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# Isolation and characterization of exosomes derived from buffalo oviductal epithelial cells

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ABSTRACT

In the present study, exosomes were successfully isolated from the conditioned medium of buffalo OECs cultured *in vitro*. Characterisation by nanoparticle analysis revealed that the size of the oviductal exosomes ranged between 40-150 nm. Imaging by Transmission electron microscopy showed that these exosomes exhibited circular/spherical morphology. The identity of isolated exosomes was further confirmed by analysing the expression of surface expressed tetraspannin markers (CD9, CD63, and CD81) by flow cytometry, and were found to be enriched with the tetraspannins analysed. As continuation of this work, further research to supplement the isolated oviductal exosomes to recreate the preimplantation physiological environment of the oviduct should be undertaken, thus improving *in vitro* culture conditions for buffalo embryos.

**Keywords:** Exosomes, *In vitro* culture, Oviductal epithelial cells (OECS)

Exosomes are the nano-sized vesicles secreted by cells which function as bioactive cargo reflecting particular cells' kind and physiological state. Just as every cell does, oviductal epithelial cells (OECs) release their molecular cargo as exosomes to their luminal microenvironment to establish the earliest maternal signal between the oviduct and the developing embryo. In the past two decades, tremendous advancements made in assisted reproductive technologies (ARTs) helped to better understand the molecular events of reproduction, from gamete development, through fertilization to early embryonic development. Even with the upswing in ARTs, the *in vitro* embryo production system is still away from the physiologically precise *in vivo* system, both in terms of success rate and the quality of embryos produced (Matsuno *et al*. 2017).

In the case of bovine *in vitro* system, usually on day seven, embryos are transferred, hence missing the oviductal life completely. Such a sub-optimal culture environment results in altered gene expression pattern, and a lower rate of transferrable embryos (Mondejar *et al*. 2013). Any attempt to mimic an oviductal microenvironment during *in vitro* fertilization (IVF) has a positive impact on embryo development, making it more suitable for transfer.

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It is already well established that co-culture of bovine early pre-implantation stage embryos with oviductal epithelial cells (OECs) improves the success rate and quality of embryos produced (Cordova *et al*. 2014). Different studies with OEC co-culture (Garcia *et al*. 2017) provided conclusive evidence on the crucial role of oviduct in pre-implantation embryo-maternal interaction. However, the OEC-based co-culture system is associated with its complexity in methodology, missing reproducibility, and bio-sanitary risks are associated with it (Ramos-Ibeas *et al*. 2014). For the reasons stated, scientists were in search of a better alternative over the years.

Recent researches invited attention towards the membrane-limited nanovesicles, called exosomes (Alimana and Bauersachs 2019). Exosomes are typically 40-150 nm in diameter and express their molecular content related to the kind of cell and their physiological status (Pavani *et al*. 2017). Exosomes affect intercellular trafficking by transferring the biomolecule(s) it carries (mRNA, miRNA, proteins, and lipids), thus modulating the activities of recipient cells by participating in active intercellular communication (Ni et *al*. 2020).

With the above background knowledge, the preliminary objective of the proposed research was to isolate and characterize the exosomes secreted by *in vitro* cultured OECs of buffaloes to further investigate on their usefulness in improving the culture conditions of *in vitro* embryo production system.

## MATERIALS AND METHODS

The present study was carried out from March 2022 to

February 2024 at Centralized embryo biotechnology lab, Department of Animal Biotechnology, Madras Veterinary College, Chennai.

*Culturing of OECs of buffalo:* The buffalo uterus with attached ovaries (in the early luteal phase) and oviduct were collected from the nearby slaughterhouse and transported to the laboratory in normal saline with antibiotics. The oviducts were dissected free of attached connective tissues and washed twice in DPBS with antibiotics.

The oviductal mucosa was gently expelled into a petri dish by squeezing the oviduct with a sterile glass slide. Following standard procedures, retrieved OECs were suspended in required volume of cell culture medium (TCM 199 with 10% FBS and antibiotics), seeded into a T25 cell culture flask, and cultured in an incubator with 5% $CO_2$ level at 37°C. Half of the spent medium was replenished every 48 h till day 4. On day 4, the whole culture medium was replaced with TCM 199, containing 10% exosome-depleted FBS (Gibco). OECs were maintained for another 48 h around which 80-90% confluency was reached. Conditioned media was harvested from the OEC culture on reaching 80-90% confluency and proceeded with exosome isolation immediately.

