ELSEVIER

International Journal of Pharmaceutics 172 (1998) 147–159

international journal of pharmaceutics

Interaction between PC12 liposomes encapsulating ATP and human spermatozoa

M. Skiba-Lahiani [a,b], M. Skiba [a,b,c,*], G. Nikas [d], J. Auger [e], E. Fattal [a], J. Delattre [f], A.-M. Courtot [d]

[a] *URA CNRS 1218, Faculté de Pharmacie, Université de Paris Xl, Laboratoire de Physico-Chimie, Pharmacotechnie et Biopharmacie, URA CURS 1218, 5 Avenue Jean-Baptiste Clément, 92290 Chatenay-Malabry, France*
[b] *Laboratoire de Pharmacie Galénique, Faculté Médecine Pharmacie de Rouen, Rouen, France*
[c] *Hall de Technologie Pharmaceutique, IUT d'Evreux, Evreux, France*
[d] *Laboratoire d'Histologie et Bâtiment Inserm G. Pincus, Centre Hospitalo-Universitaire de Bicêtre, Kremlin-Bicêtre, France*
[e] *CECOS, Groupe Hospitalier Cochin Port-Royal, Paris, France*
[f] *Laboratoire de Biochimie, Hôpital de la Salpêtrière, Paris, France*

Received 13 October 1997; received in revised form 28 May 1998; accepted 9 June 1998

Abstract

ln a preliminary report (Skiba-Lahiani, M. et al.), we showed that dilauroylphosphatidylcholine (PC12) liposomes entrapping ATP sustain sperm motion over time and in some experiments a concomittant increase in the percentage of human acrosome-reacted spermatozoa. With the present study, we confirmed an interaction between PC12 liposomes and human spermatozoa vizualizing the morphologic aspects when we used several ultrastructural techniques. Investigations using on the one hand liposomes made from fluorescently labeled phosphatidylcholine analogue, acyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylcholine (Acyl-NBD-PC), and on the other hand liposomes encapsulating a fluorochrome (calceine), allowed to approach the mode of this interaction. Electron-microscopic examination has shown on the one hand the transfer of Acyl-NBD-PC from vesicles to cells, and on the other hand the transport of encapsulated fluorochrome (calceine) into the cell, supporting the suggestion of membrane perturbation as a cause of an increase of its permeability to trigger the acrosomal exocytosis. Under conditions which stimulate the acrosome reaction by liposomes entrapping ATP (L_{ATP}) and blank liposomes (L_B) treatments, we have not shown an increase in intracellular calcium when we used the fluorescent probe [1-[2-(5-carboxyoxazol-2-yl)-6-

Abbreviations: PC12, dilauroylphosphatidylcholine; ATP, adénosine 5′ triphosphate; L_{ATP}, liposomes entrapping ATP; L_B, blank liposomes; AR, acrosome reaction; BWW, biggers Whitten-Wittingham's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; Acyl NBD-PC, acyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylcholine; Fura-2, [1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane *N*,*N*,*N*′,*N*′-tetraacetic acid]; EGTA, ethylene glycol bis (*β*-aminoethylether)-*N*,*N*,*N*′,*N*′ tetraacetic acid.

* Corresponding author. Fax: + 33 1 46619334.

PII S0378-5173(98)00203-8

aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane *N*,*N*,*N*′,*N*′-tetraacetic acid] (Fura-2) suggesting that the interaction PC12 liposomes-human spermatozoa induced an acrosome reaction different from the physiologic acrosome reaction.

Keywords: Liposomes; Adenosine triphosphate; Spermatozoa; Interaction; Microscopy

1. Introduction

It has been reported that liposomes composed of dilauroylphosphatidylcholine (phosphatidylcholine with a saturated fatty acid chain composed of 12 carbons: PC12) triggered the acrosome reaction (AR) in different mammalian species (Graham et al., 1986, 1987) including human (Holden and Trounson, 1992) but all these studies reported a simultaneous significant and rapid drop in sperm motility.

The ability of lipid vesicles (liposomes) to entrap small and large molecules, such as drugs, enzymes and also to deliver them to cells, has attracted great interest in their use as vehicles (Puisieux et al., 1994, 1995). In a previous study (Skiba-Lahiani et al., 1995), we investigated whether PC12 liposomes entrapping adénosine 5′ triphosphate (ATP) could also stimulate concomitantly sperm motility and AR. Indeed, ATP is a bioenergetic substrate. In spermatozoa, metabolism of ATP is one aspect of energy conversion and may provide the energy for sperm motility (Ford and Harrison, 1981). Our data indicated that the percentage of true acrosome-reacted spermatozoa and the values for movement characteristics were increased significantly only with liposomes entrapping ATP (L_{ATP}) treatment compared to an effect of blank liposomes (L_B), free ATP alone or L_B + free ATP treatments.

These preliminary results indicated the interest of PC12 vesicles for the targeting of ATP as a bioenergetic substrate carrier to the human spermatozoa.

An effect on spermatozoa by liposomes would imply a certain degree of interaction between them but only very little is known about this event. There are few papers presenting electron micrographs of this interaction (Arts et al., 1990, 1993) and no paper concerning the interaction PC12 liposomes entrapping ATP-human spermatozoa.

In this study, we presented some results obtained from the application of the negative staining technique, scanning electron microscopy and electron transmission microscopy. We vizualized a close contact between liposomes and spermatozoa under defined experimental conditions. We demonstrated the transfer of fluorescently labelled constituents of liposomes and finally, we compared the acrosome reaction induced by PC12 liposomes with this induced by the ionophore ionomycin using the fluorescent probe Fura-2 to measure intracellular calcium.

