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Effect of bovine follicular fluid derived exosomes on *in vitro* fertilisation rate

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Abstract
Exosomes are 30-200 nm sized small extracellular vehicles (EVs) generated by cells. Follicular fluid is one of the sources of exosomes, which has beneficial effects on *in vitro* oocyte maturation (IVM), fertilisation (IVF) and developmental competence of bovine embryos. In the present study, supplementing oocyte maturation medium with follicular fluid exosomes isolated from follicles of diameter greater than 8 mm, resulted in a significant increase in IVM, IVF and clevage rate of bovine oocytes, compared to the control group. Result suggests the possibility of improving *in vitro* embryo harvest by supplementing oocyte maturation medium with follicular fluid exosomes.

Keywords: Exosomes, follicular fluid, *in vitro* maturation, *in vitro* fertilisation, extra cellular vesicle

1. Introduction
In vitro production by assisted reproductive technology (ART) is a major tool for mass production of bovine embryos. At present, *in vitro* techniques yield only fewer embryos, due to the fact that these culture systems lack physiological pathways and essential components required for the optimum development of embryos. Over several years, researchers have been working to develop culture systems simulating biological system, so as to attain optimum *in vitro* embryo harvest. Extracellular Vesicles (EVs) have recently been identified as cell-to-cell communication mediators in physiological as well as pathological context. The EVs in follicular fluid could protect oocytes from various types of stressors and help them to increase their developmental competence.
Exosomes are 30-200 nm sized, small EVs generated by the release of intraluminal vesicles from multi-vesicular bodies (MVBs) that gets fused with the plasma membrane. They contain specific composition of proteins, lipids, RNA, and DNA and play significant roles in various biological functions, including the transfer of biomolecules such as RNA, proteins, enzymes, lipids and the regulation of numerous physiological and pathological processes. They also play a substantial role in transmitting specific molecules in the reproductive tract to modulate transcription and translational activity, granulosa cell proliferation and differentiation, cumulus expansion, gametogenesis, normal follicular growth, oocyte maturation, fertilization rate, embryo development, blastocyst formation, implantation, pregnancy outcome and fertility. In reproductive studies, follicular fluid (FF) is one of the sources of exosomes and is primarily of granulosa cell and cumulus–oocyte complex (COCs) origin. Studies indicate that exosomes derived from ovarian follicles have beneficial effects on the developmental competence of oocytes in ART. In view of the above observations, present investigation was envisaged to study the *in vitro* oocyte maturation (IVM), fertilisation (IVF) and developmental competence of bovine embryos, after supplementing oocyte maturation medium with follicular fluid exosomes, isolated from follicles of greater than 8mm size and to characterise these exosomes based on electron microscopy (EM).

2. Materials and methods
The study was carried out in two phases. During the first phase, follicular fluid was collected from the bovine slaughter ovaries and the exosomes were separated.

In the second phase, effect of exosomes on *in vitro* maturation and fertilisation was studied.

2.1. Phase I: Collection of follicular fluid and exosome separation

Ovaries of cows, procured from local abattoir were utilised for aspiration of follicular fluid and subsequent isolation of exosomes. Follicles on these ovaries having a size above 8mm were selected for the aspiration of follicular fluid. A 20-gauge needle, fitted with 5 mL syringe was used to aspirate the follicles. Follicular fluid was collected and stored at -80 °C until further processing.

Frozen follicular fluid was thawed and centrifuged at 800 x g for 10 min, followed by 2000 x g for 15 min, 12000 x g for 45 min and the supernatant fraction was filtered using a 0.2 µm syringe filter. All the centrifugations were done at 4℃. The filtered supernatant was subjected to ultracentrifugation at 100,000 x g for 70 min, so as to separate the exosomes. Ultracentrifugation was done in Sorvall WX Ultra Series Centrifuge (Thermos scientific) using a swinging-bucket Superspin Sorvall 30Ti rotor at 4 ℃, to pellet the follicular fluid exosomes. After ultracentrifugation, the supernatant was completely discarded and the tube was kept upside down for 30 seconds on a tissue paper to remove any remnants of the supernatant. The pellets obtained were suspended in 200 µL of filtered phosphate-buffered saline (PBS) and stored at 4 ℃ overnight to get uniform dilution of particles and part of it was utilised for characterisation by electron microscopy (EM). The remaining sample was further stored at -80 °C for subsequent IVM, IVF and embryo development studies.

The samples for exosome characterisation were first fixed with one per cent glutaraldehyde and then processed for EM studies (Wu *et al.*, 2015) [14]. Size (nm) and morphology of exosomes were determined with the help of Carl Zeiss Sigma field-emission scanning electron microscopic facility available at Department of Physics, CUSAT, Kerala. Images were captured after gold-sputtering at a magnification of 150 KX to 200 KX at 5.00 kV electron high tension.

