Alkaline phosphatase localization in Golden hamster epididymidis: a biochemical and histochemical investigation

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Abstract

The aim of this study was to determine alkaline phosphatase (ALP; E.C. 3.1.3.1) activity and major expression in homogenates obtained from different regions of Golden hamster epididymis, comprising the initial segment, head, body and tail, with concomitant research of this enzyme localization and activity in samples of tissues. These were collected from the same regions and investigated by histochemical conventional study performed on frozen histological sections. No significant differences in mean ALP activity, reported as U.100 mg^{-1} of tissue, were observed among the biological specimens collected from the epididymidis initial segment (0.92 ± 0.28 U.100 mg^{-1} tissue), head (1.07 ± 0.67 U.100 mg^{-1} tissue) and body (0.77 ± 0.23 U.100 mg^{-1} tissue). However, mean ALP activity was significantly higher in the epididymal tail (8.94 ± 0.40 U.100 mg^{-1} tissue) compared with the precedent segments. The findings suggested that ALP plays a significant role in the tail of the Golden hamster epididymidis, mediating androgenic segregation necessary to maintain the epithelial integrity. Furthermore, ALP acts on active transport of substances between the luminal fluid and spermatozoon membrane, and contrariwise. Thus, the high concentration of ALP in the epididymal tail helps to indicate the importance of this enzyme in the metabolism and maintenance of spermatozoa maturation and storage into the epididymidis luminal compartment, perhaps directly influencing the normal reproductive morphophysiology.

Keywords: alkaline phosphatase, epididymidis regions, hamster, histochemistry, biochemistry.

1 Introduction

The epididymis belongs to the excurrent duct system of the testis and acts directly on the maturation and storage of spermatozoa. Despite consisting of a single duct, the epididymis has shown regional differences obtained in histological, biochemical and histochemical studies, conducted on several mammalian species (ALSUM and HUNTER, 1978; ARIYARATNA, GUNAWRADANA and NAVARATNA, 1996; BHATTACHARYYA and BHATTACHARYYA, 1985; ERKMANN, 1971; HOFFMANN, KRAMER and MAIN, 1989; LINNETZ and AMANN, 1968; MANEELY, 1955; MANN, 1964; MARTAN, 1969; MONIEM, 1980; MONIEM and GLOVER, 1972; NICANDER, 1958; ORSI, MATHEUS, GREGORIO et al., 1998; RIAR, SETTY and KAR, 1973; SINOWATZ, SKOLEK-WINNISCH and LIPP, 1979; TINGARI and MONIEM, 1979). Secretion and absorption activities of the epididymal epithelial lining cells create an adequate intraluminal microenvironment for spermatozoa maturation (ROBAIRE and HERMO, 1989; SETCHELL, MADDOCKS and BROOKS, 1994) inside the tubular lumen. Besides, the epididymal luminal fluid is composed of various biochemical substances (MANN, 1964). Among them are phosphomonoesterases (BELL and LAKE, 1962) and phosphatases (FRENETTE, DUBE and TREMBLAY, 1986), enzymes which play a catalytic role in the hydrolysis of phosphoric acid esters, releasing phosphate ions (ARIYARATNA, GUNAWRADANA and NAVARATNA, 1996). Alkaline phosphatase (ALP; E.C. 3.1.3.1) is a dephosphorylative enzyme acting on many tissues and organs, including bone, liver, kidney, intestine, lung, and placenta (HOFFMANN, KRAMER and MAIN, 1989). Variable levels of ALP have also been detected in the seminal fluid of some mammals, including that of the cock and turkey (BELL and LAKE, 1962). Perhaps ALP is directly involved in some glycolytic pathways with formation of fructose (MANEELY, 1955). Therefore, histochemical studies on ALP activities carried out in various mammalian species such as buck and boar (BELL and LAKE, 1962), dog (JOHNSTON, 1991; SINOWATZ, SKOLEK-WINNISCH and LIPP, 1979), rabbit (MONIEM and GLOVER, 1972), sheep (NICANDER, 1958) and bull (BLACKSHAW and SAMISONI, 1967), have shown different patterns form this enzyme along the epididymal regions.

