RESEARCH ARTICLE
Open Access

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2023.54.2.465-471

Developmental competence of bovine oocytes in maturation media supplemented with follicular fluid exosomes#

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Citation: Arya, T., Abhilash, R.S.,Jayakumar, C., Amritha, A., Naicy, T., Harshan, H.M. andRevathy, M.M. 2023.Developmental competence of bovine oocytes in maturation media supplemented with follicular fluid exosomes. *J. Vet. Anim. Sci.* **54**(2):465-471
DOI: https://doi.org/10.51966/jvas.2023.54.2.465-471

Received: 11.11.2022 *Accepted: 20.02.2023* *Published: 30.06.2023*

Abstract

The present study evaluated the role offollicular fluid exosomes on the developmental competence of bovine oocytes. Ovaries from slaughtered crossbred cattle were collected, all visible surface follicles aspirated, and culture-grade oocytes were subjected to further study. Exosomes were isolated from bovine follicular fluid by differential ultracentrifugation. A total of 358 oocytes selected for the study were randomly divided into two groups. Group I constituted 111 oocytes, in which normal maturation was carried. Group II constituted 247 oocytes, in which in vitro maturation (IVM) medium was supplemented with follicular fluid exosomes at 1 µL/100 µL IVM medium. Maturation was assessed after 24h of culture in a CO_2 incubator maintained at 38.5°C in 95 per cent humidified atmosphere of 5 per cent CO_2. Following IVM of oocytes for 24 h, in vitro fertilisation (IVF)was carried out by co-incubating with capacitated spermatozoa for 18 h and embryo culture was carried out subsequently. In group II oocytes supplemented with exosomes, a significantly higher maturation rate (p ≤0.01) (95.80 ±1.67 vs76.10 ± 0.95), fertilisation rate (53.68 ± 3.02vs37.85 ± 7.01) and cleavage rate (p ≤0.01) (43.66 ± 2.13vs(32.47 ± 5.23) was noticed compared to oocytes in group I without any supplementation.The present study established that supplementation of follicular fluid exosomescould improve the developmental competence of bovine oocytes.

Keywords: *Exosomes*, *in vitro maturation*, *bovine oocyte, follicular fluid*

 # Part of MVSc thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala

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Running title:Developmental competence of bovine oocytes in maturation media supplemented with follicular fluid exosomes

In vitro embryo production technology (IVEP) in bovines has received great attention during the last few decades. Even though IVEP is established, the efficiency of embryo productionis still limited to 30-40 per cent (Saeki *et al*., 1991).Since the early days of embryo culture *in vitro*, various modifications were done to create the best microenvironment that simulated the biological system, to attain optimum embryo yield (Leibfried-Rutledge *et al*., 1987). Even now, the developmental competence of *invitro* cultured embryos is markedly lower due to the lack of physiological pathways and essential components in culture system as the embryonic development is highly susceptible to heat stress in *in vitro* culture environment which disrupts the success of fertilization by affecting the anti-polyspermy system of oocytes (Sakatani*et al*., 2015).

Extracellular vesicles,found in the follicular fluid are nanoparticles released by cells that facilitate cell-to-cell contact in the follicle microenvironment (de Avila and da Silveira,2020). Early endosomes are first produced by endocytosis within cells. As they develop into late endosomes, some intraluminal vesicles (ILVs) are shed by endosomes to form multivesicular bodies (MVBs), which then move to the cell membrane. The MVBs release the vesicles inside the extracellular milieu as exosomes after fusing with the plasma membrane (Chuo *et al*., 2018). Exosomes, with the size of 30-200 nm are termed as molecular cargo as they carry lipids, proteins, mRNA, miRNA and DNA and are involved in intercellular and intracellular communication (Choi *et al*., 2013). They are extremely stable, may cross the blood-brain barrier, and so open up promising possibilities for diagnostics and therapeutic interventions (Aryani and Denecke, 2016).

The “gold standard” for isolating exosomes is density gradient centrifugation, which is a variation of ultracentrifugation (Iwai *et al*., 2016). Bovine follicular fluid exosomeswere reported to have the potential to enhance the maturation and fertilisation of oocytes and development to the blastocyst stage (Bridi*et al*., 2020). These extracellular vesicles have been investigated as biomarkers and supplementing tools to replicate natural circumstances during assisted reproductive techniques. In view of the above observations,the present study was concluded to evaluate the effect of supplementation of bovine follicular fluid exosomes on *in vitro* maturation, cleavage and fertilisation of bovine oocytes.

