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ORIGINAL ARTICLE

Uterosome-like vesicles prompt human sperm fertilizing capability

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STUDY QUESTION: Does the rapid transit through the uterine environment modulate the sperm physiological state?

SUMMARY ANSWER: The uterosome-like vesicles (ULVs) secreted by endometrial epithelial cells (EECs) *in vitro* are able to fuse with human spermatozoa, prompting their fertilizing capacity.

WHAT IS KNOWN ALREADY: Early studies suggest that sperm capacitation begins in the uterus and ends in the oviduct, and that a synergistic effect of both female organs may accelerate this process. Although it has been reported that co-incubation of human spermatozoa with endometrial cell-conditioned medium (CM) stimulates sperm capacitation, the mechanism mediating this communication is unknown.

STUDY DESIGN, SIZE, DURATION: Human ULVs secreted by EECs were characterized and their effect on human sperm physiology was analysed. Spermatozoa were incubated with EEC-derived CM or ULV, after which sperm capacitation was evaluated at different time points. In addition, the interaction of spermatozoa with ULV was analysed.

PARTICIPANTS/MATERIALS, SETTING, METHODS: ULVs were isolated by ultracentrifugation and identified using electron microscopy and Western blotting to assess the presence of specific protein markers. Following seminal plasma removal, human spermatozoa were incubated CM or ULV, after which sperm capacitation was evaluated as the ability of the sperm to undergo the induced acrosome reaction and the level of protein tyrosine phosphorylation (PY) determined by Western blot and immunocytochemistry. The interaction of spermatozoa with labelled ULV was analysed by fluorescence microscopy. In all cases, at least three biological replicates from different sperm donors were performed for each set of experiments. Significant differences between mean values were determined by one-way ANOVA followed by Tukey's post hoc test. Differences between treatments were considered statistically significant at $P \leq 0.05$.

MAIN RESULTS AND THE ROLE OF CHANCE: The level of capacitated spermatozoa and those recruited by chemotaxis increased 3- to 4-fold when spermatozoa were incubated in the presence of CM for 4 h. Even a 15 min incubation of spermatozoa with CM was also enough to increase the level of capacitated cells 3- to 4-fold ($P < 0.05$). Furthermore, a short co-incubation of spermatozoa with ULV stimulates sperm capacitation, as determined by the increase in the level of induced acrosome reaction and the induction of PY. In addition, after the co-incubation of spermatozoa with fluorescent labelled ULV, the sperm cells acquired the fluorescent staining which indicates that ULV might be transferred to the sperm surface by a fusion mechanism.

LIMITATIONS, REASONS FOR CAUTION: This is an in vitro study performed with human biological material, spermatozoa and endometrial derived cells; the latter being a cell line originally isolated from a uterine adenocarcinoma.

WIDER IMPLICATIONS OF THE FINDINGS: The capability of spermatozoa to briefly interact with ULVs supports the hypothesis that any step of sperm transport may have physiological consequences, despite the interaction lasting for only a limited period of time. This way of communication of spermatozoa with cell products of uterine origin opens new frontiers of investigation (e.g. the signalling molecules involved), shedding light on the sperm processes that prepare the male gamete for fertilization, which might have implications for human infertility treatment.

LARGE SCALE DATA: N/A.

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Key words: uterine environment / endometrial cells / uterosomes / human spermatozoa / capacitation

Introduction

In order to fertilize the oocyte, mammalian spermatozoa must undergo a series of biochemical and physical changes collectively called capacitation (De Jonge, 2005). As a consequence, a fully capacitated sperm is prepared to carry out several processes occurring before fertilization, including chemotactic orientation, the acquisition of hyperactivated motility, acrosomal exocytosis, penetration of the egg vestments and the ability to bind and fuse with the oocyte membrane (Eisenbach, 1999; Stival *et al.*, 2016).

During sperm transit through the female reproductive tract, a progressive, coordinated modification of the sperm surface was observed. The removal of seminal plasma components and the subsequent interaction with molecules from the female environment thus seem to be important for inducing capacitation (Hunter and Rodriguez-Martinez, 2004). Early studies suggest that sperm capacitation begins in the uterus and ends in the oviduct, and that a synergistic effect of both female organs may accelerate this process (Adams and Chang, 1962; Hunter, 1969; Hunter and Hall, 1974a, b). Even though sperm transits quickly through the uterine environment, it seems sufficient to prompt sperm capacitation (Hunter and Rodriguez-Martinez, 2004). In line with these observations, it has been reported that co-incubation of human spermatozoa with endometrial cell-conditioned medium (CM) stimulates sperm capacitation (Laflamme *et al.*, 2005).

Although the interaction between endometrial cells and spermatozoa seems evident, the mechanism mediating this communication is unknown. Previous studies have demonstrated that extracellular vesicles can transfer proteins, lipids and nucleic acids to other cells, regulating cell function as an alternative means of cell-to-cell communication (Lo Cicero *et al.*, 2015). In recent years, some studies have reported the presence of extracellular vesicles in uterine and oviductal fluids (named uterosome and oviductosome, respectively) associated with diverse physiological effects, for instance, embryo-endometrial interaction and sperm motility (Griffiths *et al.*, 2008; Al-Dossary *et al.*, 2013; Ng *et al.*, 2013; Burns *et al.*, 2014; Machtinger *et al.*, 2016); for general information related to the role of extracellular vesicles in reproduction see (Machtinger *et al.*, 2016). However, the physiological consequences of the interaction of uterosomes with human spermatozoa have not yet been investigated. Here we show that uterosome-like vesicles (ULVs) secreted by endometrial epithelial cells (EECs) *in vitro* are able to fuse with human spermatozoa, prompting their fertilizing capacity.

