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Occurrence of prostasome-like membrane vesicles in equine seminal plasma

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Equine seminal plasma was shown to contain membrane vesicles that are similar to the well characterized prostasomes in human seminal plasma. Determination of nucleoside and nucleotide concentrations of these particles have shown that ATP, ADP and adenosine are the main components of the nucleotidic pool. 5′ nucleotidase, endopeptidase and dipeptidyl peptidase IV activities have been found on the surface of the particles. The interaction between these prostasome-like vesicles and spermatozoa was demonstrated by electron micrograph scans which revealed the steps of a fusion-like process leading to mixing of the membranes. In addition, endopeptidase activity, a marker enzyme of these seminal vesicles that is normally absent from equine spermatozoa, was shown to be acquired by these cells after interaction with the vesicles. The addition of these vesicles to equine spermatozoa resulted in the modification of adenylate catabolism. Therefore, a role in stabilizing the energy charge of the spermatozoa thus allowing longer viability is proposed for these organelles.

Introduction

Membrane vesicles have been identified in the seminal plasma of several mammals (Davis, 1973; Ronquist *et al.*, 1978a,b; Breitbart and Rubinstein, 1982; Fornes *et al.*, 1991). These vesicles are secreted from different accessory organs of the reproductive system and are named after the producing organ. Prostasomes were identified in human ejaculates (Ronquist *et al.*, 1978a,b), vesiculosomes in bovine seminal plasma (Agrawal and Vanha-Pertulla, 1987), and membrane organelles of epididymal origin in rabbit, ram and rat ejaculates (Davis, 1973; Breitbart and Rubinstein, 1982; Fornes *et al.*, 1991). These extracellular vesicles express different proteins and enzymes on their surfaces and are involved in several physiological roles, ranging from immunosuppressive activity to the enhancement of sperm cell motility (Lord *et al.*, 1977; Stegmayr and Ronquist, 1982; Kelly, 1991). These vesicles also exert opposing effects in the fertilizing process: in rabbits fertilization is inhibited (Davis and Hungund, 1976), but in bulls and humans forward sperm motility is promoted and the acrosome reaction is induced (Agrawal and Vanha-Pertulla, 1987; Ronquist *et al.*, 1990).

The present paper deals with the occurrence, isolation and characterization of these extracellular vesicles in equine seminal plasma. The occurrence of a fusion-like process between the vesicles and sperm cells might help to clarify their physiological role in the fertilizing capacity of equine spermatozoa.

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Materials and Methods

Nucleosides, nucleotides and enzyme substrates were from Sigma Co., St Louis, MO. Sephadex G-200 was from Pharmacia Biotech, Uppsala, Sweden. HPLC columns (Sulpelcosil LC 18T) were from Supelco Inc., Bellefonte, PA. Bio Rad protein assay was from Bio-Rad Lab, GmbH. All other reagents were of the highest available quality.

Semen samples and sperm cell preparations

Equine semen was obtained using an artificial vagina from stallions (trotters of proven fertility, 4–8 years old) stabled at Allevamento Spineta Trotto SaS, Sarteano, Italy. Ejaculate samples were pooled and centrifuged (800 *g* for 10 min at 22°C) to harvest spermatozoa and the supernatant (S1) was used to prepare membrane vesicles. The pellet was suspended in 10 mmol 4-morpholinepropanesulphonic acid (MOPS) l^{-1}, pH 7.4, containing 120 mmol NaCl l^{-1}, 5 mmol KCl l^{-1} and 0.5 mmol dithiothreitol l^{-1}, and was stored at 22°C for further analyses. The number of spermatozoa was determined and their intactness was assessed by eosin vital dye and by measurements of lactate dehydrogenase (LDH) (EC 3.1.1.27) activity (Keiding *et al.*, 1974; Minelli *et al*, 1997a,b).