Materials and methods

Procurement of oocytes

Bovine ovaries of unknown reproductive status were collectedfromthe Corporation Slaughterhouse, Thrissur in normal saline fortified with Penicillin (100 IU/ mL).The extra ovarian ligaments as well as excess tissues adhering to the ovaries were trimmed off using scissors. Later, ovaries were washed in normal saline 8 to 10 times until they were stain-free and maintained at 36-38°C until aspiration of oocytes were completed. All visible surface follicles between 2 and 8 mm in size were aspirated, collected in a 10 mL test tube, and left undisturbed in the incubator for 10 min to settle. Later, sediment from the bottom was pipetted out, andcumulus oocyte complexes (COCs) were morphologically gradedas A, B, C, or D under a stereozoom microscope (Ceticae*t al*., 1999).

Isolation of exosomes

Follicular fluid aspirated from ovaries using 20 guage needle wasstored in the ultra-deep freezer (-80°C) until the desired quantity is obtained for ultracentrifugation. For each batch of follicular fluid, a total of 16 mL was thawed to room temperature, diluted with an equal volume of phosphate-buffered saline (PBS), and centrifuged in a cooling centrifuge at 800g for 10 min, 2000g for 20 min and 12000g for 45 min at 4°C and the supernatant was filtered through 0.2µm syringe filter. Filtered supernatant was subjected to ultracentrifugation (SorvallWX Ultra Series Centrifuge -Thermo Fisher Scientific) at 110000g for 3 h at 4°C using swinging bucket SW30Ti rotor (SuperspinSorvall). The pellet containing exosomes was resuspended in 200 µL of sterile PBS and was stored at -80°C.

Experimental design

A total of 358 culture-quality oocytes of Grades A and B were selected for the study. Group I consisted of 111 oocytes in which maturation was carried out by standard protocol and group II (n=247) maturation was carried out by supplementing exosomes @ 1 µL/100 µL IVM medium. Cumulus oocyte complexes were identified using stereozoom microscope and transferred to the washing medium. After serial washings culture quality oocytes (grades A and B) were transferred to maturation droplet in a ratio of 10-20 oocytes per 100 µL of maturation droplet.

The maturationmedium consisted of TCM-199 (HEPES modified) supplemented with FSH, estradiol-17β, sodium pyruvate, L-glutamine, gentamicin sulphate and FBS. The culture condition set for the study was 38.5°C in 95 per cent humidified atmosphere of 5 per cent CO_2. Maturation was assessed after 24 h of culture by cumulus cellexpansion and first polar body extrusion. Fertilisation was carried out using frozen-thawed semen. Sperm oocyte co-incubation was carried out for a period of 18 h. The presumptive zygotes were then transferred to culture droplets after 18 h of incubation. The culturemedia used for the study was Vitrogen (IVC-0420220A). Cleavage and fertilisation were evaluated 48 h post-insemination under an inverted microscope at 40x magnification (Rizos, 2002).

Results and discussion

Out of the total 125 bovine ovaries of unknown reproductive statusfrom the slaughterhouse, 60 ovaries were subjected to follicular aspiration for oocyte retrieval and 65 ovaries for follicular fluid aspiration. A total of 823 visible surface follicles having 2- 8mm diameter were aspirated. The meannumber of follicles aspirated per ovary in the present study was 13.71 ± 2.16. The result was comparable with that of Garcia and Salaheddine (1998) who obtained 12.4 ± 6.1 follicles per bovineovary and was lower than the observations of Fitzpatrick and Entwistle (1996) who observed 26.40 ±1.60follicles per ovary. The difference in the number of follicles couldbe due tothe presence of CL in the ovary as it exerted a negative impact on the number of follicles and oocytes recovered (Singh*et al*., 2001) and variations in the age of animal, breed, climatic conditions, nutritional, genetic and reproductive status of the animal at the time of slaughter (Sianturi*et al*., 2002).

The recovery rate of oocytes obtained in the present study was 68.73 ± 2.71 per cent. The oocyte recovery rate found in the study was in accordance with the observations made by Singh *et al*. (2001), who obtained a recovery rate of 67 per cent. Arya*et al*. (2021) obtained an oocyte recovery rate of 75.07 per cent from caprine ovaries. Boonkong*et al*. (2012) obtained a lower recovery rate of 58.6 per cent. Rakshitha*et al*. (2019) and Minu*et al*. (2021) obtained a higher oocyte recovery rate of 86.44 per cent and 87.88 ± 1.50 per cent, respectively. A total of 358culture-grade oocytes were retrieved out of 550 oocytes, of which 111 were grade A and 247 were grade B. In the present study, meanyield of culture-grade oocytes obtained per ovary was 4.93±0.65. Rakshitha*et al*. (2019) obtained a lower yield of culture-grade oocytes per ovary which was 3.83 ± 0.23. The per cent yield of culture-grade oocytes obtained in the current investigation was 65.37 ± 3.15. Rakshitha*et al*. (2019) obtained a higher yield of culture-grade oocytes of 78.27 ± 2.08 per cent. The difference in the quality of oocytes obtained in the present study compared with other findings might be due to variability in size and functional status of the follicle, age, season, nutritional status, health condition of the animal and stage of the oestrous cycle at the time of slaughter (Nandi *et al*., 2002;Sianturi*et al*., 2002).