Materials and Methods

Ethical approval

Experiments were designed for human semen samples in accordance with the Declaration of Helsinki. The study was approved (# 2803) by the Ethics Committee of the Hospital Nacional de Clínicas (Universidad Nacional de Córdoba, Argentina). All participants gave the corresponding written informed consent.

Cell culture and conditioned media collection

Endometrial epithelial cells

Human endometrial adenocarcinoma cell line Ishikawa (a cell line which closely represents human endometrial luminal epithelium) was generously provided by Dr Oehninger (Eastern Virginia Medical School, USA). These EECs were cultured in DMEM/F12 (v/v 1:1) medium (Invitrogen, Carlsbad, CA, USA), supplemented with 100 U/ml of penicillin, 100 mg/ml streptomycin and 10% foetal calf serum (Life Technologies, Carlsbad, CA, USA), at 37°C in a humid atmosphere with 5% CO_2. When the EEC reached a cell confluence of 70–80%, they were washed with Hank's solution and then cultured in serum-depleted Ham's-F10 medium (Thermo Scientific, Rockford, IL, USA) for an additional 24 h. After that, the CM was collected, centrifuged (at 300 × *g* for 10 min, 3000 × *g* for 20 min, and 10 000 × *g* for 30 min), and the supernatant aliquoted and kept at –80°C until use.

Cell line L929

This cell line (L-929; ATCC-CCL-1), derived from non-reproductive tissue, was maintained in Eagle's Minimal Essential Medium (MEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% foetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD, USA), 100 units/ml penicillin G-streptomycin and 4 mM L-Glutamine. CM from this cell line was obtained as described above for EEC, and used as a control.

ULVs isolation

ULVs were isolated from the EEC-CM as previously described (Théry *et al.*, 2006). The last supernatant was passed through a 0.2 µm filter and then ultracentrifuged at 100 000 × *g* for 90 min, suspending the pellet in PBS, which was ultracentrifuged a second time under the same conditions. The pellet containing the ULV was stored at −20°C until use for biological assay with spermatozoa, transmission electron microscopy or Western blot. The ULV concentration was expressed as fold enrichment, calculated as the ratio of initial volume of CM over the final volume used for the physiological assay.

Transmission electron microscopy of ULVs

The pellet containing the ULV was suspended in PBS and an aliquot of 10 µl was placed on formvar/carbon-coated grids for 5 min, and then stained with 4% uranyl acetate, according to a standard protocol (Thery *et al.*, 2006). Samples were observed and photographed under a transmission electron microscope (Leo 906E, Zeiss, Oberkoshen, Germany).

Western blot for membrane markers present in EECs and ULVs

EECs were washed twice with cold PBS and kept on ice for 30 min in radioimmunoprecipitation assay buffer (RIPA) lysis buffer supplemented with a protease inhibitor cocktail (Pierce Chemical, Rockford, IL, USA). Insoluble components were removed by centrifugation at 14 000 × *g* for 30 min. The pellets containing ULV were mixed with RIPA buffer. Protein sample extracts from EEC and ULV were prepared with Laemmli sample buffer (Laemmli, 1970), separated by sodium dodecyl sulphate—polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto a polyvinylidene difluoride membrane. Nonspecific sites were blocked with 5% (w/v) skimmed milk in T-TBS (Tris-buffered saline: 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.1% Tween 20). The transfer membrane was incubated overnight at 4°C with the monoclonal anti-CD63 at 0.5 µg/ml, anti-MFGE8 (milk fat globule-EGF factor 8 protein) at 0.1 µg/ml (Abcam, Cambridge, UK) or anti-actin 0.05 µg/ml (BD Transduction Laboratories, California, USA) in 2% BSA in T-TBS. After several washes in T-TBS, the membrane was next incubated with goat anti-mouse IgG conjugated to horse-radish peroxidase (1:2000) for 1.5 h. Positive immunoreactive bands were detected by the enhanced chemiluminescence method.

Immunocytochemistry of EECs

EECs were cultured in a 12 mm round coverslip, washed with PBS and fixed with cool methanol for 10 min. Nonspecific binding sites were blocked with 10% FBS for 60 min at room temperature. The primary antibody for CD63 or MFGE8 (Acbam, Cambridge, UK) was prepared in blocking solution and then incubated overnight at 4°C. The EECs treated with a non-immune IgG (normal mouse IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as a negative control. The slides were washed three times with PBS for 10 min each and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (Thermo Scientific, Rockford, IL, USA) at a dilution of 1:500, for 60 min at room temperature. After washing with PBS, slides were mounted with Vectashield H-1100 (Vector Laboratories, Burlingame, CA, USA) containing DAPI for nuclei staining. Cell samples were observed and photographed under epifluorescence microscopy (Olympus B51, USA).