2. Materials and methods

2.1. Preparation of liposomes

Phospholipid vesicles composed of 21 mM of PC12, 6 mM of cholesterol and 3 mM of sulfatide were prepared according to a Reverse Phase Evaporation (REV) method (Szoka and Papahadjopoulos, 1978) albeit with some modifications (Laham et al., 1987) as described in a precedent report (Skiba-Lahiani et al., 1995). All chemical compounds were obtained from Sigma Chimie (MO). The prepared liposomes were extruded through 0.22 mm millipore filters to produce a sterile preparation with an homogeneous size distribution (Hope et al., 1985). They were freeze dried in a lyophilizer (USIFROID, SMH15, Maurepas, France). A batch of lyophilized liposomes was rehydrated before each experiment according to the method of Jizomoto et al., (1989). A mixture of lyophilized blank PC12 liposomes and an aqueous solution 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES buffer) with or without 100 mM ATP to obtain either L_{ATP} or L_B, was vortexed for 1 h at room temperature. Elimination of non encapsulated ATP was carried out by gel filtration on PD-10 Sephadex® columns (G-25M, Pharmacia

LKB, Sweden). After disrupting the liposomal membrane with Triton X-100 (0.2% v/v), the ATP content was determined by a bioluminescence assay (Bergmeyer, 1974) and was found to be 6 mM. The total amount of phospholipid was determined enzymatically (Takayama et al., 1977) and was found to be 8 mM. The mean diameter of liposomes, measured using a Nanosizer® (Coultronics, Paris, France), was 182 ± 18 nm.

2.2. Semen samples

Semen samples were collected from donors by masturbation at the laboratory after a recommended sexual abstinence period of 3–5 days. The semen samples were allowed to liquefy at 37°C for 30 min before routine semen analysis including the assessment of seminal volume, sperm concentration, and the percentage of motile sperm according to World Health Organization recommendations (World Health Organization, 1992).

2.3. Measurement of intracellular calcium

Fura-2 probe was used for measuring intracellular calcium (Ca^{2+}). Fura-2 chelated the cytosolic free calcium and became fluorescent when charged in Ca^{2+}. Fluorescence intensity was correlated to the quantity of Ca^{2+} complex (excitation wavelength (λ ex.) of 340 and 380 nm, emission wavelength (λ em.) of 505 nm). Intracellular Ca^{2+} was measured by spectrofluorimetry (spectrofluorimeter PTI MD-5020, Kontron Instruments, Saint-Quentin en Yvelines, France). When the calibration was done, maximal and minimal fluorescences (R_{max} and R_{min}, respectively) were determined. For the maximal fluorescence, the whole Fura-2 was charged in Ca^{2+} with an important dose of ionomycin (a Ca^{2+} ionophore) or with 0.005% triton (permeabilization of membranes and influx of Ca^{2+} which fixed on the probe). For the minimal fluorescence, Fura-Ca^{2+} binding was blocked with EGTA which chelated Ca^{2+} with a greater affinity compared to Fura. Ca^{2+} concentrations were calculated from the following equation:

$$[Ca^{2+}] = K_d(R - R_{min}/R_{max} - R)$$

Ca^{2+} is a constant of the probe: Fura-Ca^{2+} dissociation constant = 224, $R = R_{max}/R_{min}$

The sperm samples were centrifuged through a two-step Percoll gradient (47.5% and 95% of Percoll, 300 g for 20 min, Mc Clure et al., 1989), washed twice (600g for 10 min), resuspended in a no-coloured (without red phenol) Biggers–Whitten–Wittingham's medium (BWW) (Biggers et al., 1971) and incubated at 37°C under 5% CO_2 in air for 180 min to promote in vitro capacitation. The capacitated spermatozoa were incubated for 45 min at 37°C in the dark with Fura-2 (2 mM). The sperm were then washed twice (600 g for 5 min) to eliminate the free Fura-2 and resuspended in BWW medium without human serum albumin (to avoid an augmentation of the fluorescence). The final concentration of spermatozoa was adjusted to 4×10^6 sperm/ml. To test the Ca^{2+} response, the liposomes (L_{ATP}, liposomes encapsulating ATP; L_B: 0.4 mM PC12 and/or 0.5 mM ATP) or the free ATP solution (0.5 mM ATP) were added to the sperm aliquots and measurements were assessed after various periods of co-incubation: 0, 2, 10, 15, 30, and 60 min. The temperature was maintained at 37°C by means of a cuvette holder equipped with a magnetic stirring device.

2.4. Negative-staining technique

PC12 L_{ATP} characterization by electron transmission microscopy was obtained using a negative staining technique. The sperm samples were washed twice ($600 \times g$ for 10 min), resuspended at a final concentration of 30×10^6 sperm/ml in BWW medium and were incubated for 60 min at room temperature in the presence of L_{ATP} (final concentrations: 0.5 mM ATP, 0.4 mM PC12).

The samples (L_{ATP} alone or aliquots of treated sperm) were incubated for 15 min at room temperature in a 2% phosphotungstic acid solution. A drop of each preparation was rapidly dried in presence of osmic acid vapours (1% osmic acid) on a specimen grid composed of Cu and coated with a carbon-containing cellulose nitrate plastic film (Touzart and Matignon, Vitry-sur-Seine, France). After drying, grids were examined in a Philips EM 301 electron microscope.

2.5. Electron microscopy

After a washing step (two cycles of centrifugation at $600 \times g$ for 10 min), human spermatozoa were resuspended at a final concentration of 30×10^6/ml in BWW medium. Aliquots of the sperm samples were incubated in the presence of L_{ATP} (with final concentrations of 0.5, 5, 10, 25, 50, and 75 mM PC12).

2.5.1. Scanning electron microscopy

Aliquots of treated sperm samples were fixed in 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer, dehydrated very rapidly through graded acetone series, critical-point dried with carbon dioxide, mounted on the specimen holder, sputter coated with gold and examined in a Jeol 840 A scanning electron microscope.

2.5.2. Inclusion in an hydrophilic durcopan A resin

Aliquots of treated sperm samples were fixed in 2% glutaraldehyde in 0.15 M sodium cacodylate buffer for 30 min at 4°C, post-fixed in 1% osmium tetroxyde (at room temperature for 45 min). The sperm samples were washed twice in 0.15 M sodium cacodylate buffer by centrifugation ($12000 \times g$ for 3–4 min). The sperm pellets were dehydrated in a series of mixtures of water with an embedding substance: Fluka Durcupan®, a water-soluble aliphatic polypoxyde for electron microscopy (Touzart and Matignon, Vitry-sur-Seine, France), the concentration of this component increasing in consecutive stages (50, 70, 90, and 100% v/v). The dehydrated aliquots were then placed in the definitive polymerization mixture overnight at 4°C. Plastic moulds were filled with the freshly prepared polymerization mixture and were charged with the sperm cells, treated or no-treated (control) with ATP liposomes (L_{ATP}). Polymerization took place at 37–45°C and lasted 3–4 days. Ultrathin sections (500–800 Å) were cut on a Reichert OmU_3 ultramicrotome (Reichert-Jung, Roissy, France), stained with 2% uranyl acetate, then with 0.2% lead citrate and examined in a Philips EM 301 transmission electron microscope.

