Loss of protein phosphatase 1c γ (PPP1CC) leads to impaired spermatogenesis associated with defects in chromatin condensation and acrosome development: an ultrastructural analysis

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Abstract

Human male infertility affects ~5% of men, with one-third suffering from testicular failure, likely the result of an underlying genetic abnormality that disrupts spermatogenesis during development. Mouse models of male infertility such as the *Ppp1cc* knockout mouse display very similar phenotypes to humans with testicular failure. Male *Ppp1cc* mutant mice are sterile due to disruptions in spermatogenesis that begin during prepubertal testicular development, and continue into adulthood, often resulting in loss of germ cells to the point of Sertoli cell-only syndrome. The current study employs light and electron microscopy to identify new morphological abnormalities in *Ppp1cc* mutant seminiferous epithelium. This study reveals that germ cells become delayed in their development around stages VII and VIII of spermatogenesis. Loss of these cells likely results in the reduced numbers of elongating spermatids and spermatozoa previously observed in mutant animals. Interestingly, *Ppp1cc* mutants also display reduced numbers of spermatogonia compared with their wild-type counterparts. Using electron microscopy, we have shown that junction complexes in *Ppp1cc* mutants are ultrastructurally normal, and therefore do not contribute to the breakdown in tissue architecture seen in mutants. Electron microscopy revealed major acrosomal and chromatin condensation defects in *Ppp1cc* mutants. Our observations are discussed in the context of known molecular changes in *Ppp1cc* mutant testes.

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Introduction

Mammalian spermatogenesis is a complex process that results in the formation of haploid gametes from diploid spermatogonial stem cells. A key feature of this process is the coordinated progression of development through a series of stereotypical stages. This highly ordered process takes place in the seminiferous epithelium, which exhibits characteristic organization of two major cell types – somatic Sertoli cells and developing germ cells (Yan *et al.* 2007). The seminiferous epithelium is divided into basal and adluminal compartments. These two compartments are separated by the blood–testis barrier, which comprises occluding junctions formed between neighboring Sertoli cells (Dym & Fawcett 1970, Pelletier & Byers 1992). The basement compartment, which is closest to the basement membrane of the seminiferous tubule, contains diploid spermatogonial stem cells (Brinster 2007). As the developing gametes enter meiosis, they are displaced across the blood–testis barrier and enter the adluminal compartment (Russell 1977).

The adluminal compartment contains tetraploid spermatocytes, diploid secondary spermatocytes, haploid spermatids, and mature spermatozoa, with mature spermatozoa being closest to the lumen (Mruk & Cheng 2004). Developing germ cells undergo dramatic morphological changes that are used to define the different stages of spermatogenesis. Some of these morphological changes include changes to the shape of the nucleus and acrosome, which are facilitated by a microtubule structure called the manchette (Toshimori & Ito 2003).

Type 1 protein phosphatases make up a class of protein serine/threonine phosphatases that are involved in numerous cellular processes ranging from glycogen metabolism to cell cycle progression (Ceulemans *et al.* 2002). These phosphatases are made up of one catalytic subunit, which in mammals are encoded by three genes (*Ppp1ca*, *Ppp1cb*, and *Ppp1cc*) and one of many possible regulatory subunits (Ceulemans & Bollen 2004). Differential splicing of the *Ppp1cc* gene generates two isoforms, PPP1CC1 and PPP1CC2. In the testis, PP1CC2

Figure 1 *Ppp1cc* mutant mice have an overabundance of seminiferous tubules in stage VII of spermatogenesis when compared with wild-type mice of the same age. Asterisk denotes samples that are significantly different from wild type ($P=0.05$). Samples represent the mean percentage of tubules $\pm$ S.E.M. ($n=2$). Black bars denote wild-type mice, and white bars denote *Ppp1cc* mutant mice.

is the most abundant splice isoform (Kitagawa *et al.* 1990). Targeted deletion of the mouse *Ppp1cc* gene causes disruption of spermatogenesis, resulting in an almost complete absence of mature sperm and ultimately sterility (Varmuza *et al.* 1999). Previous studies have determined that this mutation causes a generalized breakdown of tissue architecture accompanied by other

anomalies such as increased levels of aneuploidy in developing germ cells (Oppedisano *et al.* 2002), increased DNA fragmentation in spermatids (Jurisicova *et al.* 1999), and premature release of germ cells during prepubertal stages (Varmuza & Ling 2003), phenotypes that mimic those observed in many human patients suffering from testicular failure, making the *Ppp1cc* knockout mouse a good model of male infertility. In order to further characterize the defects in spermatogenesis that arise as a result of the *Ppp1cc* mutation, a detailed analysis of *Ppp1cc* mutant and wild-type seminiferous epithelium was carried out using light and electron microscopy. Our study has revealed additional defects in spermatogenesis caused by the *Ppp1cc* mutation which may help to identify the primary lesion in these mutants.

