Oviduct epithelial cell-derived extracellular vesicles promote the developmental competence of IVF porcine embryos

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Abstract. Assisted reproductive technology has increased the efficiency of animal reproduction. However, polyspermy is a significant limitation of porcine *in vitro* fertilization (IVF). Therefore, reducing the polyspermy rate and improving monospermic embryos is crucial. Recent studies have reported that oviductal fluid, along with its contents of extracellular vesicles (EVs), enhanced the fertilization process and supported embryo development. Consequently, the present study investigated the effects of porcine oviduct epithelial cells (OEC-EVs) on sperm-oocyte interactions during porcine IVF and evaluated *in vitro* embryo developmental competence outcomes. During IVF embryo development, the cleavage rate was significantly higher in the group treated with 50 ng/ml OEC-EVs compared with the control group (67.6±2.5 vs. 57.3±1.9; P<0.05). Furthermore, the OEC-EV group had significantly more embryos (16.4±1.2 vs. 10.2±0.8; P<0.05), and the polyspermy rate significantly decreased (32.9±2.5 vs. 43.8±3.1; P<0.05) compared with that of the control group. Additionally, the fluorescence intensities of cortical granules (3.56±0.47 vs. 2.15±0.24; P<0.05) and active mitochondria (8.14±0.34 vs. 5.96±0.38; P<0.05) were significantly higher in the OEC-EV group compared with those in the control group. In conclusion, OEC-EV adsorption and penetration crosstalk between sperm and oocytes was observed. OEC-EV treatment was demonstrated to significantly improve the concentration and distribution of cortical granules in oocytes. Furthermore, OEC-EVs also increased oocyte mitochondrial activity, reduced polyspermy and increased the IVF success rate.

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Introduction

In vitro embryo production in pigs is a crucial tool for the development of porcine models suitable for human biomedical research due to their anatomical and physiological similarities to human organs (1). As a result, xenotransplantation using pig tissues and organs is feasible (2,3). Assisted reproductive technology has improved animal reproduction efficiency. However, polyspermy is a major limitation of porcine *in vitro* fertilization (IVF), which leads to chromosomal abnormalities in embryos (4,5). Hence, reducing the polyspermy rate and enhancing monospermic embryos are crucial for successful porcine IVF.

Standard IVF systems utilize hundreds of spermatozoa, often resulting in a rise in the number of spermatozoa that penetrate the oocyte (6). Polyspermy is strongly linked to the initial number of capacitated spermatozoa during the IVF process (7). One strategy to decrease the incidence of polyspermy is to reduce the absolute number of spermatozoa, but this results in a low oocyte penetration rate. Consequently, certain IVF conditions which mimic the oviductal environment have been examined to minimize polyspermy *in vitro*, including shortening the co-incubation time and manipulating the IVF equipment or culture conditions (8). These studies have effectively reduced the incidence of polyspermy. However, the available IVF systems do not entirely mimic *in vivo* conditions (9), and the percentage of monospermic oocytes relative to the total number inseminated during the IVF remains between 20-30% (6-8).

In mammals, the oviduct is composed of four parts; the infundibulum, responsible for collecting ovulated oocytes; the ampulla, where fertilization occurs; the isthmus, for preimplantation embryo development; and the uterotubal junction, which transits the embryos to the uterus (10). Oviductal fluid (OF) and extracellular vesicles (EVs) secreted by the oviduct epithelial cells (OEC; OEC-EVs) have been shown to enhance fertilization, protect against polyspermy and sperm entry, and aid embryonic development (11,12). EVs are nano-sized membranous vesicles containing molecular cargo, including micro RNAs (miRNAs), mRNAs, proteins, lipids, and metabolites, that can be easily delivered and fused with cell plasma

membranes (13,14). They serve a crucial role in transmitting information and organelles between living cells (15,16) and can modify the epigenetic signature by transferring small molecule RNAs between donor cells (17,18). EVs are present in the female genital tract fluids and are crucial for gamete fertilization and early preimplantation embryonic development (19-23). Furthermore, OEC-EVs reduce apoptosis and improve embryo quality by reducing reactive oxygen species (ROS) (24). The first moments of embryonic-maternal communication occur in the oviduct, where both maternal and embryonic EVs are exchanged to prepare for the maternal recognition of pregnancy (25).

Recently, the beneficial effects of OEC-EVs on porcine embryos generated through parthenogenesis and cloning technology (24) and the improved derivation of trophoblast stem cells were reported (26). Thus, the aim of the present study was to mimic the *in vivo* conditions and reduce polyspermy by investigating the effects of porcine OEC-EVs on the interaction between sperm and oocyte during IVF and subsequent *in vitro* embryonic development. The potential effects of OEC-EVs on the developmental competence of fertilized oocytes was explored by examining the uptake of EVs by both sperm and oocytes, and the effects on oocyte mitochondrial activity and cortical granule's reaction.

Materials and methods

Chemicals. All chemicals and reagents used in this work were purchased from MilliporeSigma unless otherwise specified.

