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Prostasome-Like Particles in Stallion Semen[1]

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ABSTRACT

Human semen contains membranous vesicles called prostasomes. They are secreted by the prostate gland and contain large amounts of cholesterol, sphingomyelin, and Ca^{2+}. Prostasomes enhance the motility of ejaculated spermatozoa and are involved in a number of additional biological functions.

No prostasome-like vesicles have been described in horse semen up to now. We have demonstrated the presence of prostasome-like vesicles in the equine semen and characterized them as to size, morphology, and lipid composition; we have found that they are similar to human prostasomes in many respects. We propose that these vesicles might be important for the fecundity of horse semen. This is of interest since the success of artificial insemination is limited by the fact that stallion sperm barely survive cryopreservation.

INTRODUCTION

The use of cryopreserved stallion semen for reproductive purposes is difficult [1, 2]. In addition, there are large differences among samples as to fertilizing capacity and ability to withstand cryopreservation procedures; subfertility is also common in stallions [3]. Season and other environmental parameters, such as the age and the frequency of ejaculation, may change the properties of equine semen [4–7]. Research on cryopreservation has been quite intense, which at the least indicates the difficulty of this field and its potential interest.

Spermatozoa are considered the most important components of semen; however, human seminal fluid contains elements other than sperm. These include an amorphous protein-containing fraction and a population of membranous vesicles called prostasomes [8]. Prostasomes show a number of interesting properties. They possess very high amounts of cholesterol with a very high cholesterol/lipid phosphorus molar ratio [9, 10]. In addition, sphingomyelin is abundant, whereas phosphatidylcholine is not. Therefore, these vesicles have a very unusual lipid composition compared to that of most biological membranes. Moreover, many prostasomal proteins are endowed with catalytic activity [11]. We would cite, among the physiological roles of prostasomes, the enhancement of sperm motility [12–14], the liquefaction of semen [15], and immunosuppression [16–19]. The interest in prostasome-like vesicles in horse semen originates from the facts just cited. Although the proteins of equine seminal plasma have received some attention [20, 21], no work has yet been done on the possible occurrence of prostasome-like particles. Therefore, this study was aimed at ascertaining the presence of vesicles in horse semen. Various experimental approaches permitted us to detect prostasome-like particles and to compare them to human prostasomes.

MATERIALS AND METHODS

Materials

Hepes was produced by Boehringer-Biochemie (Mannheim, Germany). Sephadex G-50 and Sephadex G-200 were obtained from Pharmacia Fine Chemicals A B (Uppsala, Sweden). Other reagents, all of reagent grade or better, were obtained from Carlo Erba (Milan, Italy) unless stated otherwise.

Biological Samples

Fresh stallion semen was collected by using an artificial vagina (AV.1; Colorado State University, Fort Collins, CO), as previously described [22]. Briefly, the horse's penis was washed with a solution of quaternary ammonium salts and rinsed with water prewarmed at 40°C. The stallions were made to ejaculate on a stuffed dummy kept close to a mare in estrus. Immediately after ejaculation, the seminal fluid was separated from jelly that was collected in a filter placed inside the collection vessel. No extenders were used for this procedure. Samples were centrifuged (800 × *g*, 10 min) to eliminate spermatozoa. The supernatant was diluted (1:1 by vol) with 30 mM Tris + 130 mM NaCl buffer (adjusted to pH 7.6 with HCl) and was centrifuged at 1000 × *g* for 20 min to eliminate cell debris and residual spermatozoa. The pellet was discarded and the supernatant used to prepare prostasome-like vesicles.

Preparation of Prostasome-Like Vesicles

The supernatants obtained as described above were centrifuged at 105 000 × *g* for 120 min. The new supernatant was discarded, and the pellet containing prostasome-like particles and amorphous material [8] was suspended in 30 mM Tris + 130 mM NaCl buffer (adjusted to pH 7.6 with HCl) so that it contained about 1–1.5 mg protein/ml. Prostasomes were purified from amorphous material by chromatography on a Sephadex G-200 column (1.5 × 30 cm) preequilibrated with 30 mM Tris + 130 mM NaCl buffer (adjusted to pH 7.6 with HCl) [12]. Prostasomes were not retained by the column and were collected with the void volume. They were finally harvested by centrifugation at 105 000 × *g* for 120 min and suspended in the same buffer.