Results

Ppp1cc mutant mice display a bottleneck in spermatogenesis

Previous data had shown that although *Ppp1cc* mutants display impaired spermatogenesis, the process is not completely arrested in these mutants (Varmuza *et al.* 1999, Varmuza & Ling 2003). Staging of mutant and wild-type testes sections was performed, using the criteria described by Russell *et al.* (1990), in order to

Figure 2 Spermatogonia are depleted in *Ppp1cc* mutants. (A) Identification of spermatogonia as visualized in plastic sections stained with methylene blue (arrows). The unique structure of the Sertoli cell nucleolus distinguishes the Sertoli cell (arrowhead) from the surrounding spermatogonia. BM indicates the basement membrane of the seminiferous tubule. Scale bar = 60 μ M. (B) Paraffin section of a wild-type seminiferous tubule. The arrows indicate spermatogonia, which can be seen at regular intervals along the basement membrane. Scale bar = 50 μ M. (C) Paraffin section of a *Ppp1cc* mutant seminiferous tubule. Asterisks indicate vacuolated regions along the basement membrane (BM). Despite the depletion of spermatogonia, these cells can still be identified in mutant tubules (arrow). Scale bar = 50 μ M. (D) Quantification of the numbers of spermatogonia present in wild-type and mutant seminiferous tubules. Bars represent the mean number of spermatogonia found in individual seminiferous tubules $\pm$ S.E.M. Asterisk represent samples that are significantly different from wild type ($P=0.05$). Black bars denote wild type, and white bars denote *Ppp1cc* mutants.

Figure 3 Basal junctions appear normal in *Ppp1cc* mutants. Electron micrographs of basal junction complexes between adjacent Sertoli cells in wild-type (A and B) and mutant (—/—) (C and D) animals. Normal appearing ectoplasmic specializations (ES) characterized by actin filaments and cisternae of endoplasmic reticulum (ER) are visible in both A and C. Tight junctions (arrowheads) occur as part of the basal junction complex both in wild-type (B) and in mutant (—/—) (D) animals. (A and C) Bars=0.5 μ m. (B and D) Bars=0.1 μ m.

determine whether any changes in the timing of events during spermatogenesis could be established. Staging of wild-type seminiferous tubules shows a stereotypical pattern in the percentage of tubules in particular stages that reflect the time spent in each stage. *Ppp1cc* mutants typically display a breakdown in cell associations that make staging a challenge (Oppedisano-Wells & Varmuza 2003). Nevertheless, mutant seminiferous epithelium contains a larger percentage of tubules in stages VII/VIII and a greatly reduced number of tubules at later stages, suggesting that a developmental bottleneck occurs in tubules containing mid-pachytene spermatocytes (Fig. 1).

Spermatogonia are depleted in *Ppp1cc* mutants

While *Ppp1cc* mutant testes become depleted of elongating and condensing spermatids, they retain spermatocytes and round spermatids. However, with age, mutant males often display evidence of complete loss of germ cells in some tubules, similar to Sertoli cell-only syndrome in human males. This suggests that the stem cell population is adversely affected in mutant testes. In order to test this possibility, we quantitated the spermatogonial stem cell population in mutant and wild-type testes. Type B spermatogonia were counted in stages IV, V, and VI tubules of similar diameters from both mutant and wild-type testes. Type B spermatogonia were chosen for analysis because they occur more frequently than type A spermatogonia. Type B spermatogonia are also present in a stage-specific manner, whereas type A spermatogonia are present in all seminiferous tubules.

Type B spermatogonia were distinguished from type A spermatogonia based on nuclear morphology. In stages IV, V, and VI, the nuclei of type B spermatogonia are roughly oval, with a prominent ring of chromatin around the nuclear membrane. In contrast, type A spermatogonia do not have heterochromatin in their nuclei. In wild-type tubules, the spermatogonia occur regularly along the basement membrane (Fig. 2A and B). In contrast, spermatogonia are seen infrequently along the basement membrane of mutant tubules (Fig. 2C). Furthermore, there occur vacuolated regions along the basement membrane of mutant tubules (Fig. 2C). To confirm these initial observations, the numbers of spermatogonia were counted in wild-type and mutant tubules (Fig. 2D).