Oocyte collection and oocyte in vitro maturation (IVM). Oocyte IVM was performed according to a previously reported method (27). Briefly, porcine ovaries and uteri including the oviducts were obtained from a slaughterhouse in Daejeon City and transported to the laboratory in normal saline solution containing 75 mg/ml penicillin and 50 mg/ml streptomycin. The temperature of the solution was maintained between 34-36˚C. Cumulus-oocyte complexes (COCs) were taken from antral follicles (3-8 mm in diameter) with an 18-gauge needle attached to a 10 ml syringe. COCs with three or more layers of cumulus cells and homogeneous ooplasm were selected and washed three times in HEPES-buffered Tyrode's media containing 0.05% (w/v) polyvinyl alcohol (PVA). COCs (n=50) were *in vitro* matured in 500 μl of maturation medium consisting of bicarbonate-buffered tissue culture medium 199 (TCM-199; Gibco, Thermo Fisher Scientific, Inc.), 10% (v/v) porcine follicular fluid, 0.91 mM sodium pyruvate, 0.57 mM L-cysteine, 10 ng/ml epidermal growth factor and 1 μg/ml insulin with 10 IU/ml equine chorionic gonadotropin (eCG), 10 IU/ml human chorionic gonadotropin (hCG) and 75 μg/ml kanamycin in 4-well dishes (SPL Life Sciences). COCs were incubated at 38.5˚C in 5% CO_2 in humidified air incubator (Astec Co., Ltd.) for 22 h and then were cultured for an additional 22 h in a hormone-free IVM medium (24).

Porcine oviductal epithelial cell (pOEC) collection. The post-ovulatory oviducts of multiparous female pigs (sows) were obtained from a local butcher and transported to the laboratory according to the aforementioned method. pOECs were isolated by mechanically scraping the oviduct with a slide and then centrifuged three times at 700 x g for 5 min at room temperature in 10 ml of DMEM supplemented with 10% (v/v) FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% (v/v) antibiotic-antimycotic solution (Gibco; Thermo Fisher Scientific, Inc.). pOECs were cultivated in a 100 mm Falcon tissue culture plate with a culture medium at 39˚C and 5% CO_2 in a humidified environment. On day 3 of the initial culture, the pOEC outgrowths were examined, after which they were grown for a further 7 days. pOECs were trypsinized in 0.05% trypsin-EDTA, washed in phosphate-buffered saline (PBS) thrice and grown in DMEM without FBS for 48 h to obtain the conditioned medium for isolating OEC-E (24).

EV isolation, purification, and characterization. The supernatant was used to separate EVs using the Total EV Isolation kit after being centrifuged at 2,000 x g for 30 min at room temperature to remove cells and cell debris from the conditioned medium (Invitrogen; Thermo Fisher Scientific, Inc.). The kit reagent was combined with the supernatant, stirred vigorously, and incubated overnight at 2-8˚C. The EV pellets were then frozen at -80˚C until future investigations. The EV pellet was resuspended in modified Tris-buffered medium (mTBM) for experimental purposes, and the final protein concentration was adjusted to 50 ng/ml using a NanoDrop 2000 (Thermo Scientific; Thermo Fisher Scientific, Inc.). The EVs were visualized using transmission electron microscopy (TEM) after negative staining with 2% uranyl acetate as described previously (24).

ZetaView nanoparticle tracking analysis (NTA). A ZetaView PMX 110 (Particle Metrix GmbH) instrument was used for NTA as previously described (28). Briefly, 1 ml of diluted EVs pellet (in 1X PBS) was loaded into the device to measure each sample at 11 different positions and two reading cycles per position. After an automated analysis and outlier removal, the mean, median, and mode sizes (indicated as diameter) and the concentrations were calculated using ZetaView SP7 software (version 8.05.14; Particle Metrix) and Microsoft Excel 365 (version 2205, Microsoft Corporation). Device calibration was performed using 100 nm polystyrene particles (Thermo Fisher Scientific, Inc.).

IVF and experimental groups. *In vitro* matured oocytes with the extruded first polar body were washed twice with mTBM, before being cultured in fresh droplets of mTBM (15 oocytes/50 μl) at 39˚C in a humidified atmosphere of 5% CO_2. In the OEC EVs group, mTBM contained EVs protein of 50 ng/ml (EVs-mTBM), while the control group was EVs-free. Chilled pig semen was commercially obtained from Darby Genetics Inc., South Korea. Oocytes were co-incubated with $5.0x10^5$ sperm/ml for 20 min. After co-incubation, the attached sperm were discarded from the zona pellucida (ZP) by gentle micro pipetting and the oocytes were washed twice in mTBM. Oocytes were then incubated in mTBM or EVs-mTBM without sperm for an additional 6 h in the same culture conditions. IVF conditions with either OEC-EVs-supplemented or plain culture mediums were compared for polyspermy, cortical granules' reaction, and mitochondrial staining. *In vitro* embryonic development was monitored after culturing the IVF oocytes in PZM-5 medium (Functional Peptides Research Institute Co. Ltd.) for 7 days at 38.5˚C in 5% CO_2 and 5% O_2 in a humidified incubator.

