

Theriogenology

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Follicular fluid-derived extracellular vesicles improve *in vitro* maturation and embryonic development of porcine oocytes

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Abstract

To optimize the most efficient method for porcine *in vitro* maturation (IVM), we compared the effects of supplementing extracellular vesicles (EVs) derived from porcine follicular fluid (pFF). The cumulus oocyte complexes were grouped into 4 groups with different supplementations as following: pFF (G1), pFF-depleted EVs (G2), EVs (G3) and control (G4) groups. After IVM with different supplementations, maturation rates and the developmental competences of porcine oocytes and blastocyst development were investigated. Additionally, glutathione (GSH) and reactive oxygen species (ROS) levels were measured in mature oocytes. The EVs were isolated and characterized with cryo-TEM and nanoparticle tracking analysis. The pFF significantly affected the maturation rate, whereas the presence of EVs did not show notable difference in the maturation rates. Although there were numerical increases in the measured parameters in EV and pFF-depleted EVs groups, no significant differences were observed between them. The EV group showed similar oocyte maturation rate for both positive and negative control groups. The GSH was not different among the groups, but ROS levels were significantly lower in pFF-supplemented group when compared with other groups with the highest level in the control group. G2 group wasn't significantly different G1 and G3 group. G3 group wasn't significantly different from G2 and G4 group. This suggests that EVs in IVM medium which probably effected partially to protect against oxidative stress and potentially enhance the quality of oocytes. This study indicates that the EVs in pFF play a significant role in improving the efficiency of oocyte maturation in porcine.

Keywords: extracellular vesicles; follicular fluid; reactive oxygen species; oocytes; swine

Introduction

In vitro maturation (IVM) of oocytes and development of embryos are essential processes in various medical fields, such as *in vitro* fertilization for infertile couples and production of animal models for disease research [1]. However, the rates of oocyte maturation and embryo development *in vitro* are significantly low com-

pared to those observed *in vivo* [2]. To overcome these challenges, various studies and experiments have been conducted at different stages of IVM and *in vitro* culture (IVC) [3,4]. The ovarian follicle plays an important role in regulating the hormones necessary for oocyte maturation [5,6]. Additionally, it provides essential nutrients and oxygen required for oocyte maturation [7]. Recent study has shown that the ingredients present in follicular fluid (FF) can affect oocyte maturation and quality [8].

Extracellular vesicles (EVs) are nanovesicles enclosed by a lipid bilayer and can be released from all living cells [9]. They contain many biologically active molecular substances such as proteins, mRNAs and miRNAs, which play active roles in regulating different physiological events [10,11]. The EVs play important roles in intercellular communication and are involved in a wide range of physiological and pathological processes [12].

The EVs are biogenic molecules known for their high stability, and compared to other additives, they enhance the efficiency of IVM. Therefore, this study aimed to investigate the effects of EVs, which have recently gained significant attention in the field of porcine follicular fluid (pFF), on the maturation and development of oocytes *in vitro*. Finally, we explored the possibility of using EVs as a natural additive for IVM.

Materials and Methods

Chemicals

All the chemicals were acquired from Sigma-Aldrich (USA) unless otherwise specified.

FF preparation and EVs isolation and characterization

Pig ovaries, contained in approximately 36°C to 38°C saline solution, are transported to the laboratory. We obtained EVs from the FF of 3 to 8 mm follicles. FF was aspirated using an 18-gauge needle and a 10 mL disposable syringe. Aspirated FF was centrifuged at 503 g for 30 minutes and filtered using 0.2 µm filter to remove cells. The filtrate was centrifuged at 120,000 g for 70 minutes, and the isolated pellets were stored at -80°C [13]. The supernatant was used as pFF-depleted EVs. The EVs were characterized using nanoparticle tracking analysis (NTA) for determining the size and concentration. For further characterization, the EVs were visualized through cryogenic transmission electron microscopy (cryo-TEM). The EVs were vitrified using Quantifoil R1.2/1.3 Cu 300 grids (Quantifoil, Germany) and Vitrobot mark IV (Thermo Fisher Scientific). Cryo-TEM images were captured using a Glacios microscope (Thermo Fisher Scientific) [14].

