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Expression of acid phosphatase in the seminiferous epithelium of vertebrates

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ABSTRACT. Acid phosphatases (AcPs) are known to provide phosphate to tissues that have high energy requirements, especially during development, growth and maturation. During spermatogenesis AcP activity is manifested in heterophagous lysosomes of Sertoli cells. This phagocytic function appears to be hormone-independent. We examined the expression pattern of AcP during the reproductive period of four species belonging to different vertebrate groups: *Tilapia rendalli* (Teleostei, Cichlidae), *Dendropsophus minutus* (Amphibia, Anura), *Meriones unguiculatus* (Mammalia, Rodentia), and *Oryctolagus cuniculus* (Mammalia, Lagomorpha). To demonstrate AcP activity, cryosections were processed for enzyme histochemistry by a modification of the method of Gömöri. AcP activity was similar in the testes of these four species. Testes of *T. rendalli*, *D. minutus* and *M. unguiculatus* showed an intense reaction in the Sertoli cell region. AcP activity was detected in the testes of *D. minutus* and *O. cuniculus* in seminiferous epithelium regions, where cells are found in more advanced stages of development. The seminiferous epithelium of all

four species exhibited AcP activity, mainly in the cytoplasm of either Sertoli cells or germ cells. These findings reinforce the importance of AcP activity during the spermatogenesis process in vertebrates.

Key words: Acid phosphatase; Reproduction; Testis; Spermatogenesis; Vertebrates

INTRODUCTION

Acid phosphatases (AcPs) are known to provide phosphate to tissues that show high energy requirements, especially during development, growth and maturation (Blum, 1970; Hurkadli et al., 1985). They are ubiquitous enzymes that catalyze the hydrolysis of orthophosphate monoesters under acidic conditions. Despite a common functional identity, these hydrolases can be differentiated according to structural, catalytic and immunological properties, as well as tissue distribution and subcellular location (Suter et al., 2001).

Five important forms of AcPs are found in humans: prostatic, lysosomal, erythrocytic, macrophagic, and osteoclastic (Bull et al., 2002). These forms were detected in erythrocytes, leukocytes, platelets, liver, spleen, kidney, bone, and other tissues (Saftig et al., 1997; Cerri et al., 1999; Bull et al., 2002). The expression of this enzyme also has been studied in the testis. It is well known that AcP activity is present in the lysosomes of Sertoli cells, which have a heterophagic function (Niemi and Kormano, 1965; Hurk et al., 1974; Chemes, 1986; Porawski et al., 2004). In rats, the digestion of residual bodies is likely to begin with autophagy in spermatids and be completed with phagocytosis by Sertoli cells. This phagocytic function appears to be hormone-independent (Chemes, 1986). This fact is corroborated by the conclusions of some studies in which some species including *Oreochromis niloticus* (Teleostei, Cichlidae) and *Odonthestes perugiae* (Teleostei, Atherinidae) showed AcP expression in the non-reproductive period (Porawski et al., 2004), whereas in other species such as *Liolemus elongatus* (Reptile, Squamata) AcP activity was detected only in the reproductive period (Grimalt et al., 1995).

Thus, the present study aimed to detect the expression pattern of the acid phosphatase in the reproductive period of four species belonging to different vertebrate groups: *Tilapia rendalli* (Teleostei, Cichlidae); *Dendropsophus minutus* (Amphibia, Anura), *Meriones unguiculatus* (Mammalia, Rodentia), and *Oryctolagus cuniculus* (Mammalia, Lagomorpha). These species were chosen because they are very useful and important models for reproduction studies in some Brazilian regions, since they are easily collected in the field (*D. minutus*) or widely raised in captivity (*T. rendalli*, *M. unguiculatus* and *O. cuniculus*) (Peruquetti, 2009). We expected to find AcP activity in the seminiferous epithelium of each species studied reinforcing the importance of this enzyme during the the spermatogenesis process in vertebrates.