Sperm preparation

Human semen samples were obtained from healthy donors after 3–5 days of abstinence. Only those samples exhibiting normal seminal parameters according to the WHO criteria (WHO, 2010) were included in the study. Semen studies were performed according to established guidelines (Sanchez-Pozo *et al.*, 2013; Bjorndahl *et al.*, 2016). Spermatozoa were separated from the seminal plasma using a discontinuous Percoll gradient (Sigma-Aldrich, St. Louis, MO, USA) in HAM F-10 medium containing L-glutamine and 25 mM Hepes (Thermo Scientific, Rockford, IL, USA), according to Aitken and Clarkson (1988). Then, the highly motile sperm population was adjusted to 10×10^6 cells/ml and incubated under different treatments at 37°C in 5% CO_2 on air, for different times as specified in the results section.

Co-incubation of labelled ULVs with spermatozoa

ULVs were fluorescently stained with the lipophilic red dye PKH26 (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. Briefly, the pellet containing ULV was suspended in diluent C and stained with 2 µM PKH26 for 5 min, stopping the reaction with 1% BSA, and then ultracentrifuged in PBS at 100 000 × *g* for 1 h to remove excess dye. Spermatozoa were co-incubated with labelled ULV at 37°C for 15 min, 1 h or 15 min followed by 3 h incubation in HAM medium. Spermatozoa were washed twice with PBS, fixed with 2% formaldehyde, transferred to slides and air dried. Slides were mounted with Vectashield H-1000 (Vector Laboratories, Burlingame, CA, USA) and observed and photographed under an epifluorescence microscope (Axioplan 2, Carl Zeiss, Toronto, Canada).

Sperm viability

Spermatozoa were stained with 0.5% (v/v) Eosin Y (Sigma, St. Louis, MO, USA), determining the percentage of viable spermatozoa as the number of cells that did not incorporate the dye over the total number of sperm counted under a light microscope at 400× (Olympus, Center Valley, USA).

Sperm motility

The percentage of motile spermatozoa and curvilinear velocity (VCL) were determined by video-microscopy and image analysis according to Teves *et al.* (2009). Briefly, an aliquot of the sperm suspension was placed in an observation chamber and then recorded at 30 Hz with the Virtualdub software (ver. 1.9.0, Avery Lee; http://www.virtualdub.org/). For determining the percentage of motile spermatozoa, the cell tracks were analysed with the ImageJ software ver. 1.47 (Sbalzarini and Koumoutsakos, 2005) and the Particle tracker plugin ver. 1.5 (Schneider *et al.*, 2012). The VCL was calculated with the SpermTrack software (ver. 4.0, UNC, Argentina).

Sperm capacitation

This parameter was evaluated in two ways: as the sperm ability to undergo the induced acrosome reaction (Jaiswal *et al.*, 1998) and by the level of protein tyrosine phosphorylation (PY) (Visconti *et al.*, 1995).

(a) *Induced acrosome reaction*. The sperm suspension was divided into two aliquots, one of which was stimulated with 8 µM of calcium ionophore A23187 (Sigma-Aldrich, St. Louis, MO, USA) while the other remained as a control, incubating both samples at 37°C for 30 min. The cells were fixed in 1% formaldehyde in PBS for 20 min at room temperature. To visualize the acrosome, spermatozoa were stained with Coomassie Brilliant Blue following the standard protocol (Larson and Miller, 1999). Sperm suspensions were washed by centrifugation in 100 mM ammonium acetate. The pellet was air dried on a slide and spermatozoa were stained for 5 min with 0.22% Coomassie Brilliant Blue G 250 (Sigma-Aldrich, St. Louis, MO, USA) in a solution of 10% acetic acid and 50% methanol. After washing with distilled water, the cell preparations were let dry and mounted with 90% glycerol in PBS. The state of the acrosome was observed at 1000× under a light microscope (Olympus, Center Valley, USA) in 200 cells counted at random, in duplicated slides. Spermatozoa were considered to have an intact acrosome when the whole sperm head was blue, while those without blue staining were considered as acrosome-reacted spermatozoa. Considering that sperm viability was higher than 90%, cell counting for acrosomal state did not discriminate live from dead cells. The percentage of capacitated spermatozoa was determined as the difference in the percentage of induced and spontaneous acrosome-reacted spermatozoa. Data were expressed as 'capacitation index' considered as a fold increase over the value obtained for the HAM medium control.

(b) PY. Sperm suspensions were incubated under different conditions: with Biggers–Whitten Whittingham (BWW) 'capacitating' medium as control of capacitating conditions (95 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM sodium lactate, 5 mM glucose, 0.25 mM sodium pyruvate and 25 mM $NaHCO_3$, pH 7.4) supplemented with HEPES (10 mM, pH 7.4) and 3 mg/ml fatty acid free bovine serum albumin (Sigma Cat # A-0281); with 'non-capacitating' medium as a negative control, which was the BWW described above but without albumin, with low $CaCl_2$ (0.2 mM) and with NaCl (120 mM) to replace the $NaHCO_3$; and with treatment medium. After seminal plasma removal, spermatozoa were washed with control medium and the concentration was adjusted to 10×10^6 spermatozoa per ml. The cells were processed for immunocytochemistry or Western blot to detect PY using the mouse anti-phosphotyrosine antibody clone 4G10 (Millipore, Billerica, MA, USA).