IVM oocytes and EVs supplementation

FF was collected by aspirating 3 to 8 mm-diameter ovarian follicles using an 18-gauge needle and a syringe. Cumulus oocyte complexes (COCs) were washed with HEPES-buffered Tyrode's medium containing polyvinyl alcohol (TLH-PVA). Only COCs with many layers of compact cumulus cells and even cytoplasm were used and washed 3 times with TLH-PVA.

The COCs were then divided into different groups and cultured for 20 to 22 hours in respective media with gonadotropin, in presence of 5% CO₂ in humidified atmosphere at 39°C. They were then transferred to media without gonadotropin and cultured for an additional 20 to 22 hours [15]. The media for all the groups contained TCM-199 (Gibco; Thermo Fisher Scientific Inc., USA) supplemented with 0.6 mM cysteine, 0.91 mM sodium pyruvate, 75 µg/mL kanamycin, 10 ng/mL epidermal growth factor, and 1 µg/mL insulin. In addition, 10 IU/mL human chorionic gonadotrophin and 10 IU/mL pregnant mare serum gonadotrophin were added to the IVM medium during the initial 20 to 22 hours.

The pFF group served as the positive control group (G1), in which 10% pFF was added to the IVM medium. The pFF-depleted EVs (G2) group was formulated by adding the supernatant after ultracentrifugation during the EV isolation step. The EV group (G3) comprised the EVs derived solely from pFF and pFF was not supplemented in negative control group (G4).

Measurement of oocyte glutathione and reactive oxygen species levels

To measure glutathione (GSH) and reactive oxygen species (ROS) in mature oocytes, oocytes (n = 10, 3 replicates) at the metaphase II stage with the first polar body were selected from each group after 40 to 44 hours of IVM. For measuring GSH levels, 4-chloromethyl 6,8-difluoro-7-hydroxycoumarin (Cell-Tracker Blue, CMF2HC; Invitrogen Corporation, USA) was used, and for ROS measurement, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen Corporation) was used. Oocytes were cultured in TLH-PVA containing 10 µM Cell-Tracker Blue and 10 µM H2DCFDA in the dark for 30 minutes [16,17]. Afterward, they were washed in Dulbecco's phosphate-buffered saline supplemented with 0.1% polyvinyl alcohol (PVA). The GSH and ROS levels were measured using an epifluorescence microscope with excitation at 370 nm for both. ImageJ software (National Institutes of Health, USA) was used to measure the fluorescence intensity.

Parthenogenetic activation after IVM and embryo IVC

After 44 hours IVM culture, cumulus cells were removed us-

ing 0.1% hyaluronidase and gentle pipetting. Denuded oocytes were washed in TLH-PVA, and only matured oocytes with the first polar body were used. The oocytes were gradually equilibrated in activation medium for parthenogenetic activation. For activation, the oocytes were activated with a double direct current (DC) pulse of 120 V for 60 μ s with a BTX electro cell manipulator (2001). The oocytes were activated with a double DC pulse of 120 V for 60 μ s. Electrically activated oocytes were washed twice in TLH medium, thrice in fresh porcine zygote medium-5 (PZM-5) and placed into 25 μ L PZM-5 droplets (10 embryos per drop). Activated oocytes were incubated at 39°C in a humidified atmosphere of 5% O₂, 5% CO₂ and 90% N₂ for 7 days. Cleavage was assessed on the day-2 and the blastocyst formation was assessed on the day-7 of IVC.

Statistical analysis

The statistical analysis was conducted utilizing IBM SPSS Statistics ver. 26.0 software (IBM Corp., USA). One-way analysis of variance and the least significant difference *post hoc* test were performed for mean comparison. The values were represented as mean \pm standard errors of the mean. Statistical significance was considered when the p-value was less than 0.05.