Figure 4 Apical ectoplasmic specializations appear normal in *Ppp1cc* mutants. Electron micrographs of ectoplasmic specializations (ES) associated with elongating spermatids in wild-type (A) and mutant (—/—) (B) animals. Actin filaments and a cistern of endoplasmic reticulum are indicated in B. Bars=0.1 μ m.

Figure 5 Electron micrograph of late spermatids and associated Sertoli cell regions from a mutant ($-/-$) animal. Even though the spermatid heads shown here are grossly abnormal, ectoplasmic specializations (arrowheads) are visible adjacent to the spermatid heads. Bar = 1 μ m. The inset is a higher magnification of the region outlined by the box. An ectoplasmic specialization (ES) containing actin filaments is indicated. Bar = 0.1 μ m.

Counting was performed on Bouin's fixed tissue, stained with periodic acid-Schiff's and hematoxylin (PAS-H). Statistical analysis of the number of spermatogonia in wild-type and mutant tubules revealed a significant difference in the mean number of spermatogonia between the two sample groups. Wild-type tubules in stages IV–VI contained an average of 14 type B

spermatogonia per tubule. In contrast, mutant tubules at the same stages contained an average of 7 type B spermatogonia per tubule ($P < 0.05$).

Cell-cell junctions are ultrastructurally normal in *Ppp1cc* mutant mice

Preliminary evidence indicated that germ cells were shed prematurely from mutant tubules as early as 3 weeks after birth (Varmuza & Ling 2003). We therefore hypothesized that the junction complexes in mutants may be abnormal.

Basal junction complexes between neighboring Sertoli cells are characterized by the presence of ectoplasmic specializations. In wild-type testes, these structures have the stereotypical ultrastructure, with actin bundles located directly beneath the Sertoli cell plasma membrane and endoplasmic reticulum located adjacent to the actin bundles. In wild-type specimens, these junctions occur in the expected basal location within the seminiferous epithelium (Fig. 3A). Occluding junctions are intercalated within the regions occupied by ectoplasmic specializations (Fig. 3B). In adult mutant testes, basal junction complexes display the same ultrastructure and localization observed in wild-type testes (Fig. 3C); that is, bundles of actin filaments occur directly beneath the Sertoli cell plasma membrane, and cisternae of endoplasmic reticulum are closely related to the actin bundles. Occluding junctions, indicated by close contacts or 'kisses' between adjacent plasma membranes, also are observed (Fig. 3D). The localization of these junction complexes within

Figure 6 *Ppp1cc* mutant mice display acrosomal defects. (A) Wild-type steps 2–3 spermatid with a typical acrosomal vesicle (arrow). The acrosomal vesicle contains an acrosomal granule indicated by an arrowhead. The acrosomal vesicle has made contact with the nucleus. (B) Mutant steps 2–3 spermatid with a normal acrosomal vesicle (arrow), containing the acrosomal granule (arrowhead). (C) Wild-type step 7 round spermatid, showing the acrosomal granule that has become flattened over the surface of the nucleus (arrowhead) and the outer acrosomal membrane (arrow). (D) Mutant step 7 round spermatid. Arrowheads show the acrosomal granule which has spread over the surface of the nucleus as seen in the wild-type step 7 round spermatid in C. In the mutant step 7 round spermatid, the vacuoles have formed in the acrosome. Vacuoles are indicated by asterisks. All scale bars = 2 μ m.

the seminiferous epithelium is similar to that observed in wild-type specimens.

Apical junctions between Sertoli cells and spermatids in wild-type specimens displayed normal junctional ultrastructure and localization. Ectoplasmic specializations display actin bundles directly beneath the plasma membrane of the Sertoli cell, and cisternae of the endoplasmic reticulum occur adjacent to the actin bundles (Fig. 4A). In *Ppp1cc* mutant adult testis, the ultrastructure of ectoplasmic specializations is similar to that of wild-type testis (Fig. 4B). Normal appearing ectoplasmic specializations were observed even in cases where there were gross abnormalities in spermatid head morphology (Fig. 5).

