J Anim Reprod Biotechnol 2024;39:131-137
pISSN: 2671-4639 • eISSN: 2671-4663
https://doi.org/10.12750/JARB.39.2.131

JARB
Journal of Animal Reproduction and Biotechnology
Check for updates

Original Article

Effects of extracellular vesicles (EVs) from uterine fluid during estrus and diestrus on porcine embryonic development

Shuntaro Miura[1,2], Heejae Kang[2,3], Seonggyu Bang[2,3], Ayeong Han[2,3], Islam M. Saadeldin[2,4], Sanghoon Lee[2], Koichi Takimoto[1] and Jongki Cho[3,*]

[1]Department of Materials Science and Bioengineering, Nagaoka University of Technology, Niigata 940-2188, Japan
[2]College of Veterinary Medicine, Chungnam National University, Daejeon 34134, Korea
[3]College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University, Seoul 08826, Korea
[4]Department of Comparative Medicine, King Faisal Specialist Hospital & Research Center, Riyadh 11211, Saudi Arabia

Received June 2, 2024
Revised June 13, 2024
Accepted June 14, 2024

***Correspondence**
Jongki Cho
E-mail: cjki@snu.ac.kr

Author's Position and Orcid no.
Miura S, MS student,
https://orcid.org/0009-0005-6658-0751
Kang H, MS student,
https://orcid.org/0000-0002-3508-6612
Bang S, PhD candidate,
https://orcid.org/0000-0002-2513-4887
Han A, PhD candidate,
https://orcid.org/0009-0006-9394-3009
Saadeldin IM, Associate professor,
https://orcid.org/0000-0002-7633-730X
Lee S, Associate professor,
https://orcid.org/0000-0002-1265-3821
Takimoto K, Professor,
https://orcid.org/0000-0002-1076-0359
Cho J, Professor,
https://orcid.org/0000-0002-8431-0457

ABSTRACT

Background: Porcine embryonic development is widely utilized in the medical industry. However, the blastocyst development rate *in vitro* is lower compared to *in vivo*. To address this issue, various supplements are employed. Extracellular vesicles (EVs) play the role of communicators that carry many bioactive cargoes. Additionally, the contents of EVs can vary on the estrous cycle.

Methods: We compared the effects of adding EVs derived from porcine uterine fluid (UF), categorized as non-EV (G1), EVs in estrus (G2) and EVs in diestrus (G3). After *in vitro* culture (IVC) was performed in three different groups, cleavage rate and blastocyst development rate were examined. In addition, glutathione (GSH) and reactive oxygen species (ROS) levels were measured 2 days after activation to assess oxidative stress.

Results: Using NTA and cryo-TEM, we confirmed the presence of EVs with sizes ranging from 30 nm to 200 nm, that the particles were suitable for analysis for analysis. In IVC data, the highest cleavage rate was observed in G2, which was significantly different from G1 but not significantly different from the next highest, G3. Similarly, the highest blastocyst development rate was observed in G2, which was significantly different from G1 but not significantly different from the next highest, G3.

Conclusions: These results indicate that estrus derived EVs contain biofactors beneficial for early blastocyst development, including GSH which protects the blastocyst from oxidative stress. Additionally, although diestrus-derived EVs are expected to have some effect on blastocyst development, it appeared to be less effective than estrus-derived EVs.

Keywords: cycle, embryo development, extracellular vesicles, reactive oxygen species, uterine fluid

INTRODUCTION

The development of porcine embryos is utilized in various medical fields, including as a model for human medical research and in organ transplantation. However, compared to *in vivo* conditions, the development rate of blastocysts *in vitro* is lower (Na et al., 2015; Mahdavinezhad et al., 2019). The reasons may include the supply of unidentified nutrients, the interaction with oxygen and carbon dioxide concentrations, temperature, and humidity, lack of hormones and growth factors, and the limitations of immune protection (Saeidnia et al., 2015). Among these, hormones and physiological signals are particularly important in the uterus during the estrous cycle.

