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Enkephalin-Degrading Enzymes in Normal and Subfertile Human Semen

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ABSTRACT: Opioid peptides have been reported to have important functions in human reproduction. Indeed, very high concentrations of enkephalins and their degrading enzymes have been reported in human semen. In the present paper, we compare the activity of two enkephalin-degrading enzymes, aminopeptidase N and neutral endopeptidase 24.11, in different fractions of semen from normozoospermic, fertile men and from subfertile patients with different abnormalities revealed by spermiogram analysis (asthenozoospermia, necrozoospermia, and teratozoospermia). High levels of activity of aminopeptidase N were found in the soluble and particulate sperm fractions of semen from patients presenting asthenozoospermia with necrozoospermia. In contrast, lower aminopeptidase N activity was measured in the soluble sperm fraction of asthenozoospermic semen. The percentage of dead spermatozoa was positively correlated with aminopeptidase N activity in both soluble and particulate sperm fractions. In contrast, the percentage of immobile spermatozoa was negatively correlated with aminopeptidase activity in soluble and particulate sperm, and in prostasome fractions. Levels of activity of neutral endopeptidase were found to be unaltered among the different conditions. In summary, the results of the present study indicate that alterations in the activity of aminopeptidase N may be one of the molecular components that contribute to male human subfertility.

Key words: Opioid peptide, peptidase, seminal fraction, sperm motility, sperm death.

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Endogenous opioid peptides participate in the regulation of reproductive functions at multiple sites (Fabbri et al, 1985; Bodnar and Hadjimarkou, 2002). However, it is presently unclear whether and how these peptides exert a direct effect on the function of sperm cells. Thus, contradictory effects of opioids on sperm motility have been reported (Foresta et al, 1985; Fujisawa et al, 1996). Nevertheless, it is quite probable that the opioid system is involved in the control of sperm movement because reduced motility (asthenozoospermia) is frequently found among opiate drugs addicts (Ragni et al, 1988). Enkephalins, which occur naturally, are endogenous opioid pentapeptides, and are also expressed in male germ cells. A possible role for these peptides in spermatogenesis has been suggested (Kew et al, 1990).

The effects of peptides can be regulated by their enzymatic hydrolysis. Due to their short chain, enkephalin pentapeptides are especially susceptible to hydrolysis by peptidases. To date, most information about enkephalin degradation has been obtained from studies of brain tissue. Two principal enzymatic pathways for the degradation of enkephalins have been characterized (Hersh et al, 1982). These are the hydrolysis of the Gly-Phe bond by the particulate enzyme neutral endopeptidase 24.11 (neutral endopeptidase, EC 3.4.24.11), also called enkephalinase (Roques et al, 1993), and the breakdown of the Tyr-Gly bond by aminopeptidases. Among this group are 2 enzymes with high affinity for enkephalins: the puromycin-insensitive alanyl aminopeptidase M or N (aminopeptidase N, EC 3.4.11.2) (Giros et al, 1986), which has been purified from human seminal plasma (Huang et al, 1997), and the puromycin-sensitive alanyl aminopeptidase (EC 3.4.11.14) (Yamamoto et al, 1998). Activity of the latter has not been determined in human semen (Fernández et al, 2002).

In seminal fractions, the activity of neutral endopeptidase and aminopeptidase N is very high, compared to other tissues (10- to 20-fold higher than in the brain). This fact is especially relevant in prostasomes, extracellular organelles present in seminal fluid, in which the activity of both peptidases is higher than in most other tissues (Fernández et al, 2002). A role for certain peptidases in re-

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production has been proposed because mice lacking puromycin-sensitive aminopeptidase are infertile and present impaired spermatogenesis (Osada et al, 2001).

Despite high levels of activity of these enzymes being present in semen, the precise role of these enzymes in semen is not known. Therefore, the aim of the present study was to measure the activity of aminopeptidase N and neutral endopeptidase in human seminal fractions, and to evaluate their possible involvement in male reproductive pathologies. To this end, we examined the level of activity of neutral endopeptidase and aminopeptidase N in seminal plasma from men with normal sperm production and in patients with asthenozoospermia (Az), asthenozoospermia + necrozoospermia (Az + Ne) and asthenozoospermia + teratozoospermia (Az + T).

Methods

Materials

Alanine-β-naphthylamide, β-naphthylamine, dansyl-D-Ala-Gly-p-Nitro-Phe-Gly (DAGNPG), dansyl-D-Ala-Gly, dithiothreitol, thiorphan, captopril, puromycin, dimethylsulfoxide (DMSO), and bovine serum albumin were purchased from Sigma Chemical Company (St Louis, Mo).

