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MM Revathy
PhD Scholar, Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala Veterinary and Animal Sciences University, Kerala, India

MP Unnikrishnan
Assistant Professor, Centre for Pig Production and Research, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala Veterinary and Animal Sciences University, Kerala, India

C Jayakumar
Associate Professor and Head, Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala Veterinary and Animal Sciences University, Kerala, India

RS Abhilash
Assistant Professor, Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala Veterinary and Animal Sciences University, Kerala, India

V Ramnath
Professor and Head, Department of Veterinary Physiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala Veterinary and Animal Sciences University, Kerala, India

AR Sreeranjini
Professor, Department of Veterinary Anatomy and Histology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala Veterinary and Animal Sciences University, Kerala, India

Corresponding Author:
MP Unnikrishnan
Assistant Professor, Centre for Pig Production and Research, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala Veterinary and Animal Sciences University, Kerala, India

Assessment of cumulus cell expansion rate and first polar body extrusion rate in bovine oocytes supplemented with bovine follicular fluid exosomes

MM Revathy, MP Unnikrishnan, C Jayakumar, RS Abhilash, V Ramnath and AR Sreeranjini

Abstract
Poor results of *in vitro* embryo production (IVEP) are typically related to deficiencies in the maturation process and culture media used for embryo development. Addition of bovine follicular fluid (FF) exosomes to *in vitro* maturation (IVM) medium is reported to improve the efficiency of IVEP system. In the present study, IVM medium was supplemented with, either the exosomes isolated from follicles 2-8 mm size or > 8 mm size and compared the first polar body extrusion and cumulus cell expansion rates of cultured oocytes in both the groups (group I and II). Exosome non-supplemented group was maintained as control (group III). Exosomes were isolated by differential ultracentrifugation of follicular fluid. Cumulus cell expansion rate was calculated by counting the number of oocytes exhibiting degree I and II cumulus expansion. The nuclear maturation was assessed by counting the number of oocytes exhibiting first polar body extrusion. Both the parameters were significantly higher in group I and II than III. Study signifies the positive effect of exosome addition to IVM medium, irrespective of the follicle size of origin.

Keywords: Exosomes, follicular fluid, *in vitro* maturation, cumulus expansion, first polar body

Introduction
Extracellular vesicles (EVs) have been recently identified as novel cell-to-cell communication mediators in physiological as well as pathological context. Exosomes are 30–200 nm sized EVs generated by release of intraluminal vesicles, following fusion of multi-vesicular bodies (MVBs) with the plasma membrane. Exosomes and other EVs are found in almost all kind of body fluids like blood, saliva, cerebrospinal fluid, urine, FF, oviduct and uterine fluid. Among these, FF is one of the sources used to investigate exosomes in reproductive studies. There is a growing interest regarding the role of exosomes in gamete / embryo-maternal communication and their potential implications in reproductive success. The pace of development of the early embryo to the blastocyst stage is decreased when the culture quality cumulus-oocyte complex is exposed to inadequate culture conditions. The EVs in FF could protect oocytes from various types of stressors and help them to increase their developmental competence, when added to the IVM medium.

Urbanelli *et al.* (2013) [10] reported that, due to the endosomal origin of exosomes, they contained proteins involved in membrane transport and fusion, MVB biogenesis or protein families primarily linked with lipid microdomains, such as integrins and tetraspanins. Sohel *et al.* (2013) [9] conducted a study to reveal the exosomal and non-exosomalmi RNA content of bovine FF and observed that majority of the extra-cellular miRNAs were associated with exosomes; they also found that these exosomes were easily taken up by the surrounding follicular cells and thereby helping to increase the endogenous miRNA levels in follicular cells; thus up regulating the pathways responsible for oocyte maturation and embryonic development. There are reports in the literature, indicating that exosomes derived from smaller follicles would be better candidates to improve developmental competence of oocytes in IVEP. The present study was designed to compare the effect of supplementation of exosomes derived from follicles of two different size groups on *in vitro* developmental competence of oocytes

Materials and Methods

Bovine slaughter ovaries were utilised for aspiration of FF and oocytes. The exosomes were isolated by ultracentrifugation of FF.

