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PROLONGED ETHANOL INGESTION DECREASES ALPHA-MANNOSIDASE ACTIVITY AND INDUCES ITS REDISTRIBUTION TO THE FLUID PHASE IN RAT CAUDA EPIDIDYMIS

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Abstract - The role of glycosidases in mammalian epididymal fluid is still a controvertial subject. There exists a body of evidence in favour of a function in remodeling the sperm surface as one step in gamete maturation, whilst others argue in favor of an extraepididymal role for these enzymes. In this study we measured the activity and distribution of four glycosidases in rat cauda epididymis after prolonged ethanol ingestion, a condition associated with fertility disturbances. We found that -mannosidase is the most sensitive enzyme to the stress caused by alcohol, since its activity in epididymis significantly decreased and partly redistributed from the spermatozoa to the fluid phase. From these results we suggested that alcohol treatment affects the expression of the enzyme and possibly induces a loss of interaction with the affinity sites on the sperm surface. Although other enzymes also underwent changes due to the alcohol treatment, we focussed on the importance of -mannosidase in the fertilizing capability of spermatozoa.

Key words*:* Glycosidases, alpha-mannosidase, epididymis, alcoholism

INTRODUCTION

The epididymis plays a crucial role in sperm maturation, since its epithelium synthesizes and secretes substantial amounts of proteins into the lumen. Amongst these proteins, the synthesis and secretion of glycosidases by the epididymal epithelium have been well documented (12,24,26), and some of them are secreted into the lumen in an androgen-dependent fashion (16,18,22,23). Although the role of such high enzyme activites in an extracellular environment is still unclear, it has been postulated that these enzymes may play a role in sperm maturation, related to the changes in cell-surface carbohydrate composition that the gametes undergo during epididymal transit (13,17). However, it has also been postulated that some lysosomal enzymes might play a role in an extraepididymal environment, since they become bound to the surface of those spermatozoa that transit through the epididymal duct (4,7,27).

Loss of fertility in males is mostly related to oligozoospermia, azoospermia, but also at times to abnormal spermatozoa unable to fertilize the oocyte (11). This latter could be due to impaired epididymal maturation.

It is known that the chronic consumption of large quantities of alcohol induces a decreased number and motiliy of spermatozoa in human semen samples, with increased teratozoospermia (15,20) and more recently it was demonstrated that ethanol ingestion affects the expression of glycosidases in rat epididymis (28). In this study we demonstrated that alcohol ingestion not only affects expression of some glycosidases but also their secretion into the lumen, and affects their distribution between the fluid and spermatozoa in cauda epididymis. We paid more attention to the response of -mannosidase to the alcohol ingestion.

MATERIALS AND METHODS

Reagents

PMSF, leupeptin, and 4-methyl-umbelliferyl substrates were purchased from Sigma Chem. (St. Louis, MO, USA). All reagents for electrophoresis were purchased from Bio-Rad (Hercules, CA).

Abbreviations: **-MAN**: -mannosidase; **-GAL**: -galactosidase; **-NAG**: N-acetyl- -D-glucosaminidase; **-GLU**: -glucuronidase; **PMSF**: phenylmethylsulfonyl fluoride

Treatment schedule

Forty-five day old male Sprague-Dawley rats were housed in a 20-25°C controlled environment at a 12L:12D cycle. They were fed and supplied with water *ad libitum*. Animals were divided into controls and ethanol-treated groups. Control rats received an isocalorific quantity of sucrose provided with the drinking water *ad libitum*. Ethanol-treated rats were administrated with alcohol with the drinking water, at a dose of 3 g/kg body weight according to the protocol of Srikanth *et al*. (29).

Preparation of spermatozoa and epididymal fluid

After 45 days of treatment the rats were sacrificed by decapitation (following the international rules for animal handling of the National Institutes of Health, USA) and the epididymidis, and testis were removed and weighed. The anatomic parts of the epididymis (caput, corpus and cauda) were separated according to White (33).