Human Semen Collection and Diagnosis

Normozoospermic (Nz) semen was obtained from healthy donors, who were known to be fertile. Samples of semen from subfertile men had been characterized and diagnosed in the Laboratory of Seminology and Clinical Embryology of the Euskalduna Clinic (Bilbao, Spain). The protocol was fully approved by the Clinical Research Ethical Committee of the local Cruces Hospital (Bilbao).

World Health Organization (WHO) criteria were used to define normozoospermia. Motility was graded using the qualitative system also proposed by the WHO: grade A, rapid and linear; grade B, slow or nonlinear; grade C, nonprogressive and grade D, nonmotile.

Semen was considered as Az when less than 50% of the spermatozoa had A + B type motility or less than 25% had A type motility during the first hour after ejaculation. Teratozoospermic semen was diagnosed when more than 85% of spermatozoa from an ejaculate had abnormal shape, and semen was considered Ne when more than 50% of spermatozoa were dead (WHO, 1999). Dead spermatozoa were determined by eosin staining. Following these criteria, samples used in this study were divided into the groups Nz (n = 24), Az (n = 16), Az + T (n = 8), and Az + Ne; (n = 24). The mean seminal characteristics of the groups selected for this study are shown in Figure 1.

Preparation of Seminal Fractions

Samples of semen were diluted (1:1, vol/vol) in Tris-buffered saline (TBS: Tris 30 mM, NaCl 130 mM, pH 7.4) and centrifuged at 600 × *g* for 10 minutes. The resulting supernatant contained seminal fluid and prostasomes, whereas the spermatozoa

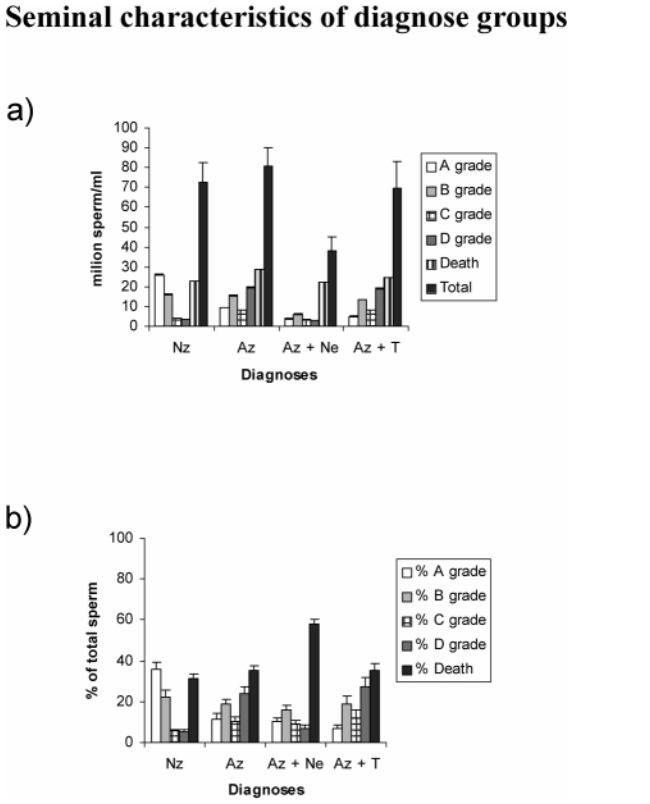

Figure 1. Characteristics of sperm of the groups selected for this study. Values are given as the number of sperm per milliliter **(a)** and as the percentage **(b)** of total sperm. Abbreviations: grade A, rapid and lineal motility; grade B, slow or nonlinear motility; grade C, nonprogressive motility; grade D, nonmotile spermatozoa.

were located in the pellet. To avoid contamination of pellets with components of seminal plasma, these pellets were washed, resuspended fully in 4 mL TBS, and centrifuged again at 600 × *g* for 10 minutes. After discarding the supernatants, pellets were washed once again by resuspension and centrifugation at 1000 × *g* for 15 minutes. The resulting pellets, containing pure spermatozoa, were resuspended in TBS and vigorously homogenized in hypotonic Tris buffer solution (10 mM Tris-HCl) to rupture the cell membranes. Finally, these homogenates were stored at −30°C until use.