2.6. Fluorescence microscopy

2.6.1. Acyl-NBD-PC

Acyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylcholine (Acyl-NBD-PC) is a fluorescent phospholipid PC12 analogue (Avanti Polar Lipids, Alabaster, λ ex., 475 nm; λ em., 530 nm). This is a marker of membranes with a part of its NBD chromophor localised at interface of the phospholipid bilayer (Chattopadhyay, 1990). L_B were prepared including 1 mol% Acyl-NBD-PC in their composition. Acyl-NBD-PC was introduced into liposomial bilayer at an adequate concentration to obtain an auto-extinction of its fluorescence. The NBD-labelled lipids are practically non-fluorescent in aqueous suspensions, but are highly fluorescent in organics solvents or at low concentrations (≤ 1 mol%) in membranes (Chattopadhyay, 1990). Human spermatozoa, centrifuged through a two-step Percoll gradient (47.5 and 95% of Percoll, 300 g, 20 min), washed and resuspended at a final concentration of 15×10^6 spermatozoa/ml in BWW medium, were incubated at room temperature for 60 min with labelled liposomes (1, 5, and 10 μl of liposomial suspension/million of spermatozoa). After coincubation, sperm samples were centrifuged through a two-step Percoll gradient (47.5 and 95% of Percoll, 300 g, 20 min), this procedure allowing the recovery of a sperm suspension totally devoid of liposomes. After a cytocentrifugation step (Cytospin®, Shandon Elliott; 1500 trs/min for 10 min) and a montage by citifluor, the rubbings were analysed by fluorescence microscopy (BH2 Olympus microscope) at 490 nm.

2.6.2. Calceine

Calceine is a water-soluble marker of internal aqueous space of liposomes. Its fluorescence (λ ex., 490–500 nm; λ em., 511–520 nm) was quenched at high concentration in intravesicular medium and reappeared with its dilution in external medium. A batch of lyophilized liposomes was rehydrated with 1 mM calceine solution (Sigma Chimie, MO) before each experiment according to the method of Jizomoto et al. (1989). Elimination of non-encapsulated calceine was achieved by twice filtration on PD-10 Sephadex® columns (G-25 M, Pharmacia LKB, Sweden).

Human spermatozoa, centrifuged through a two-step Percoll gradient (47.5 and 95% of Percoll, 300 g for 20 min), twice washed ($600 \times g$ for 10 min) and resuspended at a final concentration of 15×10^6 spermatozoa/ml in BWW medium, were incubated at room temperature for 60 min with either free calceine alone in solution (final concentration of 10 mM), PC12 liposomes entrapping calceine (1 μl of liposomial suspension/million of spermatozoa) or L_B (1 μl of liposomial suspension/million of spermatozoa + 10 mM free calceine in solution). After coincubation, the sperm pellet recovered from the 95% fraction of a two-step Percoll gradient (47.5 and 95% of Percoll, $300 \times g$ for 20 min), was washed twice ($600 \times g$ for 10 min) and resuspended at a final concentration of $15 \times l0^6$ spermatozoa/ml in BWW medium. After a cytocentrifugation step (Cytospin®, Shandon Elliott; 1500 trs/min for 10 min) and a montage by citifluor, the rubbings were analysed by fluorescence microscopy (BH2 Olympus microscope) at 490 nm.

3. Results and discussion

3.1. Intracellular calcium

The AR, that occurs after sperm capacitation, is an exocytotic event induced by a Ca^{2+} influx (Yanagimachi, 1988). It plays an essential role during fertilization by making spermatozoa able of penetrating the zone pellucida and capable of fusing with the egg plasma membrane. Other known inducers of the human acrosome reaction include the calcium ionophore ionomycin which promotes non-physiological sperm Ca^{2+} uptake. Under conditions which stimulated the acrosome reaction by L_B and L_{ATP} treatments (Skiba-Lahiani et al., 1995), we dosed the cytoplasmatic calcium concentrations ($[Ca^{2+}]_i$) utilizing the fluorescent probe Fura-2. Either with liposomes (L_B or L_{ATP}; 0.4 mM PC12) or free ATP in solution (0.5, 5, and 10 mM), we were unable to detect any change of $[Ca^{2+}]_i$ at $t = 0$ (Fig. 1) or at any concentration or incubation periods tested ($t = 0$, 2, 10, 15, 30 min, data not shown). On the contrary, the calcium ionophore (ionomycin) induced the AR producing an important Ca^{2+} influx by destabilization of sperm plasma membranes. We concluded also that PC12 liposomes induce the AR but not via an increase of Ca^{2+} influx.

3.2. Negative-staining technique

In order to vizualize the interaction PC12 liposomes-human spermatozoa, we experimented several methods using a transmission electron microscope. The negative-staining technique was used to put in the structure of PC12 liposomes of formulation: dilauroylphosphatidylcholine/cholesterol/sulfatide (7:2:1 ratio molar) encapsulating ATP (L_{ATP}) prepared by the method described here. The results indicated oligolamellar vesicles (about 3–4 phospholipidic lamella) of about 200 nm, morphologically characterized by a large aqueous core (Fig. 2). Using the negative-staining technique, after a coincubation of spermatozoa with L_{ATP}, we visualized the fixation of liposomes, mostly in the form of aggregates, on spermatozoa over both the head and tail, probably indicating the adsorption of liposomes to the surface of the cells. In Fig. 3, we pointed out the fixation of oligolamellar vesicles (liposomes) on the flagella (part of the tail) of a spermatozoon.

3.3. Scanning electron microscopy

With the scanning electron microscopy technique, we can put in a general view of the repartition of liposomes on spermatozoa. We often observed that the liposomes were together in small groups or aggregates at different levels of the sperm head or of the sperm flagella (Fig. 4A and Fig. 4B). It seems that these aggregation and conglomeration of liposomes could be accentuated by the technique of preparation which deforms and destroys a part of liposomes.

3.4. Inclusion in resin

Experiments with the transmission electron microscopy have shown a precise sight and in sections of the interaction PC12 liposomes-spermatozoa.