Female mammals, including porcine, undergo cyclic changes in physiological state during the estrous cycle. The levels of various hormones fluctuate depending on the stage of the estrous cycle (Park et al., 2022). The estrus stage elevates levels of estrogen, follicle-stimulating hormone, and luteinizing hormone secretion. During the estrus stage, the uterus undergoes cellular proliferation, increasing the thickness of the endometrium to create a suitable environment for embryo implantation. Diestrus is the stage after ovulation, characterized by increased levels of progesterone and decreased levels of estrogen. During this stage, the uterus thickens and stabilizes the endometrium with progesterone, secreting nutrients, growth factors, and immunomodulatory substances essential for embryo development (Bulletti et al., 2022). In the estrous cycle, these changes occur through interactions between cells. The factors regulated by hormones in the uterus are either directly secreted through uterine fluid (UF) or released in the form of vesicles, which are then delivered to other cells such as embryos (Beal et al., 2023).

Extracellular vesicles (EVs) are known to perform many biological functions, especially in intercellular communication (Mincheva-Nilsson and Baranov, 2010; Li et al., 2017). EVs contain RNA, proteins, enzymes, and lipids, which are important in a variety of biological functions, including the transport of these materials and the regulation of physiological and pathological processes (Gurunathan et al., 2019; Saadeldin et al., 2022). Recent research has demonstrated that EVs mediate communication between the mother and the embryo during early embryonic development (Machtinger et al., 2016).

Increased oxidative stress is known to have diverse effects, including mtDNA mutation, senescence, cell death, and reduced ATP production capacity in mitochondria (Orrenius et al., 2007). In addition, the occurrence of oxidative stress not only impairs follicle growth by inducing apoptosis of oocytes, but also decreases embryonic development, cleavage rate, and oocyte quality, thereby reducing reproductive performance (Prasad et al., 2016). oxidative stress can be evaluated by determining reactive oxygen species (ROS) levels, which are an indicator of this stress. Recent studies have revealed that EVs regulate oxidative stress (Qi et al., 2021). Additionally, oxidative stress in early embryos is emphasized because they are particularly vulnerable and it can significantly impact fetal development (Chen et al., 1999).

We hypothesized that during the estrus and diestrus stages, the EVs derived from porcine UF would contain biomolecules that prevent oxidative stress, improving the development and quality of embryos. The aim of this study was to investigate the effects of EVs derived from porcine UF at different stages of estrous cycle on embryonic development.

MATERIALS AND METHODS

Chemicals

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

EV isolation and characterization

The porcine uterus was brought from the abattoir to the laboratory, and classified into estrus and diestrus periods. The porcine UF was washed in Dulbecco's Phosphate Buffered Saline (DPBS) and the UF was collected. The UF was centrifuged at 400 × g for 10 min to remove cell debris and apoptotic bodies. The supernatant fluid was centrifuged at 2,000 × g for 30 min and filtered through a 0.22 μm filter to remove microvesicles. Finally, EVs were isolated by 180 min ultracentrifugation at 1,000 × g. Nanoparticle tracking analysis (NTA) was performed to determine the size and concentration of particles obtained through ultracentrifugation. The sample was diluted 1:1,000 in DPBS and measured by NanoSight NS 3000 (Malvern, UK) (Saadeldin et al., 2023). And the EVs were visualized through cryogenic transmission electron microscopy (cryo-TEM). Vitrobot mark IV and Quantifoil R1.2/1.3 Cu 300 grids were used. Cryo-TEM images were captured using a Glacios microscope (Kang et al., 2023).