Preparation of membrane vesicles

The supernatant (S1), diluted (1:1 v/v) with 30 mmol Tris l^{-1} and 130 mmol NaCl l^{-1}, pH 7.6 (buffer A), was centrifuged at 1000 *g* for 20 min at 4°C to eliminate cell debris and

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residual spermatozoa. The new supernatant was then centrifuged at 105 000 *g* for 120 min at the same temperature. The pellet containing vesicles and amorphous material was suspended in buffer A up to 1.0–1.5 mg protein ml^{-1}. Membrane vesicles were purified from amorphous material by chromatography on a Sephadex G-200 column (1.5 cm × 30 cm) pre-equilibrated with buffer A. Membrane organelles that were not retained by the column were collected with the void volume. Fractions were examined for absorbance at 280 nm and endopeptidase activity as the marker enzyme for prostasomes (Ronquist *et al.*, 1988) and then pooled and centrifuged at 105 000 *g* for 120 min. The pellet was suspended in buffer A and stored at –196°C until use. Vesicle concentration was determined by Bio-Rad protein assay kit (Bradford, 1976) using BSA as standard protein.

Enzyme activities

Endopeptidase (EC 3.4.2.1) hydrolysing $Succ(Ala)_3$-pNA was assayed according to Laurell *et al.* (1982). Dipeptidyl peptidase IV (EC 3.4.14.5) was assayed according to Nagatsu *et al.* (1976). Adenosine deaminase (EC 3.1.4.4) and 5′ nucleotidase (EC 3.1.3.5) were assayed according to Franco *et al.* (1986). Protein concentration was determined by Bio-Rad protein assay kit (Bradford 1976) using BSA as standard protein. The cellular volume of spermatozoa was assumed to be 25 μm^3 by electron and confocal microscopy.

Nucleoside and nucleotide determinations

Washed sperm suspensions were incubated in 188 mmol Hepes buffer l^{-1} containing 0.3 mol glucose l^{-1}, 8.33 mmol lactose l^{-1} and 50 U penicillin ml^{-1}, pH 7.5 (buffer B), at 37°C with shaking at 60 oscillations per min. At fixed intervals, aliquots containing 60–80 × 10^6 spermatozoa were withdrawn and extracted with 0.55 mol perchloric acid l^{-1}. After 10 min on ice, the samples were centrifuged at 11 000 *g* for 5 min and the supernatants were neutralized with equimolar addition of K_2CO_3.

Prostasome-like membrane vesicles were treated as described above. Nucleoside and nucleotide concentrations were determined by HPLC according to Stocchi *et al.* (1987) and Minelli *et al.* (1995).

Sperm motility analysis

Analyses of motility were performed using a computer assisted semen analyser (CASA, SCA 3.0, Microptic, Spain). The machine was equipped with a negative phase contrast optic system (Olympus CH2) and a Sony camera (Hyper HAD CCD/BN) connected to a Pentium 166 MHz. Images were analysed by sperm recognition algorithms considering sperm area and movements of each component of the sample. Equine sperm motility was assessed in 200 samples by determining total numbers of motile and static cells under appropriate experimental conditions. The method was validated with a linear model (SAS/STAT (1990) GLM, REG,CORR procedure): $n = 200$ real motile cells versus estimated, $r = 0.92$, $P < 0.001$.

Electron microscopy

Prostasome-like organelles and spermatozoa were fixed according to Ronquist *et al.* (1990). Ultrastructural examinations were carried out by Centro di Microscopia Elettronica, Università di Perugia, Italy.

Statistical analysis

The significance of variability between the mean value of the results obtained in the presence and absence of prostasome-like vesicles was determined by using Student's two-tailed (paired) *t* test. Values were considered significant for $P < 0.05$ and were expressed as mean ± SEM.