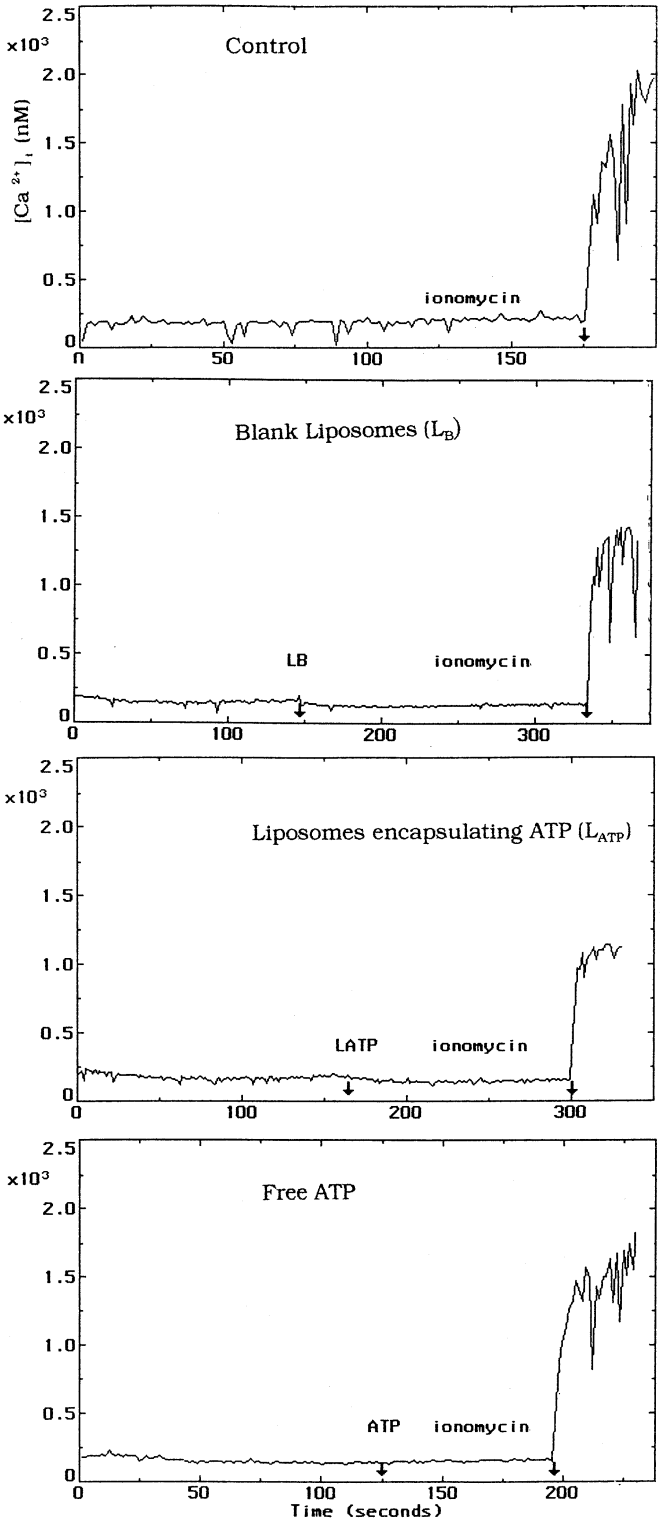

Fig. 1. Measurement of intracellular calcium ($[Ca^{2+}]_i$) by the fluorescent probe Fura-2 after addition ($t = 0$) of liposomes (L_B, blank liposomes; L_{ATP}, liposomes encapsulating ATP) or free ATP, ($n = 3$).

In a first experiment, using an hydrophobe resin, transmission electron microscopy did not reveal any liposome-sperm cell interaction when the preparatory steps included dehydration and embedding in a hydrophobic medium. Using embedding in an hydrophilic Durcopan A resin, a tight relation between spermatozoa and liposomes was then demonstrated as revealed in Fig. 5B showing liposomes that cover t h e whole surface of the sperm head. With this technique, we visualized too the changes which occured after a co-incubation human spermatozoa with ATP entrapped PC12 liposomes, translating by a destabilization effect of liposomes on the membrane of most of spermatozoa compared to the control samples (Fig. 5A). We could explain these discordant results between the two techniques because the embedding or contact of the liposomes with organics solvents would dissolve or damage the liposomes. On the contrary, an aqueous environment reasonably favored preservation of the native structure of the samples. It is less likely that the bond between liposomes and spermatozoa was constituted by only electrostatic forces since both liposomes and cells displayed a net-negative surface charge rather favoring repulsion. Vesicle adsorption (Szoka et al., 1980) and membrane fusion seem to be enhanced when negatively charged liposomes are used. However, the actual surface potential is not known in every case and we can only speculate on the effect of this factor on adsorption and fusion. Thus, these data only suggest that, under our experimental conditions, the close contact indicative of an interaction between liposomes and spermatozoa, is likely of hydrophobic character.

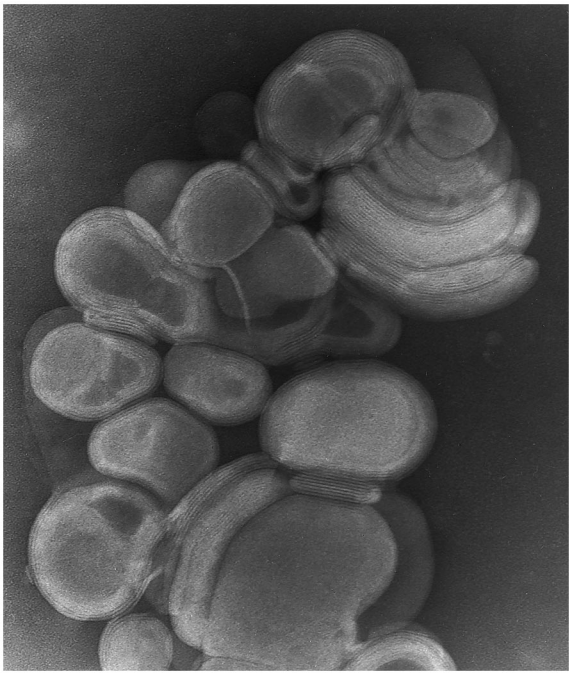

Fig. 2. Transmission electron microscopic observation from negatively stained preparations of liposomes of formulation PC12/cholesterol per sulfatide (7:2:1 ratio molar) encapsulating ATP (L_{ATP}). Liposomes were oligolamellar vesicles of about 200 nm with a large aqueous core. 70000 × .