2.2. Phase II: Oocyte maturation and embryo developmental studies after exosome supplementation

Ovaries of cows were collected from local abattoirs and were transported to the laboratory in PBS (maintained at 37±2 ℃), within 2h of slaughter. All the surface follicles measuring 2-8 mm in diameter were aspirated, oocytes separated, COCs were identified, washed and prepared for grading, following standard procedure (Gordon, 2003) [4]. These COCs were graded as described by Saleh (2017) [9]; Grade A and B oocytes were selected for IVM and randomly allocated to two groups (I and II). A total of 226 oocytes were selected into group I and 223 oocytes were selected in to group II. These oocytes were subjected to IVM in modified Tissue Culture Medium-199 (mTCM 199). The culture medium of group I oocytes was supplemented with exosomes, collected from follicle having a size above 8mm after quantifying the total protein concentration of exosome pellet to 200 µg/mL of IVM medium. Group II was maintained as the control without any exosome supplementation. The mean IVM rate (per cent) was calculated by counting the number of oocytes having degree I and II cumulus expansion out of the total culture grade oocytes kept for IVM. The oocytes having degree I and II cumulus cell expansion, as described by Nandi *et al*. (2002) [7] were selected for IVF studies. The IVF was carried out as per standard procedure, using frozen-thawed semen (Gordon, 2003) [4]; The IVF rate was calculated by counting the number of oocytes bearing male and female pro-nuclei, second polar body and cleavage, out of the total matured oocytes and expressed as per cent.

3. Results and Discussions

Field emission scanning electron microscopy (FESEM) revealed agglomeration of round nanoparticles in the ultra-centrifuged follicular fluid sample, ranging from 36.43 to 171.60 nm size. Sharma *et al*. (2010) [10] and Sokolova *et al*. (2011) [11] reported that agglomeration of vesicles occurs due to the drying process during SEM. Wu *et al*. (2015) [14] reported similar sized, round structured exosomes under EM and noted that sputtering enhanced the conductivity of the sample and helped to obtain better image quality. Rodrigues *et al.* (2019) [8] and Zhao *et al.* (2020) [15] reported exosomes of size ranging from 30-200nm from bovine and human follicular fluid.

Table1. *In vitro* bovine oocyte maturation and embryo developmental competence with and without exosome supplementation to oocyte maturation medium

	IVM rate (%)	Fertilisation rate (%)	Cleavage rate (%)
Group I	95.07±1.16^{a}	83.09±0.99^{a}	47.03±1.62^{a}
Group II (control)	85.57±1.98^{b}	74.39±1.55^{b}	40.56±5.80^{b}
P- value	< 0.01	< 0.01	< 0.01

Means with different superscripts differ significantly between rows at 1% level

The mean IVM rate (per cent), as assessed by cumulus cell expansion rate was significantly higher in exosome added group than control (95.07±1.16 vs 85.57±1.98; p<0.01). Higher cumulus expansion and oocyte maturation observed in the present study for the exosome-supplemented group is in agreement with the reports of Hung *et al.* (2015) [5] and Uzbekova *et al.* (2020) [13]. They reported that treatment of COCs with follicular fluid exosomes resulted in the expansion of cumulus cells during IVM. Similar observations were made by Rodrigues *et al.* (2019) [8], who stated that supplementation of follicular fluid EVs to oocyte maturation medium can promote cumulus cell function and enhance the oocyte developmental competence. It was explained that, the addition of follicular fluid exosomes increased the expression of prostaglandin-endoperoxide synthase- 2 (Ptgs 2), pentraxin-related protein- 3 (Ptx3) and tumour necrosis factor alpha (TNFα)-induced protein- 6 (Tnfaip6) genes in the COC (Hung *et al.*, 2015) [5]. Higher maturation rate observed in group I in the present study might be due to increased expression of these genes which support cumulus expansion. In the *in vivo* conditions, higher expressions of these genes are associated with higher luteinizing hormone (LH) concentration in the serum during LH surge, which triggers ovulation (Carletti and Christenson, 2009) [1]. Inside an antral follicle, majority of LH receptors are present in the theca and mural granulosa cells, whereas early cumulus cells do not possess receptors. There occurs a bidirectional communication across the follicular fluid between the mural granulosa and the COC. For the cumulus expansion and oocyte maturation, the signals are initially produced by the mural granulosa cells and then

