



Factors Affecting the Absorption of Exosomes by Sertoli Cells

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ABSTRACT

Exosomes play an important role in the signal transmission process of Sertoli (ST) cells. However, what factors affect the ability of ST cells to take up exosomes remains unclear. To investigate the influences of time, temperature, cytochalasin D (Cyt D), and Fingolimod (FTY720) on exosome absorption by ST cells, ST cells and exosomes were co-incubated under different environmental conditions. Then after exosome uptake by ST cells, cell surface fluorescence was observed and mean fluorescence intensity was measured. Furthermore, the cytoskeleton disruption and cell apoptosis after treatment by Cyt D and FTY720 treatment were detected, respectively. Our results showed that the time and temperature for co-incubation had a direct effect on the ability of ST cells to take up exosomes. Under our experimental conditions, co-incubation at 37°C for 4 hours gave maximum absorption, indicating that this was the optimal co-incubation condition. The Cyt D destroyed cytoskeletal structure of ST cells, while FTY720 induced ST cells apoptosis. With the increase of Cyt D and FTY720 concentrations, the ability of ST to absorb exosomes is significantly reduced. It is concluded that the factors like time, temperature, Cyt D and FTY720 have important impacts on the ability of exosome absorption by ST cells, but different factors affect it in different ways.

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Authors' Contribution

MH presented the concept of the study and wrote manuscript. FB, WL and WF conducted the experiment. FB and GB performed analysis. LD did acquisition of research funds.

Key words

Sertoli cells, Exosomes, Temperature, Cytochalasin D, Fingolimod

INTRODUCTION

Exosomes are small extracellular vesicles (sEV) secreted by cells. They have a unilamellar lipid bilayer membrane with a diameter of 30–150 nm. Exosomes can transfer mRNAs, miRNAs, and proteins from donor cells to recipient cells and thereby affect the gene expression of the recipient cells (Zhang *et al.*, 2015). Almost all cell types can secrete exosomes. The exosomes are released into the extracellular space and are transported to distant locations by various body fluids, for example the plasma, semen, urine, lymph, and cerebrospinal fluid. They are then taken up by recipient cells and function to regulate gene expression of recipient cell (Lu *et al.*, 2022).

Studies have shown that the absorption of exosomes by recipient cells occurs either through extracellular receptors, or via direct fusion with the plasma membrane, or by internalization (Schorey *et al.*, 2015). Many biological processes are involved in uptake of exosomes, and these

processes are affected by various external factors. Sertoli (ST) cells in testicular seminiferous tubules can provide a suitable external environment for the development of spermatogenic cells. ST cells not only provide a stable microenvironment for sperm development but also need to be adjusted according to different stages of sperm development (Yan *et al.*, 2020; Zhao *et al.*, 2020). Therefore, maintaining information exchange and communication between ST cells and spermatogenic cells is important for sperm development. The ability of ST cells to take up exosomes play important roles in the information exchange and self-adjustment.

The ability of ST cells to take up exosomes is affected by a variety of factors. In this study we investigated the effects of time, temperature, cytochalasin D (Cyt D), and Fingolimod (FTY 720) on this ability. thus to explore the factors which affect the exosome absorption ability of ST cells, and to improve the conditions for sperm development.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise indicated.

Abbreviations

sEV, small extracellular vesicles; ST, Sertoli; Cyt D, cytochalasin D; FTY720, Fingolimod; DMEM/F12, Dulbecco's modified Eagle's medium /Nutrient Mixture F-12

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Cell culture

Swine Sertoli (ST) cells were cultured in DMEM/F12 (Life Technologies Inc., Rockville, MD, USA) containing 10% exosome-free fetal calf serum (Merck, Germany). The cells were incubated at 37°C in a humidified incubator with 5% CO₂ and 95% air until 70–80% confluence. Then, the cells were treated according to the experimental design.

Exosomes isolation, purification, and identification

The ST cells were cultured in DMEM/F12 supplemented with 10% exosomal-free fetal calf serum (Merck, Germany) at 38°C, 5% CO₂ and 95% air in a humidified incubator for 24 h. Then, the extracellular vesicles in the medium were collected using ultracentrifugation. Briefly, the medium was centrifuged at 2,000×g for 10 min at 4°C twice to remove cell debris. Then, the supernatant was filtered using 0.2-µm filters and centrifuged at 10,000×g for 30 h at 4°C to remove cell debris. The pellets were suspended and centrifuged at 120,000×g for 70 min at 4°C. Finally, the pellet containing sEV was resuspended in PBS. The collected sEV were stored at –80°C.