***Ppp1cc* mutant mice display acrosomal defects and ectopic manchettes**

At the light microscopic level in PAS–H-stained sections, acrosome dysmorphology becomes evident around steps 7–8, where the most obvious defect is fusion of acrosomes between two or three spermatids. Ultrastructural examination supported this earlier observation and revealed additional acrosomal defects not evident at the light microscopic level. Wild-type steps 2–3 spermatids have a rounded acrosomal vesicle, containing an acrosomal granule (Fig. 6A). At this stage of development, this structure will have made contact with the surface of the nucleus but the acrosomal vesicle remains rounded and does not begin to spread over the nucleus until subsequent stages. Mutant steps 2–3 spermatids have acrosomal vesicles and granules that display all of the characteristics described above (Fig. 6B). The obvious defects do not appear until the later stages of spermatid development, and can be seen in spermatids as early as step 7 (Fig. 6C and D). The severity of these defects varies, but the majority of defects fit into one of the two following categories: acrosomes with one large vacuole and acrosomes with multiple small vacuoles.

Acrosomes in the first category generally have a large vacuole that has unknown contents. Interestingly, with this type of acrosomal defect, the acrosomal granule sometimes remains identifiable, if not entirely normal. Acrosomes in the second category generally have multiple smaller vacuoles, which appear to be empty. These vacuoles are spherical in shape and appear in clusters of 2–5.

In addition to abnormal acrosomes, spermatids also had manchettes that were in the wrong position with respect to the nucleus. Often parts of the manchette appeared to be within clefts or folds in the nucleus (Fig. 7).

Very few mutant germ cells progress past the round spermatid stage, probably due to extensive sloughing of damaged cells; the few elongating spermatids that can be found in mutant seminiferous epithelium have significant morphological abnormalities. Mutant

Figure 7 *Ppp1cc* mutant mice display ectopic manchettes. Electron micrograph of elongating spermatids from a mutant animal. Although the manchette is normally positioned in some areas (arrows), it is abnormally positioned in others (arrowheads) where it appears to be in a nuclear fold or indentation. Bar=1 μ m.

elongating spermatids display an indentation in the anterior aspect of the sperm head that gives the sperm head a 'bowling pin' shape. In addition, the caudal aspect of mutant sperm heads does not display the expected dorsal angle that forms between the caudal and dorsal surfaces of the sperm head. Instead, the sperm tail is allowed to insert on a flat caudal surface in mutant spermatids (Fig. 8).

***Germ cells in Ppp1cc* mutant mice display chromatin condensation defects**

The most obvious defect in *Ppp1cc* mutant germ cells at the ultrastructural level was the appearance of the chromatin. Spermatids in particular displayed evidence of non-uniform chromatin condensation. These defects were evident in both round spermatids (Fig. 9A–C) and elongating spermatids (Fig. 9D and E). Subtle chromatin defects can also be seen in spermatocytes (Fig. 10). Unusual dense inclusions and other types of unidentifiable matter are commonly found in mutant spermatocytes. In addition, very heavily condensed chromosomes can occasionally be found in mutant sections, but never in wild-type sections (Fig. 10C).

Discussion

The *Ppp1cc* mutation causes male infertility through the loss of elongating and condensing spermatids, and eventual breakdown in testicular tissue architecture. Whether the overall effect is a function of one primary defect or several operating in the testis is unknown. The nature of the gene product, a serine threonine phosphatase, suggests the possibility of pleiotropic

Figure 8 *Ppp1cc* mutant elongating spermatids display abnormalities in sperm head shape. (A) Wild-type elongating spermatid showing characteristic morphology. (B) Mutant elongating spermatid demonstrating abnormal morphology. Arrowheads indicate manchette microtubules; large arrow denotes misshapen acrosome with multiple vesicles. Asterisk denotes the area of sperm tail insertion that lacks the expected dorsal angle. (C) Mutant elongating spermatid with a pronounced indentation in the sperm head. Arrow indicates the perinuclear ring of the manchette, arrowheads indicate manchette microtubules. Scale bars = 3 μ M.

activity; however, the expression in the testis of two other very closely related isoforms, PPP1CA and PPP1CB, is also consistent with a single PPP1CC-dependent event going awry. The purpose of the present study was to evaluate ultrastructural images for evidence of the earliest departure from normal development of sperm in mutant testes.