For immunocytochemistry, spermatozoa were washed with PBS, fixed with 2% formaldehyde, smeared on slides and air dried. Cells were permeabilized for 15 min in 0.2% Triton X-100 in PBS at room temperature. To block nonspecific sites, the slides were incubated for 1 h in 5% BSA-0.2% Triton X-100 in PBS and then incubated with the monoclonal anti-phosphotyrosine mouse antibody (1:150) overnight at 4°C, in a humidified chamber. After that, the sperm were incubated with an anti-mouse Alexa-Fluo 488 antibody (1:300; Invitrogen, Carlsbad, CA, USA). Negative controls were prepared by using a non-immune mouse IgG instead of the anti-phosphotyrosine antibody. Finally, cells were mounted with Vectashield H-1100 (Vector Laboratories, Burlingame, CA, USA) containing DAPI nuclear stain, and were observed and photographed under a fluorescence microscope (Olympus, Center Valley, USA). The

state of PY was observed at 1000× in 200 cells counted at random, in duplicated slides. The percentage of spermatozoa showing immunoreactivity on the principal piece of the flagellum was determined.

For Western blot, total protein extracts were isolated as previously described (Suhaiman *et al.*, 2010). Briefly, once the incubation time ended, the medium was removed by washing sperm cells with PBS supplemented with 2 mM sodium orthovanadate. Sperm pellets were resuspended in sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl) without disulphide reducing agents. Proteins were extracted by heating the pellet in sample buffer twice, at 95°C, for 6 min each. Extracts were centrifuged (12 000 × *g*) for 10 min, and the supernatants were adjusted to 5% β-mercaptoethanol, boiled for 3 min, and used immediately or stored at −20°C until use. Western blotting procedure was performed as described above, except that membrane blocking was performed with 5% BSA. Monoclonal anti-phosphotyrosine antibody was diluted 1:5000 in 2% BSA. Membranes were stripped and re-probed with anti-tubulin beta (1:1000) as total protein loading control (Developmental Studies Hybridoma Bank, University of Iowa, IA, USA).

Sperm selection assay

This assay recruits capacitated spermatozoa on the basis of sperm chemotaxis toward progesterone as previously described (Gatica *et al.*, 2013). Briefly, spermatozoa at a concentration of 6×10^6/ml were placed in one of the wells of the sperm selection assay (SSA) device, and a 10 pM solution of progesterone (Sigma-Aldrich, St. Louis, MO, USA) diluted in culture medium, in the other well. The device was incubated at 37°C in 5% CO_2 on air for 20 min. The sperm suspension from the well containing progesterone was recovered and then used to evaluate the level of induced acrosome reaction as described above.

Statistical analysis

Significant differences between mean values were determined by one-way ANOVA followed by Tukey's post hoc test with the SPSS 14.0 software for Windows (SPSS, Inc., Chicago, USA). Differences between treatments were considered statistically significant at $P \leq 0.05$.

Results

Identification of ULVs secreted by EECs

Electron microscopy analysis showed that ULV are present in the pellet obtained from the last ultracentrifugation of the EEC-CM. These vesicles show cup-shape morphology with a diameter size from ~50 to 200 nm (Fig. 1A and B), comprising exosomes (≤100 nm) and microvesicles (≥100 nm to 1 μm), considered, as a whole, as uterosomes by others (Griffiths *et al.*, 2008), suggesting that they might be generated by multivesicular bodies as well as by apocrine secretion.

The proteins CD63 and MFGE8, which are commonly used as markers for extracellular vesicles (Escola *et al.*, 1998; Thery *et al.*, 1999), were also observed on the apical surface of endometrial epithelium *in vivo* (Franchi *et al.*, 2011; Ng *et al.*, 2013). Therefore, we first verified whether these proteins were present in EEC and then in ULV. Immunostaining of EEC showed that CD63 as well as MFGE8 was located in the cell surface and cytoplasm (Fig. 1C and D). CD63 was mainly observed as a punctate staining within the cells (Fig. 1C), while MFGE8 was detected on the cell surface, with some cells also showing a punctate intracellular staining (Fig. 1D). Both MFGE8 and CD63 proteins were detected by Western blot in EEC (Fig. 1E) and ULV (Fig. 1E)

