Investigating the Internalization of Hybrid Structures Comprising Cationic Liposomes and Extracellular Vesicles from Follicular Fluid in Bovine Granulosa Cells

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Abstract

The development of hybrid vesicles combining liposomes and extracellular vesicles (EVs) has garnered significant interest in the field of drug delivery and cellular uptake due to their scalability, versatility and distinct advantages over conventional delivery systems, enabling precise control over payload delivery and dosage. Liposomes are lipid-based synthetic vesicles that can offer stability to EVs, which are natural extracellular vesicles derived from biological fluids, by safeguarding them against degradation in biological environments. The lipid bilayer present in liposomes may also present a formidable barrier for the controlled release of cargo carried by EVs, such as miRNAs, mRNAs and proteins, facilitating their sustained delivery over extended periods. Also, EVs possess inherent biocompatibility, minimizing the risk of immune responses and mitigating the cytotoxicity usually related to liposomes. This study investigated the production and internalization of hybrid vesicles consisting of cationic nanoliposomes and EVs isolated from bovine follicular fluid into bovine granulosa cells. The nanoliposomes were first produced using the thin-film lipid method followed by extrusion through 100-nm polycarbonate membranes (Avanti Polar Lipids, UK). The liposomal composition consisted of phospholipids, cholesterol, and rhodamine-PE, proportioned in an 80:19:1 molar% ratio to ensure optimal stability. Concurrently, EVs were isolated from follicular fluid via serial centrifugation at 4 °C (300 x g for 10 min to remove live cells, 2,000 x g for 10 min to remove residual cells and cell debris, and at 16,500 x g for 30 min to remove large microvesicles) followed by double ultracentrifugation at 119,700 x g for 70 min (Beckman 70TI rotor, USA). Extracellular vesicles were then labeled with calcein 1 mM for subsequent visualization. The synthesis of hybrid vesicles involved the strategic combination of liposomes and extracellular vesicles in a 4:1 v/v ratio, followed by a series of processing steps including five cycles of freezing and thawing using liquid nitrogen and 15 cycles of extrusion. Samples were maintained at 4 °C until further

analysis. Characterization studies employing nanoparticle tracking analysis (NS3000, Malvern Panalytical, UK) confirmed the production of hybrid vesicles with a size diameter of up to 150 nm, and a concentration reaching 10^9 vesicles/mL. Nanoflow cytometry (CytoFLEX, Beckham Coulter, USA) analysis served as a pivotal tool for validating the hybridization process, revealing a substantial population of hybrid vesicles exhibiting dual staining characteristics for both rhodamine and calcein. Toxicity tests using propidium iodide carried out via cytometry showed that hybrids were not harmful to granulosa cells in a greater extent when added at a concentration of 10^8 vesicles per well, showing viability of over 90%. Additionally, fluorescence microscopy (THUNDER Imaging System, Leica Microsystems, Germany) provided visual confirmation of the efficient internalization of hybrid vesicles into granulosa cells within a relatively short incubation period of 6h. All the analyses were carried out at least in triplicates. The significance of these findings lies in their potential applications in reproductive medicine and assisted reproductive technologies. By harnessing the synergistic properties of hybrid vesicles, our approach offers a promising avenue for targeted delivery of bioactive molecules to somatic cells, thereby facilitating the modulation of key molecular pathways involved in follicular development and oocyte maturation.

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