Ethical approval

Experiments were conducted in accordance with the guidelines for the management of laboratory animals at Chungnam National University (approval number: 202303-CNU-105).

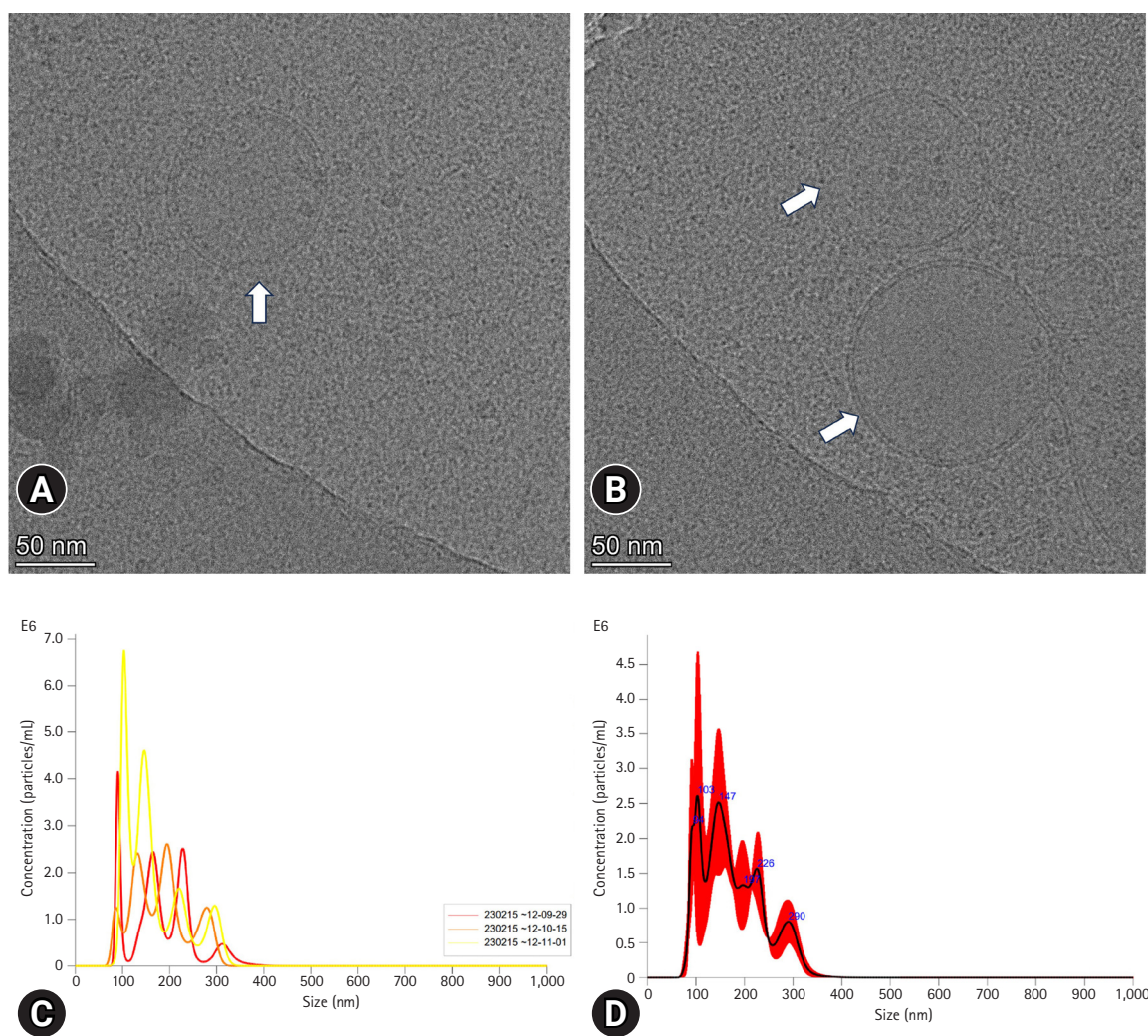


Fig. 1. Characterization of extracellular vesicles (EVs). (A, B) Image of cryo-transmission electron microscopy. The arrows point to the EVs from porcine follicular fluid. (C, D) Nanoparticles tracking analysis of porcine follicular fluid-derived EVs.