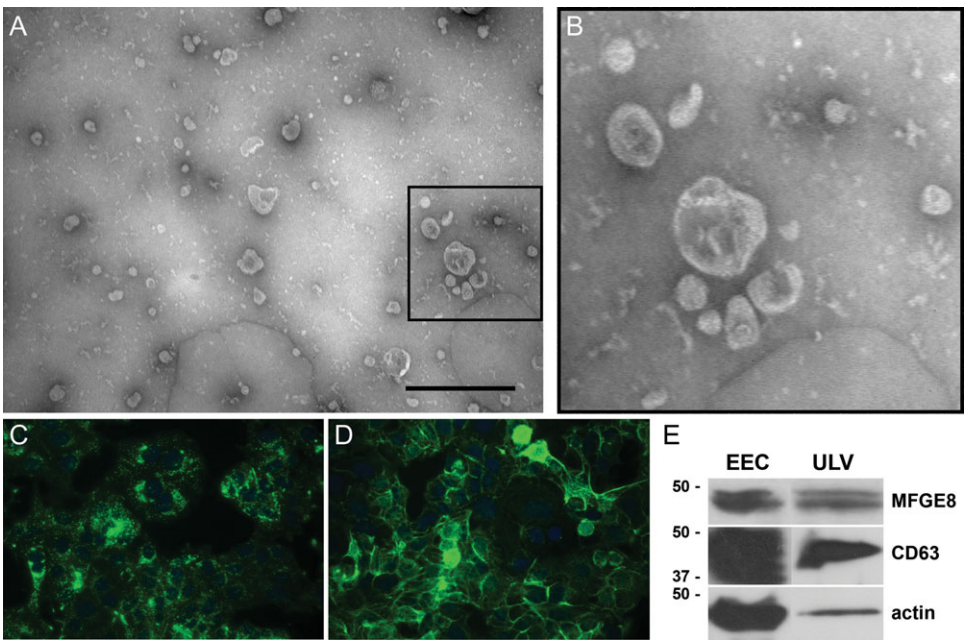

Figure 1 Identification and characterization of ULVs derived from an EEC line. (**A**) Transmission electron microscopy images of ULV isolated from EEC-CM at 36 000×, and a magnified image at 100 000× (black box) shown in (**B**); the scale bar corresponds to 0.5 μm. (**C**) and (**D**) Immunostaining of EEC labelled with CD63 and MFGE8 antibodies, respectively; (**E**) Western blot showing the presence of MFGE8 and CD63 in EEC and ULV protein extracts; actin was used as a loading control. The position of molecular weight markers (kDa) is indicated at the left. The data shown are representative of three experiments. ULVs, Uterosome-like vesicles; EEC, endometrial epithelial cell; CM, conditioned medium.

protein extracts. These results show that EEC are able to secrete extracellular vesicles in the absence of serum or other stimulants. These vesicles were called 'uterosome-like' vesicles since they are secreted by a cell line, and have similar characteristics to those isolated from uterine fluid in humans (Ng *et al.*, 2013) and mice (Griffiths *et al.*, 2008).

ULVs stimulate human sperm capacitation even after a short co-incubation time

To test the possible effect of ULV on sperm capacitation, several experimental settings were used. Firstly, spermatozoa were incubated with control medium (HAM), conditioned medium from EEC (CM) or from a cell line derived from non-reproductive tissue (L929), for 4 h. As shown in Fig. 2A, the capacitation index was significantly increased about 4-fold under the presence of CM, in comparison to HAM culture medium (control). In contrast, L929 CM had no effect on sperm capacitation (nor cell motility or viability, data not shown), suggesting that the effect observed is specific to the CM derived from EEC. Moreover, when CM-treated spermatozoa were exposed to a 10 pM gradient of progesterone (an assay that selects capacitated spermatozoa by chemotaxis, Gatica *et al.*, 2013), the sperm capacitation index was significantly increased in the selected sperm population after the SSA assay (Fig. 2B). In addition, sperm viability and motility were not affected by CM (Supplementary Fig. S1). Since the transit of spermatozoa through the uterus takes only a few minutes (Suarez and Pacey, 2006), the effect of CM on the capacitation index was assessed after a short co-incubation time. Spermatozoa were incubated with CM (or HAM medium as a control) during 15 min, then washed by centrifugation for CM removal and further incubated for 0, 2 or 4 h in HAM medium. The brief 15 min contact with CM was sufficient to prompt sperm capacitation, but a significant, more than 3-fold increase was observed after 2 h of incubation (Fig. 2C; the inset shows the percentage of capacitated sperm after 4 h of incubation in culture medium alone).

Next, we verified whether ULVs were able to stimulate sperm capacitation. After seminal plasma removal, spermatozoa were incubated with different dilutions of the ULV fraction for 15 min. The capacitation index significantly increased as a function of the ULV dilution, with a maximum value at 20× ULV fraction, in comparison to HAM medium