The extracellular vesicles were observed using a scanning electron microscope (SEM). Briefly, 7.5 µL of the sEV suspension was top loaded on grids and dried. The grids were stained with 2% uranyl acetate and visualized with an energy-filtering SEM (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) at 120 kV.

Size and concentration distributions of isolated EVs were determined by a Nanosight NS500 nanoparticle analyzer (Malvern Instruments) according to the manufacturer's instructions. sEVs samples were evaluated in units of concentration, and values were averaged from triplicate experiments.

Finally, immunofluorescence of isolated EVs was performed as described previously (Marie-Nicole *et al.*, 2021). Briefly, after ultracentrifugation the sEVs suspension was incubated with 5 µL of 4-µm aldehyde/sulfate latex beads (Life Technologies Corp., Grand Island, NY, USA) according to the manufacturer's instructions to obtain the exosome–bead complexes. Then, the exosome–bead complexes were incubated with anti-CD63 (MEM-61, Thermo Fisher Scientific, Rockford, IL, USA) for 1 h at room temperature. The labeled exosome–bead complexes were centrifuged and washed twice, then resuspended in 20 µL of PBS with 0.5% BSA. The final complexes (10 µL) were spread on a microscope slide, air-dried, and sealed with a cover-slip. The slides were examined using a fluorescence microscope (Carl Zeiss Microscopy Axioptan 2 Imaging Stand). Experiments were repeated at least three times.

Exosome labeling and observation of its absorption by ST cells

To examine whether exosomes could be taken up by ST cells, the exosomes were labeled using PKH67 (Sigma-Aldrich Co., St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, the exosomes were diluted in 200 µL of PBS to a concentration of 100 µg/mL. PKH67 dye (2.0 µL) was diluted in 1.0 mL of Diluent C (PKH67 solution). Then, 1 mL PKH67 solution and 200 µL diluent exosomes were mixed. The diluted and suspended exosomes were mixed for 5 min at 37°C; then, 2 mL 1% BSA was added to bind the excess PKH67 dye. The PKH67-stained exosomes were washed three times to remove the excess dye and PKH67-labeled exosomes were collected using an ExoEasy Maxi Kit. Finally, PKH67-labeled exosomes were resuspended in DMEM/F12 containing 10% exosomal-free fetal calf serum. ST cells were cultured at a mean density of 5×10⁵ cells/well. According to the experimental design, ST cells and 10 µL PKH67-labeled exosomes were co-incubated under indicated conditions.

After co-incubated, the cells were washed with PBS three times, then fixed with 4% paraformaldehyde solution for 15 min at room temperature and washed three times with PBS. Subsequently, the nuclei were stained using DAPI. Finally, the wells with stained cells were viewed and imaged under a fluorescence microscope (Carl Zeiss).

Impacts of factors and experimental design

The ST cells were cultured for 24 h. In order to detect the effects of time, temperature, Cyt D and FTY 720 on the exosome adsorption by ST cells and exosomes were co-incubated under the following conditions: (1) the exosomes were added to the ST cells culture dish and incubated at 37°C for 1 h, 2 h, and 4 h. (2) The exosomes and ST cells were co-incubated at 16°C, 28°C and 37°C for 4 h. (3) The exosomes were added to the ST cell culture dish, and then 1 µM, 2 µM, or 4 µM Cyt D were added and incubated at 37°C for 4 h. (4) The exosomes and ST cells were co-incubated, then 5 µM, 10 µM, or 20 µM FTY 720 were added and incubated at 37°C for 4 h.

Cytoskeleton staining

ST cells incubated with or without exosomes and Cyt D were washed three times with PBS. The subsequent staining procedure was as follows: 10 mL of a 4% paraformaldehyde solution was used to fix cells for 20 min at 4°C. Following fixation, cells were permeabilized using Triton X-100 in ASB for 10 min. Then, 5 µg/mL FITC labeled phalloidin (Sigma-Aldrich Co., USA) was added for 30 min. The cells were subsequently stained with 0.5 mg/mL DAPI at 37°C for 5 min. Finally, the stained cells

were used for flow cytometric analysis.

Analysis of apoptosis by flow cytometry

The apoptosis rate of ST cells was evaluated using an Annexin V-FITC/PI Apoptosis Detection Kit according to the manufacturer's instructions. After being treated with FTY720, the ST cells were collected, and washed using PBS. Then, cells were resuspended in 500 μ L binding buffer. The mixture of 5 μ L Annexin V-FITC and 5 μ L PI was added to the binding buffer and incubated for 30 min at 4°C in dark. The mixed cell solution was centrifuged at 1000 rpm/min for 5 min and were resuspended in PBS. The apoptosis of ST cell was analyzed by flow cytometry (BD FACSCanto).