Measurement of Size

The homogeneity of the prostasome-like vesicle preparations was checked by quasi-elastic light scattering

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(QELS). Light-scattering measurements were made using about 0.6 ml of the vesicle-containing suspensions. Samples were placed in 6-mm-diameter Pyrex (Corning Glass Works, Corning, NY) glass culture tubes, protected from dust by Parafilm (American Can Company, Greenwich, CT) caps. Tubes were then centrifuged at 1000 × *g* for 5 min immediately before use, to sediment any particles of dirt/impurity. The cylindrical glass sample tube was firmly placed at the center of a toluene-filled cuvette to provide refractive index matching against stray light reflections. The cuvette was housed in a black-anodized aluminum cell whose temperature was kept at 37°C by a custom-made thermoelectric element. The light source was a Coherent Innova 70–3 argon-ion laser (Coherent Laser Products, Palo Alto, CA) operating at 488 nm. Light scattered at 90° was collected from approximately one coherence area and imaged onto the slit of a photomultiplier tube. A 64-channel Nicomp Model 370 computing autocorrelator (Particle Sizing Systems, Santa Barbara, CA) was used to calculate and display the diffusion coefficient (D) and associated derived parameters from cumulants analysis [23] and nonlinear least-squares fits (method of inverse Laplace transform [24]) to the intensity autocorrelation function. All measurements showed excellent goodness-of-fit values and were not disturbed by scattering due to large dust and/or dirt particles.

The high ionic strength of the samples allowed us to reasonably rule out any electrostatic repulsion effects on the measured diffusion coefficients; therefore, hydrodynamic radii (Rh) could be estimated by applying the Stokes-Einstein relation (for stick-boundary conditions [25]): $D = kT/6\pi\eta R_h$, where η is the buffer solution viscosity that can be approximated to that of water (approximately 0.89 centipoise at 25°C [26]).

Lipid Analysis

The extraction of lipid from membranes was performed as described by Folch et al. [27]. In some instances, chloroform extracts were used to determine the distribution of phosphorus among lipid classes. The chloroform phase was dried under a gentle stream of nitrogen and dissolved in known amounts of chloroform:methanol (2:1, v:v). Phospholipids were separated by two dimensional thin-layer chromatography (6.5 × 6.5 cm, PE SIL G 250 m; Whatman Ltd., Maidstone, UK) with a) chloroform:methanol:1.6 M ammonia (70:30:5, v:v) and b) chloroform:acetone:acetic acid:methanol:water (75:30:15:15:7.5, v:v). Spots were visualized by exposure to I_2 vapors and identified with pure reference standards. After the sublimation of I_2, spots were scraped off the plate and their phosphorus content was determined as described by Bartlett [28].

Electron Microscopy

Isolated prostasome-like vesicles were examined by electron microscope as described previously [29]. Briefly, they were fixed for 6 h in 2% glutaraldehyde in 0.1 M PBS buffer, pH 7.4. They were postfixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in ethanol, and embedded in the epoxy resin Epon-Araldite (Fluka, Buchs, Switzerland). Ultrathin sections were contrasted with uranyl acetate and lead citrate. Ultrastructural examination was done in a Philips TEM 400 (Philips Eindhoven, The Netherlands) electron microscope.

Analyses

Protein was determined as described by Lowry [30]; phospholipid phosphorus was assayed after digestion with 70% (w:w) perchloric acid [28], and cholesterol was assessed as described previously [31].

RESULTS

QELS

To obtain prostasome-like vesicles from horse semen, we used the method described for human seminal fluid [12]. The fraction precipitating at 105 000 × *g* contained vesicular material that was then purified on Sephadex columns and again centrifuged at 105 000 × *g* as described above.

This material was studied by using the QELS technique, which permitted us to demonstrate the presence of particles by measuring the light scattering of the preparations. Equine semen contained a population of vesicles having an average diameter of 160–180 nm. The statistical distribution of size was normal, and therefore we argue that only one vesicle population was present in stallion semen. The coefficient of variation in each sample was about 40%.

Electron Microscopy

The microscopic appearance of the fraction enriched in vesicular material, prepared as described in *Materials and Methods*, is reported in Figure 1. Two magnifications are shown. Electron-dense vesicles could be distinguished at low magnification (Fig. 1a). However, no images referring to material derived from spermatozoa could be observed. This indicated that the contamination of our preparation with residual sperm cells was minimal or absent. The fragments may derive from vesicles breaking during the preparation procedures.