Results

EVs isolation and characterization

The pFF-derived EVs were first characterized using NTA to measure the size and concentration of the particles. Results showed that they have an average size of 176.7 ± 5.5 nm, and the concentration of those particles was $3.01 \times 10^8 \pm 1.95 \times 10^7$. Furthermore, for EV characterization (a second method), we measured the shape and size of the particles using cryo-TEM. The results revealed the presence of lipid bilayer vesicles with an average size of < 150 nm (Fig. 1A and B).

Comparison of maturation rates based on the presence of pFF or EVs

Results showed that the maturation rate of the pFF group (G1) was $82.5\% \pm 1.0\%$. G2 (pFF-depleted EVs) had a maturation rate of $80.5\% \pm 1.1\%$, the EV group (G3) had a maturation rate of $79.7\% \pm 1.8\%$, and the control group (G4) showed a maturation rate of $77.4\% \pm 1.6\%$ (Table 1). A significant difference was observed depending on the presence of pFF. No significant differences between G1 and G2 groups while there was a tendency toward numerical improvement in the measured parameters in both groups when compared with G3 and G4.

Measurement of oxidative stress in matured oocytes

No significant differences were observed among all the groups in terms of GSH levels (Fig. 2). In contrast, ROS levels was reduced in G1 and G2 groups when compared with G3 and G4 groups. G1 group differed significantly from G3 and G4 groups, whereas the G2 group showed significant differences only with the G4 group (Fig. 3).

Comparison of Embryonic development based on the presence of pFF or EVs

Cleavage rate was significantly reduced in G4 group when compared with the other 3 groups, while blastocyst formation rate was partially increased with the presence of EVs (G3) when

compared with the lowest rate in G4 or with the highest rate in G1 and G2 (Table 2).