The site of ALP formation and action has been determined in some species and seems to have a species-specific dependence. Previous biochemical studies (TINGARI and MONIEM, 1979) had demonstrated that ALP activity lev-

els in normal stallions reproductive tract were significantly higher in testis and in epididymis than in any other tissues of the seminal pathway of this species. Data obtained in the same research support the statement that ALP activity is not uniform in the epididymis, but is concentrated at high levels in the lumen of the epididymal tail (TURNER and MACDONELL, 2003).

With regard to the ALP relevance in mammalian and avian epididymidis (BELL and LAKE, 1962), viewing the spermatozoa maturation and fertility ability in the epididymidis luminal compartment functions previously emphasized (ROBAIRE and HERMO, 1989), the objective of this study was to determine ALP activity along the epididymal duct in the initial segment, head, body and tail of the adult Golden hamster epididymidis.

2 Material and methods

Biochemical analysis for determination of ALP kinetics was performed on epididymal tissue samples obtained from 14 young 60 day old adult Golden hamsters (*Mesocricetus auratus*), proceeding from the Central Animal House of UNESP at Botucatu. The rodents were maintained under a 12 hours light/dark cycle at a controlled temperature, with water and food available *ad libitum*. The rodents were sacrificed with an anesthetic overdose of Ketalar®. Afterwards, the epididymis was collected and divided into initial segment, head, body and distal tail (ROBAIRE and HERMO, 1989). The epididymal tissues obtained were weighed and frozen until the necessary time for biochemical analysis. Three samples from each epididymal region were used for biochemical analysis, each sample with an average weight of 135 mg. The samples were homogenized in 5 mL sodium phosphate buffer pH 7.4 with a Potter-Elvehjem homogenizer (Curtin Matheson Scientific, Houston, TX, USA), using a Teflon® pestle. The homogenates obtained were centrifuged in a refrigerated centrifuge at 10,000 rpm for 15 minutes at –4 °C, and the resulting supernatants were used for the biochemical determinations.

ALP was determined in 3 µL supernatant of each sample using the CELM alkaline phosphatase kit (Modern Laboratory Equipment Company, Sao Paulo, Brazil). Kinetic analysis of ALP was carried out at 25 °C and absorbance at 405 nm, read after 1, 2 and 3 minutes with an ELISA microplate reader (Bio-tech Instruments, Inc., Winooski, VT, USA). The results are reported as unit per 100 mg tissue (U.100 mg^{-1} tissue). Statistical analysis consisted of a completely random design, with four treatments and three replicates. The data were submitted to analysis of variance (ANOVA) and mean values were compared by the Tukey test at 5% of significance.

Histochemical analysis of ALP activity was performed in 10 µm cryostatic sections of the same epididymidis regions biochemistry analyzed, utilizing tissue samples obtained from 4 hamsters. The samples were previously frozen in n-hexane (–60 °C), and the sections were used for histochemical localization of ALP, as described in a previous similar study (ORSI, MATHEUS, GREGORIO et al., 1998). As customary, the specific tissular reactivity at light microscopy was signalized as strong, medium and low (ORSI, MATHEUS, GREGORIO et al., 1998).

3 Results

The results of the biochemical analysis of ALP activity in the epididymal regions of the Golden hamster are shown in Figure 1. The lowest ALP activity was observed in the epididymal body (0.77 ± 0.23 U.100 mg^{-1} tissue), while the highest activity was detected in the tail (8.94 ± 0.40 U.100 mg^{-1} tissue). Intermediate values were observed in the initial segment (0.92 ± 0.28 U.100 mg^{-1} tissue) and in the head (1.07 ± 0.67 U.100 mg^{-1} tissue). Statistical analysis revealed a significant difference in mean ALP activity between the tail and the precedent epididymal segments, while no significant differences were observed among the initial segment, head, and body of the epididymis.