Staging revealed an apparent bottleneck around stages VII/VIII. This did not represent a complete block, since seminiferous epithelium could proceed to later stages, and even mature sperm, albeit grossly abnormal, can be found in mutant testes (Davies & Varmuza 2003). The transition between stage VIII and IX coincides with the first appearance of elongating spermatids. These are the cells that become rapidly depleted in mutant testes, possibly through premature sloughing. Examination of both basal and apical ectoplasmic specializations revealed no defects in the ultrastructural appearance of these structures in mutant testes, suggesting that loss of germ cells proceeds via some other mechanism, most likely cell death (Varmuza *et al.* 1999). What triggers the cell death?

The most dramatic difference between mutant and wild-type testes at the ultrastructural level is the morphology of the developing spermatids. Abnormal nuclear shape, improper development of the acrosome, misshapen tails, and ectopic manchettes all point to a failure to proceed through the complex cell reorganization that embodies spermiogenesis. Many of these defects may be related to malformed nuclei resulting from improperly condensed chromatin. The appearance of the chromatin in spermatids is abnormal, with uneven condensation clearly evident. However, abnormalities in chromatin appearance are also evident in spermatocytes, suggesting that some aspect of germ cell chromatin remodeling during meiosis may depend on PPP1CC2, presumably through dephosphorylation of a target protein that mediates some early aspect of chromatin change necessary for the major condensation and packaging that follows during spermiogenesis.

This interpretation is consistent with several other lines of evidence. First, *Ppp1cc* mutant spermatids retain histones to a greater extent than wild-type spermatids (Varmuza *et al.* 1999). Second, the earliest time during development when mutants can be phenotypically distinguished from wild-type littermates is at 3 weeks of age, when testes are undergoing the first wave of spermatogenesis, and are at the pachytene stage of meiosis (Varmuza & Ling 2003). In the same study, subtle defects in recombination were also observed, although meiosis proceeded relatively normally. Third, a phosphoproteome analysis of mutant testes revealed that HSPA2 (Hsp70.2) may be a target of PPP1CC (Henderson *et al.* 2010). HSPA2 protein decorates the synaptonemal complex of pachytene spermatocytes, and is also present in a cloud of puncta over the chromatin (Govin *et al.* 2006, Henderson *et al.* 2010);

Figure 9 *Ppp1cc* mutant spermatids display abnormalities in chromatin condensation. Electron micrographs of nuclei in round (A–C) and elongating (D and E) spermatids from wild-type (A and D) and mutant (B, C, and E) animals. Chromatin condensation is clearly abnormal in the nuclei of spermatids from mutant animals. Bars = 1 μ m.

this latter pattern is missing from *Ppp1cc* mutant spermatocytes, supporting the intriguing possibility that dephosphorylated HSPA2 performs a second function, different from that identified by the null mutation and related to conditioning of the germ cell chromatin in preparation for the major morphological changes the cells are about to embark upon. Furthermore, HSPA2 is highly expressed by spermatocytes and spermatids in the human testes (Son *et al.* 1999). Reduced expression of HSPA2 in the testes has been associated with human male infertility (Feng *et al.* 2001).

Other potential chromatin remodeling proteins may play similar roles in human spermatogenesis. Our laboratory recently found a large cluster of non-synonymous substitutions in the *SBF1* gene in men suffering from testicular failure (Kuzmin *et al.* 2009). *SBF1*, or *MTMR5*, is a pseudophosphatase, and interacts with the histone methyltransferase SUV39H. The *Sbf1* knockout mouse model of male infertility yields a phenotype very similar to that observed in *Ppp1cc* $-/-$ mice; impaired spermiogenesis and

progressive loss of germ cells, starting during puberty (Firestein *et al.* 2002).

Another potential target of PPP1CC identified in our phosphoproteome survey is β -tubulin. This is an interesting observation because tubulin is a component of the manchette, the structure that reshapes the nucleus. In addition, acrosome biogenesis is dependent on cytoskeletal transport. In the current study, we observed ectopic or misplaced manchettes and acrosomal defects in *Ppp1cc* mutants. Acrosome biogenesis involves the transport of Golgi-derived vesicles along the actin and microtubule cytoskeleton (Kierszenbaum & Tres 2004). Thus, acrosomal defects could be indicative of problems with the cytoskeletal structures such as the manchette and acroplaxome. Microtubule-based motor proteins such as kinesin and dynein have been shown to play a role in the transport of proacrosomic vesicles (Kierszenbaum & Tres 2004). Therefore, acrosomal defects could be attributed to malfunctioning motor proteins, or to defects in the manchette microtubules that act as transport pathways for the motor proteins and their cargo. Furthermore, recent studies in which acrosomal defects can be induced by microtubule depolymerizing agents such as nocodazole have reinforced the concept that proper acrosome formation is dependent on cytoskeletal elements (Moreno *et al.* 2006).