*Isolation of exosomes secreted by the cultured OECs:* Initially, the harvested conditioned media was centrifuged at 2000 ×g for 30 min (at 4°C) to remove the cells and debris. The cell-free supernatant was used for exosome isolation using Total Exosome Isolation kit (Invitrogen) following the manufacturer's instructions. The final exosome pellet obtained was resuspended in $Ca^{2+}$/$Mg^{2+}$ free PBS as recommended by MISEV (Minimal Information on the Study of Extracellular Vesicles 2018), and stored at -20°C until used.

*Characterization of oviduct derived exosomes*

The isolated oviductal exosomes were characterised for their physical integrity and biological characteristics by the most widely employed techniques: Nanoparticle size analysis, Transmission electron microscopy and Flow cytometry.

*Nanoparticle size analysis:* Isolated exosomes were evaluated for their size and homogeneity in distribution by the Nanoparticle size analyser (Nano partica SZ-100, Horiba scientific) facility available at the institute. For the same, isolated exosomes were resuspended in $Ca^{2+}$/$Mg^{2+}$ free PBS (1:500) and loaded into the cuvette used with the instrument. The sample was scanned at random position throughout the cuvette. Recorded measurements were processed by the software giving final results. After outlier removal, the measurements were recorded in terms of mean, median, and mode which indicated the diameter of the vesicles. Using quadratic interpolation, the software also represented frequency distribution of the vesicles according to their size.

*Physical characterization by transmission electron microscopy:* For TEM analysis, the exosome sample was fixed with 2% glutaraldehyde (1:1) for 30 min. A fixed sample of 6 µl was pipetted onto a 200-mesh copper grid with carbon-coated formvar film and incubated for 10 min. Then negative staining with 2% uranyl acetate was carried out for 1 min and visualized under the Transmission electron microscope (FEI Tecnai G2 Spirit Bio-Twin TEM,120 kV) facility available at Indian Institute for Science Education and Research (IISER), Thiruvananthapuram.

*Flow cytometry for CD9, CD63 and CD81:* Flow cytometry analysis was carried out for ultimate confirmation of exosomes, based on the surface expressed tetraspannin proteins (CD9, CD63, and CD81). Isolated exosomes were resuspended in 50 µl of $Ca^{2+}$/$Mg^{2+}$ free PBS in an eppendorf tube and were tagged with the recommended quantity of fluorophore conjugated monoclonal antibodies by the manufacturer's end (BD, USA). The exosomes were incubated with respective positive and negative antibodies separately for 45 min in dark at refrigerated temperature. The process was followed by centrifugation at 4500 ×g for 10 min. The exosomes were washed once with PBS after passing through 0.1 µ syringe filter. Again, the centrifugation step was repeated, and the pellet was analysed on BD FACSaria. Anti CD34 monoclonal antibody was used as negative control in the experiment.

## RESULTS AND DISCUSSION

Extracellular vesicles (EVs) are the general term used to represent vesicles of different kinds, including exosomes, microvesicles, apoptotic bodies, and pathologically derived necrotic debris. Among all, exosomes are the vesicles of interest and are circular/spherical shaped phospholipid bilayer vesicles of size range 40-150 nm (Pavani *et al*. 2017).

It is evidenced that, exosomes derived from the conditioned medium upon the culture of OECs improve embryo development and quality in bovines, to the same extent as the conventional co-culture with fresh OEC does (Lopera-Vasquez *et al*. 2016). In this study, exosomes were isolated from the conditioned media upon *in vitro* culture of buffalo OECs (Fig. 1). FBS used for culture were reported to have their own exosomes, contaminating the oviduct-

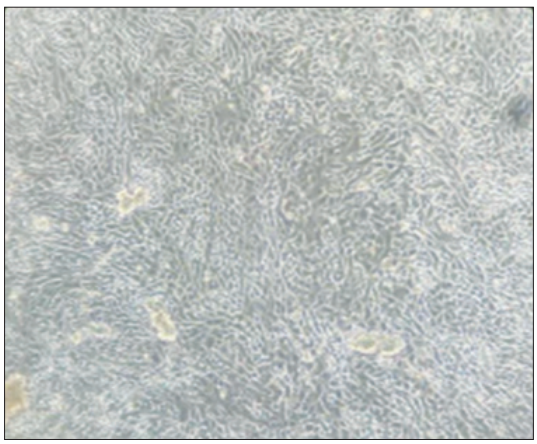

Fig. 1. Fully confluent monolayer of OECs when conditioned medium was harvested for exosome isolation (100× magnification).