MATERIAL AND METHODS

Five adult male specimens of *T. rendalli* (Teleostei, Cichlidae) taken from fish farming tanks of the São Paulo State University (UNESP/IBILCE), São José do Rio Preto, SP, Brazil, were housed in asbestos tanks (500 L, 1 fish, 5L-1) for 15 days before experimentation. Water

was kept at 25°C and light at 12-h dark:light cycle (from 7:00 to 19:00 h) and constant aeration was supplied. Fish were fed pellets for tropical fish (28% protein) offered *ad libitum* 1 h after starting the light period and 1 h before ending the light period. The animals were killed by excess anesthesia (benzocaine) and their gonads were removed. Fifteen adult males in calling activities of *D. minutus* (Amphibia, Anura) were captured at the Morro do Diabo State Park (Municipality of Teodoro Sampaio, State of São Paulo, Brazil) during nocturnal field work, acclimatized in plastic bags, and transported to the laboratory. The amphibians were anesthetized and killed by ether inhalation, and their gonads were removed. Five adult male specimens of *M. unguiculatus* (Mammalia, Rodentia) were obtained from the Biology Department Animal House (IBILCE/UNESP). Five adult male specimens of *O. cuniculus* (Mammalia, Lagomorpha) were obtained from the FAMERP Animal House (São José do Rio Preto School of Medicine, São José do Rio Preto, SP, Brazil). These animals were housed under standard conventional conditions (25°C, 40-70% relative humidity, 12-h light/dark cycle) and allowed access to chow and water *ad libitum*. The animals were anesthetized and killed by carbon dioxide gas (CO_2) inhalation and their gonads were removed. All animals were obtained during their respective reproductive periods (Figure 1). This study followed the ethical principles of animal research adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Biosciences Institute/UNESP Animal Research Ethics Committee (CEEA) under protocol number 054/06. The animals were treated according to the recommendations of the Committee on Care and Use of Laboratory Animals (1980) from the Institute of Laboratory Animal Resources. All sacrifice procedures were in accordance with Resolution 714 - Brazilian Veterinary Medicine Federal Council.

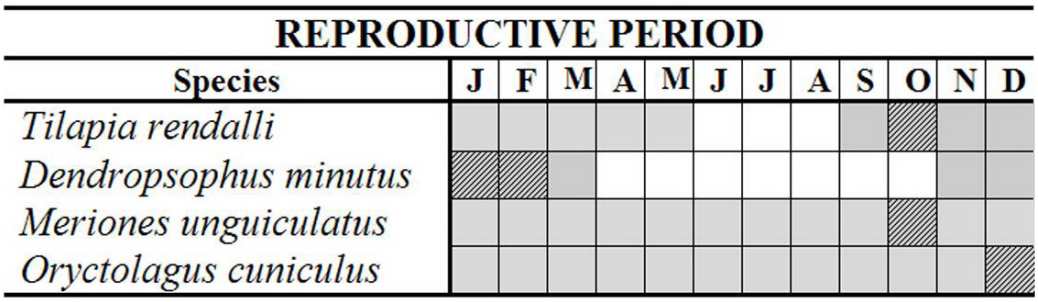

Figure 1. Reproductive periods of the four species studied. The open squares indicate the non-reproductive period of each species, the light gray squares demonstrate the reproductive period of each species, and the striped squares show the months during which each species was collected.

Hematoxylin-eosin stain

The testes of each animal were removed and fixed by immersion in Bouin's fixative solution for 24 h. The testes were embedded in glycol-methacrylate historesin. Sections (1 to 3 µm thick) were obtained using a Leica RM 2155 microtome. Tissue sections were submitted to hematoxylin-eosin staining according to Ribeiro and Lima (2000). The sections of seminiferous tubules were evaluated with an Olympus BX 60 photomicroscope and documented by Image Pro-Plus-Media Cybernetics, version 6.1, for the Windows computer software for image analysis.

Acid phosphatase technique

The testes were removed after sacrifice and immediately frozen in liquid nitrogen and sectioned (10 to 15 μM) in a Leica CM 1850 cryostat microtome. For demonstration of AcP activity, the cryosections were processed for enzyme histochemistry by a modification of the method of Gömöri (1950). Frozen sections were incubated for 60 min at 37°C in a medium composed of sodium ß-glycerophosphate substrate and lead nitrate. The control for the histochemical reaction was obtained using the incubation medium without substrate (ß-glycerophosphate). After incubation, the sections were washed in distilled water, treated with 1% ammonium sulfide for 1 min, rinsed in distilled water and then counterstained with 0.1% methyl-green aqueous solution for 10 min. Forty sections of seminiferous epithelium containing experimental reactions and 40 sections of seminiferous epithelium containing control reactions from each species studied were evaluated with an Olympus BX 60 photomicroscope and documented by Image Pro-Plus-Media Cybernetics, version 6.1, for the Windows computer software for image analysis.