In vitro maturation (IVM) oocytes

Cumulus oocyte complexes (COCs) aspirated oocytes from follicles 3-6 mm in diameter using an 18-gauge needle attached to a 10 mL syringe. After discarding the supernatant, the precipitate was washed with HEPES-buffered Tyrode's (TLH) containing 0.05% (w/v) polyvinyl alcohol (PVA). Only COCs with homogeneous cytoplasm, with layers of compact cumulus cells, were used and washed three times with TLH-PVA. The COCs were then divided into different groups and incubated in their respective medium supplemented with hormones for 20-22 hours in a humidified atmosphere at 39℃ in the presence of 5% CO_2 (Fang et al., 2023). The COCs were then transferred to hormone-free medium and incubated for another 20-22 hours. The medium was 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. The hormones included 10 IU/mL hCG and 10 IU/mL PMSG (Fang et al., 2022).

Parthenogenetic activation & *in vitro* culture (IVC) with supplement of EVs

After 44 hours of IVM, COCs were denuded by gentle pipetting with 0.1% hyaluronidase. Oocytes were washed twice in TLH medium, and only oocytes with the first polar body were selected. The oocytes were then gradually equilibrated in activation medium for parthenogenetic activation. Denuded oocytes were placed between electrodes covered with activation medium connected to the BTX Electro-Cell Manipulator 2001. The oocytes were activated with a double direct current (DC) pulse of 120 V for 60 μs (Bang et al., 2023). The activated oocytes were washed twice in TLH medium and three times in fresh Porcine Zygote Medium-5 (PZM-5) and then placed in non-EVs (G1), EVs in estrus (G2) and EVs in diestrus (G3), respectively. The concentration of EVs during culture was 3×10^5 particles/mL (Leal et al., 2022). The activated oocytes were cultured at 39℃ in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2 for 7 days.

Measurement of embryos glutathione and reactive oxygen species levels

To measure glutathione (GSH) and ROS, 2 cell and 4 cell stage embryos from each group were selected 2 days after activation. To measure GSH, embryos were incubated with 10 μM Cell Tracker Blue in TLH-PVA for 30 minutes in the dark (Kwak et al., 2012). They were then washed with Dulbecco's phosphate-buffered saline supplemented with 0.1% polyvinyl alcohol (PVA).

In addition, to measure ROS, embryos were incubated with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen Corporation) in TLH-PVA for 30 minutes in the dark (Fang et al., 2023). They were then washed with Dulbecco's phosphate-buffered saline supplemented with 0.1% polyvinyl alcohol (PVA). Subsequently, the images were captured using a fluorescence microscope (Leica DE/DM 2000, Wetzlar, Germany). ImageJ software (National Institutes of Health, USA) was used to measure fluorescence intensity.

Statistical analysis

IBM SPSS Statistics ver. 26 software (IBM Corp.) was used for statistical analysis. One-way analysis of variance and LSD post hoc tests were performed to compare means. Values were presented as mean ± standard error of the mean. Statistical significance was defined as a p-value less than 0.05.

RESULTS

EVs isolation and characterization

EVs in estrus and EVs in diestrus from porcine UF were first measured for their respective particle sizes and concentrations using nanoparticle tracking analysis (NTA). The results showed that the mean size and concentration of EVs in estrus were 253.9 ± 48.0 nm and $2.42 \times 10^9 \pm 4.80 \times 10^7$ particles/mL, respectively; the mean size and concentration of EVs in diestrus was 111.7 ± 23.7 nm and $5.41 \times 10^8 \pm 3.51 \times 10^7$ particles/mL, respectively. To further characterize the EVs, the shape and size of the particles were measured using TEM (Fig. 1).