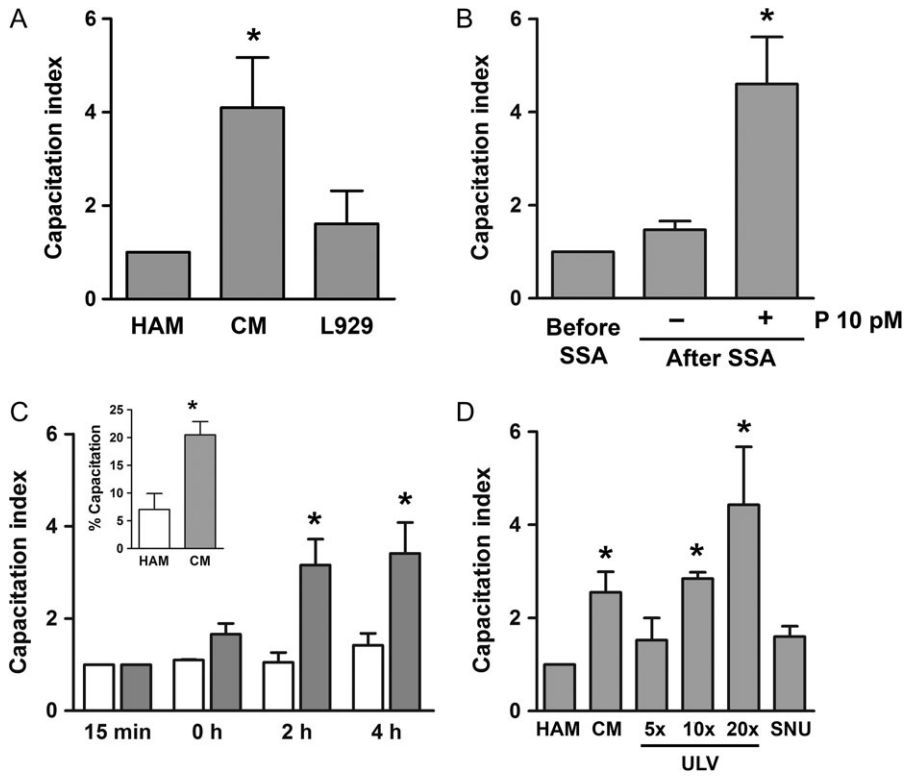

Figure 2 ULVs stimulate sperm's induced acrosome reaction. After seminal plasma removal, spermatozoa were used for different experimental settings, where the capacitation index was determined. (**A**) Spermatozoa were incubated in HAM medium, CM from EEC or L929 cells for 4 h; *significant difference vs. Ham medium. (**B**) Spermatozoa were exposed to EEC-CM for 4 h and then were selected by a chemotactic gradient of progesterone, determining the capacitation index before and after the SSA; *significant differences vs. after SSA without progesterone and before SSA. (**C**) Spermatozoa were exposed to HAM (open bars) or EEC-CM (filled bars) for 15 min, then the CM was removed by centrifugation and the spermatozoa were further incubated for 0, 2 or 4 h with HAM medium; the percentage of capacitated sperm after 4 h is shown in the inset; *significant differences vs. the corresponding 15 min and time 0. (**D**) Spermatozoa were incubated with several concentrations of the ULV suspensions (5×, 10× or 20×) for 15 min, as well as with CM, the supernatant of ULV ultracentrifugation (SNU) or HAM medium; *significant differences vs. HAM. Data are expressed as mean ± SEM of three to five experiments performed with samples from different donors. ULVs, Uterosome-like vesicles; CM, conditioned medium; EEC, endometrial epithelial cell; SSA, sperm selection assay.

and ULV supernatant controls (Fig. 2D), without affecting sperm viability or motility (Supplementary Fig. S2). As a whole, these results suggest that even a short interaction of spermatozoa with EEC-derived ULV prompts sperm capacitation.

In order to verify the results described above, we next assessed the effect of CM and ULV on sperm PY by Western blot and immunocytochemistry. Spermatozoa were incubated with non-capacitating medium, capacitating medium, CM, or medium containing ULV, for 15 min or 4 h. At the end of each incubation time, cells were processed for Western blotting. About 15 min of sperm incubation with CM or ULV was enough to stimulate PY, an effect that was enhanced after 4 h (Fig. 3A).

At the same time points, a sperm sample was used for PY detection by indirect immunofluorescence. Most of the cells were positive for PY, either with a light staining on the neck or a strong labelling in the flagellum principal piece (Fig. 3B), considering the latter pattern associated to capacitation (Barbonetti *et al.*, 2008). After 4 h of incubation, a significant increase was observed in the percentage of principal piece labelled cells in spermatozoa incubated in CM or ULV compared to non-capacitating medium (Fig. 3C). These results suggest that CM and ULV secreted by EEC induce an increase in the levels of PY in human spermatozoa, after even a brief stimulus contact.

ULVs interact with human spermatozoa

ULVs were first labelled with the fluorescent red lipophilic membrane dye PKH-26 and then co-incubated with human spermatozoa for 15 min or 15 min followed by 3 h incubation in HAM medium. After exposing spermatozoa to labelled ULV for 15 min, a red fluorescence spotting was detected in the sperm head, tail or both regions (Fig. 4A). Moreover, the interaction of spermatozoa with ULV was also observed when spermatozoa were incubated for 15 min with ULV and then further exposed to HAM medium without ULV for 3 h (Fig. 4B). In contrast, when assays were performed with no labelled ULV or PBS, spermatozoa showed no fluorescent labelling (Fig. 4C). These results suggest that the interaction of ULV with spermatozoa might be mediated by membrane fusion, and that a brief interaction time is enough for this process to occur.