derived exosomes. Also, earlier studies pointed out that exosomes from FBS have deleterious effects on embryo quality. Therefore, exosome-depleted FBS was used to support the cell growth from the log phase to ensure the desired oviductal origin of exosomes (Thery *et al*. 2018).

Even though several methods are available to isolate exosomes, the classical ultracentrifugation approach is still widely used in many laboratories. However, possible mix-up with other EV subtypes, requirement of long hours of the procedure, difficulties like availability of the instrument and need of technical expertise, demands the use of alternative approaches for exosome isolation. Hence, a kit-based procedure was used for exosome isolation in the present study (Total Exosome Isolation reagent, Invitrogen), which was based on the property of the reagent to tie up with water molecules and henceforth excluded less-soluble components (i.e. exosomes) out of the solution, which in turn was collected upon low-speed centrifugation. This approach allowed easy and definitive recovery of exosomes, and was advocated by Capra and Lange-Consiglio (2020) for high throughput biological applications.

*Characterization of exosomes:* The isolated exosomes were initially investigated for their physical and functional integrity. Nanoparticle sizing and Transmission electron microscopy (TEM) analysis are the preliminary investigations suggested for the physical characterisation of exosomes.

Nanoparticle size analysis provides general information on size distribution of the EVs as it is based on the Brownian motion of the particles. As shown in Fig. 2, the vesicles isolated in the study constituted of a homogenous population of EVs of size range 100-150 nm, suggestive of the enrichment with exosomes among other EV subtypes. Among EV subtypes, microvesicles were reported to be in the size range of 100-1000 nm, exosomes were of 40-150 nm, and apoptotic bodies were within the range of 1000-5000 nm (Pavani *et al*. 2017).

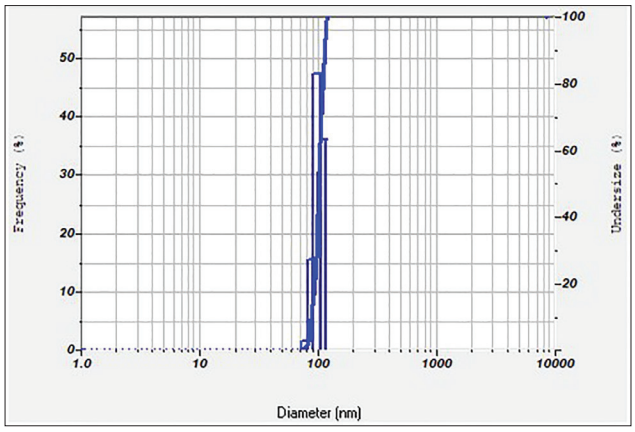


Fig. 2. Size distribution of oviductal exosomes by Nanoparticle size analysis.

Characterisation by TEM provides high resolution imaging (Fig. 3) based on the morphology and size of EVs. As per the categorization of EVs by Pavani *et al*. (2017),

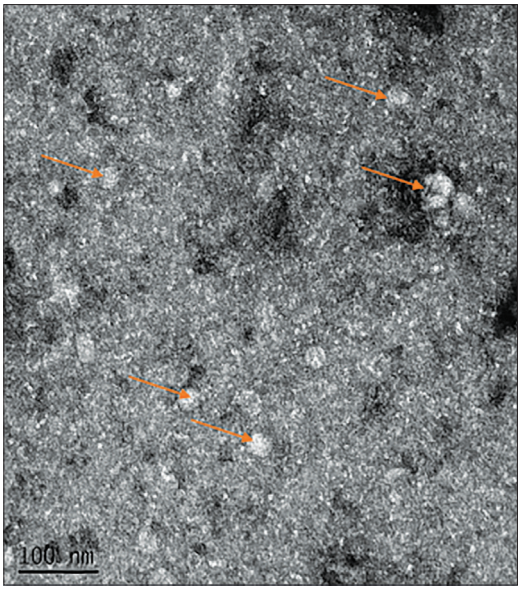


Fig. 3. TEM images of oviductal exosomes showing its spherical morphology.

microvesicles were of rounded shape with an upper size limit and apoptotic bodies represent vesicles of heterogenous morphology and size. At the same time, exosomes were reported to have spherical/circular morphology and lower size range (Pavani *et al*. 2017 and Hasan *et al*. 2021).