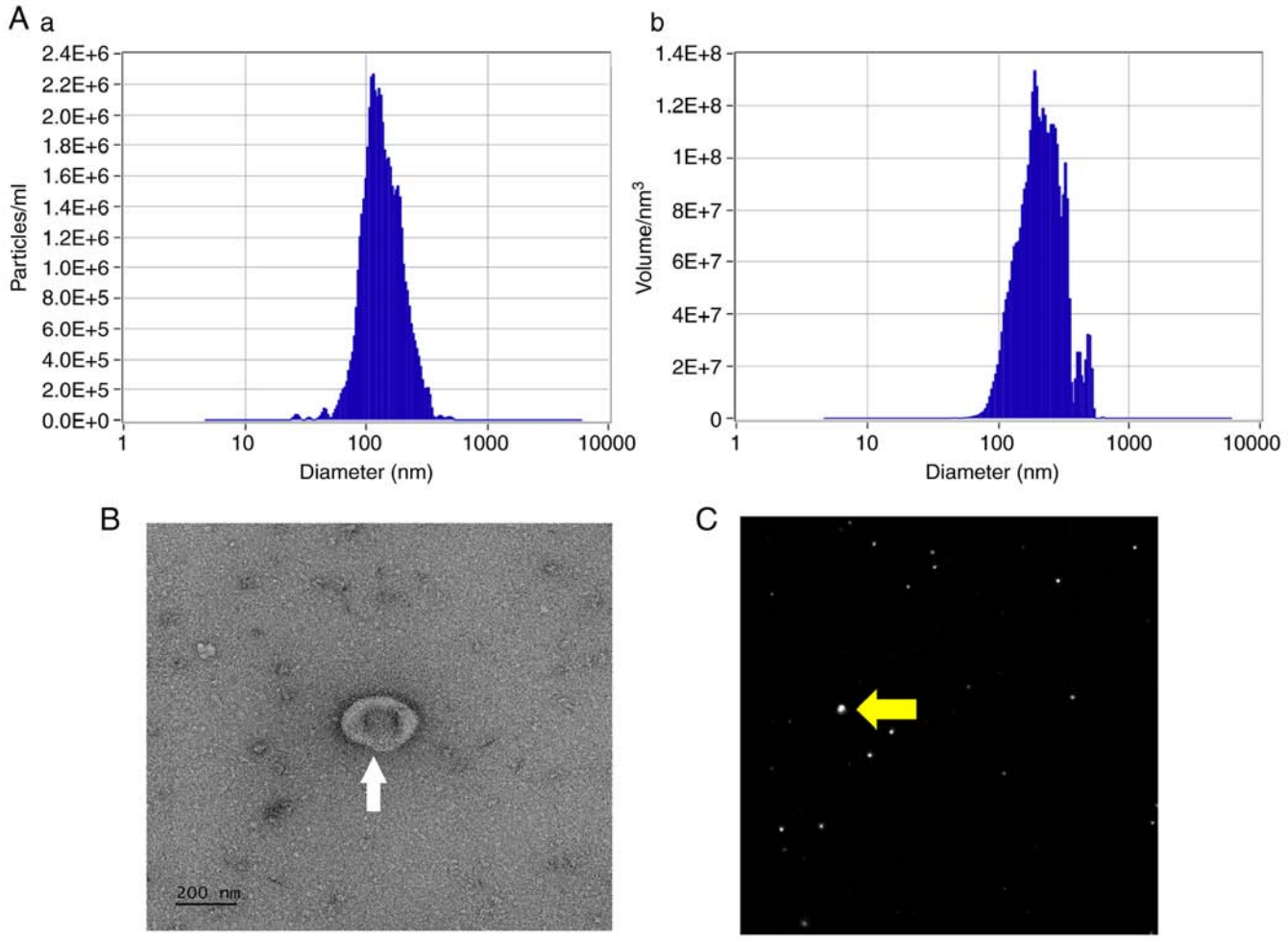

Figure 1. Concentration and visualization of EVs isolated from pOECs. (A) ZetaView nanoscale tracking analysis was used to assess the concentrations of the isolated pOECs EVs (particle/ml) (a) and the size or volume (nm^3) (b). (B) Transmission electron microscope image of pOECs EVs (white arrow), scale bar, 200 nm. (C) Nanovesicles were visualized using fluorescent nanoparticle tracking analysis and showed as fluorescence signals (yellow arrow). pOECs, porcine oviduct epithelial cells; EV, extracellular vesicles.

Penetration and polyspermy. After 18-20 h of IVF, the embryos were fixed using 3.7% (w/v) paraformaldehyde (PFA) for 30 min and washed in PBS/PVA three times at room temperature. The embryos were stained with 10 μg/ml Hoechst 33342 in PBS/PVA for 30 min at room temperature. After staining, the embryos were washed three times in PBS/PVA. The embryos were examined for penetration and pronucleus (PN) formation using a fluorescence microscope. Embryos with one nucleus and polar body (Fig. 1A) were considered non-penetrated. Embryos with one female PN and one male PN in the cytoplasm were considered to be monospermic (Fig. 1B). Embryos with more than one nuclear staining (i.e., sperm or male PNs) were considered to be polyspermic (Fig. 1C).

EV labeling and uptake. After OEC-EV isolation, the OEC EVs were mixed with the lipophilic PKH67 dye according to the manufacturer's instructions, and OEC-EVs were isolated to remove the excess free PKH67 dye according to the manufacturer's protocol (29,30). EVs were then supplemented with sperm or oocytes for 6 h to monitor their uptake using a confocal microscope. For negative control staining, the plain conditioned medium was mixed with PKH67 and processed through the same EV labeling procedure.

Cortical granule (CG) staining. Three times, *in vitro* matured oocytes, were washed with PBS containing 0.1% (PBS/PVA). The OEC EVs group oocytes were treated for 20 min with OEC EVs in mTBM and the control group was kept in mTBM without EVs. The oocytes were then fixed using 3.7% (w/v) PFA at room temperature for 30 min, followed by three 10 min washes in PBS/PVA per oocyte. This was followed by a 1% Triton X-100 treatment for 30 min and three 10-min washes in PBS/PVA. Oocytes were then cultured for 30 min at room temperature in FITC-labeled Peanut Agglutinin (PNA) diluted 1:500 in PBS in a dark box. Following staining, the oocytes were washed for 10 min in PBS/PVA three times. Finally, the nuclei were stained with DAPI for 10 min, mounted on slides, and images were acquired using a DMi8 confocal microscope (Leica Microsystems GmbH).

