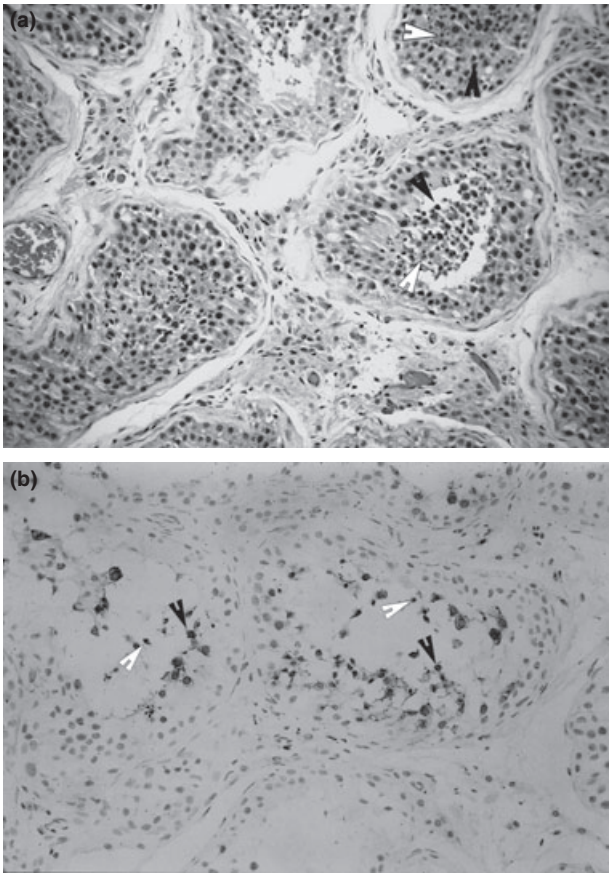

Fig. 2 (a, b) Grade 2 varicocele testis of a 15-year-old boy. (a) Together with abnormal expansion of exceeding endotubular fluid (ETF), seminiferous tubules show sloughing h-GCs and depleted adluminal compartments. (b) AQP-9 immunolabelling focally marks cell membrane and cytoplasm of sloughing h-GCs and occasional primary spermatocytes (original magnification 250×) (black arrows: primary spermatocytes; white arrows: secondary spermatocytes).

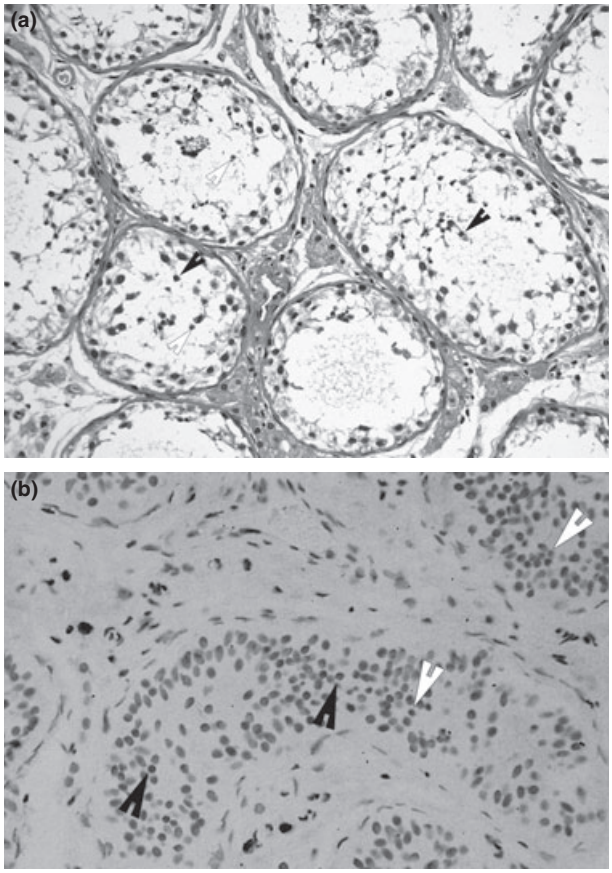

Fig. 3 (a, b) Grade 3 varicocele testis of a 18-year-old boy. (a) An extracellular matrix (ECM) expansion is associated with dispersed Leydig cell and peritubular fibrosis. Endotubular cell lines are highly immature. (b) AQP-9 cell labelling is lacking inside the seminiferous tubules, but it is *focal* in dispersed Leydig cells (original magnification 250×); (black arrows: primary spermatocytes; white arrows: secondary spermatocytes).

Discussion

Rat AQP-9 was pointed out to have the greatest homology with human AQP-9, allowing the anti-rat AQP-9 antibody to cross-react with the human protein (Tsukaguchi *et al.*, 1998; Pastor-Soler *et al.*, 2001; Li *et al.*, 2004, 2005; Wang *et al.*, 2004).

This corroborates the above results that document an unprecedented AQP-9 expression in human normal testis and prove AQP-9 to be expressed diffusely in primary spermatocytes and h-GCs, but focally in Leydig cells. Instead, AQP-9 expression is commonly lacking in spermatogonia and Sertoli cells, outside the blood-testis barrier. AQP-9 is known to be a developmentally regulated lactate channel (Tsukaguchi *et al.*, 1998; Li *et al.*, 2004), promoting cell influx of lactate (Carbrey *et al.*, 2003). As a consequence, a physiological role for AQP-9 is conceivable in supporting germ-cell metabolism and maturation. In fact, peritubular microvessels normally supply glucose to both spermatogonia and Sertoli cells (Boussouar & Benahmed, 2004). The latter metabolise glucose to lactate and export it to primary spermatocytes and h-GCs, as a substrate for glycolytic pathway (Erkkila *et al.*, 2002).

Unlike observed in normal testes, adolescent varicocele testes show AQP-9 immunostaining to be progressively reduced in sloughing endotubular cells. AQP-9 up-regulation was previously reported in extra-testicular sites as a consequence of interstitial tissue dehydration (Arima *et al.*, 2003). Similarly, the present results suggest an AQP-9 downregulation to occur in varicocele testes, agreeing with the observed expansion of both ETF and ECM.

Moreover, the defective AQP-9 cell staining in varicocele testes might be due to varicocele-associated hypoxia, as otherwise reported under experimental hypoxic condition (Fujita *et al.*, 2003).

Above reported AQP-9 immunostaining of primary spermatocytes and h-GCs in human pubertal testes suggests a direct role of AQP-9 in water and neutral solute transmembrane exchanges, from Sertoli cells to spermatocytes, spermatids and sperm cells, inside the blood barrier. Such immunocytochemical feature agrees with AQP-9 role in preventing energetic unbalance and cell death in germ cells (Erkkila *et al.*, 2002; Boussouar & Benahmed, 2004). In this way, it might be reasonable AQP-9 downregulation to be co-related to ETF- and ECM-expansion, low osmolarity and tissue hypoxia, which are common features of varicocele testes, as responsible for impairing spermatogenesis.

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