The yieldof follicular fluid per ovary in the present study was 0.82 ± 0.07mL. The result was comparable with that of Grimes and Ireland (1986) and Hazeleger (1995) who found that the volume of follicular fluid collected from each ovary ranged from 0.04 to 2.4 mL and 0.03 to 2.3 mL, respectively. The volumeof follicular fluid aspirated depends on the size of the follicles, skill of the person, pressure exerted and gauge of the needle used for aspiration.

A significantly higher maturation rate ($p \leq 0.01$) was observed in group II (Fig. 2) compared to the control group (95.80 ±1.67

Table 1. Effect of exosome supplementation on maturation rate of oocytes recovered from slaughter house ovaries

Treatment group	No of oocytes kept for maturation	Number of matured oocytes	Maturationchanges observed		Maturation rate (%)
			Cumulus cell expansion (%)	First polar body extrusion (%)	
Group I	111	85	65.92 ± 3.23 (70)	10.19 ± 3.44^{a} (15)	76.10 ± 0.95^{a}
Group II	247	239	71.79 ± 2.5 (182)	24.01 ± 2.30^{b} (57)	95.80 ± 1.67^{b}

*** Significant at 0.01 level. Means having different letter as superscript in column differ significantly. Test done:One-way ANOVA with 5 replicates in each group.*

Table 2. Effect of exosome supplementation on cleavage and fertilisation rate of oocytesrecovered from slaughter house ovaries

Treatment group	Total no of oocytes kept for maturation	Fertilisation changes observed			
		Second polar body (%)	Male and female pronuclei (%)	Cleavage rate (%)	Fertilization rate (%)
Group I	111	3.54 ± 1.74^{a} (3)	3.54 ± 1.75 (3)	32.47 ± 5.23^{b} (36)	37.85 ± 7.01^{b} (42)
Group II	247	8.61 ± 0.92^{b} (19)	2.26 ± 0.70 (6)	43.66 ±2.13^{c} (106)	53.68 ± 3.02^{c} (131)

**Significant at 0.05 level. Means having different letter as superscript differ significantly. Test done:One-way ANOVA with 5 replicates in each group*

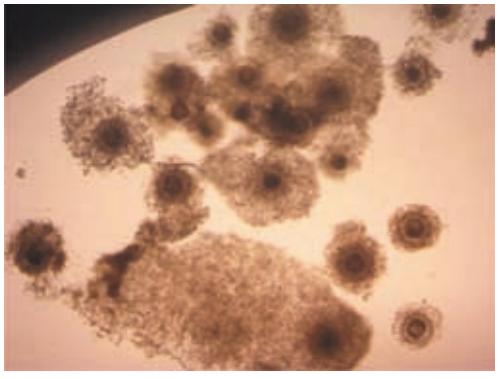

Fig.1. Cumulus expansion noticed in control group (4X)

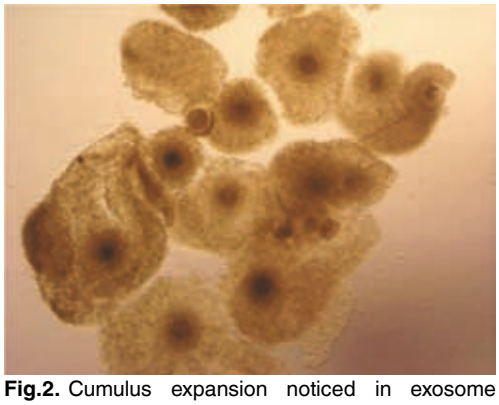

Fig.2. Cumulus expansion noticed in exosome supplemented group (4X)

vs 76.10 ± 0.95) (Table 1 and Fig. 1). The advantage of exosome supplementation was significantly evident (p≤0.01) in cleavage rate also (43.66 ± 2.13vs32.47 ± 5.23) (Table 2 and Fig. 3). Similarly, in the fertilisation rate also, the benefit was obvious with a significant increase in the fertilisation rate (p ≤0.05)in group II (53.68 ± 3.02) as against (37.85 ± 7.01)in group I (Table 2).