transferred through the follicular fluid to affect the cumulus cells. Accordingly, the LH surge promotes the release of mural granulosa epidermal growth factor (EGF) ligands, which must then pass through the follicular fluid to activate the cumulus cells, causing the COC to expand and the typical alterations in gene expression (Carletti and Christenson, 2009; Conti, 2010) [1]. All these communications in the *in vivo* conditions happen through follicular fluid exosomes and hence supplementation of follicular fluid exosomes helps in cumulus expansion and oocyte maturation. Tanghe *et al.* (2003) [12] reported that cumulus cells serve as a direct channel for the transport of chemicals to the oocyte, preventing meiotic arrest and maintaining the quality of the oocyte. They have a beneficial effect during fertilisation by providing a microenvironment favouring sperm capacitation, acrosomal reaction and penetration

The mean fertilisation rate in the exosome-added and the non-added group were 83.09±0.99 and 74.39±1.55 per cent, respectively and the difference was statistically significant ($p< 0.01$). Da Silveira *et al.* (2017) [3], reported that exosomes isolated from follicular fluid have a significant role in fertilisation and in the early stages of embryonic development. The authors reported that supplementation of follicular fluid exosomes helps in the global DNA methylation and hydroxyl methylation process in the cells and there by alter the gene expression. Exosomes modulate the mRNA and miRNA levels in the gametes and early embryos, and also induce changes in genes called epigenetic modifiers, which are associated with embryo development. These changes were attributed to the increased fertilisation rate and higher embryonic development rate after exosome supplementation. Hung *et al*. (2015) [5] and Uzbekova *et al.* (2020) [13] reported that, exosomes from small follicles have greater effect on oocyte developmental competence compared to larger follicles.

The mean cleavage rate of oocytes kept for maturation in the current study was 47.03±1.62 and 40.56±5.80 per cent, respectively. There was a significantly higher ($p<0.01$) cleavage rate in the exosome-supplemented group than the control group. Hung *et al.* (2015) [5], Da Silveira *et al.* (2017) [3] and Rodrigues *et al*. (2019) [8] reported higher cleavage rate after supplementation of follicular fluid exosomes isolated from bovine ovarian follicles. The present finding is not in agreement with the findings of Lopera-Vasquez *et al.* (2016) [6] who reported non- significant difference in the cleavage rate between the control and EV supplemented groups.

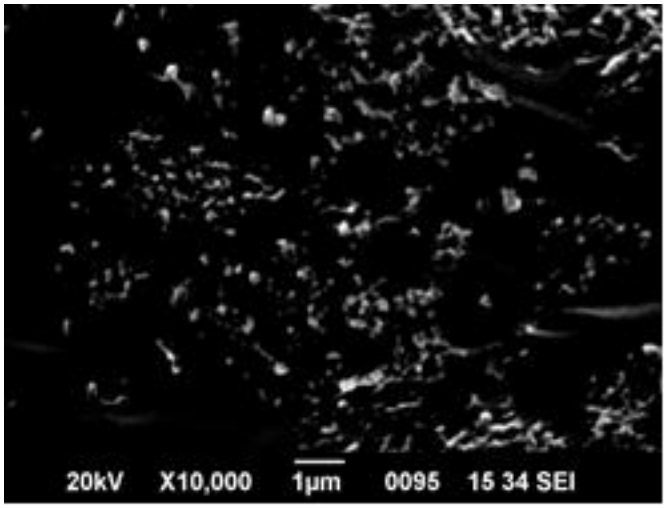

Fig 1: SEM image of exosomes (10 KX) showing round structure

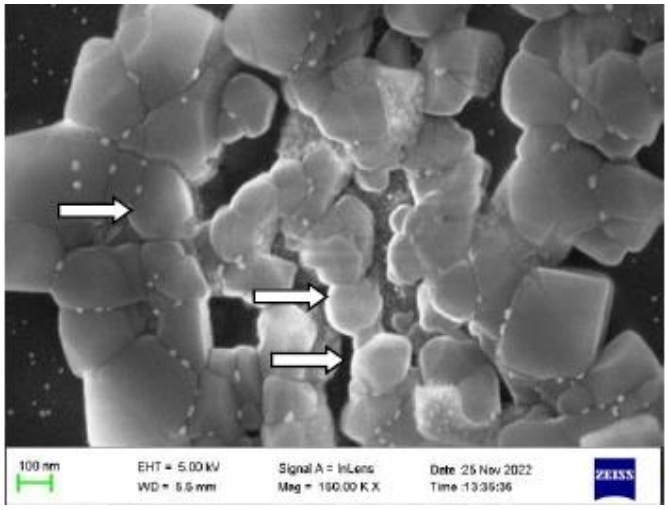

Fig 2: FESEM image of exosomes (150KX) showing round structure (Arrows indicating exosomes)