Active mitochondria staining. After 18-20 h, the IVF embryos were washed with PBS/PVA three times and were then cultivated for 30 min at 37˚C in 500 nM MitoTracker Red CMXRos (cat. no. M7512; Invitrogen; Thermo Fisher Scientific, Inc.) in a dark box. After staining, the embryo was washed three times for 10 min in PBS/PVA. Each embryo was fixed in 3.7% PFA at room temperature for 30 min, followed by three 10 min

Table I. Effect of OEC-EVs during penetration and polyspermy after *in vitro* fertilization.

Group	Total number	Penetration (% ± SD)	Polyspermy (% ± SD)
Control	203	151 (74.4±4.2)	90 (43.8±8.7)
OEC-EVs	209	155 (74.2±6.6)	67 (32.9±7.0)[a]

[a]P<0.05 (Control vs. OEC-EVs; unpaired Student's t-test). OEC-EV, oviduct epithelial cell-derived extracellular vesicles; SD, standard deviation. A total of eight replicates were performed (24-27 oocytes each replicate).

Table II. Effect of OEC-EVs on the development of the *in vitro* fertilized embryo.

Group	Total number	Cleaved (% ± SD)	Developed to Bl (% ± SD)
Control	293	168 (57.3±6.2)	30 (10.2±2.8)
OEC-EVs	318	215 (67.6±8.4)[a]	52 (16.4±4.0)[a]

[a]P<0.05 (Control vs. OEC-EVs; unpaired Student's t-test). Bl, blastocyst; OEC-EV, oviduct epithelial cell-derived extracellular vesicles; SD, standard deviation. A total of 12 replicates were performed (24-27 oocytes each replicate).

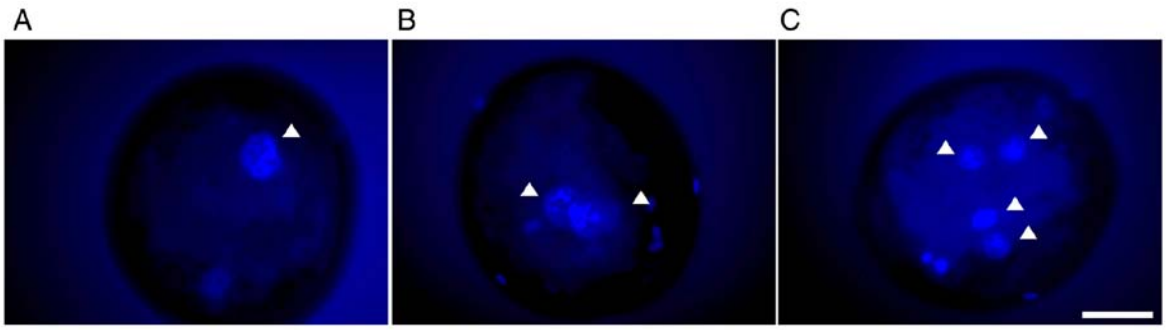

Figure 2. Representative micrographs of porcine *in vitro* fertilized oocyte nuclei (white arrows) stained with DAPI. (A) Non-penetration oocyte, (B) monospermic fertilized and (C) polyspermic fertilized oocytes. Scale bar, 50 μm.

washes in PBS/PVA. Finally, the nuclei were stained using DAPI for 10 min at room temperature, mounted on slides, and images were acquired using a DMi8 confocal microscope (Leica Microsystems GmbH).

Statistical analysis. Lieven's test and Kolmogorov-Smirnov test were used to confirm the homogeneity of variance and the normality of distribution, respectively. At least 10-20 replicates were performed per experiment. For data normalization, the mean expression level value was calculated from the control group, and the means were normalized to an arbitrary unit (1-fold) by dividing the values against the control group. The image intensities were analyzed using ImageJ (version 1.53k; National Institutes of Health). Data were analyzed using unpaired Student's t-test using SPSS software (SPSS 22.0; IBM Corp). P<0.05 was considered to indicate a statistically significant difference.

Results

Porcine OEC culture and EV characterization. OEC confluent cells were achieved as reported previously (24) and the expression of oviduct epithelial cells' specific gene expression [oviduct-specific glycoprotein (OVGP1)] and proteomics were performed for further confirmation. OEC-EVs were isolated, visualized, and characterized using TEM and NTA, the mean diameter of the isolated EVs was 146.2±57.2 nm, and the mean concentration was $5.3x10^9$ particles/ml (Fig. 1A-C).

Effects of OEC-EVs on sperm penetration and polyspermy. Sperm penetration into the oocyte was detected 18-20 h after IVF. Overall, non-penetration and non-fertilized oocytes (Fig. 2A), penetrated and monospermic fertilized embryos (Fig. 2B), and penetrated and polyspermic abnormally fertilized embryos (Fig. 2C) were observed. The penetration rate did not differ between the OEC-EV and control groups (74.4±1.5 vs. 74.2±2.3, P=0.27). However, the polyspermy rate was significantly lower in the OEC-EV group when compared with the control group (32.9±2.5 vs. 43.8±3.1; P=0.0026; Table I).

Effects of OEC-EVs on IVF embryo development. The cleavage rate differed significantly between the OEC-EV and the control groups (67.6±2.5 vs. 57.3±1.9; P=0.0028). Furthermore, the OEC-EV group had significantly more embryos than the control group (16.4±1.2 vs. 10.2±0.8; P=0.0001; Table II).