Results

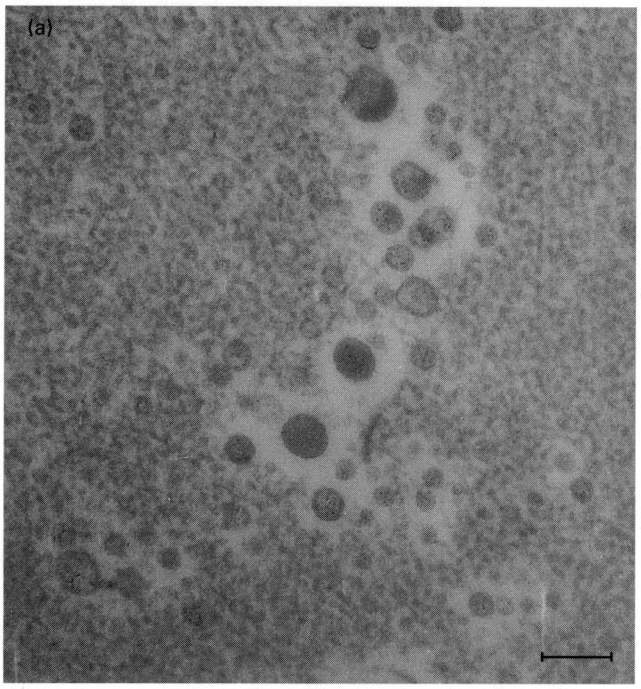
Electron microscope observations

The pellet mainly consisted of vesicles that were roughly round in shape with a diameter ranging from 75 to 175 nm (estimated average 100 nm) as observed from sections at different levels. The particles were surrounded by a bilaminar unit membrane and contained an amorphous matrix (Fig. 1). These particles showed membrane-bound 5′ nucleosidase (7.3 nmol mg^{-1} min^{-1}), endopeptidase (3.38 nmol mg^{-1} min^{-1}) and dipeptidyl peptidase IV (6.8 nmol mg^{-1} min^{-1}) activities.

Nucleoside and nucleotide determinations

The nucleoside and nucleotide concentrations of equine seminal prostasome-like vesicles were determined (Fig. 2). Only adenosine and its tri- and diphosphate derivatives were detected in the vesicles. AMP as well as other non-adenylic nucleotides were absent.

Nucleoside and nucleotide concentrations of equine spermatozoa kept in buffer B at 37°C for 120 min in the presence and absence of seminal plasma vesicles were also determined (Fig. 3). Total degradation of ATP accompanied by the appearance of its degradative products, that is, ADP, AMP and adenosine, was observed in equine spermatozoa kept in glucose–lactose medium without the seminal plasma vesicles. In the presence of the vesicles, the intracellular ATP concentration remained high and AMP did not accumulate. Consequently, the energy charge of the cell was stabilized at vital physiological values by these prostasome-like vesicles, which is in contrast to the situation observed with spermatozoa alone. After 60 min of incubation in buffer B, the energy charge values, ranging between 0.5 and 0.4, were indicative of the deficient vitality of the cells. In these conditions, spermatozoa were almost totally immotile, whereas the same cells kept in the presence of the vesicles



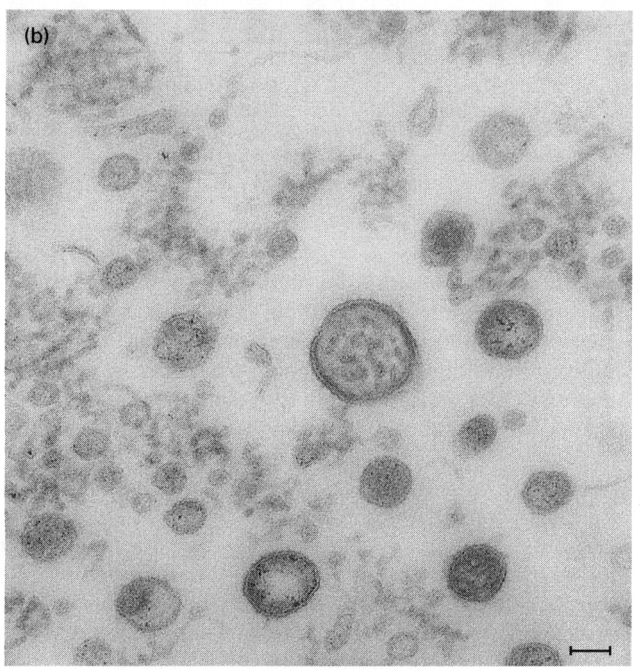


Fig. 1. Transmission electron micrograph of equine seminal plasma membrane vesicles (a) before and (b) after gel filtration on Sephadex G 200. Scale bars represent 100 nm.

were as motile after 60 min as at the beginning of the experiment (sperm motility analysis by the CASA system).