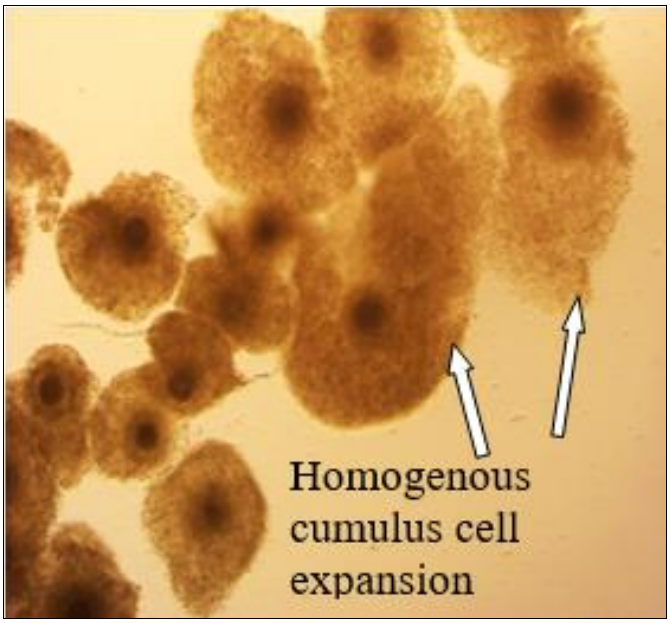

Fig 3: IVM with exosome supplementation (Inverted microscope-10X)

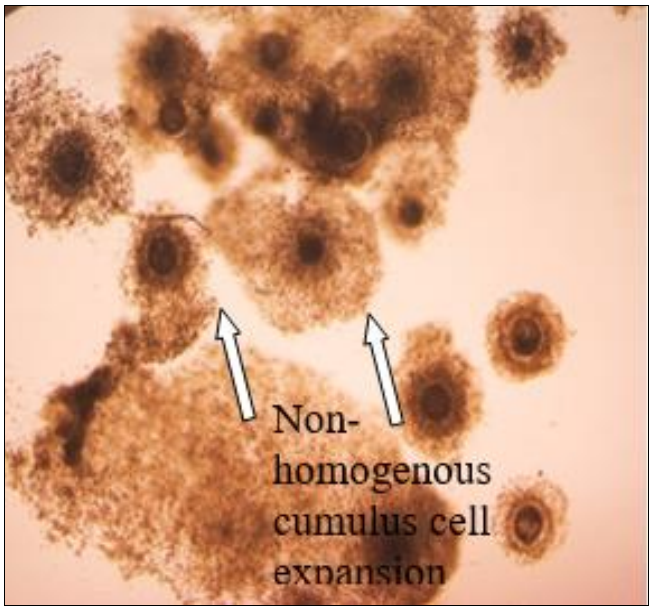

Fig 4: IVM without exosome supplementation (Inverted microscope-10X)

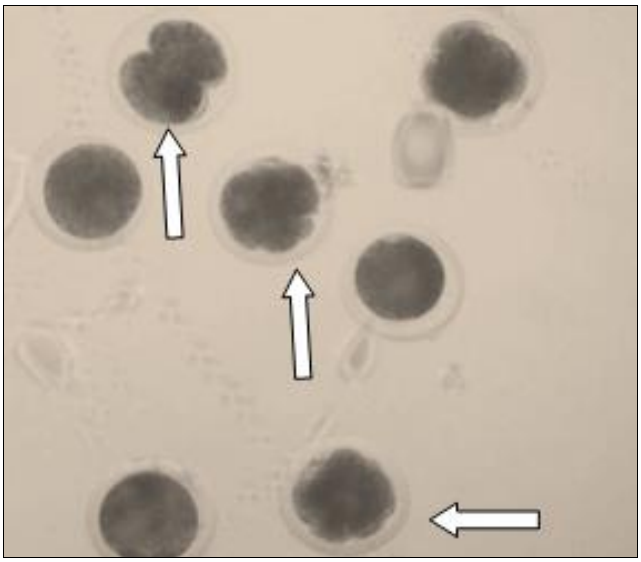

Fig 5: IVC of *in vitro* produced embryos showing cleavage (Inverted microscope- 10X)

4. Conclusion

Supplementation of follicular fluid exosomes to oocyte maturation medium has a positive effect on the overall development of oocytes; such as IVM, fertilisation rate, and cleavage rate. Thus, exosomes can be considered as a physiological resource for supplementation in ART processes to obtain better embryo yield.

5. Acknowledgement

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