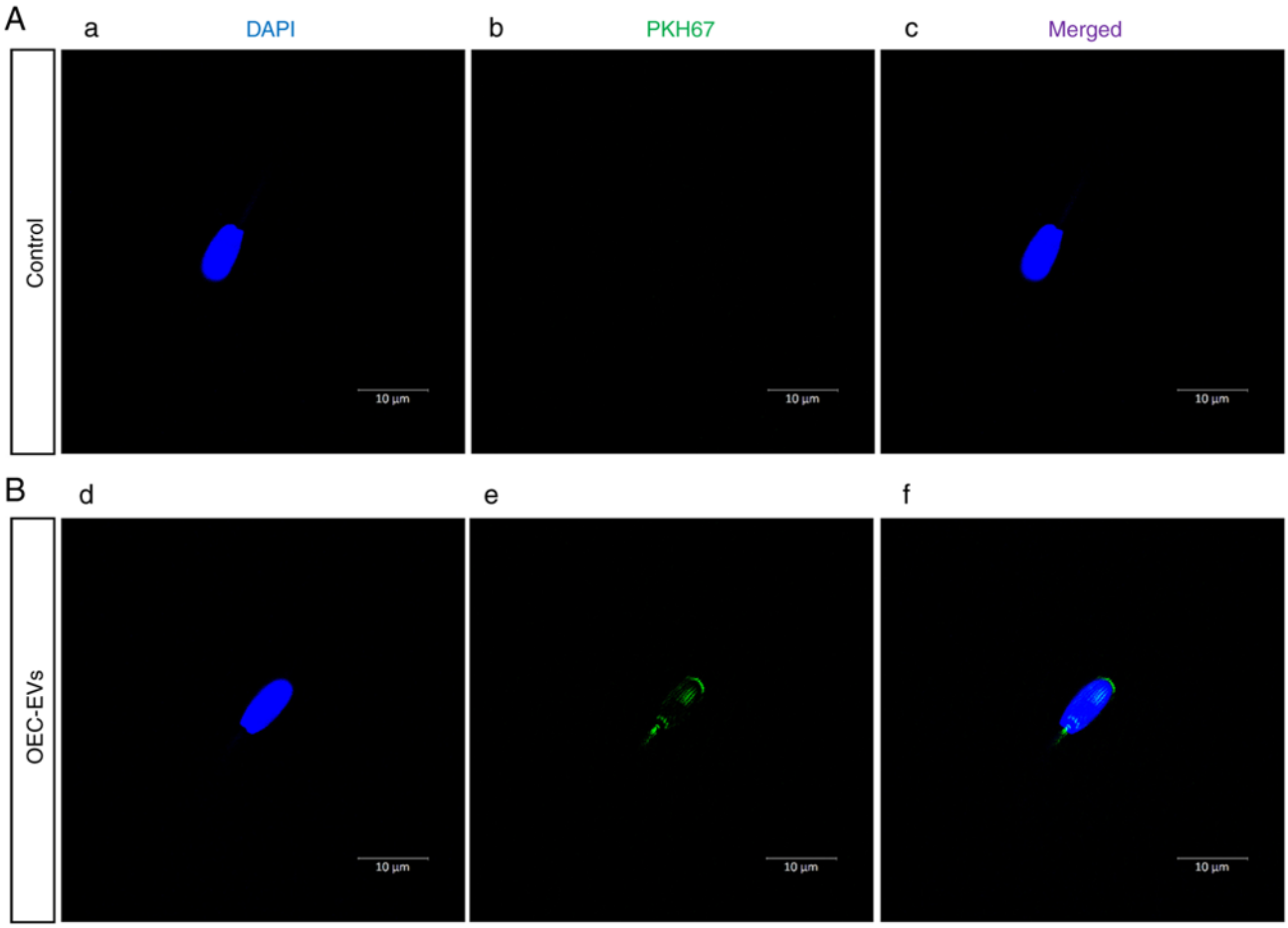

Figure 3. PKH67 (green fluorescent)-labeled OEC-EVs bound with sperm. Images were captured in control (A) and (B) after co-incubation of labeled EVs with oocytes for 6 h (a and d) sperm nuclear staining with DAPI, (b and e) PHK67-labeling of the extracellular vesicles and (c and f) Merged DAPI and PKH67 images. Scale bar, 10 μm. OEC-EVs, oviduct epithelial cell-derived extracellular vesicles.

Effects of OEC-EVs on sperm and oocyte communication. In the OEC-EV group, it was observed that OEC-EVs attached to the heads and tails of sperm (Fig. 3) and OEC-EVs that entered the oocyte through the cell membrane (Fig. 4), which was not observed in the control group.

Effects of OEC-EVs on CGs. The CG distribution in the control and OEC-EV groups after FITC-PNA staining were presented (Fig. 5A). The OEC-EV group had significantly higher fluorescence intensity values than those in the control group (3.56±0.47 vs. 2.15±0.24; P<0.05; Fig. 5B).

Effects of OEC-EVs on mitochondrial activity. Mitochondrial activity was detected in both control and OEC-EV-supplemented groups using MitoTracker Red staining (Fig. 6A). The fluorescence values of active mitochondria were higher in the OEC-EV group compared with those in the control group (8.14±0.34 vs. 5.96±0.38; P=0.00007; Fig. 6B).

Discussion

The results of the present study indicated that OEC-EVs support successful porcine IVF by enhancing oocyte mitochondrial activity, reducing polyspermy, and supporting the CG reaction. Polyspermic oocyte penetration is a common reason for decreased IVF success and low blastocyst rates in porcine species (4,9,31). Additionally, previous studies have reported that oxidative stress can alter CG distribution (32-34).