RESULTS

ST cells secrete exosomes

EVs were isolated from the medium of ST cells using a described procedure based on ultracentrifugation. The images obtained by the scanning electron microscope (SEM) showed that EVs were membrane structures, hemispherical concave on one side (Fig. 1A). Nanoparticle Tracking Analysis (NTA) showed that most of the vesicles were in a size range of 60–100 nm, peaking at 73 nm (Fig. 1B). Immunofluorescence characterization revealed that exosomal marker protein CD63 signals were strongly enriched in EV–bead complexes (Fig. 2A), while the opposite was observed in the beads without EVs (Fig. 2B). All these results indicate that the EVs secreted by ST cells had exosomal characteristics; in other words, ST cells are able to secrete exosomes.

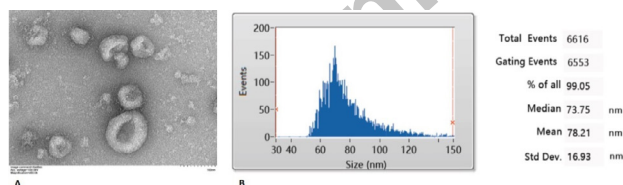


Fig. 1. EVs are secreted by STs. (A) Electron microscope image of ST EVs. The scale bar represents 100 nm. (B) Size distribution profile of ST EVs as determined by NTA.

Co-incubation time affects the absorption of exosomes by ST cells

The absorption capacity of ST cells for exosomes is affected by many factors. In order to examine the influence of co-incubation time on the uptake of exosomes by ST cells, we adjusted the co-incubation time of ST cells and PKH 67 stained exosomes for 1 h, 2 h, or 4 h at 37°C. Then, the distribution of green fluorescence on the surface of ST cells was observed under a fluorescence microscope.

In addition, flow cytometry was used to detect the average fluorescence intensity of PKH 67 in the ST cells after co-incubation. The level of fluorescence intensity directly reflects the number of cells that have absorbed exosomes, thereby indicating the influence of co-incubation time on the ability of ST cells to absorb exosomes.

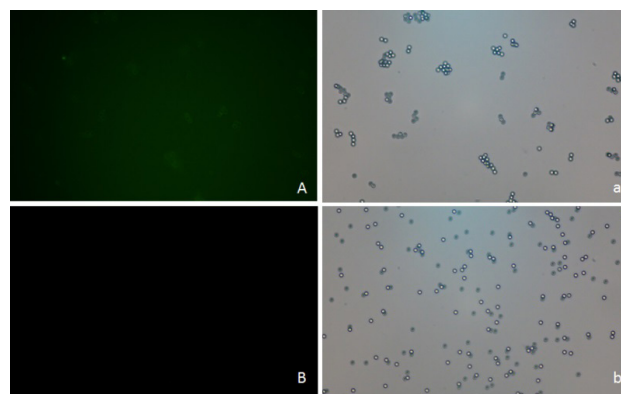


Fig. 2. ST-derived EVs were identified by immunofluorescence. The EVs were bound to a 4- μ m-diameter latex beads that were in the detection range of the fluorescence microscope. Images were taken under epifluorescence (A, B) and DIC (a, b). (A and a) The EV–bead complexes were bound to fluorescence-conjugated antibody against CD 63. (B) The beads were incubated with fluorescence-conjugated antibody against CD63. (b) Negative control.

The results showed that when the co-incubation time of ST cells and exosomes increased from 1 h to 2 h to 4 h, the number of absorbed exosomes increased with time (Fig. 3A). It can be seen in the fluorescence microscope images showed that there are only few green fluorescent dots in ST cells at 1 h, and when the co-incubation time was increased to 2 h, more fluorescent spots appeared on the cell surface. The highest density of fluorescent spots appeared in the 4 h incubation treatment. The cell surface fluorescence intensity at 1 h, 2 h, and 4 h was measured by flow cytometry, and the average fluorescence intensity value were 50.5, 93.7 and 131, respectively (Table I). All results indicated that the co-incubation time was positively correlated with the number of exosomes absorbed by the ST cells.

Co-incubation temperature affects the absorption of exosomes by ST cells

When the co-incubation temperature of ST cells and exosomes was increased from 16°C to 28°C and to 37°C for 4 h, the exosomal signal on the surface of ST cells under the fluorescence microscope was significantly increased (Fig. 3B). There were only few green fluorescent dots at

16°C. However, when the temperature was increased to 37°C, the number of exosomal signal was the highest. The average fluorescence intensity was 24.3, 77.6 and 135, respectively (Table I).