Interaction of equine spermatozoa with seminal prostasome-like vesicles

The suspension of washed spermatozoa was mixed with purified vesicles and incubated at 37°C for 120 min. These

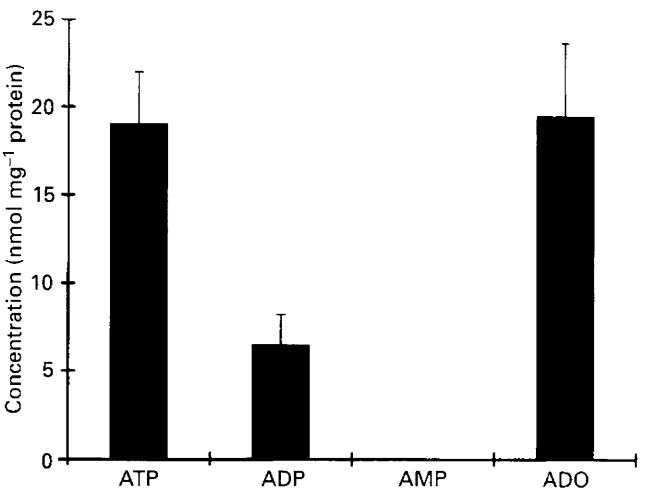


Fig. 2. Concentration of adenylic nucleotides and nucleosides of equine prostasome-like seminal plasma vesicles as determined by HPLC. Values are the means ± SEM, $n = 20$. ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ADO, adenosine.

preparations were observed under the electron microscope. At time 0 (the time of addition of the vesicles), the washed spermatozoa were organelle-free (Fig. 4a), indicating that the fusion-like process is time dependent. After 15 min incubation, the start of the fusion-like process was observed as the formation of a bridge between the membranes (Fig. 4b) and after 30 min complete mixing of the membranes was apparent (Fig. 4c).

Endopeptidase assays were carried out with washed spermatozoa at several time intervals (Fig. 5). These results show that this enzyme, which is used as a prostasome marker (Ronquist and Brody, 1985), is not normally present on surface of spermatozoa. After the addition of the prostasome-like vesicles, equine spermatozoa displayed endopeptidase activity, which increased up to 60 min, supporting the suggestion of a stable interaction between the organelles and the cells of 30–35%.

The percentage of prostasome-like vesicles undergoing a fusion-like process with spermatozoa, that is, the percentage of enzyme activity transferred to the sperm cells, as calculated from the endopeptidase assays, was 30–35%. This finding was supported by electron microscopic observations.

Discussion

It has been known for a long time that the seminal plasma of several mammals contains membrane particles. Therefore, the occurrence of membrane vesicles in equine seminal plasma was not unexpected.

In man, these particulate elements are derived from the prostate by exocytosis and diacytosis and are named prostasomes (Brody *et al.*, 1983). In bovine seminal plasma, the organelles originate from seminal vesicles and are appropriately referred to as vesiculosomes. The seminal vesicles in different species differ not only in their site of