Cumulus cells refer to a group of cells that surround and nourish the oocytes. Cumulus expansion mainly occursas a result of extracellular matrix synthesis of

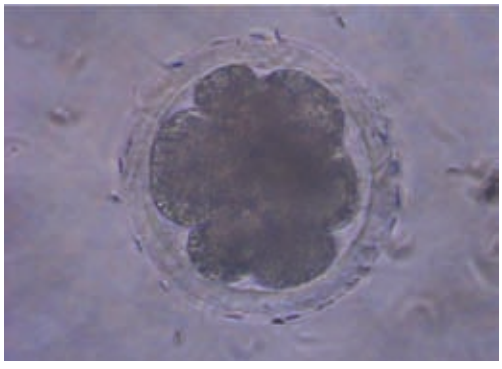

Fig.3. Eight-cell stage embryo (10X)

hyaluronic acid by cumulus cells (Hung *et al*., 2015). For maintaining the meiotic arrest and developmental competence of oocytes, cumulus cells are a necessary factor. The number of cumulus cell layers, cumulus quality and cumulus expansion intensity are important for the success of *in vitro* oocyte maturation and for the attainment of viable matured oocytes. The expansion of cumulus cells happens at the time of maturation of oocytes in both *in vitro* and *in vivo* conditions (Nevoral*et al*., 2014).

In the present study, cumulus expansion was found to be greater in exosome supplemented group compared to the control group. Also, there is a significant difference noticed in the IVM rate. It clearly depicts that exosome supplementation in IVM medium is having a positive influence in increasing the cumulus cell expansion and maturation rate. During *in vivo*, at the time of oestrus, the surge of luteinizing hormone triggered ovulation and increased the expression of genes that support cumulus cell expansion. They areprostaglandin-endoperoxide synthase 2 (Ptgs2), pentraxin-related protein 3 (Ptx3), and tumour necrosis factor alpha-induced protein 6 (Tnfaip6)(Hung *et al*., 2015). The theca and mural granulosa of antral follicles contain the majority of LH receptors, whereas early cumulus cells do not. It is known that bidirectional communication takes place across the antral follicular fluid between the mural granulosa and the cumulus-oocyte complex (COC). Accordingly, the luteinizing hormone (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 in order to cause the COC to expand and the typical alterations in gene expression. Thus, when bovine oocytes are supplemented with follicular fluid exosomes, it resulted in increased cumulus cell expansion and maturation rate (Hung *et al*., 2015).An increased maturation rate of significance in exosome supplemented oocytes compared to the oocytes in the non-supplemented medium corroborates to the findings of da Silviera*et al*. (2017) and Lopez *et al*.(2019) who observed that supplementation of exosomes enhanced cumulus cell expansion and *in vitro* maturation rate of bovine oocytes.

The fertilisation rateand cleavage rateof exosome supplemented oocytes in the present study showed a significant difference incomparison with the control group. Extracellular vesicles (EVs) have a significant role in fertilisation and the early stages of embryonic development and are found in the oviductal and uterine fluid. Small EVs from the oviduct and endometrium can be taken up by embryonic cells, and EVs from the embryo can alter ovarian and uterine function (Bridi*et al*., 2020). According to Qu*et al*. (2017) supplementation of exosomes to the culture medium significantly improved blastocyst formation and quality as well as their growth using bovine somatic cell nuclear transfer (SCNT) embryos as a model system. They found that during *in vitro* culture, SCNT embryos are capable of secreting exosomes into the culture medium which is essential for an increase in blastocyst formation rate, total cell numbers of the blastocyst and ratio of inner cell mass/ trophectoderm. Exosomes secreted by early-stage embryos canincrease the expression of early pleuripotent genes like *Oct4, Sox2, Klf4, c-Myc*, and *Nanog*(de Avila and da Silveira., 2020).

The results of the present study signify that exosome termed molecular cargo, contain mRNA, miRNA, lipids, proteins and DNA involved in both intercellular and intracellular communication. This communication of exosomes within and between cells could have resulted in increased cumulus cell expansion, maturation, cleavage and fertilisation rate (Bridi*et al*., 2020).

Conclusion

From the above findings, it can be concluded that the supplementation of exosomes in the IVM mediumhas a positive effect on the developmental competence of bovine oocytesas signified by increase in the cleavage and fertilisation rate in the exosome-supplemented group. Hence,the presentstudy reportsthat the efficiency of IVEP could be increased by incorporating follicular fluid exosomes in the IVM medium of bovine oocytes.

Acknowledgements

The authors are thankful to the Dean, College of Veterinary and Animal Sciences, Mannuthy, Kerala Veterinary and Animal Sciences University for providing the facilities needed for carrying out this research work.

Conflict of interest

The authors declare that they have no conflict of interest.

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