Comparison of embryonic development based on the presence of EVs

The cleavage rate was highest in the order of G2 (74.7 ± 1.6), G3 (73.5 ± 2.8), and G1 (71 ± 1.5). The highest cleavage rate in G2 was significantly different from G1, but it was not significantly different from the next highest, G3. Similarly, the blastocyst development rate was highest in the order of G2, G3, and G1. The highest blastocyst development rate in G2 was significantly different from G1,

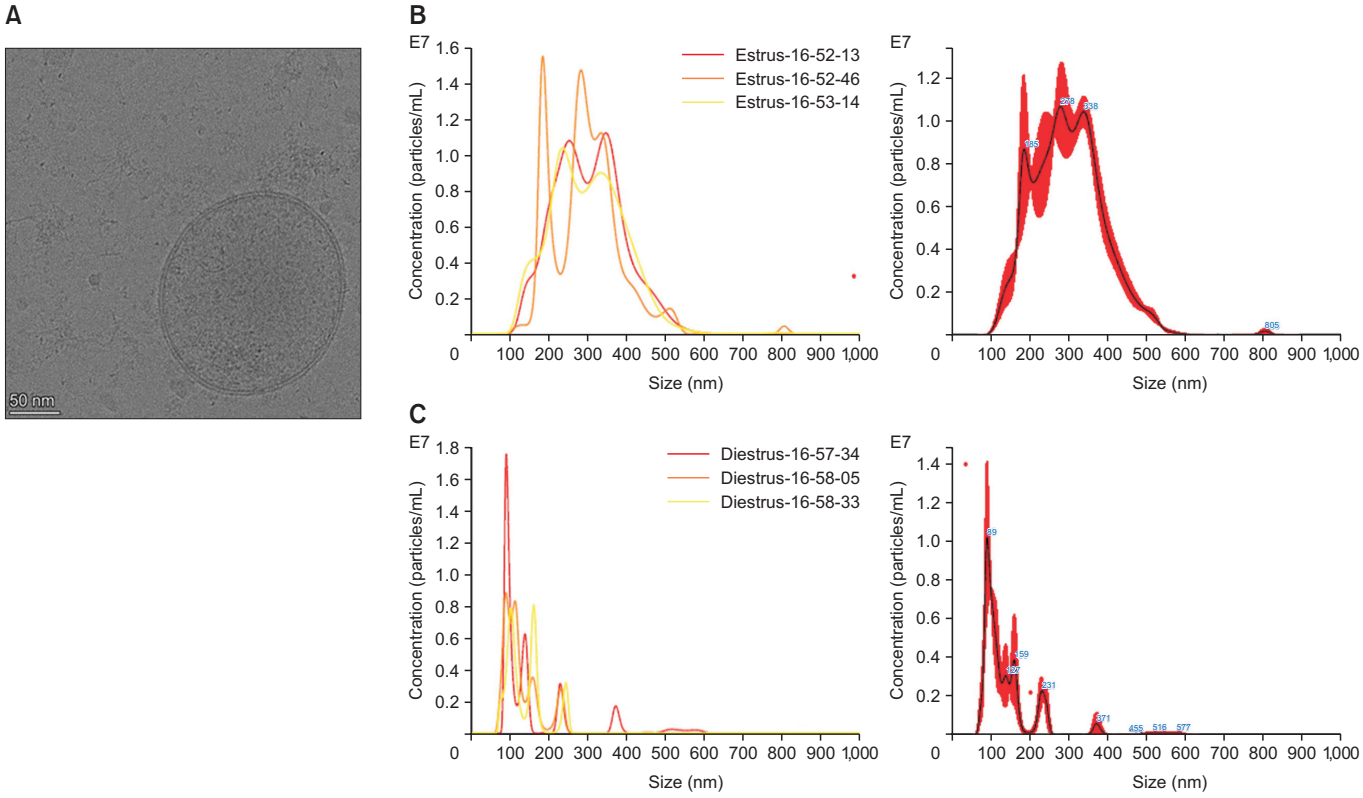

Fig. 1. Characterization of extracellular EV. (A) Cryo-TEM image of UF-derived EVs. The scale bar represents 50 nm. (B) NTA results of EVs derived from UF during the estrus stage. (C) NTA results of EVs derived from UF during the diestrus stage. Cryo-TEM, cryo-transmission electron microscopy; NTA, nanoparticles tracking analysis.