Mutant tubules displayed a reduction in the numbers of type B spermatogonia. This may be a secondary consequence of testicular failure. Older males often show evidence of complete loss of germ cells in some or all tubules, reminiscent of the human Sertoli cell-only syndrome associated with some forms of male infertility. The loss of more mature germ cells within a tubule may create an environment that is inimical to stem cell survival over the long term.

We have performed an ultrastructural analysis of *Ppp1cc* mutant testes, and have found evidence that the earliest defects are apparent at the spermatocyte stage, before the major morphological defects become readily visible, although there also appears to be a loss of spermatogonia as well. It remains to be determined whether the defects in *Ppp1cc* mutant testes are truly pleiotropic, or the consequence of a single, early event with catastrophic downstream consequences.

Materials and Methods

Animals

Mice were bred using standard animal husbandry. The *Ppp1cc* mutant allele has been propagated in a CD-1 background (Charles River Laboratories, Dorval, Quebec, Canada). Mutant and wild-type mice were identified by PCR genotyping as described (Varmuza *et al.* 1999). Adult males between 3 and 9 months of age were used for all experiments. All procedures involving laboratory animals were approved by the Canadian Council on Animal Care.

Figure 10 Chromatin condensation is abnormal in *Ppp1cc* mutant spermatocytes. Electron micrographs of spermatocyte nuclei from wild-type (A) and mutant (B–D) animals. In addition to unusual inclusions (arrowheads), irregular and uneven patterns of chromatin condensation (arrows) are present in the nuclei of mutant cells. Bars = 1 μ m.

Tissue processing and staining for light microscopy

Both paraffin- and plastic-embedded tissues were used for light microscopy analysis. Paraffin-embedded tissue was fixed for 48 h in Bouin's fixative. The tissue was then dehydrated in a graded ethanol series, cleared with xylene, and infiltrated with molten paraplast wax. All paraffin-embedded tissue was stained with PAS–H. PAS staining identifies glycogen-containing structures, and is therefore used as an acrosomal stain to facilitate staging of tubules. Hematoxylin is a chromatin stain used to distinguish nuclear morphology. Plastic sections were obtained from tissue that was first perfusion fixed with 3% glutaraldehyde, and then embedded in Spurr's or Poly/Bed 812 epoxy resin. Thick sections were obtained using an ultramicrotome, fixed to glass slides and stained with methylene blue or toluidene blue. All light microscopy was performed using an Olympus BX60 light microscope and ImagePro 4.1 image capture software.

Staging of tubules

In order to count the number of tubules in each stage of spermatogenesis, three different pairs of mice were used. Each pair consisted of one mutant and one wild-type mouse of the same age; all mice were between 3 and 9 months of age. Testis from each mouse was fixed in Bouin's fixative and processed as described above. Paraffin sections were stained with PAS–H.

One testis cross section per mouse was analyzed under the light microscope. The total number of tubules in each section was counted, and all tubules were assigned a stage based on the criteria outlined by Russell *et al.* (1990). Based on the total number of tubules, the percentage of tubules at each stage in a given testis cross section was determined.

Statistical analysis

In order to determine the statistical significance for the numbers of spermatogonia in wild-type and mutant tubules, an unpaired two-tailed *t*-test was performed ($P=0.05$).

Tissue processing for electron microscopy

Testes were fixed by perfusion fixation followed by an overnight immersion fixation in 4% glutaraldehyde. The tissue was washed in 0.15 M sodium cacodylate buffer (pH 7.3) to remove excess glutaraldehyde. Tissue was then post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.3) for 1 h on ice. After post-fixation, tissue was washed in ddH₂O, and dehydrated in a graded ethanol series, infiltrated with Spurr's or Poly/Bed 812 epoxy resin, and embedded at 65 °C overnight. Thin sections were mounted on copper grids for transmission electron microscopy (TEM) observation. Grids were stained with uranyl acetate and lead citrate. Stained sections were observed using a Hitachi 7100 STEM (scanning transmission electron microscope) or a Philips 300 electron microscope.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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