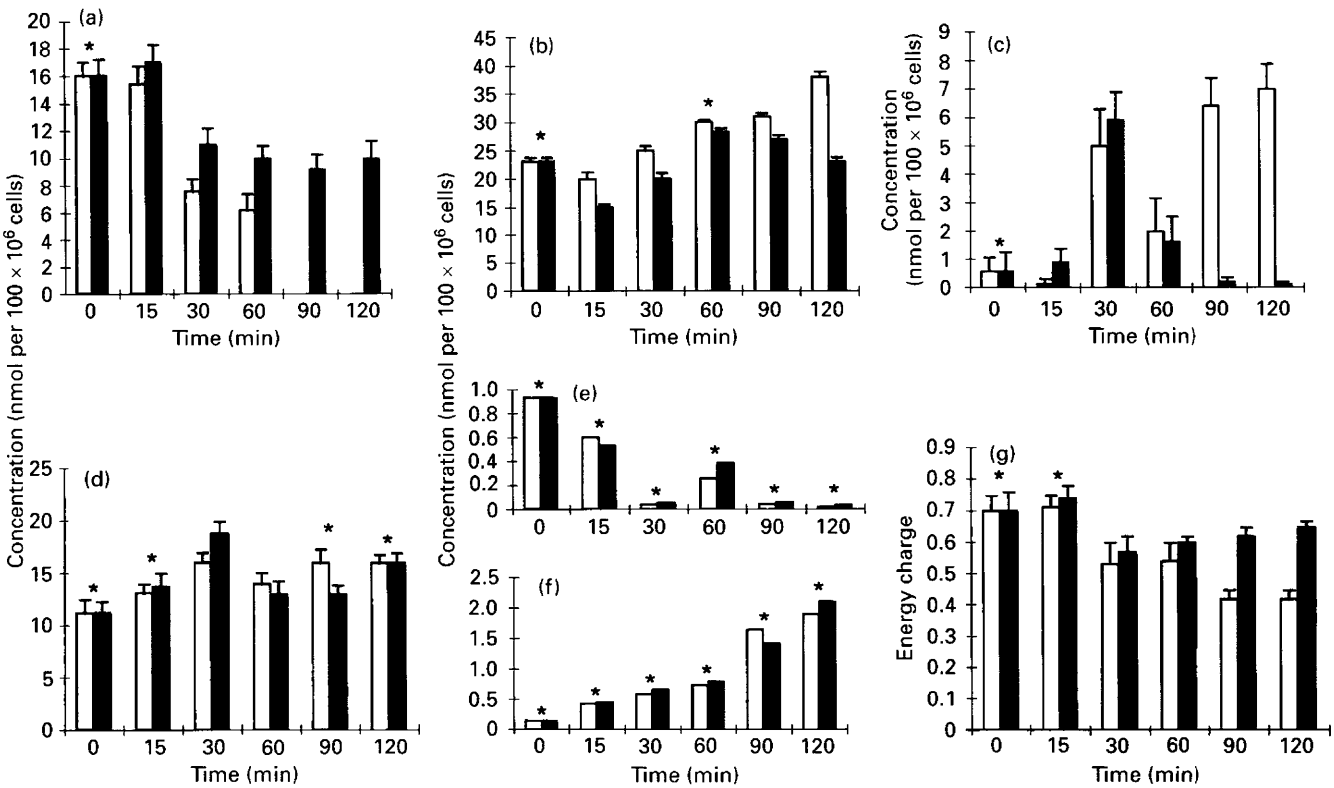


Fig. 3. Energy charge and nucleoside and nucleotide concentration of equine spermatozoa in the absence (□) and in the presence (■) of prostasome-like seminal plasma vesicles as determined by HPLC. Values are the means ± SEM, $n = 15$. Asterisks denote values are not significantly different at $P > 0.05$. (a) adenosine triphosphate, (b) adenosine diphosphate, (c) adenosine monophosphate, (d) adenosine, (e) inosine monophosphate, (f) inosine and (g) energy charge.

origin but also in their biochemical composition. Bovine vesiculosomes contain aminopeptidase A (Agrawal and Vanha-Pertulla, 1985) and alanyl-aminopeptidase (Agrawal and Vanha-Pertulla, 1986a,b). In addition, the particles show Mg–Ca ATPase, γ-glutamyl transpeptidase and dipeptidyl peptidase IV activities, which are all well known membrane-bound enzymes in cells and tissues (Agrawal and Vanha-Pertulla, 1987). However, the endopeptidase active on Succ(Ala)$_3$-pNA is not present in bovine vesiculosomes, whereas it characterizes human prostasomes (Laurell *et al.*, 1982; Krassnig *et al.*, 1985).

The vesicles identified in equine seminal plasma in this study present nucleotidic components (Ronquist and Frithz, 1986) and enzyme activities similar to those found in human prostasomes (Laurell *et al.*, 1982; Fabiani and Ronquist, 1993; De Mester *et al.*, 1996). Moreover, there is a strong anatomical resemblance between human and equine genito-urinary tracts. Thus, there is a strong possibility that these equine particles may be of prostatic origin. However, further investigations are required to confirm their anatomic derivation.