First, the effects of EVs on polyspermy were investigated. Amongst other things, oocytes prevent polyspermy by producing CGs that contain enzymes to harden the ZP and prevent subsequent spermatozoa fusion. Recently, EVs have emerged as an important component of several biological fluids, serving a crucial role in communication among different reproductive cells (35). The present study demonstrated that OEC-EVs significantly increased CG content (Fig. 5), which supported previous findings (12,36-38). Coy *et al* (38) and Batista *et al* (37) reported that incubation of oviductal fluid with porcine IVM oocytes caused ZP hardening, increased ZP resistance to proteolytic digestion, and reduced sperm binding to ZP, subsequently decreasing the polyspermy incidence. The protein cargo contents of OEC-EVs, specifically OVGP1 (22), could be related to increasing monospermy by masking the protease-cleaving sites of ZP proteins and causing ZP hardening (39-41). Furthermore, the molecular mechanisms regarding CG formation and release are not well-known. Nonetheless, six genes have been associated with CG distribution after IVM: Rho/Rac guanine nucleotide exchange factor 2, microtubule-associated protein 1B, C-X-C motif chemokine ligand 12, fibronectin 1, DAB adaptor protein 2, and SRY-box transcription factor 9. However, the expression

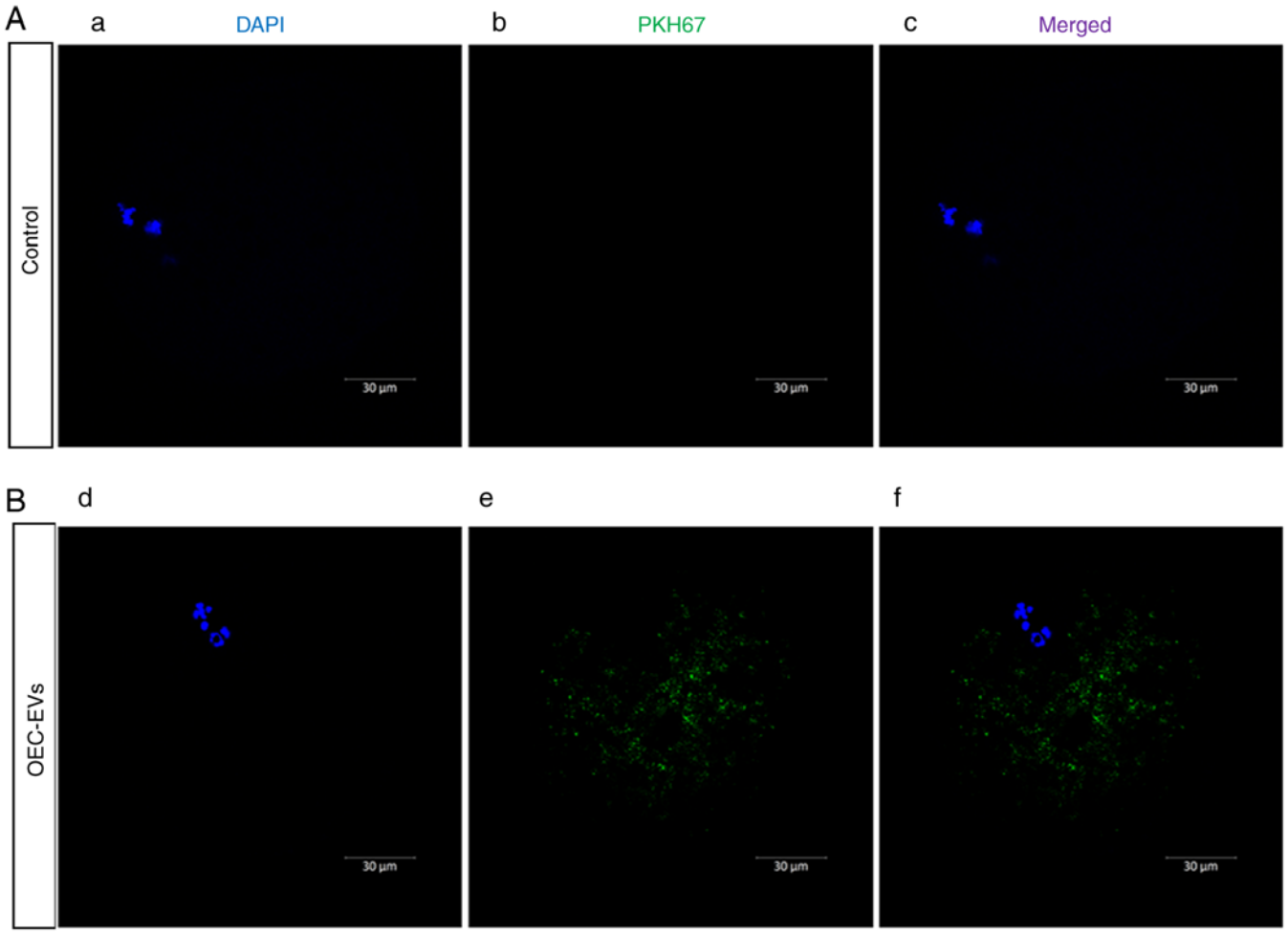

Figure 4. PKH67 (green fluorescent)-labeled OEC-EVs bound with oocytes. Images were captured in control (A) and after co-incubation of labeled EVs with oocytes for 6 h (B). The images present (a and d) oocyte nuclear staining with DAPI, (b and e) PHK67 labeling of the extracellular vesicles, and (c and f) merged DAPI and PKH67 images. Scale bar, 30 μm. OEC-EVs, oviduct epithelial cell-derived extracellular vesicles.

of these genes decreased after IVM (42). The main component of CG is metalloproteinase ovastacin (43), and it is hypothesized that the OEC-EV contents indirectly enhance ovastacin mRNA expression. Importantly, evidence suggests that EVs penetrate the ZP and release their cargo contents inside the ooplasm (36,44), similar to what was observed in the present study (Fig. 4).