Table 1. Effect of EVs derived from UF in estrus and diestrus stage in porcine embryonic development

Groups	No. of embryos		
	Culture	Cleaved (%)	Develop to Bl. (%)
Non-EVs (G1)	267	170 (71.7 ± 1.5)[a]	39 (16.5 ± 2.6)[a]
EVs in estrus (G2)	267	177 (74.7 ± 1.6)[b]	47 (19.8 ± 2.3)[b]
EVs in diestrus (G3)	267	174 (73.5 ± 2.8)[a,b]	43 (18.2 ± 3.0)[a,b]

[a,b]Values with different superscript letters within a column vary significantly among the three groups ($p < 0.05$, n = 8). Bl, Blastocyst.

but it was not significantly different from the next highest, G3 (Table 1).

Measure of oxidative stress in embryos

The results of ROS level measurements showed significant differences among all groups. Among them, it was observed that G1 exhibited the highest expression of ROS, while G2 showed lower expression compared to G3 (Fig. 2). The results of the GSH level measurement showed significant differences among all groups. The expression of GSH was lowest in G1, and G3 exhibited lower expression than G2 (Fig. 3).

DISCUSSION

The aim of this study was to investigate the effect of porcine UF-derived EVs on embryonic development. NTA and Cryo-TEM were used to confirm the presence of EVs. Next, the IVC medium was divided into three groups: non-EVs (control, G1), EVs in estrus (G2), and EVs in diestrus (G3). Then, IVC was performed under three conditions, and the embryonic development rate was measured.

The IVC results showed that the presence of EVs in estrus had a significant effect on embryonic developmental potential cleavage rate, and developed to blastocysts rate. In the group with EVs in estrus (G2), both the cleavage rate and blastocyst development rate were the highest among the groups. This suggests the presence of physiological factors derived from the UF environment during estrus, which support cleavage. As estrus is characterized by elevated estrogen levels, it is anticipated that factors maintained by estrogen would be abundant. Factors regu-

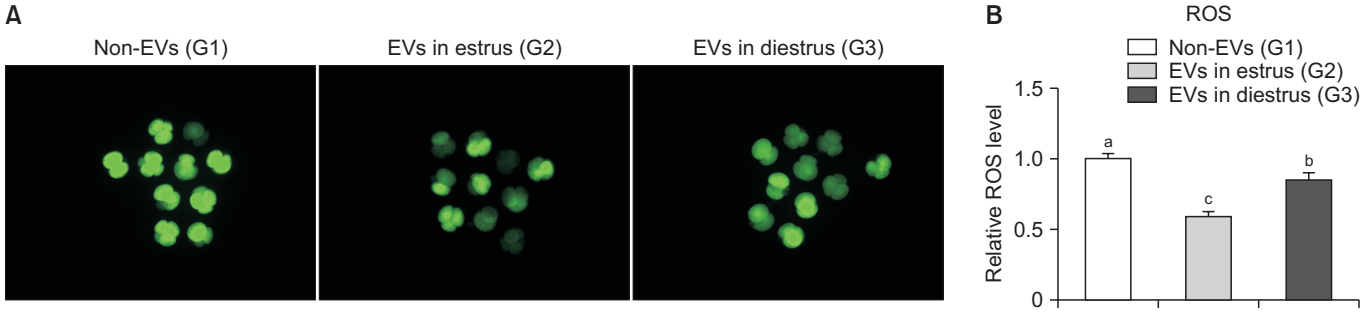

Fig. 2. The influence of EVs on the levels of ROS in early pig embryos. (A) Images of ROS measurements for each group. (B) The relative levels of ROS in early-stage embryos. The staining was performed on embryos at the 2-cell and 4-cell stages at 2 days after parthenogenetic activation. Bars with different alphabetical letters are significantly different among the groups ($p < 0.05$).