RESULTS

The hematoxylin-eosin-staining technique was employed for specific analysis of seminiferous epithelium structure and for general analysis of seminiferous epithelium. This general analysis effectively located the sites of AcP action in the testes of each animal studied. In *T. rendalli*, Sertoli cells surround germ cells in the same developmental stage, forming germ cysts, and this species has an unrestricted type of testicular arrangement, in which spermatogonias appear along the length of the tubules (Figure 2A). In *D. minutus*, Sertoli cells also surround germ cells to form germ cysts, although spermatogonias appear in cysts instead of along the tubule length (Figure 2D). In *M. unguiculatus* and *O. cuniculus*, the germ cells were found at different levels from the base of the tubule to the lumen and are surrounded by Sertoli cell cytoplasm (Figure 2G and J). This Sertoli cell cytoplasm extends the entire height of the epithelium because the cell serves to nurture the germ cells through their development cycles. As the germ cells divide and develop into different cell types, they move from the basement membrane region through the tight junctional complex of adjacent Sertoli cells until they reach the adluminal compartment. Synchrony of germ cell development results in large areas of the seminiferous tubule containing vast numbers of cells at the same level of development, the same stage of spermatogenesis. This sequential order of stages and their repetition along the length of the tubules constitutes the "wave" of spermatogenesis in mammalian seminiferous epithelium.

The testes of *T. rendalli* submitted to the experimental reaction (incubation medium with sodium β-glycerophosphate) showed an intense AcP activity at the base of the seminiferous epithelium and at the base of the germ cysts, where the Sertoli cells are located (Figure 2B). This activity was moderate or absent in the lumens of both seminiferous epithelium and germ cysts (Figure 2B). AcP activity was detected predominantly in the cytoplasm and not in the nuclear interior. The testes of *T. rendalli* submitted to the control reaction (incubation medium without sodium β-glycerophosphate) did not display enzymatic activity either at the base of seminiferous tubules and germ cysts or inside these structures (Figure 2C). The testes of *D. minutus* submitted to the experimental reaction showed AcP activity at the base of both seminiferous epithelium and germ cysts where the Sertoli cells are located (Figure 2E) and in the germ cysts with cells in more advanced stages of development (later spermatids and spermatozoa)