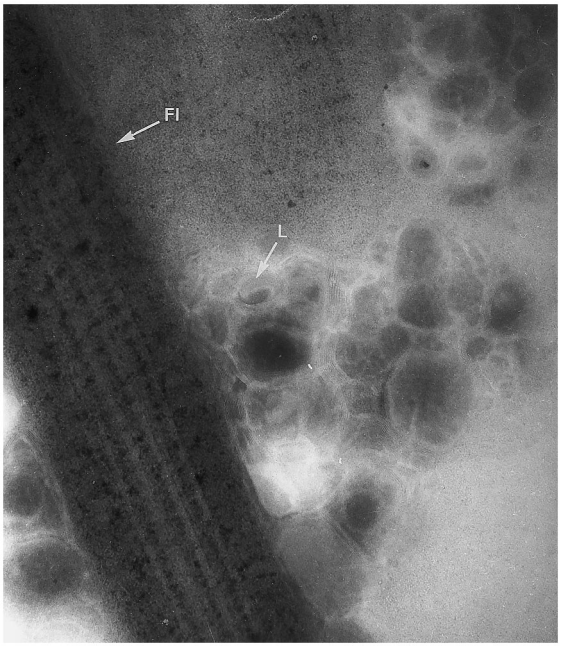

Fig. 3. Transmission electron microscopic observation from negatively stained preparations of human spermatozoa coincubated with L_{ATP}, showing the fixation of oligolamellar vesicles, mostly in the form of aggregates, on the flagella (part of the tail) of a spermatozoon. L, liposomes; Fl, flagella of spermatozoon. 70000 × .

3.5. Acyl-NBD-PC

The interaction of ATP liposomes with individual spermatozoa was characterized using NBD-

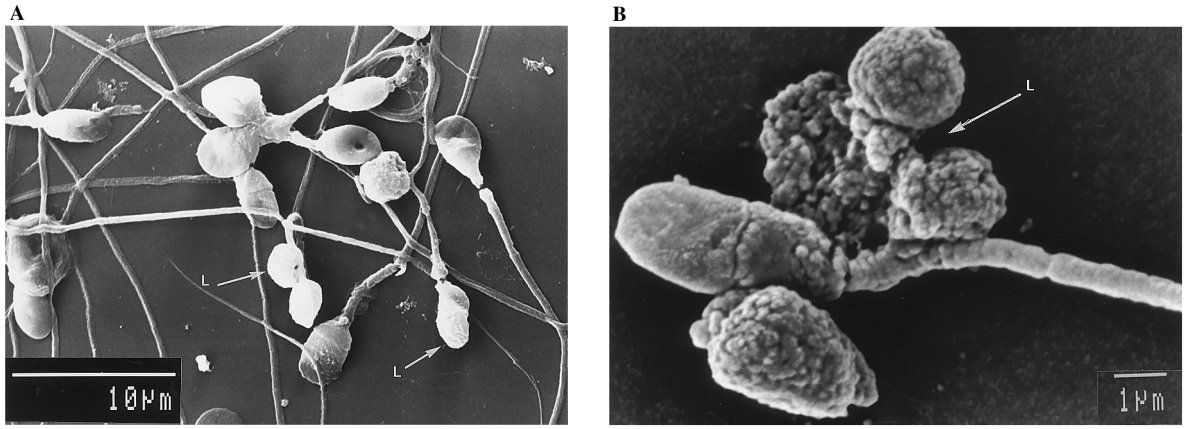

Fig. 4. Scanning electron micrograph of human spermatozoa coincubated with L_{ATP} after fixation, dehydration and critical-point drying. L, liposomes; A, general view showing the liposomes in aggregates bound to different level of sperm cells (arrows). Bar = 10 μm. B: precise view of liposomes in aggregates (arrow) that interacted with a spermatozoon. Bar = 1 μm.

PC labelled liposomes and visualized by fluorescent microscopy. NBD-labelled lipids are currently one of the most widely used fluorescent lipid analogues in membrane studies. Fluorescence distribution was mainly restricted to sperm plasma overlying the head and the midpiece (located in the middle part close to the tail) of spermatozoa (Fig. 6). Thus, these observations suggest the incorporation of the exogenous NBD-labelled lipids into cellular membranes after coincubation of the spermatozoa with liposomes containing NBD-labelled lipids. We could suppose that the head and sperm midpiece were the preferent domains for an interaction with PC12 because each being the siege of an intensive activity. Indeed, the acrosome, a membrane-limited organelle covering the anterior portion of the sperm head, is implicated in an exocytotic event (AR) which plays an essential role during fertilization (Sathananthan et al., 1986). In the midpiece, the mitochondria, that synthetize ATP, provide the energy metabolism for sperm motility (Ford and Harrison, 1981). But much more investigations were necessary to determine precisely the sites of this interaction PC12 liposomes-human spermatozoa.

A kinetic model based on transfer of soluble lipid monomers has been yet developed (Nichols and Pagano, 1982). According to this model, the transfer of lipids in liposomes occurs by dissociation of lipid monomers from the donor membrane, diffusion through the

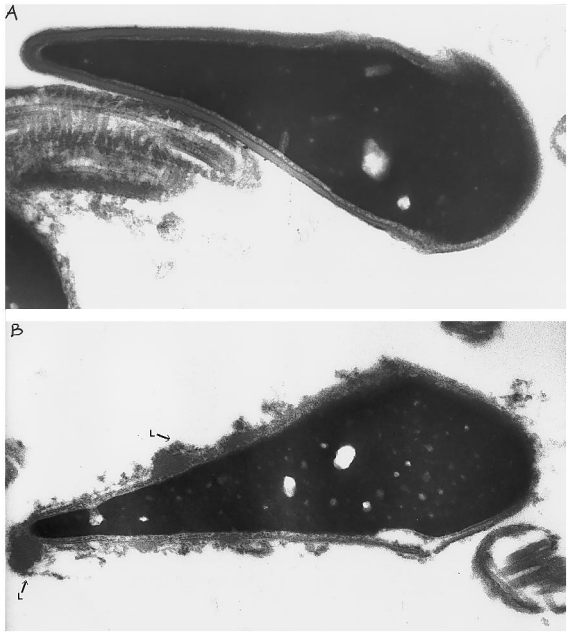

Fig. 5. Transmission electron micrograph of spermatozoa after an embedding in Durcopan A, a water-soluble epoxy resin. 45000 ×. L, liposomes; A, control (not treated spermatozoa with L_{ATP}) B: After coincubation of spermatozoa with L_{ATP}, liposomes (arrows) cover the whole surface of the sperm head.