The epididymal fluid and spermatozoa were obtained from the cauda epididymis as previously described (7). Briefly, the caudal region of each epididymis was retroperfused from the efferent duct with 20 mM phosphate buffer (pH 7.2) containing 150 mM NaCl, 5 mM glycerophosphate, 0.1 mM PMSF, and 1 g/ml leupeptin (buffer P). The spermatozoa were sedimented at 800 g for 10 min, washed twice with the same buffer and the final pellet was resupended in a small volume of the same buffer. The washings did not release significant amounts of enzyme. The supernatants were clarified by centrifugation at 100,000 g for 30 min, corresponding to the epididymal fluid. The remained epididymis (after perfusion) was weighed and homogenized in 1:5 (w/v) of Tris-acetate buffer (pH 7.2) containing 0.1 mM PMSF, 0.1% Triton X-100 and 0.4 M NaCl with a Teflon/glass homogenizer, and centrifuged at 100,000 g for 30 min. The pellet was newly submitted to homogenization in buffer P and centrifuged as before. The supernatants from these centrifugations were recovered and stored at -20°C for up to 3 weeks before use.

Enzymatic activity in spermatozoa

The sperm obtained as mentioned above were washed twice with buffer P, and the remaining spermatozoa were washed once with the same buffer containing 0.3 M NaCl. The enzymatic activities bound to spermatozoa were measured in the supernatants. Under these conditions the spermatozoa remained intact, and this concentration of NaCl did not interfere with enzymatic activity. The residual enzymatic activitiy was less than 1% for each enzyme. All values (number of spermatozoa, protein content and enzymatic activity) were corrected for the perfusion volumes.

Enzymatic activity in the fluid

The epididymal fluid was measured without prior treatment.

Preparation of samples for optical microscopy

Cauda epididymis from either control or treated rats were fixed in Bouin's solution and embedded in parafin; 5 m thick sections were cut, stained with haematoxylin-eosin and observed with a Nikon light microscope.

Measurements

Protein concentration was measured according to Lowry *et al.*(21). -galactosidase, N-acetyl- -D-glucosaminidase, -glucuronidase and -mannosidase were measured spectrofluorometrically with the corresponding 4-methylumbelliferyl substrate according to Barret and Heath (6). For each enzyme, one unit of activity represents the amount of enzyme that catalyzes the release of 1 nmol of 4-methylumbelliferone/hr.

Statistical analysis

Enzymatic activities were subjected to Student's *t*-test and the level of significance was set at $p \leq 0.001$.

RESULTS

In this work we studied the effect of the prolonged ingestion of alcohol on rat epididymal glycosidases, which might explain the observed decrease of fertility in males. We tested this effect in young rats (45 days old rats), and the treatment was prolonged until adulthood (90 days). The level of serum testosterone was significantly decreased by the treatment from means of 2.2 ± (SD) 0.43 in the controls to 1.7 ± (SD) 0.52 g l^{-1} in treated rats (n= 12 and 11, respectively, $p<0.02$). As shown in Table 1, the weight of testis and epididymis did not change significantly with the treatment, although the corpus showed a certain increase. Interestingly, the number of spermatozoa rescued from the cauda epididymis was reduced by up to 50%, indicating that the treatment could also affect spermiogenesis (Table 2).

We also observed that alcohol ingestion affects the activity of some glycosidases in cauda epididymis; the activity of N-acetyl- -D-glucosaminidase (-NAG) and

Table 1 Weight of the whole organs from adult male rats (90 days old) after 45 days of alcohol ingestion

Tissues	Control (n= 12)	Treated (n= 12)
Testes (g)	1.54 ± 0.07	1.61 ± 0.08
Epididymis (mg)	530 ± 80	550 ± 50
Caput (mg)	220 ± 20	210 ± 20
Corpus (mg)	36 ± 6	50 ± 20*
Cauda (mg)	230 ± 30	260 ± 40

Values are expressed as means ± SD from the number of indicated measurements (n). *p<0.001

Table 2 Number of spermatozoa obtained from cauda epididymis from 90 days old rats after prolonged (45 days) alcohol ingestion