Collection of follicular fluid

Bovine slaughter ovaries were collected from Corporation slaughterhouse, Kuriachira, Thrissur and Meat Technology Unit, Kerala Veterinary and Animal Sciences University. The ovaries were transported to laboratory within 2 h of slaughter, in a thermos flask containing 0.90 per cent normal saline at 37±2 ℃, supplemented with of gentamicin sulphate (1mL/Litre). Initially the ovaries were rinsed 3 - 5 times in normal saline to remove the blood stains and contaminants. Subsequently, extra ovarian ligaments and remaining excess tissues were trimmed off. The ovaries were then washed in normal saline 8 - 10 times, until no stains were visible. They were subsequently maintained at a temperature of 36 – 38 ℃ until the aspiration was completed.

Follicles on the ovaries were classified into two groups: group A with size up to 8 mm and group B, with size above 8 mm. A 20 gauge needle, fitted with 5 mL syringe was used to aspirate all the visible follicles on the ovarian surface. The FF was collected separately from the two groups of follicles and stored at -80 °C until a minimum volume of 16 mL was attained in each group. The FF was thawed at room temperature and diluted with an equal volume of Phosphate Buffered Saline (PBS) prior to centrifugation. To eliminate remaining residual granulosa cells and oocytes, samples were spun in a cooling centrifuge at 4˚C (Eppendorf centrifuge – 5804R) at 800 g for 10 min followed by 2000 g for 20 min. The fluid was then centrifuged at 12000 g for 45 min to remove cell debris and large particles. The samples were then filtered through a 0.20 µm pore syringe filter to remove particles larger than 200 nm. Ultracentrifugation was performed at 1,10,000 g for 3 h at 4˚C in a Sorvall WX Ultra Series Centrifuge (Thermos scientific) using a swinging-bucket SuperspinSorvall30Ti rotor, to pellet the follicular extracellular vesicles. After ultracentrifugation, the supernatant was completely discarded and kept the tube upside down on tissue paper to remove any remnants of the supernatant. The pellets obtained were re-suspended in 200µL of filtered PBS (0.22 µm pore syringe filter) and kept overnight at 4 °C before storage at -80 °C.

3.2 *In vitro* oocyte maturation

Ovaries of cows were collected, transported and cleaned as described earlier. All the surface follicles measuring 2-8 mm size were aspirated, oocytes separated, COCs were identified, washed and prepared for grading, following standard procedure. These COCs were graded as described by Saleh (2017); Grade A and B oocytes only were considered as culture grade and hence selected for IVM and randomly allocated to three groups (I, II and III). The number of oocytes subjected to IVM in group I (supplemented with exosomes from 2-8 mm follicles), II (supplemented with exosomes from >8 mm follicles) and III (control) were 228, 226 and 223, respectively, forming a total of 677. The number of sessions of IVM cycles in group I, II and III were 6, 7 and 9, respectively, forming a total of 22 sessions during the study period.

Total protein concentration measurement of exosomes was carried out by BCA assay and exosomes supplementation was done in such a way that protein concentration was maintained at the rate of 200 µg/mL of IVM medium. The mean cumulus cell expansion 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 subjected to IVM. The oocytes with first polar body were identified after removing the cumulus cells by vortexing. First polar body extrusion rate was calculated by counting the number of oocytes with first polar body, out of total culture grade oocytes subjected to IVM.

4. Results and Discussion

In group I, out of the total 228 oocytes subjected to IVM in six sessions (mean 38.00±4.55 oocytes per session), 223 oocytes (mean 37.17±4.55 oocytes per session) matured, as indicated by cumulus cell expansion, yielding a mean cumulus cell expansion rate of 97.69±0.52 per cent. Total number of oocytes exhibiting first polar body extrusion was 78 (mean 13.00±1.85 per session); yielding first polar body extrusion rate of 34.10±1.24 per cent. Details are presented in Table 4.1.

Table 4.1: Session-wise details of cumulus cell expansion and first polar body extrusion rates of culture grade oocytes supplemented with exosomes from 2-8 mm follicle (Group I)

Session number	Number of culture grade oocytes in serial cultures (a)	Number of oocytes showing degree I and II cumulus cell expansion (b)	Number of oocytes showing first polar body (c)	Cumulus cell expansion rate (%) (b/a)	First polar body extrusion rate (%) (c/a)
1	56	55	20	98.21	35.71
2	38	38	12	100.00	31.58
3	39	38	14	97.44	35.90
4	29	28	10	96.55	34.48
5	34	33	10	97.06	29.41
6	32	31	12	96.88	37.50
Total (n=6)	228	223	78		
Mean±S. E	38.00±4.55	37.17±4.55	13.00±1.85	97.69±0.52	34.10±1.24

In group II, out of the total 226 oocytes subjected to IVM in seven sessions (mean 32.29±9.87 oocytes per session), 218 oocytes (mean 31.14±9.74 oocytes per session) matured, as indicated by cumulus cell expansion, yielding a mean cumulus cell expansion rate of 95.07±1.16 per cent. Total number of oocytes exhibiting first polar body extrusion was 70 (mean 10.00±2.63 per session); yielding first polar body extrusion rate of 32.29±1.47 per cent. Details are presented in Table 4.2.