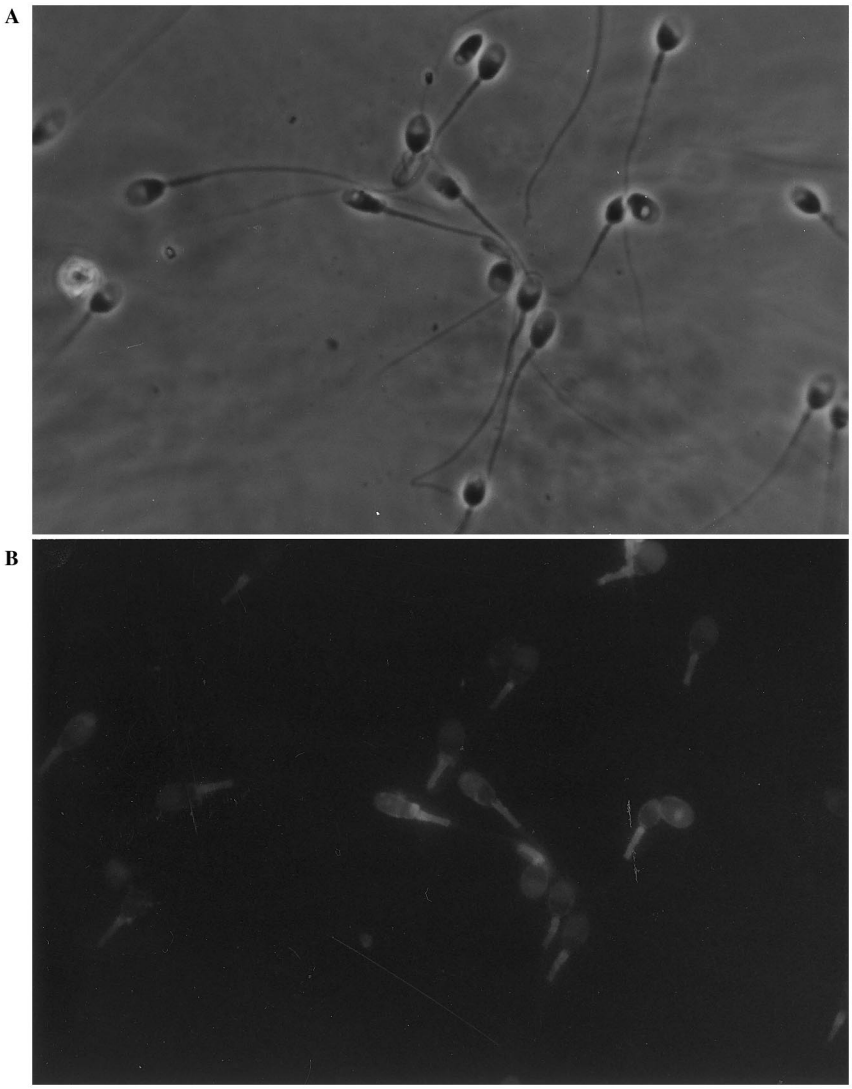

Fig. 6. Fluorescence labelling patterns of human sperm after incubation with Acyl-NBD-PC labelled liposomes (10 μl of liposomial suspension/million of spermatozoa). Fluorescence distribution is mainly restricted to sperm plasma overlying the head and the midpiece of spermatozoa.

aqueous phase, followed by association with the acceptor membrane. The current study demonstrates that PC12 vesicles action on sperm cells could involve diffusion of monomeric PC12 from vesicles to cells. Once incorporated into the sperm plasma membrane, monomeric PC12 could alter the properties of the membranes and increase the permeability to trigger the acrosomal exocytosis.

3.6. Calceine

To support the precedent hypothesis, in a series of experiments, liposomes were loaded with fluorescence labelled calceine and their interaction with human spermatozoa was examined in a fluorescent microscope. Calceine, a water-soluble marker of internal aqueous space of liposomes (Kendall and Mac Donald, 1983) was used as a