In order to obtain soluble and particulate sperm fractions, the purified homogenates were thawed and incubated on ice with hypotonic Tris buffer solution for 1 hour. After a brief sonication (6 bursts of 30 seconds with standby intervals of 15 seconds), samples were centrifuged at 100 000 × *g* for 35 minutes. The resulting supernatants were centrifuged again (100 000 × *g* for 35 minutes), and the soluble sperm fraction was obtained from the new supernatants. In order to obtain the particulate sperm fraction, pellets from centrifugation of the sonicated samples mentioned above were washed and homogenized in hypotonic Tris buffer solution. This homogenate was centrifuged again at 100 000 × *g* for 35 minutes, and the resulting pellet was washed and homogenized with hypotonic TBS.

In order to obtain seminal fluid and prostasome fractions, we employed the method described by Ronquist and Brody (1985).

The supernatants obtained after the first centrifugation (600 × *g*, 10 minutes), containing seminal fluid and prostasomes, were centrifuged at 1000 × *g* for 15 minutes to eliminate cell debris and residual spermatozoa. The resulting pellets were discarded, while the new supernatants were frozen at −30°C until use. In all cases, samples were maintained frozen for no more than 2 weeks before use. Thawed supernatants were centrifuged at 100 000 × *g* for 2 hours. Resulting supernatants were centrifuged again (100 000 × *g*, 2 hours), and the resulting prostasome-free supernatants were used as the seminal fluid fraction. Pellets obtained from the previous centrifugation (100 000 × *g*, 2 hours) were washed and resuspended with TBS, and were used as the prostasome fraction. This fraction contained prostasomes and amorphous substance.

Measurement of Peptidase Activities

Aminopeptidase N activity was fluorometrically measured according to the method described by Fernández et al (2002). The assay is based on the fluorescence of β-naphthylamine generated from the hydrolysis of an appropriate substrate by the enzyme. The components of the assay mixture (in a total volume of 1 mL) included 50 mM sodium phosphate buffer (pH 7.4), 0.1 mg bovine serum albumin, and 0.5 mM Ala-β-naphthylamide. This substrate was chosen because previous studies carried out in our laboratory have demonstrated that the enzyme responsible for the hydrolysis of Ala-β-naphthylamide by samples of human semen is aminopeptidase N (APN) (Fernández et al, 2002). The reaction was initiated by adding 10 μL of sample to the assay mixture. After a 30-minute incubation at 37°C, 1 mL of 0.1 M sodium acetate buffer (pH 4.2) was added to the mixture to terminate the reaction. The released β-naphthylamine was determined by measuring the fluorescence intensity at 412 nm with excitation at 345 nm. Tubes without samples were used to determine background fluorescence. The relative fluorescence was converted into picomoles of product using a standard curve, constructed with increasing concentrations of β-naphthylamine.

Neutral endopeptidase activity was measured according to the method described by Florentin and coworkers (1984) with some modifications. Addition of DMSO to stop the reaction also increased fluorescence, thereby improving the sensitivity of the assay. Briefly, the enzyme cleaved the Gly-Phe(p-Nitro) peptide bond of the DAGNPG substrate, leading to an increase in fluorescence, which is proportional to the reduction of intramolecular quenching of dansyl fluorescence by the nitrophenyl residue. The enzymatic reaction was initiated by addition of 10 μL of sample to the assay mixture (total volume of 1 mL) containing; 50 mM Tris-HCl buffer (pH 7.4), 0.1 mg bovine serum albumin, and 90 μM DAGNPG as substrate. After a 30-minute incubation at 37°C, the reaction was stopped by adding 1 mL DMSO. Tubes without samples were used to determine background fluorescence. The excitation and emission wave lengths were 342 nm and 562 nm, respectively. Relative fluorescence was converted into picomoles of product using a standard curve, prepared with increasing concentrations of product and decreasing concentrations of substrate.

In both cases (aminopeptidase N and neutral endopeptidase), one unit of enzyme activity is considered as the amount of enzyme that hydrolyzes 1 pmol of substrate per minute. Protein concentration was measured in triplicate using the method described by Bradford (1976) using bovine serum albumin as the standard. All activities are expressed as units of peptidase activity per milligram of protein.