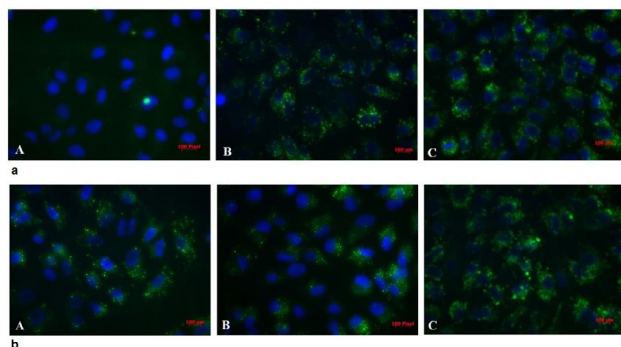


Fig. 3. Effect of co-incubation time (a) and temperature (b) the uptake of exosomes by ST cells. (A) DAPI stained nuclei of ST cells; (B) PKH67 stained exosomes; (C) DAPI and PKH67 merged.

Table I. The effect of time, temperature and Cyt D on mean fluorescence intensity measured by flow cytometry.

Sample	Count	Mean:FL2
Time		
Control	11858	4.80
Co-incubation 1 h	11874	50.5
Co-incubation 2 h	12646	93.7
Co-incubation 4 h	13923	131
Temperature		
Control	11820	7.70
Co-incubation 16°C	10474	24.3
Co-incubation 28°C	11002	77.6
Co-incubation 37°C	10905	135
Cyt D		
Control	15206	8.11
Cyt D 1.0 μ M	12581	142
Cyt D 2.0 μ M	13482	134
Cyt D 4.0 μ M	11426	92.3

Cyt D reduces ST cell uptake of exosomes

In order to investigate the influence of cytoskeleton on the ability of cells to absorb exosomes, we examined the exosome absorption of ST cells after treatment by cytoskeleton disruptor Cyt D. The distribution of cytoskeleton in the cells. Then, the absorption of exosomes was measured after ST cells were treated with different

concentrations of Cyt D. ST cells cultured under different Cyt D concentrations were stained with phalloidin to show the cytoskeleton structure. As shown in Figure 4A, the fibrous cytoskeleton was evenly distributed throughout the cell without Cyt D treatment. However, with the increase of the Cyt D concentration, the fibrous structure of the cytoskeleton gradually disappeared, and clustered fluorescent plaques were observed. With the increase in the Cyt D concentration the green fluorescent spots of exosome on the surface of ST cells gradually decreased (Fig. 4B), and the mean fluorescence intensity detected by flow cytometry were 142, 134 and 92.3 (Table I). These results indicate that when the cytoskeleton is destroyed, the ability of ST cells to absorb exosomes will decrease, suggesting that the ability of ST cells to absorb exosomes was positively related to the integrity of their cytoskeleton.

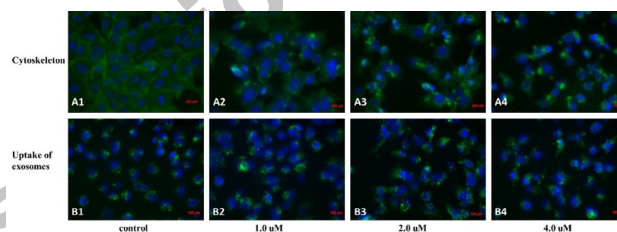


Fig. 4. Effect of co-incubation of Cyt D concentrations on PKH67 stained exosomes with ST cells. (A) DAPI stained nuclei of ST cells. (B) PKH67 stained exosomes. (C) DAPI and PKH67 merged.

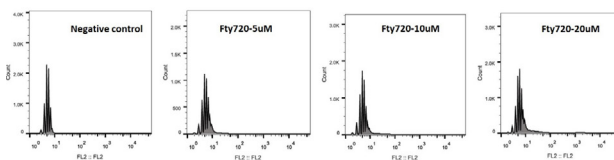


Fig. 5. Effects of different concentrations of FTY720 on ST cell apoptosis.

FTY720 reduced the absorption of exosomes by ST cells

In the ST cell culture process, 5 μ M, 10 μ M, or 20 μ M FTY720 were added to the medium. After four hours of incubation, DAPI was used for cell staining, and the cells were tested for apoptosis by flow cytometry. With the increase in the concentration of FTY720, the average fluorescence intensity of increased 26.1, 44.2 and 80.5. The results indicate that FTY720 induced apoptosis through a certain mechanism (Fig. 5 and Table II).