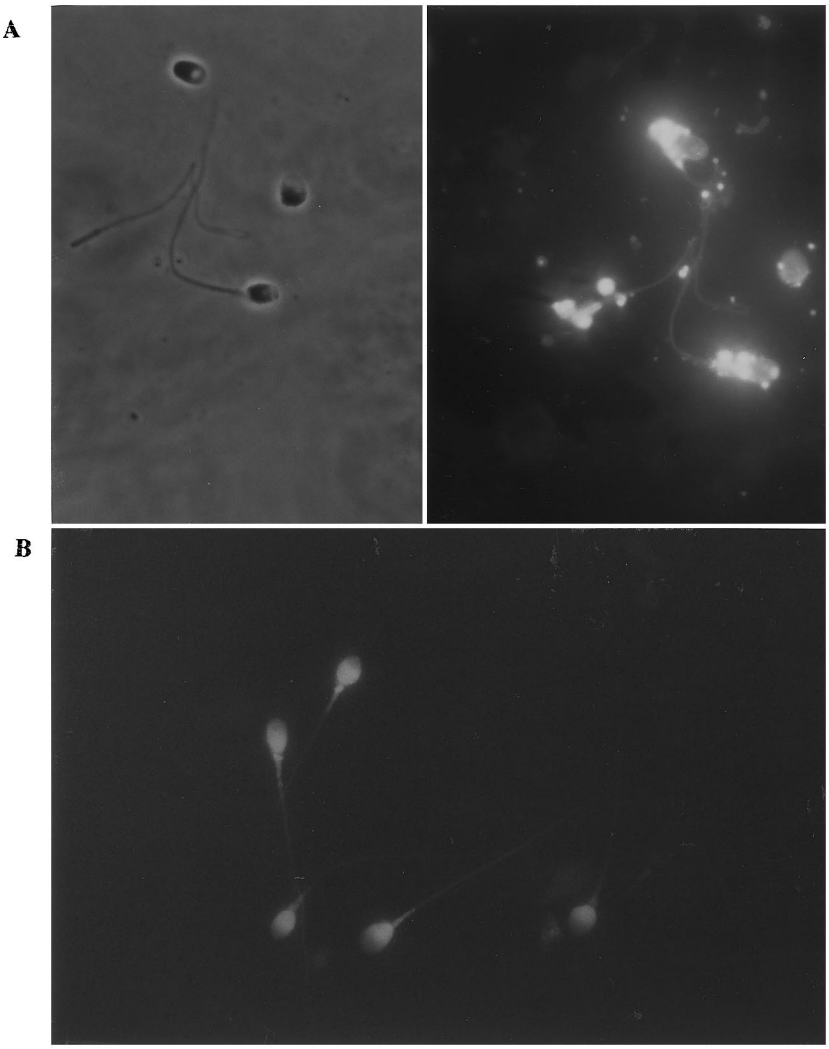

Fig. 7. Fluorescence labelling patterns of human sperm after incubation with liposomes loaded with fluorescence labelled calceine (1 μl of liposomial suspension/million of spermatozoa). A, adsorption of calceine liposomes to the surface of the spermatozoa; B, after an extensive washing of the cells, a permanent fluorescence of the sperm head and of the midpiece are observed.

model instead of ATP drug because apart from its fluorescent properties, the calceine molecule is a polyanion as ATP. These experiments were carried out to visualize a possible entrance of encapsulated fluorochrome from liposomes into the sperm cells and to confirm our hypothesis that incubation with PC12 liposomes might change the membrane permeability of spermatozoa, thus improving the transport of encapsulated compounds into the cells.

Fluorescence became unevenly distributed along the body of spermatozoa, mostly in the form of aggregates, indicating adsorption of calceine liposomes to the surface of the spermatozoa. After an extensive washing of the cells, the fluorescent aggregates were no longer apparent. A permanent fluorescence of the sperm head and of the midpiece suggested that the fluorescent label may have entered the cells and become evenly distributed inside the cells or that adsorption may

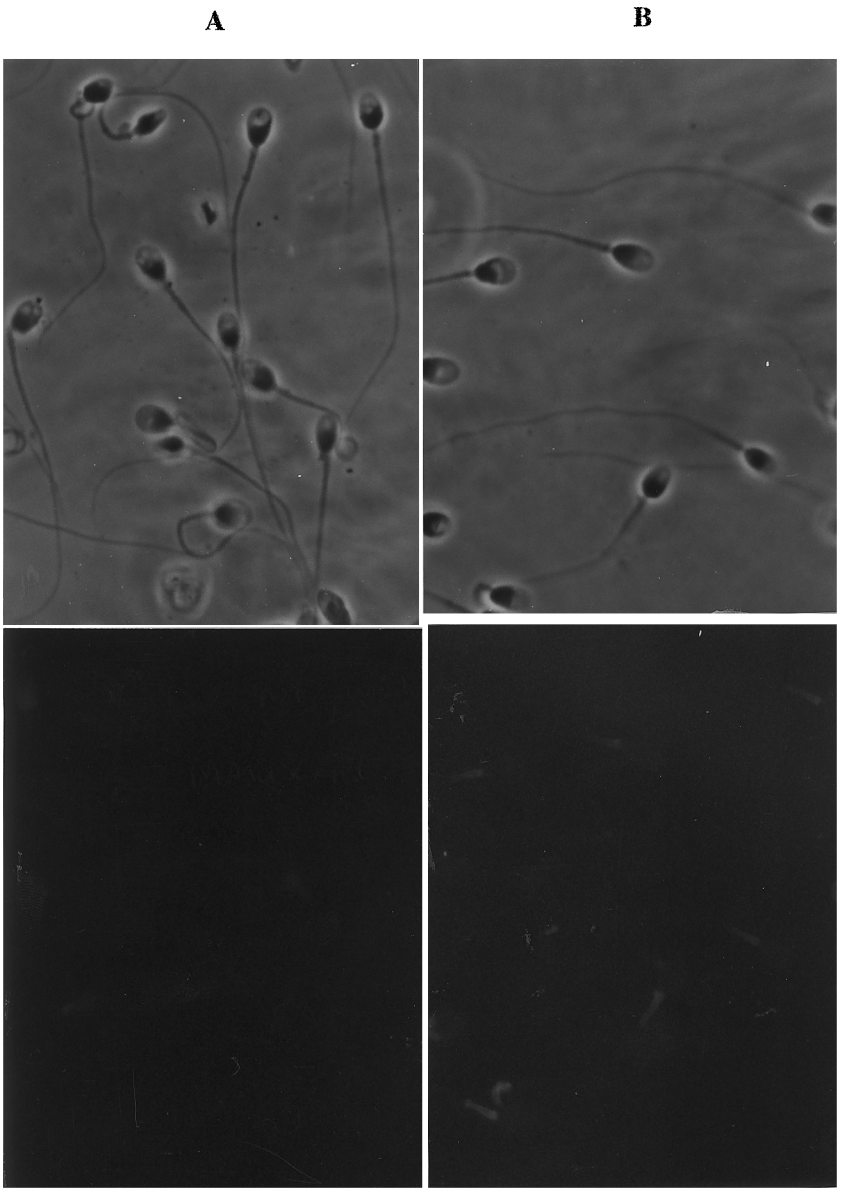

Fig. 8. Fluorescence labelling patterns of human sperm after incubation with free calceine in solution. (A) after coincubation spermatozoa-free calceine in solution (10 mM), no fluorescence is observed; (B) after coincubation spermatozoa with both blank liposomes (L_B: 1 μl of liposomial suspension/million of spermatozoa) + free calceine in solution (10 mM), a very reduced fluorescence is observed.

be strong (Fig. 7). After the same experimental conditions, when spermatozoa were coincubated with free calcein in solution (Fig. 8A), no fluorescence was observed. We could also suppose that external calceine have not a specific effect. A very reduced fluorescence after coincubation of spermatozoa with both L_B and free calceine in solution (Fig. 8B) suggested that liposomes were essential to destabilize the sperm membrane and to facilitate the entrance of encapsulated

fluorochrome (calceine) into the sperm cells. These data are in agreement with our precedent results (Skiba-Lahiani et al., 1995) showing an increase of the values for movement characteristics and simultaneous measurements of intracellular ATP content of spermatozoa maintained at a high concentration only when spermatozoa were incubated with L_{ATP}. In contrast, when using a solution of free ATP, all of the movement characteristics remained unchanged, suggesting that under these conditions, ATP cannot enter the cells. These results indicated the interest of PC12 vesicles for introducing highly hydrophilic compounds into spermatozoa. A more direct evidence for the entrance of liposome-entrapped ATP into the sperm cells could be provided by autoradiography upon their incubation with liposomes containing labelled ATP.