In group III, out of the total 223 oocytes subjected to IVM in nine sessions (mean 24.78±2.69 oocytes per session), 193 oocytes (mean 21.44±2.65 oocytes per session) matured, as indicated by cumulus cell expansion, yielding a mean cumulus cell expansion rate of 85.57±1.98 per cent. Total number of oocytes exhibiting first polar body extrusion was

38 (mean 4.22±0.46 per session); yielding first polar body extrusion rate of 17.18±1.21 per cent. Details are presented in Table 4.3

Table 4.2: Session-wise details of cumulus cell expansion and first polar body extrusion rates of culture grade oocytes supplemented with exosomes from >8 mm follicle (Group II)

Session number	Number of culture grade oocytes in serial cultures (a)	Number of oocytes showing degree I and II cumulus cell expansion (b)	Number of oocytes showing first polar body (c)	Cumulus cell expansion rate (%) (b/a)	First polar body extrusion rate (%) (c/a)
1	19	18	6	94.74	31.58
2	16	15	5	93.75	31.25
3	31	30	10	96.77	32.26
4	30	29	12	96.67	40.00
5	9	8	3	88.89	33.33
6	88	86	24	97.73	27.27
7	33	32	10	96.97	30.30
Total (n=7)	226	218	70		
Mean± S. E	32.29±9.87	31.14±9.74	10.00±2.63	95.07±1.16	32.29±1.47

Table 4.3: Session-wise details of cumulus cell expansion and first polar body extrusion rates of culture grade oocytes without exosome supplementation (Group III)

Session number	Number of culture grade oocytes in serial cultures (a)	Number of oocytes showing degree I and II cumulus cell expansion (b)	Number of oocytes showing first polar body (c)	Cumulus cell expansion rate (%) (b/a)	First polar body extrusion rate (%) (c/a)
1	25	22	5	88.00	20.00
2	25	20	6	80.00	24.00
3	35	32	6	91.43	17.14
4	25	22	4	88.00	16.00
5	40	36	5	90.00	12.50
6	19	15	3	78.95	15.79
7	21	19	4	90.48	19.05
8	17	15	3	88.24	17.65
9	16	12	2	75.00	12.50
Total (n=9)	223	193	38		
Mean± S. E	24.78±2.69	21.44±2.65	4.22±0.46	85.57±1.98	17.18±1.21

Mean cumulus cell expansion rates in group I, II and III were 97.69±0.52, 95.07±1.16 and 85.57±1.98, respectively. Mean first polar body extrusion rates in group I, II and III were 34.10±1.24, 32.29±1.47 and 17.18±1.21, respectively. Statistically, both the rates in group III were significantly lower ($p<005$) than group I and II. There existed no significant difference between groups I and II, in either of the rates. Results are presented in Table 4.4.

Table 4.4: Comparison of cumulus cell expansion and first polar body extrusion rates (Mean± SE) of culture grade oocytes from different groups, with/without exosome supplementation to maturation medium

Group	Mean no. of culture grade oocytes subjected to IVM (Mean± SE)	Mean no. of oocytes showing degree I and II cumulus cell expansion (Mean± SE)	Mean number of oocytes showing first polar body extrusion (Mean± SE)	Cumulus cell expansion rate (%) (Mean± SE)	First polar body extrusion rate (%) (Mean ±SE)
I	38.00±4.55	37.17±4.55	13.00±1.85	$97.69^{a\pm}$0.52	34.10^{a}±1.24
II	32.29±9.87	31.14±9.74	10.00±2.63	$95.07^{a\pm}$1.16	32.29^{a}±1.47
III	24.78±2.69	21.44±2.65	4.22±0.46	$85.57^{b\pm}$1.98	17.18^{b}±1.21
P- value				<0.05	

Means having different superscripts within a column differ significantly at 5% level