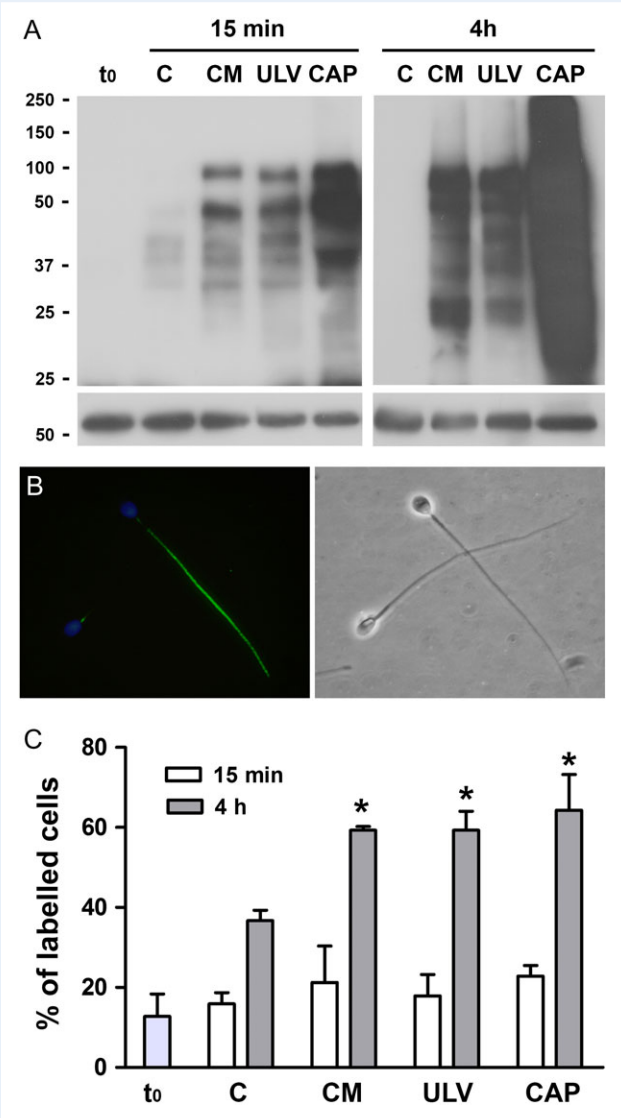

Figure 3 ULVs induce an increase in sperm protein tyrosine phosphorylation. Spermatozoa were incubated with non-capacitating medium, CM, ULV or capacitating medium for 15 min or 4 h. (**A**) The level of PY was analysed by Western blot using anti-phosphotyrosine antibody (upper panels) and anti-tubulin as protein loading control (lower panels). (**B**) Sperm immunostaining showing two cells nuclei stained with DAPI, one of them labelled for PY in the principal piece, showing the corresponding phase contrast image. (**C**) The percentage of sperm showing the principal piece stain was determined immediately after seminal plasma removal (t_0) and after 15 min or 4 h incubation in non-capacitating medium (C), CM, non-capacitating medium containing ULV, or capacitating medium (CAP) as positive control. *Significant differences vs. non-capacitating medium. Data are expressed as mean ± SEM of three experiments performed with samples from different donors. ULVs, Uterosome-like vesicles; PY, tyrosine phosphorylation; CM, conditioned medium.

Discussion

Even though the first description of sperm capacitation a long time ago was associated with sperm residence in the uterus, it was long believed that spermatozoa might not be affected by the uterine environment due to their rapid transit through this region of the reproductive tract. Here we show that extracellular vesicles secreted by EECs rapidly interact with the sperm plasma membrane, prompting the fertilizing capability of human spermatozoa.

Early studies suggested that sperm interaction with the uterus might influence sperm fertilizing capacity and that the uterus and oviduct may synergistically accelerate capacitation (Adams and Chang, 1962; Hunter, 1969; Hunter and Hall, 1974a, b). For instance, when boar spermatozoa first remained in the uterus for several hours and then were used to fertilize eggs *in vitro*, most of them were activated; however, sperm residence in the caudal isthmus caused the activation of half of the eggs (Hunter and Rodriguez-Martinez, 2004). It was also found that the co-incubation of endometrial cells with human sperm may support capacitation and the penetration of hamster oocytes (Fusi *et al.*, 1994; Lai *et al.*, 1996).

To evaluate a possible influence of EEC secretion on sperm physiology, human spermatozoa were incubated for 4 h in CM derived from

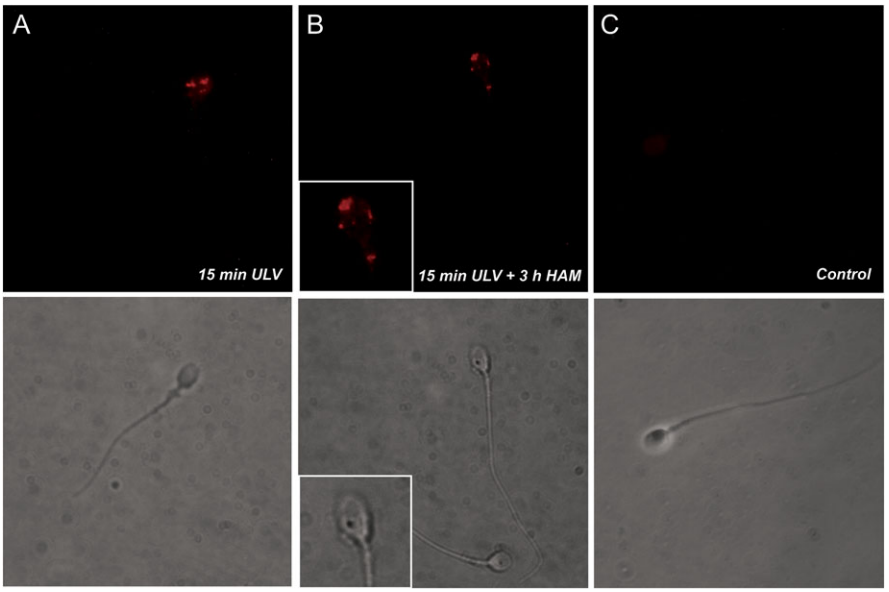

Figure 4 ULVs are able to interact with human spermatozoa. Spermatozoa were incubated with PKH26-labelled ULV for 15 min (**A**) or for 15 min followed by 3 h incubation in HAM medium without ULV (**B**); inset: magnified region of the sperm head. Sperm samples were incubated with non-labelled ULV as negative controls (**C**). The corresponding phase contrast images are shown at the bottom panel. The images shown are representative of three experiments performed with samples from different donors. ULVs, Uterosome-like vesicles.

these cells. The level of capacitated spermatozoa and those recruited by chemotaxis increased 3- to 4-fold in the presence of CM. Even a short, 15 min incubation of spermatozoa with CM was enough to increase the level of capacitated cells 3- to 4-fold. These results suggest that even a fast passage through the uterus environment might prompt sperm capacitation.