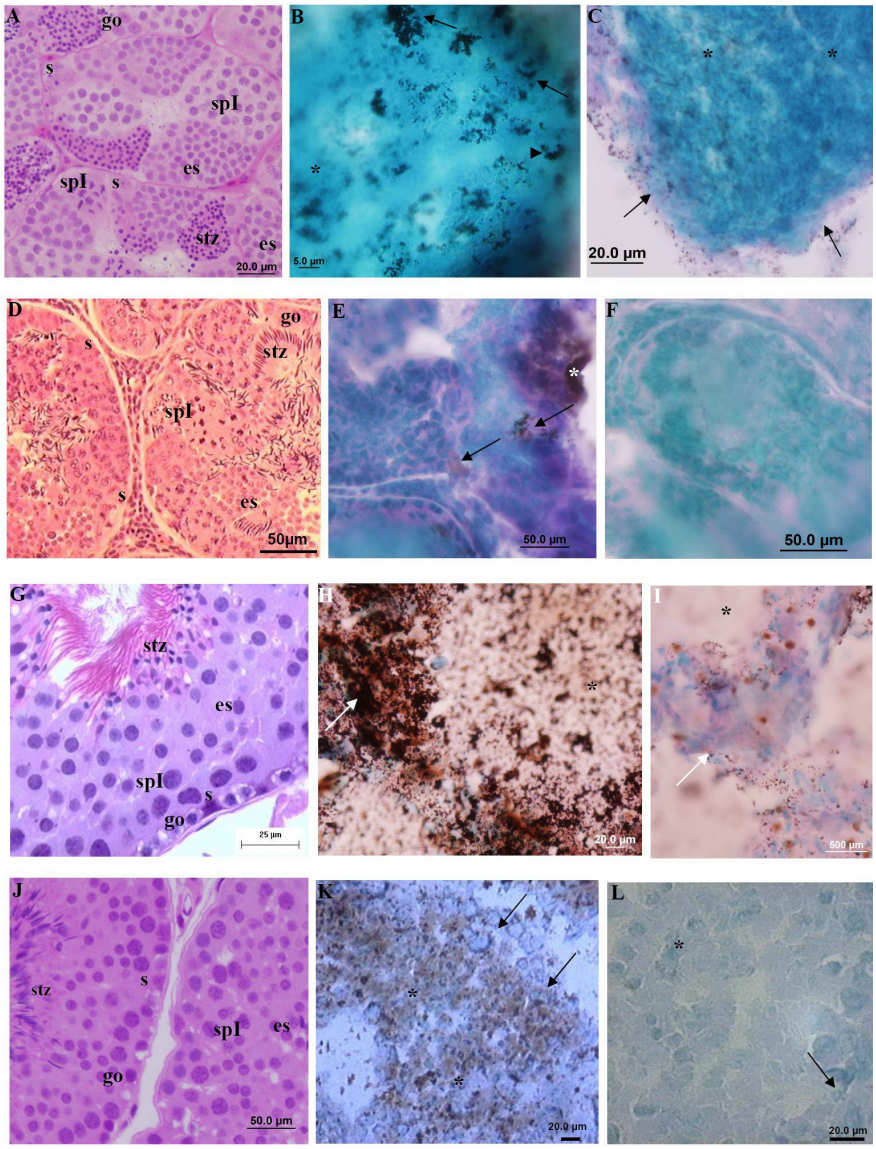

Figure 2. A. to **C.** *Tilapia rendalli* germ epithelium; **D.** to **F.** *Dendropsophus minutus* germ epithelium; **G.** to **I.** *Meriones unguiculatus* seminiferous tubules; **J.** to **L.** *Oryctolagus cuniculus* seminiferous tubules. A, D, G, and J: Hematoxylin-eosin stain. General analysis of the germ epithelium: go = spermatogonium; spI = primary spermatocyte; es = spermatids; stz = spermatozoon; s = Sertolli cells. B, E, H, and K: Detection of acid phosphatase (AcP) activity (experimental reaction). Arrows: Base of germ epithelium, base of germ cysts or base of seminiferous tubules. Asterisk: Lumen of germ epithelium, lumen of germ cysts or lumen of seminiferous tubules. B: AcP activity in germ cell cytoplasm (arrowhead). C, F, I, and L: Detection of AcP activity (control reaction). Arrows: Base of germ epithelium, base of germ cysts or base of seminiferous tubules. Asterisk: Lumen of germ epithelium, lumen of germ cysts or lumen of seminiferous tubules.

(Figure 2E). The *D. minutus* testes submitted to the control reaction showed a total absence of enzyme activity at the base of the seminiferous tubules and germ cysts as in the interior of these structures (Figure 2F). The testes of *M. unguiculatus* submitted to the experimental reaction exhibited intense AcP activity at the seminiferous tubule base, the location of the Sertoli cells (Figure 2H). This activity was moderate in the seminiferous tubule lumen (Figure 2H). AcP activity was detected predominantly in the cytoplasm and not in the nuclear interior. Testes of *M. unguiculatus* submitted to the control reaction showed no enzymatic activity either at the base or in the lumen of seminiferous tubules (Figure 2I). The *O. cuniculus* testes submitted to the experimental reaction displayed AcP activity throughout the seminiferous epithelium; however, in the lumen this reaction was more intense (Figure 2K). Testes of *O. cuniculus* submitted to the control reaction showed a total absence of enzyme activity both at the base and in the lumen of the seminiferous tubules (Figure 2L). The main results of this study are summarized Table 1.

Table 1. Expression of AcP activity during experimental reaction (incubation medium with sodium β-glycerophosphate) and control reaction (incubation medium without sodium β-glycerophosphate). ++ = intense AcP expression; + = moderate AcP expression; - = AcP expression absent.

Reaction	Germ epithelium position	Species			
		T. rendalli	*D. minutus*	*M. unguiculatus*	*O. cuniculus*
Experimental (with sodium β-glycerophosphate)	Base (Sertoli cells)	++	++	++	+
	Lumen (later spermatids and spermatozoon)	+	++	+	++
Control (without sodium β-glycerophosphate)	Base (Sertoli cells)	-	-	-	-
	Lumen (later spermatids and spermatozoon)	-	-	-	-

DISCUSSION

The Gömöri method has been widely used for identification of AcP activity in different tissues and diagnosis of lesions (Sternberg, 1996; Zaviacic, 1999). AcPs are ubiquitous enzymes that catalyze the hydrolysis of orthophosphate monoesters under acidic conditions (Suter et al., 2001). AcP activity has been demonstrated in the liver of rats (Siebert, 1966), nucleoli of human cells (Soriano and Love, 1971), *Allium cepa* (Sánchez-Pina et al., 1980), maize roots (Deltour et al., 1981), salivary glands of *Drosophila melanogaster* (Jones and Bowen, 1993), gerbil (*M. unguiculatus*) prostate glands of both sexes (Custódio et al., 2004), and salivary glands of Triatomines (Anhê et al., 2007) and during spermiogenesis of insects (Báo and Dolder, 1990; Fernandes, 1999).