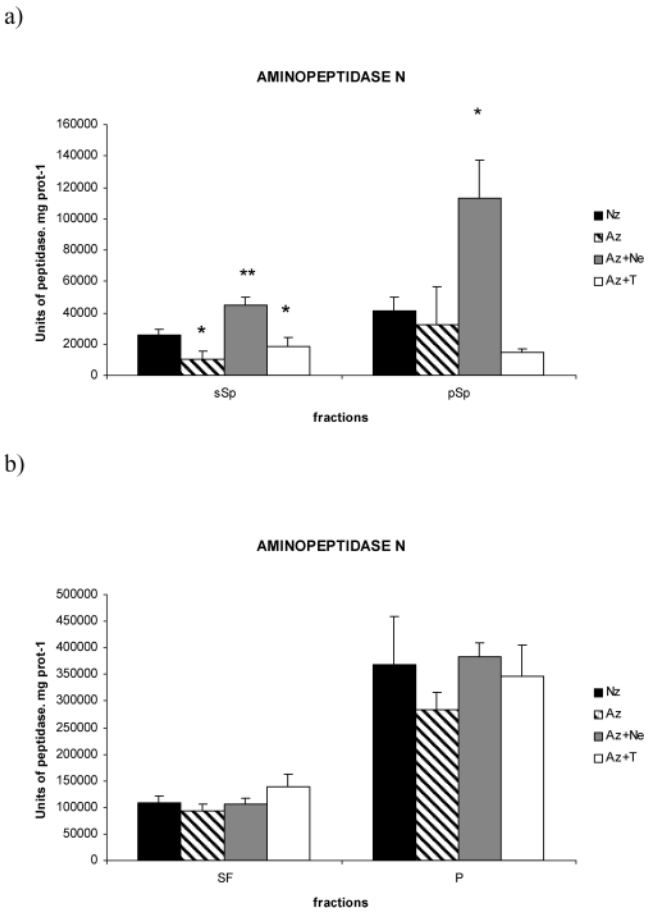

Figure 2. Specific aminopeptidase N activity in soluble (sSp) and particulate (pSp) sperm fractions **(a)** and in the prostasome fraction and seminal fluid **(b)** of men with Nz (n = 24), Az (n = 16), Az + Ne (n = 24), and Az + T (n = 8). Values are expressed as the mean (units of peptidase·mg prot^{-1}) ± SEM. *$P < .05$ compared with normozoospermic semen; **$P < .005$ compared with normozoospermic semen.

Statistics

Results were analyzed using the analysis of variance test, followed by the protected least significant difference test to compare 2 × 2 diagnoses. Bivariate and partial correlations (controlling other factors) between peptidase activities and semen characteristics were evaluated.

Results

Aminopeptidase N activity in different fractions of human semen from fertile and subfertile patients is illustrated in Figure 2. In all the studied samples, the highest level of activity of aminopeptidase N was found in the prostasome fraction (Figure 2b). Moderate activity was found in the

Table 1. *Aminopeptidase N activity and seminal characteristics*

Parameter	Correlation	Soluble Sperm Fraction	Particulate Sperm Fraction	Seminal Fluid	Protasome Fraction
A grade (%)	Pearson *R*	−.016	−.012	.067	.079
	Significance	.903	.926	.572	.520
B grade (%)	Pearson *R*	.028	.010	.075	.089
	Significance	.830	.936	.530	.470
A + B grade (%)	Pearson *R*	.029	−.003	.091	.107
	Significance	.823	.980	.442	.383
C grade (%)	Pearson *R*	−.243	−.183	.118	−.199
	Significance	.064	.157	.323	.106
D grade (%)	Pearson *R*	−.326	−.278	−.197	−.239
	Significance	.011	.029	.096	.049
C + D grade (%)	Pearson *R*	−.417	−.342	−.108	−.321
	Significance	.001	.007	.366	.008
Dead sperm (%)	Pearson *R*	.461	.339	−.061	.165
	Significance	<.001	.007	.609	.178
Sperm number/mL	Pearson *R*	−.390	−.283	.093	−.132
	Significance	.002	.026	.432	.282

seminal fluid (Figure 2b), and the lowest levels of activity were found in particulate and soluble sperm fractions (Figure 2a). The level of activity of aminopeptidase N in semen fractions is at least 50-fold higher than in human blood plasma (data not shown).

Increased levels of activity of aminopeptidase N were found in the soluble ($P < .001$) and particulate ($P < .05$) sperm fractions of Az + Ne semen compared with normal semen. In contrast, reduced levels of aminopeptidase N activity were measured in the soluble sperm fraction ($P < .05$) of Az and Az + T semen compared with normal semen. Lower activity of aminopeptidase N was also measured in the prostasome and particulate sperm fractions of Az semen, but these differences were not statistically significant. In seminal fluid, no statistically significant differences were observed among the studied pathologies.