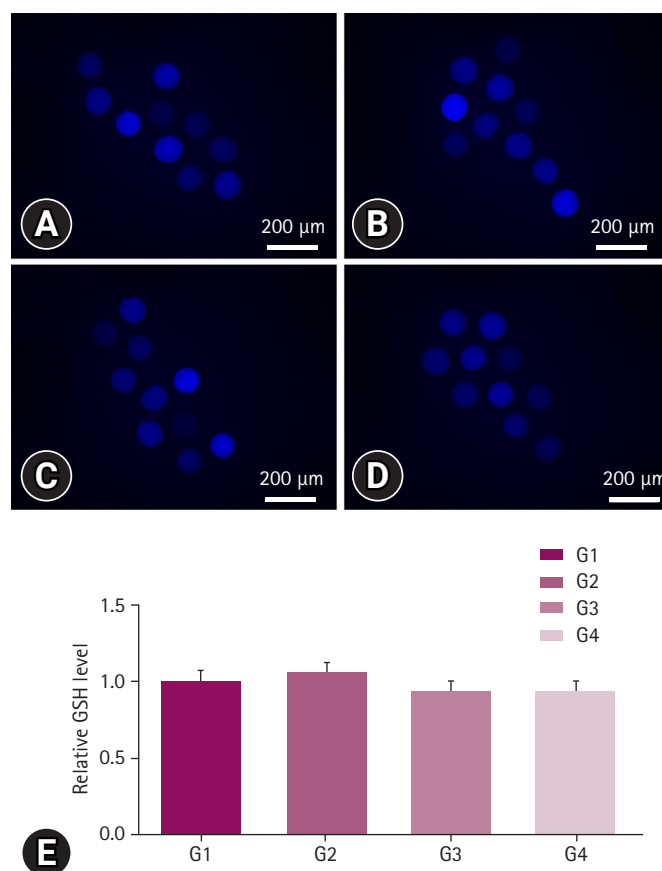


Fig. 2. Glutathione (GSH) staining for oxidative stress measurement. The staining was performed on oocytes after 40 to 44 hours of *in vitro* maturation, at the stage when the first polar body was released. Oocytes were cultured in Tyrode's medium containing polyvinyl alcohol containing 10 μ M CellTracker Blue (Invitrogen Corporation, USA) in the dark for 30 minutes. At least 3 replicates were performed, and more than 30 oocytes were used. (A) Image of porcine follicular fluid (pFF) supplementation (positive control, G1) group, (B) image of pFF w/extracellular vesicle (EV) (G2) group, (C) image of EVs group (G3), and (D) image of no pFF supplementation (negative control, G4) group. (E) The GSH levels in each group were expressed relative to the mean of the positive control. Scale bar = 200 μ m.

Table 1. Maturation rate according to follicular fluid and/or EVs supplementation

Group	No. of cumulus oocyte complexes (%)			
	Cultured	Matured (%)	Immature (%)	Dead (%)
G1	440	363 (82.5 \pm 1.0)*	38 (8.6 \pm 1.0)*	39 (8.9 \pm 1.5)
G2	438	352 (80.5 \pm 1.1)**†	46 (10.5 \pm 1.1)**†	40 (9.0 \pm 1.4)
G3	437	348 (79.7 \pm 1.8)**†	53 (12.1 \pm 1.2)**†	36 (8.1 \pm 1.3)
G4	438	339 (77.4 \pm 1.6)†	68 (15.5 \pm 1.4)†	31 (7.1 \pm 1.3)

EV, extracellular vesicle; pFF, porcine follicular fluid; G1, pFF supplementation; G2, pFF-depleted EVs; G3, EVs only; G4, no pFF supplementation. **†† Values with different superscript letters within the column vary significantly among the 4 groups ($p < 0.05$, $n = 10$).