4 Conclusion

Alkaline phosphatases in mammals are ectoenzymes bound to the plasma cell membrane through phosphatidylinositol glycan anchors (LOW and SALTIEL, 1988); they are glycosylated and possess variable amounts of sialic acid, which is responsible for their electrophoretic mobility (ECKERSALL and NASH, 1983). The isomer of ALP catalyzes the hydrolysis of monophosphate esters and is mainly a plasma membrane enzyme located on the absorptive or secretory surface of cells. Alkaline phosphatases are found in various tissues and organs, but tend to be biochemically unique in each tissue (HOFFMANN, KRAMER and MAIN, 1989). High ALP activity can be detected in absorptive epithelia such as those of the intestines and proximal convoluted tubules of the kidneys (HOFFMANN, KRAMER and MAIN, 1989). With regard to the reactivity of these epithelia to ALP, a relationship between ALP activity and transport of fluid across the plasma membrane has been proposed (ERKMANN, 1971). Other investigations have suggested that ALP plays a role in the transport of sugars and other organic molecules across biological membranes, and also contributes in the epididymal histoarchitecture, in the transport of molecules between the epithelium principal cells and capillaries of the subepithelial connective tissue (ADAMS, 1983). These con-

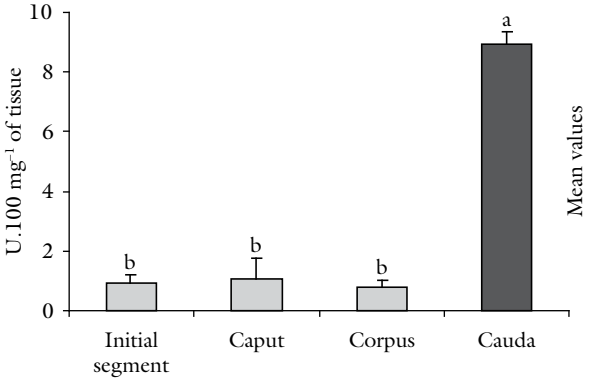

Figure 1. Alkaline phosphatase (ALP) activity in the initial segment, head, body and tail of the Golden hamster epididymidis. Data are reported as ± SD means. The same letters indicate that the values did not differ significantly from one to another epididyml region (Tukey test, $p < 0.05$).

siderations help to support the results here verified on ALP activity in hamster epididymis, which showed that the principal cells of the epididymal epithelium are directly involved in some process such as endocytosis, protein secretion and absorption, and apparent transluminal transport of different molecules between the interstitium and the tubular lumen (HERMO, OKO and MORALES, 1994; ROBAIRE and HERMO, 1989; SETCHELL, MADDOCKS and BROOKS, 1994).

Another hypothesis suggests a possible role of ALP in androgen transfer between the peritubular interstitium and the seminiferous tubules of the fowl testis (GUNAWARDANA, 1990). Moreover, the epididymis receives a double androgen hormonal supply through the luminal fluid and by the peritubular blood vessels, and it has been assumed that maintenance of the integrity of the lining tubular epithelium is dependent on intraluminal androgens (GOYAL and VIG, 1984). Thus, it is expected that the enzymatic activity of ALP present in the luminal margins and in the basement membrane of the epididymal epithelium supplies the epithelium with androgens necessary for maintenance of the epithelial integrity (GOYAL and VIG, 1984). This thesis may support our observations, since the epithelium of the epididymal tail, a storage region for epididymal spermatozoa, is theoretically submitted to a higher pressure regarding the large caudal volume of luminal content and by the strong emission of spermatozoa during sperm ejaculation (PABST, 1969).