Rabbit seminal vesicles have been shown to block fertilization (Davis and Hungund, 1976). In contrast, prostasomes promote forward motility (Ronquist and Brody, 1985) and vesiculosomes stimulate motility and the normal acrosome reaction (Agrawal and Vanha-Pertulla, 1987). Motility-promoting activity by prostasomes requires membrane integrity. Therefore, this effect is not due to the protein composition of the vesicles (Stegmayr and Ronquist, 1982).

It has been hypothesized that prostasomes may assist the fertilizing potential of spermatozoa by adhering to them (Ronquist *et al.*, 1990) and modifying their microenvironment. In the present study, before the computerized analysis of the effects of these prostasome-like vesicles on the motility of equine spermatozoa was undertaken, the occurrence of this interaction was investigated by electron microscopy and biochemical studies. Under the electron microscope the interaction between the sperm cells and the vesicles appeared to be a fusion-like process. This event starts with the formation of a clearly visible bridge between the membranes and then gradually proceeds until the vesicle is completely embedded in the sperm cell membrane. A similar process occurs between human spermatozoa and prostasomes, although fusion was not demonstrated (Ronquist *et al.*, 1990). Arienti *et*

Fig. 4. Scanning electron micrographs of equine spermatozoa incubated in the presence of prostasome-like seminal vesicles for (a) 0 (b) 15 and (c) 30 min. Arrows indicate (b) the bridge between the sperm and vesicle membranes, and (c) the vesicle completely embedded in the sperm membrane. Scale bars represent 1 μm.