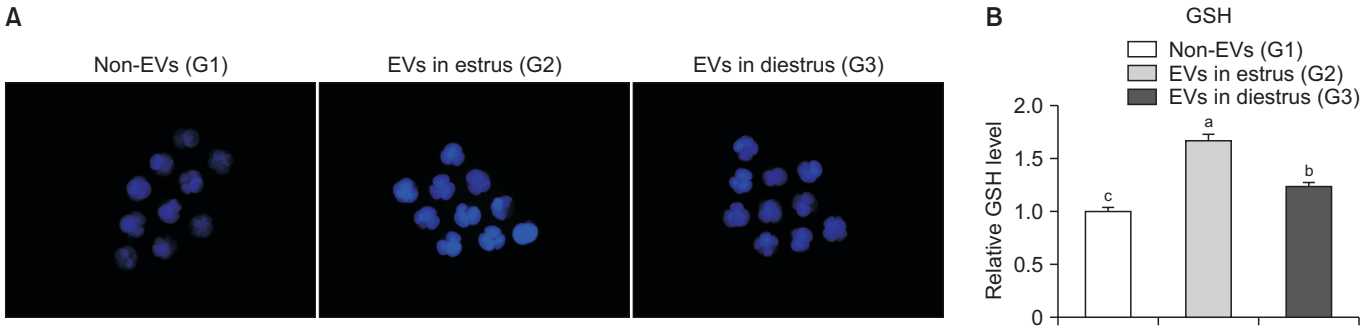

Fig. 3. The influence of EVs on the levels of GSH in early pig embryos. (A) Images of GSH measurements for each group. (B) The relative levels of GSH in early-stage embryos. The staining was performed on embryos at the 2-cell and 4-cell stages at 2 days after parthenogenetic activation. Bars with different alphabetical letters are significantly different among the groups ($p < 0.05$).

lated by estrogen include growth factors such as Insulin-like Growth Factor (IGF) and Epidermal Growth Factor (EGF) (Filardo et al., 2000; Fujimoto and Kitamura, 2004). It is expected that various proteins and mRNA enhancing the activation of pathways such as AP-1, STAT, Elk-1, CREB, NF-κB, mediated by estrogen receptors and estrogen response elements, were transferred from the uterus to the blastocyst through EVs (Fuentes and Silveyra, 2019). This could have led to the activation of metabolism in the early blastocyst.

EVs during diestrus are expected to contain many factors regulated by progesterone. Progesterone also regulates factors such as fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and IGF which influence early embryo growth and division, as well as Leukemia Inhibitory Factor (LIF) and Transforming Growth Factor-beta (TGF-beta), involved in endometrial maintenance and immune regulation (Satterfield et al., 2008). However, in the IVC, the cleavage rate and blastocyst development rate were lower in the diestrus EVs group (G3) compared to the estrus EVs group (G2). This suggests that progesterone-derived EVs may contain fewer factors involved in early blastocyst development compared to estrogen-derived EVs and may contain more factors involved in immune response generation and other roles necessary for maintaining pregnancy. Further investigation is needed to explore this phenomenon.

In the results of oxidative stress, compared to G1, both G2 and G3 showed a decrease in ROS levels. This is attributed to the activation of intracellular antioxidant defense systems by factors activated by estrogen and progesterone, as discussed earlier (Xiang et al., 2021). This is consistent with the high expression of the antioxidant GSH in G2 and G3, compared to G1. Therefore, it is expected that estrogen and progesterone activate the antioxidant defense system, leading to the delivery of associated downstream factors via EVs to the blastocyst, thereby increasing the levels of the antioxidant GSH and reducing oxidative stress (Park et al., 2022).

CONCLUSION

In this experiment, we observed the effects of estrus and diestrus-derived EVs on blastocyst development. The results showed that while diestrus EVs did not significantly differ from the control in terms of cleavage and blastocyst development rate, they exhibited a numerical increase. In contrast, the estrus EVs group showed a significant effect compared to the control. This suggests that estrus derived EVs may contain more factors aiding in early embryonic development compared to diestrus EVs, including GSH, which protects against ROS.