The sites of ALP formation in the male reproductive tract have been determined for some species, and there seem to be species-specific variations. In dogs, most ALP is produced in the epididymis (FRENETTE, DUBE and TREMBLAY, 1986). In rabbits, it has been shown that the testis, epididymis, deferent duct and the ampulla of vas deferens synthesize significant amounts of ALP (MÜLLER, 1983), while in bulls most ALP originates from the seminal vesicles and, to a lesser extent, from the testes and epididymis (ALEXANDER, ZEMJANIS, GRAHAM, et al., 1971). In stallions (TURNER and MACDONELL, 2003), a large part of ALP was found originating from the seminal plasma and not from the spermatozoa, an observation that is based on the analysis of non-processed ejaculates and spermatozoa-free ejaculates (TURNER and MACDONELL, 2003). This finding agrees with studies on human sperm in which ALP activity was lower in isolated spermatozoa compared to the higher activity observed in seminal plasma (FRENETTE, DUBE and TREMBLAY, 1986; ECKERSALL and NASH, 1983; JOHNSTON, 1991; SINGER, BARNET, ALLALOUF et al., 1980). In the Golden hamster, a strong expression of ALP production was observed in the epididymis, in agreement with the present biochemical and histochemical results having previous and similar histochemical findings earlier reported for this rodent (MONIEM and GLOVER, 1972); also a strong activity of ALP was histochemically verified in the epididymal cauda of the black isogenic mouse (ORSI, MATHEUS, GREGORIO et al., 1998).

ALP has been demonstrated in various mammalian species but its distribution shows species-specific variations (ALSUM and HUNTER, 1978; ARIYARATNA, GUNAWRADANA and NAVARATNA, 1996; BHATTACHARYYA and BHATTACHARYYA, 1985; ERKMANN, 1971; HOFFMANN, KRAMER and MAIN, 1989; LINNETZ and AMANN, 1968; MANEELY, 1955; MANN, 1964; MARTAN, 1969; MONIEM, 1980; MONIEM and GLOVER, 1972; NICANDER, 1958; ORSI, MATHEUS, GREGORIO et al., 1998; RIAR, SETTY and KAR, 1973; SINOWATZ, SKOLEK-WINNISCH and LIPP, 1979; TINGARI and MONIEM, 1979). In this respect, ALP was observed at very high concentrations (>10,000 $U.L^{-1}$) in the epididymal fluid of dogs, while only small amounts of ALP were detected in the canine prostate and testis (JOHNSTON, 1991). Also in dogs, histochemical analysis has shown ALP activity in epithelial cells of the head, body and tail, emphasizing a higher reactivity in the last segment (KUTZLER, SOLTER, HOFFMAN et al., 2003), in agreement with the ALP activity characterized here for the epididymal tail of the Golden hamster. ALP reactivity was also demonstrable in the seminiferous tubules, although the reaction in the testis was less intense than that observed in the epididymis. Results concerning the general epididymal ALP activity in the hamster are in accordance with those reported for dogs (KUTZLER, SOLTER, HOFFMAN et al., 2003) and rabbits (MÜLLER, 1983). In humans, ALP reactivity has been found to be weak in the epididymal epithelium, while in goats ALP activity decreased during the emission of epididymal spermatozoa (BHATTACHARYYA and BHATTACHARYYA, 1985). These findings disagree with the present results showing a weak reaction in the initial segment, head and body and a marked increase in the epididymal tail, thus revealing a parallelism between an increase in the activity of this enzyme and the emission and storage of epididymidis spermatozoa in Golden hamsters.

Regarding the more specific location of ALP in the epididymal epithelia, differences were found among some species. In dogs, the ALP activity was detected mainly in the luminal margin of epithelial cells and also in stereocilia, but not at the basement membrane level (KUTZLER, SOLTER, HOFFMAN et al., 2003). In contrast, in swamp buffaloes, strong activity was observed along the basal region of the epithelium throughout the epididymis, while in the luminal border, reactivity was only detected in zone II of the epididymal head (ARIYARATNA, GUNAWRADANA and NAVARATNA, 1996). Observations made on bulls showed strong positive reactions in the stereocilia and apical brush border of zones I to III, in the base membrane and in peritubular smooth muscle cells, along the epididymal duct (GOYAL and VIG, 1984). Positive ALP reactivity in epididymal microvilli has also been reported for sheep and bulls (NICANDER, 1958), hamsters (MALONE and BOWER, 1962) and rabbits (SETCHELL, MADDOCKS and BROOKS, 1994), but not for rats (SINGER, BARNET, ALLALOUF et al., 1980). In guinea pigs, ALP reactivity was only observed in the basement membrane of the epididymal epithelium (ADAMS, 1983). The studies on swamp buffaloes (ARIYARATNA, GUNAWRADANA and NAVARATNA, 1996) and bulls (GOYAL and VIG, 1984), in which strongly positive reactions were demonstrated in the proximal regions of the epididymis, differ significantly from those reported in the present investigation, whose ALP activity in the epididymal duct was higher in the distal part, e.g., at the distal tail level.