Control (n= 11)	43.39 ± 4.86 x 10^6
Treated (n= 12)	22.85 ± 3.73 x 10^6

Values are expressed as the mean of the number of cells/mg tissue ± SD. (p<0.01)

Table 3 Activity of glycosidases in cauda epididymis from adult rats (90 days old) after prolonged (45 days) alcohol ingestion

Enzyme	Control (n= 12)	Treated (n= 12)
-MAN	6081.6 ± 227.3	1150 ± 144.6 *
-GAL	379.2 ± 18.5	374.1 ± 29.5
-GLU	10.1 ± 0.2	7.7 ± 0.6
-NAG	3622.5 ± 344	2506.9 ± 258.5**

Values are expressed as means of total activity/mg tissue ± SD. *, **p< 0.001 (compared to controls)

Table 4 Compartmentalization of glycosidases in cauda epididymis from 90 days old rats after prolonged (45 days) alcohol ingestion

Enzyme	Tissue		Spermatozoa		Fluid	
	Control	Treated	Control	Treated	Control	Treated
-GAL	17.4 ± 3.8	16.5 ± 8.3	6.7 ± 0.4	13.2 ± 9.2	75.9 ± 3.4	70.3 ± 12.6
-NAG	38.1 ± 1.4	40.8 ± 6.9	51.1 ± 6.1	46.3 ± 5.5	10.8 ± 5.2	12.9 ± 1.4
-GLU	59.9 ± 1.1	56.6 ± 9.4	0.4 ± 0.15	0.67 ± 0.23	39.7 ± 1	42.73 ± 9.1
-MAN	8.35 ± 2.4	8.6 ± 3.35	87.8 ± 6.9	78.9 ± 4.8	3.85 ± 0.92	12.5 ± 2.9

Values are expressed as percentages of the total activity ± SD (n= 12 for treated or controls).

-mannosidase (MAN) was significantly reduced, whilst -glucuronidase (-GLU) and b-galactosidase (-GAL) were not affected (Table 3).

Although the distribution of these enzymes between the tissue and those secreted did not change significantly, in the lumen we observed a redistribution of two of these enzymes between the fluid and spermatozoa, as -GAL redistributed to the spermatozoa, and -MAN to the fluid (Table 4). No major changes were observed with -GLU and -NAG. This redistribution may be mostly due to a change in the capability of the spermatozoa to bind those enzymes, as the activity of -MAN bound to the gametes was reduced three times, whilst -GAL was increased about 4 times in the alcohol treated rats with respect to the controls (Table 5). The morphological appearance of the epididymal tissue remained intact after treatment, as shown in Fig. 1. Moreover, no major changes were observed in the testis germinal epithelium (Fig. 2).

DISCUSSION

The compartmentalization of glycosidases in epididymis can be considered as a parameter to test the functionality of the organ. Under conditions where male

Fig. 1 Morphology of control rat cauda epididymis (**A**) or after prolonged treatment with alcohol (**B**). The epithelium (ep), spermatozoa (s) and interstitium (*i*) are indicated.

Fig. 2 Morphological aspects of the control rat testis (**A**) or after prolonged treatment with alcohol (**B**). The germinal epithelium (ep), spermatozoa (s) and interstitium (*i*) are indicated.

Table 5 Activity of glycosidases bound to caudal spermatozoa in 90 days old rats after prolonged alcohol ingestion

Enzyme	Control	Treated
-MAN	1.23 x 10^{-4}	0.31 x 10^{-4}
- GAL	0.57 x 10^{-6}	2.1 x 10^{-6}
-NAG	4.2 x 10^{-5}	4.6 x 10^{-5}
-GLU	1.1 x 10^{-8}	1.25 x 10^{-8}