Instead of merely concluding the identity of exosomes by their size and morphology, the International Society for Extracellular Vesicles (ISEV) recommends their conformation based on the expression of transmembrane proteins (tetraspanins; CD9, CD63, and CD81). These markers are not expressed exclusive to exosomes, but are expressed by other EV subtypes as well. Even then, ISEV recommends the use of these markers for exosomes, due to their more abundance on exosomes compared to other EV sub-types. These markers can be detected by either flow cytometry or western blotting. As flow cytometry allows for quantification of exosomes, in this study flow cytometry using antibodies against CD9, CD63, and CD81 was preferred over the western blotting technique (Kowal *et al*. 2016).

Flow cytometry analysis confirmed that the oviductal exosomes studied were found to be enriched with the tetraspannins: CD9, CD63, and CD81. CD34 was used as the negative marker for exosomes. The result showing the enrichment of exosomes with the specified markers identified by flow cytometry analysis is represented in Fig. 4, where respectively 50.5%, 46.4%, and 45.3% of the total EV population was found positive for CD9, CD63, and CD81 expressed exosomal subtypes (Supplementary Figs. 1-4).

The nanoparticle size of exosomes makes it difficult to be analysed under conventional flow cytometry. Thus, it is recommended to conjugate the exosomes with latex/ polystyrene beads to make it detectable by flow cytometry (Szatanek *et al*. 2017). Also, the bead-based procedure is of added advantage in preventing the aggregation of

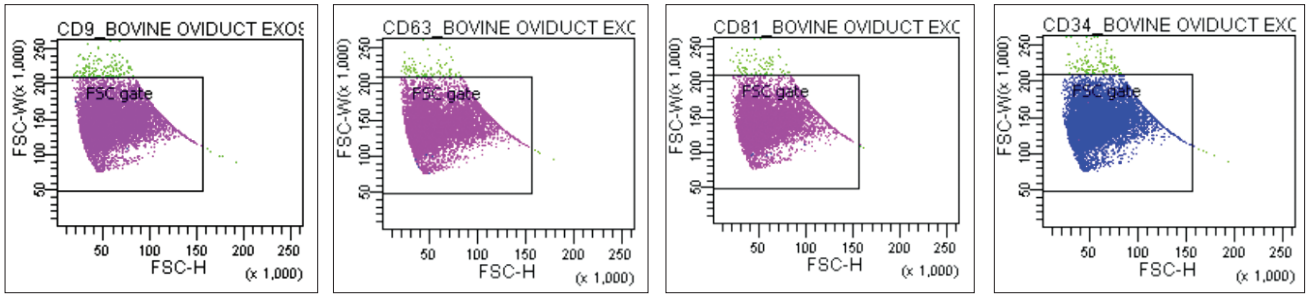


Fig. 4. Flow cytometry analysis of the oviductal exosomes for the surface tetraspannins, CD9, CD63, and CD81. CD34 used as a negative marker for exosomes.

vesicles. In our preliminary study, even with bead-assisted flow cytometry, only a lower level of detection of CD9, CD63 and CD81 could be achieved. Similar observation was earlier made by Mellisho *et al*. (2017) reporting CD9, CD63 and CD81 positive exosomes at 16, 3, and 19%, respectively by conventional bead-based flow cytometry. Hence, it was attempted to utilise high resolution flow cytometry for the analysis. The results were in consistent with the findings of Melo-Baez *et al*. (2020) who reported an occurrence of 59% CD81 positive exosomes in their study. The negative exosomal marker CD34 was also included in the study, alongside the samples. Absence of expression of the negative marker CD34 in the identified exosomal population further favoured the results.

In this study, exosomes were successfully isolated and characterised from the conditioned medium of buffalo oviductal epithelial cells cultured *in vitro*. The study will further follow *in vitro* co-culture of buffalo embryos with the isolated oviductal exosomes, aiming at improving the success rate and quality of so-produced buffalo embryos.

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