Author Contributions: Conceptualization, S.M. and J.C.; methodology, S.M., H.K., S.B. and A.H.; data curation, S.M., H.K., S.L.; writing - original draft preparation, S.M., H.K., S.B. and A.H.; writing - review and editing, A.H., S.B., I.M.S., S.L., K.T. and J.C.; supervision, K.T. and J.C.; project administration, K.T., J.C.; funding acquisition, J.C.

Funding: This work was supported by the Ministry of Science and ICT through the National Research Foundation of Korea (NRF) (grant numbers: 2021R1A2C2009294). The Research Institute for Veterinary Science at the Seoul National University partially funded this study.

Ethical Approval: Not applicable.

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Availability of Data and Materials: Not applicable.

Acknowledgements: The authors would like to thank to Seongja Kim for providing the porcine ovaries from the Daejeon Metropolitan Slaughterhouse.

Conflicts of Interest: No potential conflict of interest relevant to this article was reported.

REFERENCES

Bang S, Qamar AY, Fang X, Kim H, Han A, Kang H, Cha D, Shim J, Kim JH, Choi K, Yun SH, Lee H, Park HS, Kim SI, Kim JY, Saadeldin IM, Lee S, Cho J. 2023. Effects of extracellular vesicles derived from steroids-primed oviductal epithelial cells on porcine in vitro embryonic development. Theriogenology 209:213-223.

Beal JR, Ma Q, Bagchi IC, Bagchi MK. 2023. Role of endometrial extracellular vesicles in mediating cell-to-cell communication in the uterus: a review. Cells 12:2584.

Bulletti C, Bulletti FM, Sciorio R, Guido M. 2022. Progesterone: the key factor of the beginning of life. Int. J. Mol. Sci. 23:14138.

Chen EY, Fujinaga M, Giaccia AJ. 1999. Hypoxic microenvironment within an embryo induces apoptosis and is essential for proper morphological development. Teratology 60:215-225.

Fang X, Bang S, Tanga BM, Seo C, Zhou D, Seong G, Saadeldin IM, Lee S, Cui XS, Cho J. 2023. Oviduct epithelial cell-derived extracellular vesicles promote the developmental competence of IVF porcine embryos. Mol. Med. Rep. 27:122.

Fang X, Tanga BM, Bang S, Seong G, Saadeldin IM, Qamar AY, Shim J, Choi K, Lee S, Cho J. 2022. Vitamin C enhances porcine cloned embryo development and improves the derivation of embryonic stem-like cells. Reprod. Biol. 22:100632.

Filardo EJ, Quinn JA, Bland KI, Frackelton AR Jr. 2000. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol. Endocrinol. 14:1649-1660.

Fuentes N and Silveyra P. 2019. Estrogen receptor signaling mechanisms. Adv. Protein Chem. Struct. Biol. 116:135-170.

Fujimoto N and Kitamura S. 2004. Effects of environmental estrogenic chemicals on AP1 mediated transcription with estrogen receptors alpha and beta. J. Steroid Biochem. Mol. Biol. 88:53-59.

Gurunathan S, Kang MH, Jeyaraj M, Qasim M, Kim JH. 2019. Review of the isolation, characterization, biological function, and multifarious therapeutic approaches of exosomes. Cells 8:307.

Kang H, Bang S, Kim H, Han A, Miura S, Park HS, Saadeldin IM, Lee S, Cho J. 2023. Follicular fluid-derived extracellular vesicles improve in vitro maturation and embryonic development of porcine oocytes. Korean J. Vet. Res. 63:e40.

Kwak SS, Cheong SA, Jeon Y, Lee E, Choi KC, Jeung EB, Hyun SH. 2012. The effects of resveratrol on porcine oocyte in vitro maturation and subsequent embryonic development after parthenogenetic activation and in vitro fertilization. Theriogenology 78:86-101.