Even though there seems to be an interaction between endometrial cells and spermatozoa, the mechanism mediating this communication is unknown. A few studies have attempted to identify the intrauterine effectors that stimulate spermatozoa to acquire their functional competence during their transit toward the oocyte (Banerjee and Chowdhury, 1994; Banerjee and Chowdhury, 1995). For instance, the cytokine interleukin-6 secreted by EECs (whose expression increases upon ovulation; von Wolff *et al.*, 2002) stimulates the induced acrosome reaction in human sperm and PY after 4 h of incubation (Laflamme *et al.*, 2005).

The role of extracellular vesicles in cell-to-cell communication has only recently been investigated in several cell types. Such interaction may be mediated by direct binding with the cell surface or by fusion with the target cell plasma membrane (Raposo and Stoorvogel, 2013). Specifically, the interaction of somatic cells with gametes via extracellular vesicles has been recently reported in animal models. For instance, epididymosomes (vesicles secreted by epithelial cells of the cauda epididymis) can transfer molecules to bovine epididymal spermatozoa *in vivo* (Caballero *et al.*, 2013). Murine oviductosomes produced by oviductal epithelial cells during the oestrus cycle show in their membrane the calcium efflux pump PMCA4a (plasma membrane Ca^{2+} ATPase 4a), which is also associated with calcium homoeostasis in spermatozoa (Al-Dossary *et al.*, 2013). Moreover, oviductosomes and exosomes are able to fuse with the sperm membrane, delivering the PMCA4a (Al-Dossary *et al.*, 2015). In addition, human uterosomes have been implicated in embryo-endometrial cross-talk during implantation (Ng *et al.*, 2013).

Here we showed that EEC release extracellular vesicles to the culture medium after 24 h of incubation in the absence of serum or any other supplement, indicating that ULV are secreted by these cells in a constitutive manner. These ULV have a characteristic cup-shape morphology with a diameter from 50 to 200 nm, showing the presence of the membrane proteins CD63 and MFGE8, the usual markers for most extracellular vesicles (Mathivanan *et al.*, 2010). In addition to their use as extracellular vesicle markers, these proteins may also have a specific role in endometrium and sperm physiology. Indeed, the expression of MFGE8 in the human endometrium is upregulated during the implantation window (Franchi *et al.*, 2008) and its secretion by EECs via extracellular vesicles was stimulated by human chorionic gonadotropin (Sarhan *et al.*, 2013). It is also involved in mouse sperm–egg binding (Shur *et al.*, 2004) as well as in other cell communication processes (Raymond *et al.*, 2009). These observations suggest that this multi-domain protein might participate not only in the regulation of sperm preparation for fertilization but also in embryo-endometrium cross-talk during implantation.

Our results showed that even a short co-incubation of spermatozoa with ULV stimulates sperm capacitation determined by the increase in the level of induced acrosome reaction and the induction of PY. The latter is in agreement with a previous report showing the effect of primary endometrial cell-derived CM on sperm PY (Laflamme *et al.*, 2005).

We hypothesized that the sperm capacitation induced by EEC products could be mediated through the fusion of the ULV with spermatozoa. After a short co-incubation of spermatozoa with fluorescent

labelled ULV, the sperm cells acquired the red fluorescent staining which persisted even after a further incubation with culture medium in the absence of labelled ULV. These results show that ULV might be transferred to the sperm surface by a fusion mechanism, with long-lasting consequences for the spermatozoon, since vesicle labelling can be observed in the sperm surface for at least several hours. A similar fast material transfer was observed between prostasomes and spermatozoa (Ronquist *et al.*, 2011), where the stained DNA transference was maximum at 15 min, with the signal remaining constant for 105 min. In agreement with the effect of ULV on capacitation priming, others observed that the interaction of spermatozoa with the uterus may remove cholesterol and binding proteins from their membrane, which are events associated with sperm capacitation (Lalumiere *et al.*, 1976; Hobkirk, 1985; Banerjee and Chowdhury, 1995; Griffiths *et al.*, 2008). Even though sperm transit through the uterus takes just a few minutes (Suarez and Pacey, 2006), it seems to be sufficient to promote sperm physiological processes mediated by uterus-derived extracellular vesicles, as observed in other cellular models (Parolini *et al.*, 2009).

The capability of spermatozoa to briefly interact with ULVs supports the notion that any step of sperm transport may have physiological consequences, despite the interaction lasting for only a limited period of time. This way of communication of spermatozoa with cell products of uterine origin opens new frontiers of investigation (e.g. the signalling molecules involved), shedding light on the sperm processes that prepare the male gamete for fertilization, which might have implications for human infertility treatment.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

A.F. and L.C.G. conceived and designed the experiments, A.B. performed experiments, A.F. and M.C. performed the experiments and analysed the data, M.C. helped in drafting the article and A.F. and L.C.G. wrote the article. H.A.G. interpreted data and revised the article.

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Conflict of interest

None declared.

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