A significantly positive correlation was found between the percentage of dead sperm and aminopeptidase N activity in the soluble and particulate sperm fractions of all studied samples (Table 1). In contrast, this correlation was not found in the prostasome and seminal fluid fractions.

We found a significantly negative correlation between the percentage of nonmotile (D grade) spermatozoa in semen samples and aminopeptidase N activity in the soluble and particulate sperm fraction, and in the prostasome fraction; the same negative correlation was also detected in pooled C + D grade semen samples. The number of sperm per milliliter was negatively correlated with aminopeptidase N activity in the soluble and particulate sperm fractions.

The activity of neutral endopeptidase in the prostasome fraction of semen from patients with different pathologies is shown in Figure 3. No neutral endopeptidase activity was detected in the other semen fractions. Neutral endopeptidase activity was not found to be significantly different among the different pathologies. Significant correlations between the activity of neutral endopeptidase and seminal characteristic of the sample (Table 2) were not detected.

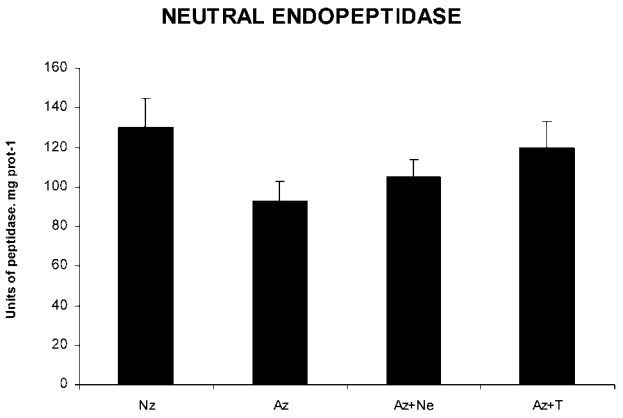

Figure 3. Specific neutral endopeptidase 24.11 activity in the prostasome fraction of Nz (n = 24), Az (n = 16), Az + Ne (n = 24), and Az + T (n = 8) semen. Values are expressed as the mean (units of peptidase·mg prot^{-1}) + SEM.

Discussion

In this work, we have studied the activity of aminopeptidase N and neutral endopeptidase in various fractions of human semen. Aminopeptidase N activity was detected in all the studied semen fractions of fertile and subfertile patients. In contrast, neutral endopeptidase was found to be present only in the prostasome fraction. The distribution of peptidase activities between the different seminal fractions of men with normozoospermia that we measured in the present study is consistent with that reported by

Table 2. *Neutral endopeptidase 24.11 activity and seminal parameters*

Parameter	Correlation	Protasome Fraction
A grade (%)	Pearson *R*	.078
	Significance	.521
B grade (%)	Pearson *R*	−.125
	Significance	.305
A + B grade (%)	Pearson *R*	−.032
	Significance	.795
C grade (%)	Pearson *R*	−.144
	Significance	.239
D grade (%)	Pearson *R*	−.043
	Significance	.724
C + D grade (%)	Pearson *R*	.05
	Significance	.861
Dead sperm (%)	Pearson *R*	−.145
	Significance	.240
Sperm number/mL	Pearson *R*	−.010
	Significance	.935

others (Arienti et al, 1997; Fernández et al, 2002). The high level of activity of these peptidases is also consistent with the high levels of met-enkephalin and other opioid peptides in human semen, which have been reported to have values 6–12 times higher than in plasma (Fraioli et al, 1984).

Increased activity of aminopeptidase N was found in the soluble and particulate sperm fractions of Az + Ne semen in comparison to Nz semen. Moreover, the activity of aminopeptidase N in both soluble and particulate sperm fractions was found to be significantly correlated with the percentage of dead sperm (analysis of bivariate correlations), indicating that necrozoospermia may be associated with high aminopeptidase N activity. The association of high peptidase activity with cell death has been reported in other tissues. Thus, in the brain, the administration of selective inhibitors of peptidases protected cells from death (Shishido et al, 1999), and murine T cells with high levels of activity of prolyl endopeptidase are susceptible to activation-induced cell death (Odaka et al, 2002). The expression of aminopeptidase N is increased in apoptotic neutrophils, and ectoprotease activities are increased preferentially in apoptotic HeLa cells, whereas little change occurs in viable cells (Piva et al, 2000). Nevertheless, it is presently unclear whether high levels of activity of aminopeptidase N associated with necrozoospermia represents a cause or consequence of the pathology.