Values are expressed as units bound per cell

fertility is affected, the activity of certain glycosidases in epididymis changes (7,28). Here, we studied the distribution of four glycosidases in rat cauda epididymis after the prolonged ingestion of ethanol, condition that affects the fertility in male mammals (2,3,15,20,29). Interestingly, the activity of -NAG and -MAN decreased in cauda epididymis after the treatment, whilst -GLU and -GAL activities did not change significantly. Since the synthesis and secretion of -NAG and -MAN are androgen-dependent (1,7,10), we could postulate that the effect of ethanol is due to the decrease of the testosterone levels observed after the treatment. However, synthesis and secretion of other androgen-dependent enzyme, -GAL, was not affected by ethanol. It is thus possible that ethanol acts by a mechanism apart from that mediated by androgens. To date, other studies have shown, in non-reproductive tissues, that alcohol and its metabolite, acetaldehyde, exert their effects directly on the endoplasmic reticulum and Golgi apparatus, impairing the synthesis and release of glycoproteins (14). It is possible that a similar mechanism of action occurs in the epididymal epithelium.

Interestingly, the ethanol ingestion induced a redistribution of secreted -MAN and -GAL between the fluid and the caudal spermatozoa. It is known that, in normal conditions, a percentage of these secreted enzymes become bound to high affinity sites on the surface of caudal spermatozoa (8,27). The epididymal -MAN is a ligand for the cation-independent mannose-6-phosphate receptor (CI-MPR) (8), which is present on the sperm surface (5). Mannose-6-phosphate receptors are required for the selective transport of lysosomal enzymes to lysosomes, and two forms of these receptors have been described for several cell types; the CI-MPR and the cation-dependent receptors (19,32). Their existence on sperm surface is still poorly understood. It has been thought that expression and distribution of the CI-MPR on sperm surface might respond to maturation status of the gametes (9). The fact that the secreted -MAN redistributed from the spermatozoa to the fluid may be due to a decreased number of CI-MPR on sperm surface, changes in the binding activity of those receptors, or to modifications in the carbohydrate residues on the enzyme molecule. This question should be elucidated in the future. We discarded the possibility of sperm disruption, as the gametes rescued from the treated rats remained intact (data not shown). Interestingly, -MAN activity is also decreased in rat epididymis after a prolonged treatment with tamoxifen, another condition that affects male fertility (7). From this concordance, -MAN is a candidate for the selection of the gametes for fertilization. Although it is believed that some lysosomal enzymes in the lumen play a role in the maturation of spermatozoa by modifying their membrane glycoproteins (17), an extraepididymal hydrolytic activity for human -MAN in rats has been suggested (30). In addition, in mice it has been shown that -MAN inhibitors cause a decrease in the number of spermatozoa bound per egg (25). Thus, the spermatozoa may be a vehicle for the enzyme via the mannose-6-phosphate receptors.

In contrast to -MAN, -GAL redistributed from the fluid to spermatozoa. Although this enzyme is recognized by affinity sites different from mannose-6-phosphate receptors (27), the fact that -GAL is predominantly soluble in the fluid supports the possibility of a hydrolytic function in the epididymis. In addition, other authors have presented evidence for a role of this enzyme in the modification of sperm glycoproteins (31). The redistribution of -GAL caused by ethanol ingestion might be explained by an increase in the binding capability of the sperm, or changes in the molecular structure of the enzyme.

This work shows certain inconsistencies with the results obtained by other authors, who found a decrease in all the studied enzymes (28). However, these differences may be explainded by either different methods of fluid collection, or the different rat strain used in our measurements. In our model we foccused on the sensitivity of the reproductive tract to certain stresses before rats reach maturity.

In any case, epididymal -MAN proved to be the enzyme most susceptible to stress conditions that affect fertility in males, and its real function in the epididymis and/or spermatozoa should be the aim of further studies.

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-In the Materials and Methods you should note how many animals have been used for the study (controls / treated)
In the Tables mostly numbers = 12 / 12
in table 2 it is 11 / 12 ?

- National Institutes of Health, USA Argentina ??
After 45 days of treatment the rats were sacrificed ALL animals ?

to J. Dávila for their valuable technical assistance and Dr. A. Penisi
Affiliations ?