Histochemical studies on the epididymis of the camel have shown a positive reaction in subepithelial connective tissue, blood vessels and stereocilia, with subepithelial and vascular reactions being common to almost all species (SINOWATZ,

SKOLEK-WINNISCH and LIPP, 1979). The location of ALP in stereocilia and subepithelial connective tissue is consistent with a possible function of active transport between vascular structures and epithelial cells on the one hand, and between epithelial cells and luminal content on the other (BHATTACHARYYA and BHATTACHARYYA, 1985). Similar results are verified here with histochemistry analysis of the hamster epididymal duct, being predominately strong on the caudal region, similar to the biochemical findings. In some species, ALP reactivity has been detected in the epididymal lumen and in the cytoplasmic droplet of the spermatozoon. In Rhesus monkeys (ALSUM and HUNTER, 1978), ALP activity was higher in the lumen than in the epididymal epithelium lining, whose most intense reaction was observed in the epididymal body. ALP activity has been demonstrated in cytoplasmic droplets of sheep and rabbit spermatozoa (TINGARI and MONIEM, 1979). However, no positive reaction was observed in spermatozoa of rodents despite the high levels of this enzyme in epididymal plasma (SINGER, BARNET, ALLALOUF et al., 1980). The location of ALP in cytoplasmic droplets of spermatozoa raises the hypothesis that ALP catalyzes dephosphorylation and transport of phosphate groups between the luminal fluid and spermatozoa and, consequently, ALP is related to the maturation of spermatozoa into the luminal compartment of the epididymis (SINGER, BARNET, ALLALOUF et al., 1980).

Furthermore, results of ALP activity in Golden Hamsters showed that its activity was strong in the distal tail, while it was low in the initial segment, head and body of the hamster epididymidis. These findings are in contrast to a previous study performed on some mammals, including the hamster itself, which showed no ALP activity in the initial segment, head and body, and maximum to moderate activity present in proximal tail and distal tail, respectively (BELL and LAKE, 1962). Those results obtained in the hamster's ALP activity also disagree with comparative findings reported for sheep, rabbits and rats (BELL and LAKE, 1962), in which moderate ALP activity was observed in the initial segment and head of the sheep epididymis, and no activity was present in the epididymal body and tail. In albino rats, no ALP activity was detected in any of the epididymal segments, while in rabbits all the segments showed reactivity to ALP. This enzyme, including in hamsters, perhaps derive from cytoplasmic droplets of the spermatozoa "pool" stored at the luminal level of the epididymidis tail. This hypothesis has been based on studies conducted on Rhesus monkeys epididymidis (ALSUM and HUNTER, 1978) but disagrees with the statement following the analysis of ALP in cytoplasmic droplets (MONIEM, 1980). On the other hand, according to Moniem and Glover (1972), ALP could be separated by cytoplasmic droplets and afterwards released into the lumen during spermatozoa maturation. Therefore, the stronger positive reaction detected in the epididymal lumen would originate from the cytoplasmic droplets and not from the lining epithelium, but the cytoplasmic droplet suffer endocytosis by clear cells along the course of spermatozoa through the epididymal lumen (HERMO, OKO and MORALES, 1994; ROBAIRE and HERMO, 1989). Thus, those droplets are theoretically not present in the lumen of the epididymal tail.

In conclusion, the biochemical and histochemical results obtained in this study, together with basis on previous histochemical findings obtained for cytoplasmic droplet pattern in mammalian spermatozoa (MONIEM and GLOVER, 1972), support the hypothesis that in the hamster epididymis ALP is secreted by the epididymal epithelium and accumulates in the luminal microenvironment of the epididymal tail. ALP seems to be an essential enzyme related to the epididymidis metabolism, acting on the maintenance of spermatozoa stored in the luminal compartment of epididymal tail of Golden hamsters, and perhaps also in other mammalian epididymidis.

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