The effects of OEC-EVs on oocyte mitochondrial activity were investigated to identify possible mechanisms related to EVs and to improve porcine IVF success. The results of the present study demonstrated that EV treatment increased mitochondrial activity. A previous study suggested that fertilization levels are related to oocyte quality, particularly mitochondrial activity (45). The number and distribution of active mitochondria in an oocyte serve a crucial role in regulating the fertilization of sperm by providing ATP and Ca^{2+} ion responses (46). Abnormal changes in mitochondria, lipid droplets, calcium release after electro-activation, and the adenosine triphosphate (ATP) and glutathione content in oocytes during aging may result in poor developmental competence of parthenotes (47). OEC-EVs enhanced mitochondrial activity (Fig. 6), providing a better ATP environment for nuclear reprogramming and good conditions for chromosome expansion into chromatin (48,49). Therefore, EVs may initiate several activities in the cytoplasm, which allow the egg to acquire energy and reach a mature state. *In vivo* and *in vitro*, oviduct EVs have large amounts of protein related to ATP, adenyl ribonucleotide, purine nucleotide binding, glucose and hexose catabolic processes, and glycolysis (22). Thus, it was hypothesized that OEC-EVs increase mitochondrial activity (16,50) and help provide the large quantity of ATP required for successful fertilization.

Furthermore, the results of the present study indicated that OEC-EVs mediate sperm-oocyte interaction within an *in vivo* environment. Fallopian tube-secreted EVs contribute to improving the developmental competence and the fertilization of oocytes by reducing the ROS levels, eliciting anti-apoptotic effects, improving lipid metabolism, and increasing the expression of reprogramming-related genes (11,24). Many studies have presented isolation, characterization, and microRNA analyses of oviduct EVs in various species (12,25,35,36,51). Oviduct EV characterization identified proteins with critical roles in the gamete/embryo-oviduct interactions, such as OVGP1, heat shock protein (HSP) 90, HSPA8, HSP70, gelsolin and ezrin (22). In addition, spermatozoa undergo several modifications in the female reproductive tract for the capacitation process to acquire a complete fertilizing ability (52,53). Porcine oviduct EVs attach to sperm membranes (54) and participate in the maintenance of sperm viability and reducing motility, functions associated with the oviduct sperm

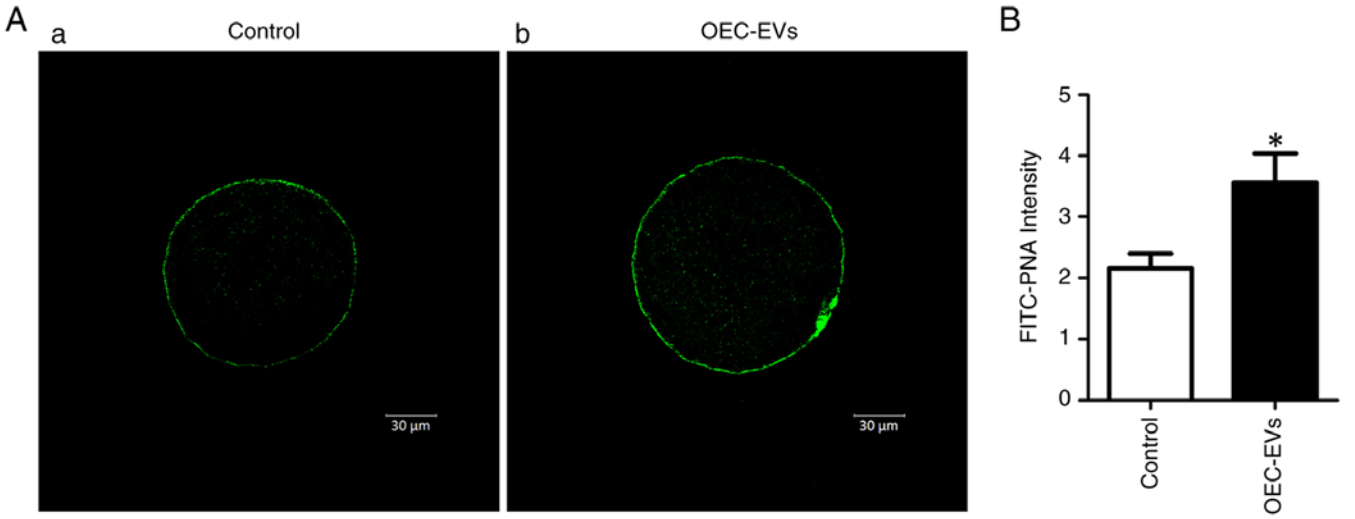

Figure 5. Detection of cortical granules (CG) in porcine oocytes after EVs supplementation. (A) Porcine oocyte cortical granules stained by FITC-PNA, (a) control, and (b) OEC-EV co-cultured oocytes. Scale bar, 30 μm. (B) The FITC-PNA intensities differed between the control and OEC-EV oocyte membranes. Three replicates (19 oocytes each) were compared. *P<0.05 (Control vs. OEC-EVs; unpaired Student's t-test). FITC-PNA, fluorescein isothiocyanate-labeled peanut agglutinin; OEC-EV, oviduct epithelial cell-derived extracellular vesicle.