Increased susceptibility to death of cells with high aminopeptidase activity may be related to their hydrolysis of enkephalins because, in different tissues and cell lines, enkephalins and other opioids increased cell survival, and protected cells from necrosis and apoptosis (Hayashi et al, 2002). Thus, the high levels of activity of aminopeptidase N that we detected in soluble and particulate sperm fractions of Ne semen may be responsible for reduced levels of enkephalins, with the consequence of sperm cells being more susceptible to death. Nevertheless, other studies have assigned a protective role to peptidases. Thus, in lymphocytes and neutrophils, opioids facilitate apoptosis (Singhal et al, 2001; Sulowska et al, 2003), and cytosolic aminopeptidase seems to be essential for maintaining cell viability (Constam et al, 1995). In the same way, leukemic cells with high aminopeptidase N activity are protected from undergoing apoptosis (Mishima et al, 2002), and aminopeptidase inhibitors induce apoptosis in tumor cell lines (Sekine et al, 2001).

Lower aminopeptidase N activity was consistently measured in Az semen in comparison to Nz semen. These differences were statistically significant in the soluble sperm fraction. Accordingly, a significantly negative correlation was detected between aminopeptidase N activity and the percentage of nonmotile sperm in the soluble sperm fraction, and in the prostasome fraction, which is indicative of an association between aminopeptidase N activity and sperm motility. In this regard, it is interesting to note that prostasomes have been reported to enhance the motility of ejaculated spermatozoa (Carlsson et al, 1997). Moreover, it has been reported that aminopeptidase N can be transferred from prostasomes to spermatozoa in order to modify the biological properties of semen (Arienti et al, 1997). The lower activity of aminopeptidase N in semen samples with reduced motility, together with the high activity of this enzyme in prostasome fractions, indicate that aminopeptidase N may be involved in the motility enhancer effects of prostasomes.

Asthenozoospermia is a very common seminal abnormality in heroin addicts (Ragni et al, 1988), indicating that the motility of sperm cells may be modulated by the opioid system, and, in particular, that chronic heroin use can lead to reduced motility. Indeed, mice with altered enkephalin expression have been reported to present altered sperm motility (O'Hara et al, 1994). Our results are consistent with the hypothesis that increased levels of opioid peptides have an antimotility effect, because samples with high levels of nonmotile sperm are associated with low levels of aminopeptidase N, and thus increased levels of enkephalins. Nevertheless, in some variants of infertility, changes in the levels of spermatic peptidases have been reported to be compensatory (Neimark et al, 1998); the decrease of aminopeptidase activity could be a consequence of low enkephalin levels associated with asthenozoospermia (Fujisawa et al, 1996). Indeed, enkephalins have been reported to both reduce (Fraioli et al, 1984; Foresta et al, 1986) and increase sperm motility (Fujisawa et al, 1996).

Because enkephalin peptides exert their activity principally via the activation of extracellular receptors, the significance of the increased activity of these enzymes in

the soluble sperm fraction, which contains mainly the cytosol of sperm cells (Fernández et al, 2002), is not immediately apparent. Nevertheless, the possibility that enkephalins may play an important intracrine role, as has been proposed for opioid peptides in other locations (Ventura et al, 1998), should not be ruled out. In addition, aminopeptidases may act on enkephalins that are stored in acrosomes; these are depleted from sperm following the acrosome reaction (Kew et al, 1990).

In our study, we found a negative correlation between the total number of spermatozoa per milliliter and aminopeptidase N activity in the soluble sperm fraction, indicating that defects in spermatogenesis may be associated with increased levels of aminopeptidase N activity in the sperm cytosol. Opioid peptides have been implicated in paracrine interactions between germ and Sertoli cells, which are important for the maintenance of spermatogenesis (Fabri et al, 1985), and may be relevant in this regard.

Neutral endopeptidase was found only in the prostasome fraction. This specific location indicates that seminal neutral endopeptidase originates in the prostate, because these extracellular organelles are of prostatic origin (Ronquist and Brody, 1985). The absence of alterations in neutral endopeptidase activity levels among the studied pathologies, together with its low and restricted activity, indicate that aminopeptidase N may be more relevant than neutral endopeptidase in determining sperm motility or viability. In addition, the relatively constant levels of activity of neutral endopeptidase among the different pathologies support the specificity of the changes observed in the levels of activity of aminopeptidase N.

In summary, the results of the present study indicate that alterations in the activity of aminopeptidase N may contribute to the subfertility of human semen in terms of sperm motility, viability, and spermatogenesis. More detailed studies of the role of aminopeptidase N activity in the male genital tract may contribute to the development of therapeutic strategies for the treatment of male subfertility.

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