Leal CLV, Cañón-Beltrán K, Cajas YN, Hamdi M, Yaryes A, Millán de la Blanca MG, Beltrán-Breña P, Mazzarella R, da Silveira JC, Gutiérrez-Adán A, González EM, Rizos D. 2022. Extracellular vesicles from oviductal and uterine fluids supplementation in sequential in vitro culture improves bovine embryo quality. J. Anim. Sci. Biotechnol. 13:116.

Li P, Kaslan M, Lee SH, Yao J, Gao Z. 2017. Progress in exosome isolation techniques. Theranostics 7:789-804.

Machtinger R, Laurent LC, Baccarelli AA. 2016. Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation. Hum. Reprod. Update 22:182-193.

Mahdavinezhad F, Kazemi P, Fathalizadeh P, Sarmadi F, Sotoodeh L, Hashemi E, Hajarian H, Dashtizad M. 2019. *In vitro versus in vivo*: development-, apoptosis-, and implantation-related gene expression in mouse blastocyst. Iran J. Biotechnol. 17:e2157.

Mincheva-Nilsson L and Baranov V. 2010. The role of placental exosomes in reproduction. Am. J. Reprod. Immunol. 63:520-533.

Na S, Lee E, Kim G, Min K, Yu Y, Roy PK, Fang X, Hassan BMS, Yoon K, Shin S, Cho J. 2015. Study on embryo transfer system for production of transgenic pigs. J. Emb. Trans. 30:345-350.

Orrenius S, Gogvadze V, Zhivotovsky B. 2007. Mitochondrial oxidative stress: implications for cell death. Annu. Rev. Pharmacol. Toxicol. 47:143-183.

Park JE, Lee ST, Lee GS, Lee E. 2022. *In vitro* maturation on a soft agarose matrix enhances the developmental ability of pig oocytes derived from small antral follicles. J. Anim. Reprod. Biotechnol. 37:34-41.

Park Y, Park YB, Lim SW, Lim B, Kim JM. 2022. Time series ovarian transcriptome analyses of the porcine estrous cycle reveals gene expression changes during steroid metabolism and corpus luteum development. Animals (Basel) 12:376.

Prasad S, Tiwari M, Pandey AN, Shrivastav TG, Chaube SK. 2016. Impact of stress on oocyte quality and reproductive outcome. J. Biomed. Sci. 23:36.

Qi H, Wang Y, Fa S, Yuan C, Yang L. 2021. Extracellular vesicles as natural delivery carriers regulate oxidative stress under pathological conditions. Front. Bioeng. Biotechnol. 9: 752019.

Saadeldin IM, Tanga BM, Bang S, Fang X, Yoon KY, Lee S, Cho J. 2022. The theranostic roles of extracellular vesicles in pregnancy disorders. J. Anim. Reprod. Biotechnol. 37:2-12.

Saadeldin IM, Tanga BM, Bang S, Maigoro AY, Kang H, Cha D, Lee S, Lee S, Cho J. 2023. MicroRNA profiling of royal jelly extracellular vesicles and their potential role in cell viability and reversing cell apoptosis. Funct. Integr. Genomics 23:200.

Saeidnia S, Manayi A, Abdollahi M. 2015. From in vitro experiments to in vivo and clinical studies; pros and cons. Curr. Drug Discov. Technol. 12:218-224.

Satterfield MC, Hayashi K, Song G, Black SG, Bazer FW, Spencer TE. 2008. Progesterone regulates FGF10, MET, IGFBP1, and IGFBP3 in the endometrium of the ovine uterus. Biol. Reprod. 79:1226-1236.

Xiang D, Liu Y, Zhou S, Zhou E, Wang Y. 2021. Protective effects of estrogen on cardiovascular disease mediated by oxidative stress. Oxid. Med. Cell. Longev. 2021:5523516.