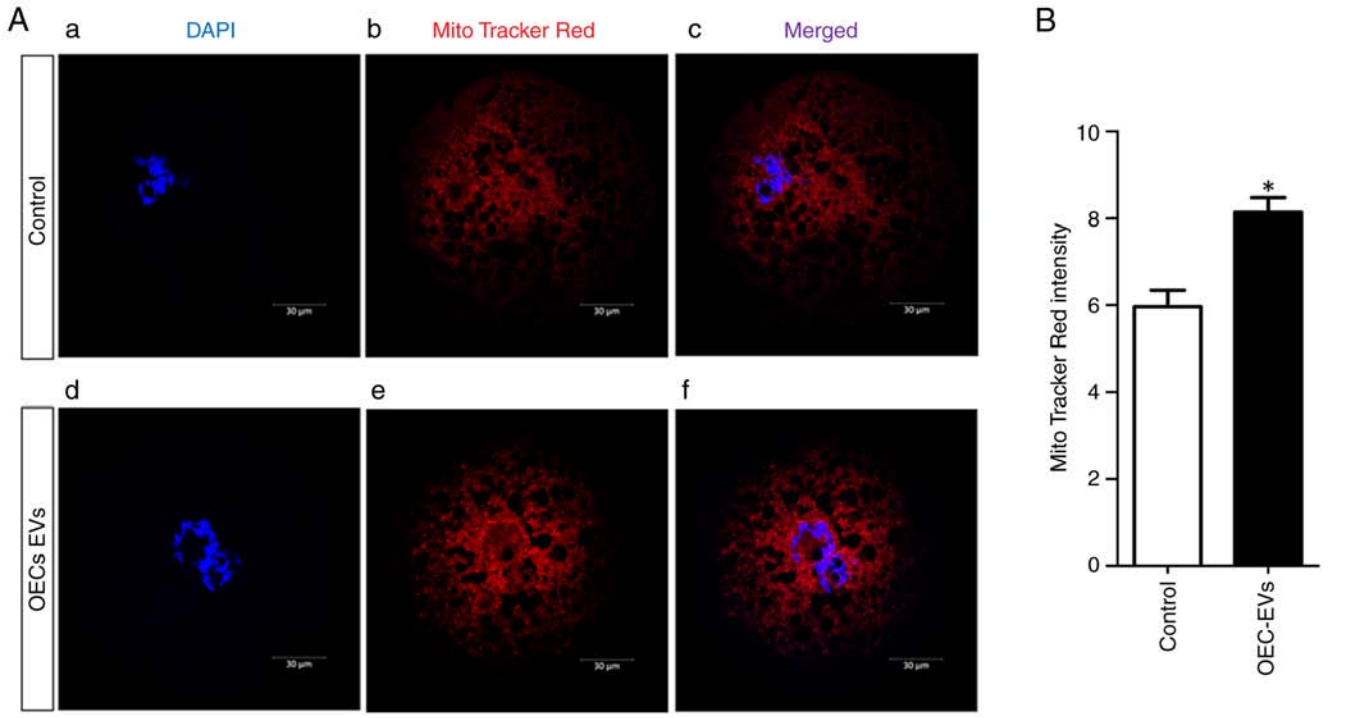

Figure 6. Detection of mitochondrial activity in porcine oocytes after EVs supplementation. (A) Confocal microscopy of embryo immunostaining 18 h after *in vitro* fertilization using MitoTracker Red, (a and d) embryonic nuclear staining with DAPI, (b and e) mitochondrial activity staining with MitoTracker Red, and (c and f) merged DAPI and MitoTracker Red images. Scale bar, 30 μm. (B) The MitoTracker Red intensities differed between the control and OEC-EV oocyte membranes. Three replicates (19 oocytes each) were compared. *P<0.05 (Control vs. OEC-EVs; unpaired Student's t-test). OEC-EV, oviduct epithelial cell-derived extracellular vesicle.

reservoir (36,55). Oviduct EVs also regulate plasma membrane Ca^{2+}-ATPase 1 to promote sperm motility (7). Moreover, proteins and miRNAs in OEC-EVs may enhance cross-talk in oocyte fertilization (53,56). Ferraz *et al* (57) reported that oviductal EV supplementation to fresh epididymal spermatozoa enhanced sperm motility and functions. Furthermore, Al-Dossary *et al* (58) demonstrated that oviductal EVs primarily enhance sperm motility, viability, capacitation, and acrosome reactions during oviduct transit. Therefore, it is hypothesized that OEC-EVs affect sperm penetration and capacitation via the molecular cargo contents, such as OVGP1, myosin heavy chain 9, valosin-containing protein, and annexin A5, or at least in part. Previous studies have observed interactions with these proteins, which are responsible for regulating sperm functions, membrane scaffold/trafficking processes, fertilization, and acrosome reaction (12,22,38,40).

In conclusion, it can be hypothesized that the primary reason for the lower quality of IVF embryos compared to *in vivo* embryos is due to differences between synthetic medium and pure natural biological secretions. Furthermore, it is suspected that bioactive EVs are a crucial missing link in artificial synthesis. Therefore, it is important to maintain a state as close to that found *in vivo* as possible to increase the success rate of embryos *in vitro*. Results of the present study support these hypotheses, as they demonstrated that the addition of OEC-EVs improved porcine IVF by enhancing CG distribution, increasing mitochondrial activity and reducing polyspermy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XF conceived the study. XF and JC designed the study. XF, BMT, SB, CS, GS, DZ, IMS, SL, XSC, and JC performed the experiments. XF and IMS performed data analysis. XF, IMS, SL, and JC wrote the manuscript. XF and JC confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethical approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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