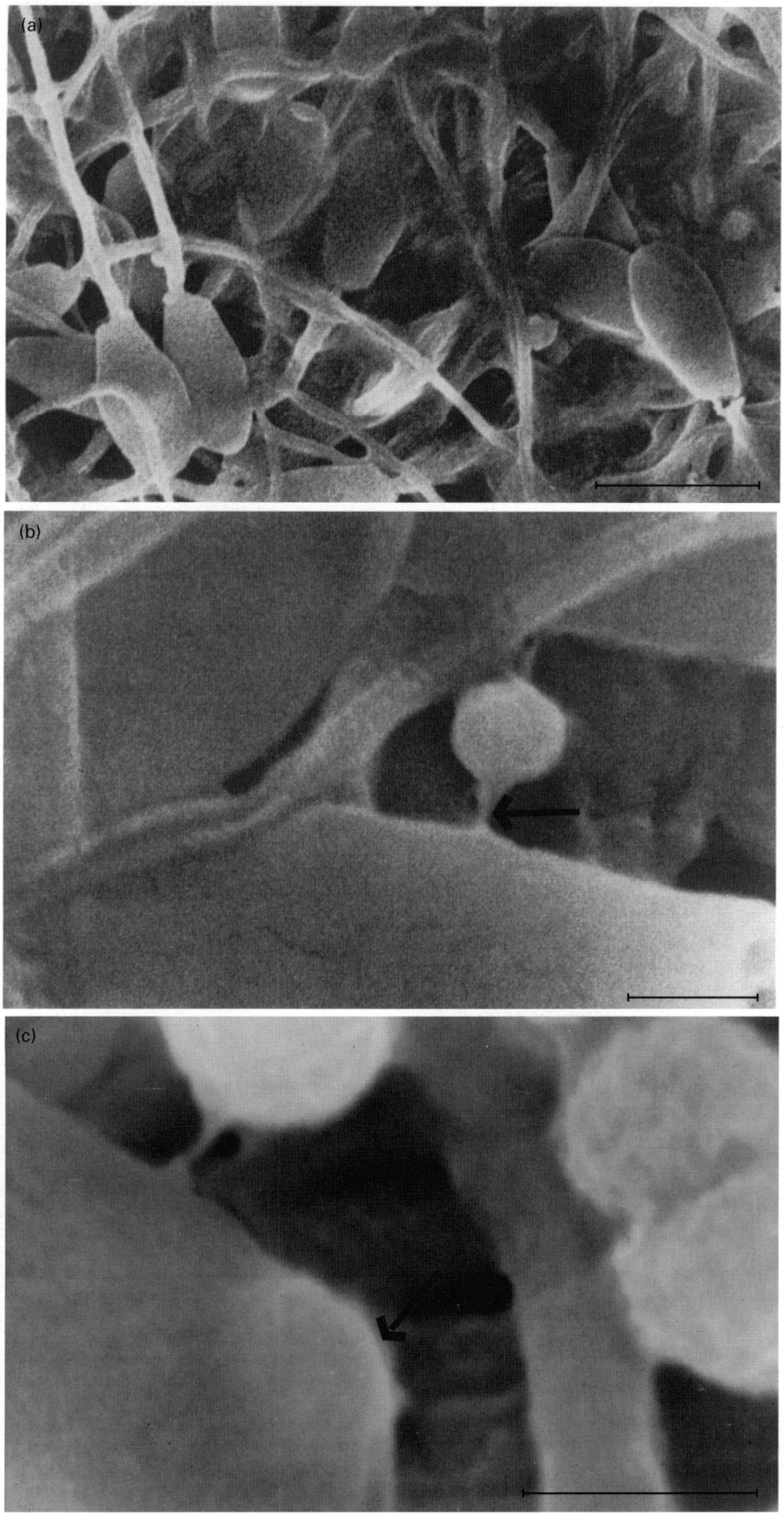


Fig. 4.

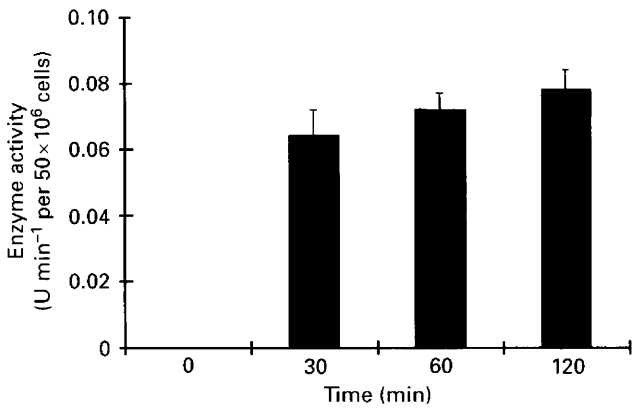


Fig. 5. Endopeptidase activity on equine spermatozoa (200×10^6) incubated in the presence of seminal plasma vesicles. At fixed intervals, aliquots of 50×10^6 cells were withdrawn and assayed for enzyme activity at 410 nm.

al. (1997) reported fusion between human prostasomes and spermatozoa in acidic conditions (pH 4–5). This pH-dependent fusion required at least one protein on the sperm surface, but proteins on the prostasomal surface also appeared to be important. Several methods, namely the release of octadecyl-rhodamine G fluorescence self-quenching, fluorescence microscopy and flow cytometry, were used to investigate the fusion process in the study of Arienti *et al.* (1997). However, the ultrastructural results from the present study showed the interaction between equine spermatozoa and prostasome-like vesicles occurred at pH 7.5, which is in contrast to human spermatozoa and prostasomes.

The presence of endopeptidase activity on the membrane of washed spermatozoa that had previously been incubated with the prostasome-like vesicles supports the proposed interaction between the two membranes. This enzyme activity, which is a marker of the equine seminal vesicles, is not normally present on the sperm cells. The equine spermatozoa acquire the enzyme by the fusion-like process with the added vesicles. Furthermore, the variation in the nucleoside and nucleotide concentration of the sperm cells upon the addition of the membrane vesicle preparation indicates that the nucleotidic and nucleosidic content of the vesicles is incorporated into the cells, thus stabilizing their energy charge. This fusion-like process may also result in the acquisition of new properties by the sperm cells. Therefore, the fusion process may take part in the activation and viability of the spermatozoa, especially as they enter the female genital tract.

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