In the present study, AcP activity was demonstrated in the seminiferous epithelium during the reproductive period of four vertebrate species. AcP activity was similar in the testes of these four species studied, all of which were in their respective reproductive periods. According to Porawski et al. (2004) in Nile tilapia (*O. niloticus*) and kingfish (*Odonthestes perugiae*), positive AcP activity was observed only in the non-reproductive period: winter (Nile tilapia) and summer (kingfish). This difference can be related to hormonal control of AcP expression in these organs. Acid hydrolases in lizard epidymis showed distinct activity in the reproductive cycle. Acid hydrolytic activity was intense in the non-reproductive period of *Phymaturus palluma*. However, in another

lizard species, *Liolemus elongatus*, the activity of acid hydrolases was intense in the reproductive period (Grimalt et al., 1995). These authors postulated that the epididymal acid hydrolases may be under hormonal control in the lizards studied. On the other hand, AcP activity is not hormone-dependent in rats (Chemes, 1986) and the human testicular AcP gene was found to be upregulated by androgens and downregulated by estrogen (Yousef et al., 2001). In spite of these observations, it had been postulated that, in general, AcP activity is very important to the spermatogenetic process in vertebrates during the reproductive period as much as in the non-reproductive period. In germ cells during the reproductive period of some animals, the study of acid phosphatase has been principally related to the lysosomes of Sertoli cells (Niemi and Kormano, 1965; Hurk et al., 1974; Chemes, 1986; Porawski et al., 2004), the acrosome (Anderson, 1968; Sousa et al., 1988; Báo et al., 1989) and the axoneme (Anderson, 1968; Baccetti et al., 1971, 1973).

Testes of *T. rendalli*, *D. minutus* and *M. unguiculatus* showed an intense reaction in the Sertoli cell region. This finding indicates a prominent/significant autophagic function of this cell type in this tissue during the reproductive period in these species. It is well known that AcP activity is present in heterophagic lysosomes of Sertoli cells (Niemi and Kormano, 1965; Hurk et al., 1974; Chemes, 1986; Porawski et al., 2004). AcP and lysosomal contents of Sertoli cells are greater than those of germ cells, and the residual body disposal is probably initiated by autophagy and completed by Sertoli cell phagocytosis (Chemes, 1986). In the present study, intense AcP activity was detected in testes of *D. minutus* and *O. cuniculus* in regions of the seminiferous epithelium where cells were found in more advanced stages of development: germ cysts of later spermatids and spermatozoa (*D. minutus*) and lumen of the seminiferous tubules (*O. cuniculus*). These findings indicate that the Sertoli cell lysosomes had started to act in testes of these species by assisting in the digestion of residual bodies and cytoplasmic remains in these seminiferous-tubule regions and that AcP activity is present during acrosome and axoneme formation. It is well known that AcP activity - present in spermatogonial cells, primary and secondary spermatocytes, spermatids and spermatozoon bundles - may readily provide phosphate to meet their high energy requirements (Blum, 1970; Hurkadli et al., 1985).

The seminiferous epithelium in all the species studied showed AcP activity, mainly in the cytoplasm of either Sertoli cells or germ cells. This finding reflects a strong relation between AcP activity and lysosomes in testicular cells. In some somatic organs including salivary glands of Triatomines a role for acid phosphatase in the nuclear matrix could be assumed, because AcP was detected in both the nucleolus and chromatin. Thus, phosphatase activity during rRNA transcription, possibly in the nucleolar fibrillar center, is suggested (Anhê et al., 2007). However, it is impossible to conclude that AcP is not present in the nucleus of some germ cell epithelium, since at some sites of the organ the reaction was so intense that it hid the cell nuclei.

In conclusion all species studied showed AcP activity expressed in the testes during their respective reproductive periods. This activity presented was partially distinct among the four species and was detected mainly in the cytoplasm of the cells. These findings reinforce the importance of AcP activity during the spermatogenesis process of vertebrates.

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