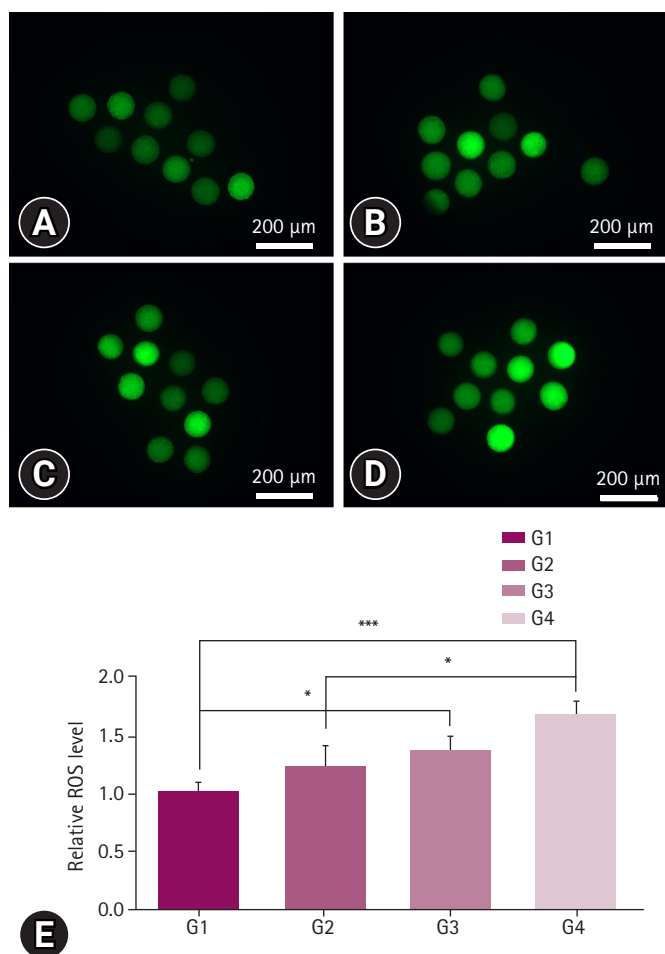


Fig. 3. Reactive oxygen species (ROS) staining for oxidative stress measurement. The staining was performed on oocytes after 40 to 44 hours of *in vitro* maturation, at the stage when the first polar body was released. Oocytes were cultured in Tyrode's medium containing polyvinyl alcohol containing 10 μM H2DCFDA in the dark for 30 minutes. At least 3 replicates were performed, and more than 30 oocytes were used. (A) Image of porcine follicular fluid (pFF) supplementation (positive control, G1) group, (B) image of pFF w/extracellular vesicle (EV) (G2) group, (C) image of EVs group (G3), and (D) image of no pFF supplementation (negative control, G4) group. (E) The ROS levels in each group were expressed relative to the mean of the positive control. Scale bar = 200 μm. Significant differences among the 4 groups were indicated with * when $p < 0.05$, and *** when $p < 0.001$.

Discussion

This study aimed to investigate the effects of EVs derived from pFF on oocyte maturation. Initially, pFF was separated using ultracentrifugation, and the pellets obtained from pFF were confirmed to be EVs using NTA and cryo-TEM. Thereafter, the COCs were divided into 4 groups: pFF (positive control, G1), pFF-depleted EVs (G2), EVs (G3), and pFF was not supple-

Table 2. Embryonic development rate after follicular fluid and/or EVs supplementation in *in vitro* maturation stage

Groups	No. of embryos (%)		
	Cultured	Cleaved (%)	Developed to blastocyst (%)
G1	172	140 (81.5 ± 1.5)*	40 (23.0 ± 1.5)*
G2	163	132 (81.1 ± 2.9)*	38 (23.4 ± 1.5)*
G3	162	117 (72.3 ± 2.5) [†]	31 (18.8 ± 2.2)* [†]
G4	161	116 (72.0 ± 1.6) [†]	24 (14.5 ± 1.7) [†]

EV, extracellular vesicle; pFF, porcine follicular fluid; G1, pFF supplementation; G2, pFF-depleted EVs; G3, EVs only; G4, no pFF supplementation.

*[†]Values with different superscript letters within a column vary significantly among the 4 groups ($p < 0.05$, $n = 5$).

mented (negative control, G4). IVM was conducted, and oocyte maturation rates were compared among the groups.

The results showed that the presence of pFF significantly affected the maturation rate of oocytes, and compared with the 2 control groups, there was no significant difference between the G2 and G3 groups. This suggests that both EVs and pFF-depleted EV may have an impact on oocyte maturation. EVs and other components in FF work together to improve oocyte maturation. The pFF contains steroid hormones, such as follicle stimulating hormone, estrogen, and related RNA molecules, which play a role in oocyte maturation. The pFF enhances the maturation rate [18]. Some of these molecules may exist in the form of EVs and potentially contribute to oocyte maturation [13].

ROS levels as indicators of oxidative stress were the lowest in G1 and G2 groups. Increased oxidative stress has various effects on cells, including insufficient ATP production in the mitochondria, cell death, and DNA damage [19,20].

It has been speculated that these effects may also impair oocyte quality, including cleavage and blastocyst development rates [21]. Although there was no statistically significant difference between the G3 and G4 groups in terms of cleavage and blastocyst development rates, there was numerical improvement in G3 when compared with G4. Additionally, blastocyst development rates and ROS level in G3 was not significantly different from G2 or G1. This suggests that pFF-derived EVs are partially contributed to the mechanism of action of pFF and can be considered as synergistic cargo carriers of the pFF. EVs contain factors that mitigate oxidative stress and are involved in cellular maturation, contain steroid hormones, and may contribute to oocyte maturation and quality enhancement. Our results coincide with Singina et al. [22], who found a high rate of blastocyst formation after supplementing bovine oocyte with FF-derived EVs.

In summary, FF-derived EVs are partially contributing to improving the oocyte maturation and blastocyst formation.

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