The pictures taken at higher magnification (Fig. 1b) showed that the particles were surrounded by lipid bilayer figures enclosing a scarcely organized, electron-dense material.

Lipid Analysis

The vesiculated material described above was similar to human prostasomes in many respects. However, the recovery from semen was much lower for the horse; i.e., the prostasome protein:sperm protein ratio, which is about 2 in humans [10], was 0.7 (± 0.2, 10 determinations) in the horse.

The lipid composition of horse semen prostasome-like vesicles is reported in Table 1. The lipid:protein ratio in horse vesicles was about 570 nmol lipid/mg protein, i.e., about half the value found in human semen prostasomes [10]. Therefore, horse vesicles contain more protein than the corresponding human vesicles. The comparatively low lipid:protein ratio is attributable to both cholesterol and phospholipid. For this reason the cholesterol:phospholipid phosphorus molar ratio is very high (1.7), although it does not attain the extraordinary value of 2 found in human prostasomes [10].

Phosphatidylcholine is the main lipid in most membranes. A characteristic of human prostasomes is the replacement of phosphatidylcholine with sphingomyelin. This was true also for horse semen vesicles, although to a slightly lesser extent.

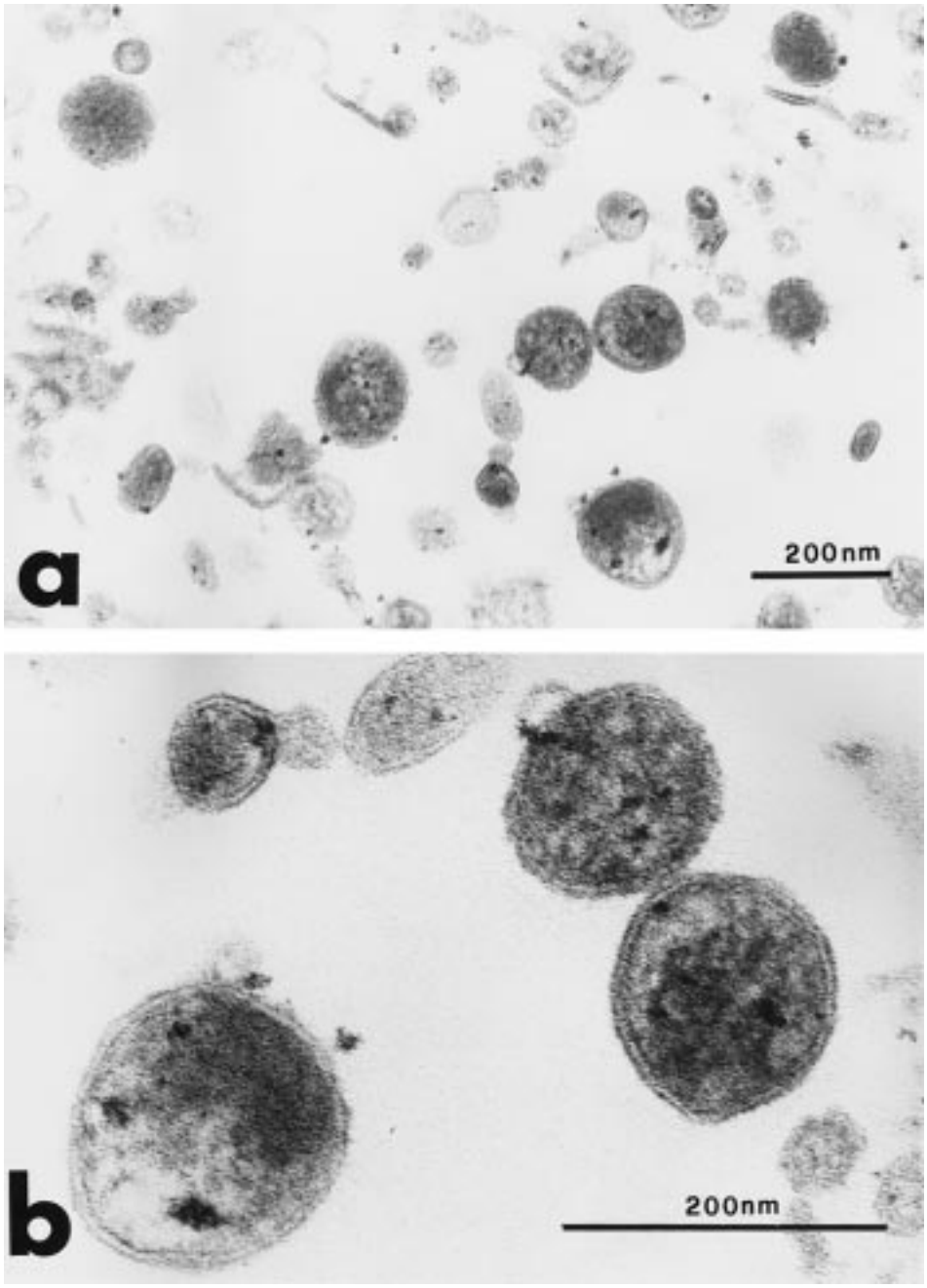

FIG. 1. Transmission electron micrographs of horse semen prostasome-like vesicles showing round, membrane-surrounded organelles, containing an unorganized matrix.

TABLE 1. Lipid composition of stallion semen components.

Component	Horse seminal vesicles	Human prostasomes*
Cholesterol	357 ± 34 (10)†	800 ± 100†
Lipid P	210 ± 47 (10)†	400 ± 100†
Lipid:protein ratio	567†	1200†
Cholesterol:phospholipid molar ratio	1.7	2.0
Phosphatidylethanolamine	32 ± 5%‡	27 ± 2%‡
Phosphatidylcholine	13 ± 1%‡	12 ± 3%‡
Phosphatidylserine + phosphatidylinositol	17 ± 2%‡	20 ± 3%‡
Sphingomyelin	38 ± 7%‡	53 ± 12%‡

* Data from [10].
† Data expressed as nmol/mg protein; number of determinations in brackets.
‡ Data expressed as percentage of total lipid phosphorus.

DISCUSSION