The process of maturation of oocyte can be divided into cytoplasmic and nuclear maturation. Nuclear maturation of oocyte is completed after resumption of meiosis, germinal vesicle break down and progression of meiosis to metaphase II. The completion of nuclear maturation is indicated by the presence of first polar body in the peri- vitelline space of oocyte (Combelles *et al*., 2009) [2]. Better cumulus expansion rate observed in the present study for the exosome-supplemented groups agrees with the reports of Hung *et al.* (2015) [5] and Uzbekova *et al.* (2020) [11]. Similar observations were made by Rodrigues *et al.* (2019) [8], who stated that supplementation of EVs of FF origin to oocyte maturation medium can promote cumulus cell function and enhance oocyte developmental competence. It was explained that, the addition of FF exosomes increased the expression of prostaglandin-endoperoxide synthase- 2 (Ptgs2), pentraxin-related protein- 3 (Ptx3) and tumour necrosis factor alpha - induced protein- 6 (Tnfaip6) genes in the COC (Hung *et al.*, 2015) [5], which are responsible for the cumulus cell expansion and oocyte maturation. The improved cumulus cell expansion in turn promotes nuclear maturation of oocyte (Lee *et al*., 2020; Javadi *et al*., 2022; Han *et al*., 2023) [7, 6, 4].

In the *in vivo* conditions, higher expressions of these genes are associated with higher 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 FF between the mural granulosa and the COC. Accordingly, LH surge promotes release of mural granulosa epidermal growth factor (EGF) ligands, which must then pass through the FF to activate the cumulus cells, causing the COC to expand and the typical alterations in gene expression (Carletti and Christenson, 2009; Conti, 2010) [1, 3]. Thus FF acts as a channel for bidirectional communication between COC and granulosa cells.

Significantly higher cumulus cell expansion rate and first polar body extrusion rate recorded in the exosome added groups during the study might be due to increased expression of such genes responsible for cumulus expansion.

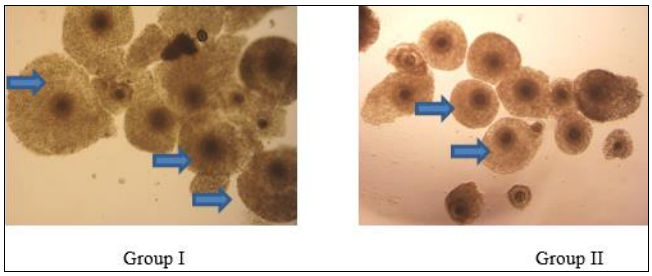

Plate 4.1: *In vitro* maturation with exosome supplementation (Inverted microscope- 10X, arrows indicating oocytes with degree I and II cumulus cell expansion)

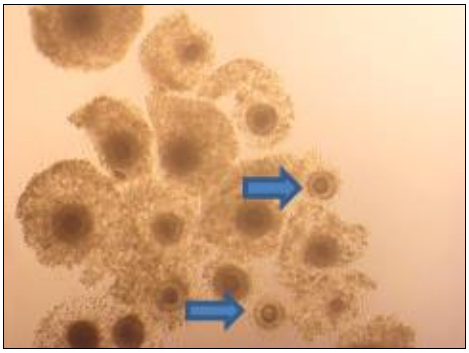

Plate 4.2: *In vitro* maturation without exosome supplementation (Inverted microscope- 10X, arrows indicating oocytes with degree zero cumulus cell expansion)

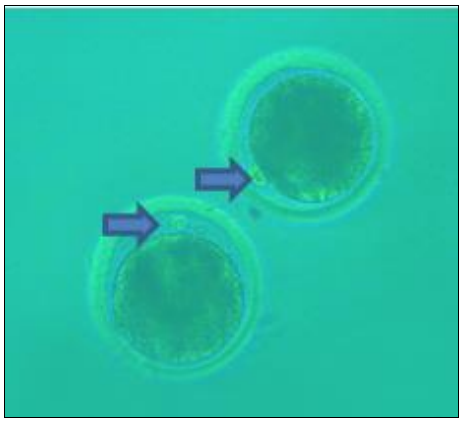

Plate 4.3: Oocytes with first polar body (Inverted microscope- 20X)

5. Conclusion

Supplementation of FF exosomes to oocyte maturation medium has a positive effect on cumulus cell expansion rate and first polar body extrusion rate, indicating the beneficial effects on *in vitro* oocyte developmental competence.

6. Acknowledgement

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