4. Conclusion

This is the first report as regards the visualization of interaction ATP liposomes-human spermatozoa from different electron microscopy techniques used. We visualized a liposome-sperm cell close relation but no identified specific site for liposomes adhesion to the spermatozoon. By immunofluorescence, investigations have demonstrated on the one hand the transfer of Acyl-NBD-PC, fluorescently labelled PC analogues, from liposomes to spermatozoa, and on the other hand the transport of encapsulated calceine into the cells. We have shown that acrosome reaction of human spermatozoa induced by PC12 liposomes occured after a tight interaction between liposomes and spermatozoa, a transfer of PC12 monomers but not accompanied by a variation of intracellular calcium as in the physiologic conditions. Nevertheless, the results could be attractive for some cases of male infertility related to an inability of spermatozoa to effect a normal acrosome reaction because of a no mobilization of calcium. The results as a whole indicate the interest of PC12 vesicles for introducing compounds into spermatozoa as well as for modulating membrane structures and functions required for fertilization.

Acknowledgements

We thank Catherine Serres for her technical assistance to measure intracellular calcium by the fluorescent probe Fura-2.

References

Arts, E.G.J.M., Hoekstra, D., Jager, S., 1990. Domain specific membrane fusion of phospholipid vesicles with bovine spermatozoa. In: Evers, J.L.H., Heineman, M.J. (Eds.), From Ovulation to Implantation: Proceedings of the VIIth Reinier de Graaf Symposium Maastricht Netherlands. Excerpta Medica, Amsterdam, pp. 315–322.

Arts, E.G.J.M., Kuiken, J., Jager, S., Hoekstra, D., 1993. Fusion of artificial membranes with mammalian spermatozoa: specific involvement of the equatorial segment after acrosome reaction. Eur. J. Biochem. 217, 1001–1009.

Bergmeyer, H.U., 1974. Methods in Enzymatic Analysis. Verlag Chemie Weinheum Academic Press, New York, p. 2112.

Biggers, J.D., Whitten, W.K., Whittingham, D.G., 1971. Methods in Mammalian Embryology. Freeman Press, San Francisco, pp. 86–116.

Chattopadhyay, A., 1990. Chemistry and biology of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-labelled lipids: fluorescent probes of biological and model membranes. Chem. Phys. Lipids 53, 1–15.

Ford, W.C.L., Harrison, A., 1981. The role of oxidative phosphorylation in the generation of ATP in human spermatozoa. J. Reprod. Fertil. 63, 271.

Graham, J.K., Foote, R.H., Parrish, J.J., 1986. Effect of dilauroylphosphatidylcholine on the acrosome reaction and subsequent penetration of bull spermatozoa into zone-free hamster eggs. Biol. Reprod. 35, 413–424.

Graham, J.K., Foote, R.H., Hough, S.R., 1987. Penetration of zone-free hamster eggs by liposome-treated sperm from the bull, ram, stallion and boar. Biol. Reprod. 37, 181–188.

Holden, C.A., Trounson, A.O., 1992. Effect of dilauroylphosphatidylcholine on human spermatozoa. J. Androl. 13, 260–265.

Hope, M.J., Bally, M.B., Webb, G., Cullis, P.R., 1985. Production of large unilamellar vesicles by a rapid extrusion procedure: characterization of size distribution, trapped volume and ability to maintain a membrane potential. Biochim. Biophys. Acta 812, 55–65.

Jizomoto, H., Kanaoka, E., Hirano, K., 1989. Encapsulation of drugs by lyophilized, empty dipalmitoylphosphatidylcholine liposomes. Chem. Pharm. Bull. 37, 1895–1898.

Kendall, D.A., Mac Donald, R.C., 1983. Characterization of a fluorescence assay to monitor changes in the aqueous volume of lipid vesicles. Anal. Biochem. 134, 26–33.

Laham, A., Claperon, N., Durussel, J.J., et al., 1987. Liposomally entrapped ATP: improved efficiency against experimental brain ischemia in the rat. Life Sci. 40, 2011–2016.

Mc Clure, R.D., Nunes, L., Tom, R., 1989. Semen manipulation: improved sperm recovery and function with a two-layer Percoll gradient. Fertil. Steril. 51, 874–877.

Nichols, J.W., Pagano, R.E., 1982. Use of resonance energy transfer to study the kinetics of amphiphile transfer between vesicles. Biochemistry 21, 1720–1726.

Puisieux, F., Fattal, E., Skiba-Lahiani, M., et al., 1994. Liposomes, an interesting tool to deliver a bioenergetic substrate (ATP), in vitro and in vivo studies. J. Drug Target 2, 443–448.

Puisieux, F., Couvreur, P., Delattre, J., Devissaguet, J.P., 1995. Liposomes, New Systems and New Trends in their Applications. Editions de Sante, Paris France.

Sathananthan, A.H., NG, S.G., Edirisinghe, R., Ratnam, S.S., Wong, P.C., 1986. Human sperm-egg interaction in vitro. Gamete Res. 15, 317–326.

Skiba-Lahiani, M., Auger, J., Terribile, J., et al., 1995. Stimulation of movement and acrosome reaction of human spermatozoa by PC12 liposomes encapsulating ATP. Int. J. Androl. 18, 287–294.

Szoka, F., Papahadjopoulos, D., 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reversephase evaporation. Proc. Natl. Acad. Sci. USA 75, 4194–4198.

Szoka, F.C., Olson, F., Heath, T., Vail, W., Mayhew, E., Papahadjopoulos, D., 1980. Preparation of unilamellar liposomes of intermediate size (0, 1–0, 2 mm) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes. Biochim. Byophys. Acta 601, 559–571.

Takayama, M.N., Itoh, S., Nagazaki, T., Tanimizu, I., 1977. A new enzymatic method for determination of serum choline-containing phospholipids. Clin. Chim. Act. 79, 93–98.

World Health Organization, 1992. Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. Cambridge, Cambridge University Press.

Yanagimachi, R., 1988. Mammalian fertilization. In: Knobil, E.-et-al. (Ed.), The Physiology of Reproduction. Raven Press, New York.