It has been reported that human prostasomes are vesicles with a very high lipid:protein molar ratio [9, 11, 12]. The morphology of these vesicles is similar in human males [29] and horses (Fig. 1). The vesicles found in stallion semen appear as round bodies surrounded by bilayer structures. Inside the vesicles there is an electron-dense and scarcely organized material. Some vesicles appear paler, and fragments of electron-dense material can be distinguished. We interpret this datum as due to the fragmentation of the vesicles during the procedures necessary for their preparation. The size of the vesicles as measured by electron microscopy was in good agreement with the findings obtained by the QELS technique. Therefore we conclude that stallion semen contains vesicles morphologically similar to prostasomes found in human semen.

However, the amount of prostasome-like vesicles in horse semen, with reference to spermatozoa, is lower in the stallion. The sperm:prostasome protein ratio indicates that the prostasome-like vesicles are about three times less abundant in the horse.

The lipid composition of human prostasomes is peculiar [9, 10, 32]. Indeed, the molar ratio of cholesterol:lipid phosphorus is very high (about 2); and sphingomyelin replaces phosphatidylcholine, which is the major lipid in most membranes.

Table 1 shows that the most peculiar difference with respect to the lipid composition of human prostasomes and horse semen vesicles is the lipid:protein ratio. Horse vesicles contain much less lipid than human prostasomes [10] on a protein basis. Differences can be appreciated also with respect to the lipid composition, but these are comparatively minor.

Therefore the vesicles found in stallion semen share many properties with human prostasomes, but not all. These differences may be connected to the possible functional roles of these particles in the reproductive physiology of the human male and the horse.

As far as we know, this is the first report describing prostasome-like vesicles in stallion semen. A question immediately arises as to the physiological role of these particles and particularly their connection to the fertility of equine semen. The data obtained from human material cannot be easily extrapolated to horses, since the physiology of reproduction is different in the two species, especially with respect to the vaginal milieu and the modalities of semen deposition [33]. The concentration of prostasomes in stallion semen (as compared to human) is low. This fact might, in the authors' opinion, be relevant to equine fertility. These particles, made of lipid and protein, may act as a seminal fluid extender [34], and the description of prostasome-like particles in horse semen may open new prospects in this field of research.

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