Furthermore, the changes in the exosome fluorescence intensity on the ST cell surface were measured with addition of different concentrations of FTY720. The changes in the fluorescence intensity of the ST cell surface

were observed under a fluorescence microscope, and the mean fluorescence intensity was measured by flow cytometry. The results showed that with the increase of the concentration of FTY720, the bright exosome fluorescent spots decreased (Fig. 6). When the concentration of FTY720 reached 20.0 μM , there were only a few bright spots. At the same time, the average fluorescence intensity detected by flow cytometry also decreased, showing 209, 139 and 95.6 (Table II) with 5.0 μM , 10.0 μM , and 20.0 μM of FTY720, respectively. The above results indicated that as the concentration of FTY720 increased the absorption of exosomes by ST cells decreased.

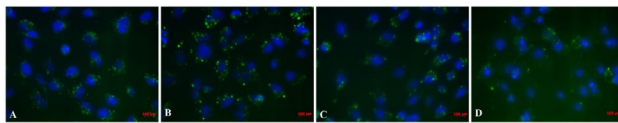


Fig. 6. Co-incubation of FTY720 concentrations on PKH67 stained exosomes with ST cells. (A) DAPI stained nuclei of ST cells. (B) PKH67 stained exosomes. (C) DAPI and PKH67 merged.

Table II. The effect of different concentrations of FTY720 on ST cell apoptosis and mean fluorescence intensity measured by flow cytometry.

Sample	Count	Mean:FL2
ST cell apoptosis		
Control	12938	5.53
FTY720 5.0 μM	10722	26.1
FTY720 10.0 μM	14641	44.2
FTY720 20.0 μM	17938	80.5
Mean fluorescence intensity		
Control	12347	8.37
FTY720 5.0 μM	10832	209
FTY720 10.0 μM	10144	139
FTY720 20.0 μM	11259	95.6

DISCUSSION

Exosomes are unilamellar vesicles with a diameter of 30-150 nm. Most cell types can secrete exosomes and release them into the extracellular space (Luz and Rosanna, 2020). ST cells exist in the testes of male animals and are in the shape of irregular tall cones. The base of a ST cell is attached to the basement membrane, and the top extends to the lumen surface of the seminiferous tubule, thus providing a suitable environment for the development of spermatogenic cells. Recent research shows that Sertoli cells regulate the differentiation and

development of spermatogonia stem cells by secreting exosomes (Jackson *et al.*, 2016; Quan *et al.*, 2021). Liang also found that Sertoli-derived exosomes can regulate and inhibit Leydig cell steroidogenesis (Liang *et al.*, 2021). In this experiment, we extracted extracellular vesicles from the ST cell culture medium by ultracentrifugation. Scanning electron microscopy, particle size analysis, and immunoassay of these vesicles confirmed that they have typical exosomal characteristics, i.e., a single-sided concave cup-shaped structure with a diameter of 60–100 nm and being immunoreactive with CD63 antibody. Therefore, the results demonstrated that ST cells secrete a large number of exosomes during in vitro culture. These exosomes may also transmit information between cells and regulate the physiological functions of neighboring cells.

The absorption of exosomes involves many steps, including receptor-mediated binding, activation of downstream signals, protease-mediated cleavage of receptor cells to release soluble ligands, and lipid membrane fusion (Pegtel and Gould, 2019). Therefore, the ability of ST cells to absorb exosomes could be affected by many factors. In this experiment, the time, temperature, cytoskeletal state, and degree of apoptosis of recipient cells were chosen to examine their effects on the uptake of exosomes by ST cells.

Among all the influencing factors, the temperature and time of recipient cells and exosomes had direct impact. Temperature is one of the key factors in maintaining the normal physiological function and behavior of cells (Kanduser *et al.*, 2008; Francis and Barlow, 1998). At lower temperatures, the fluidity and permeabilization of the cell membrane decreases, and the cytoskeleton depolymerizes (Vishard *et al.*, 2010). However, higher temperatures can cause enzymes in cells to inactivate and even cause cell death (Nisar *et al.*, 2016). The co-incubation time determines whether the recipient cells and exosomes can fully contact and finish the absorption process. In this experiment, the co-incubation time of 4 h ensured that the cells could fully absorb the exosomes to obtain the maximum absorption, and 37°C was also the most suitable temperature. The shorter time and lower temperature will lead to the decline of ST cells' ability to absorb exosomes.

The cytoskeleton plays an important role in the uptake, release, and transport of exosomes. Exosome internalization was dependent on the actin cytoskeleton and phosphatidylinositol 3-kinase (Mathieu *et al.*, 2019; Feng *et al.*, 2010). Cyt D inhibits actin polymerization, leading to cytoskeleton depolymerization (Meng *et al.*, 2017). When we treated recipient ST cells with Cyt D, the cytoskeletal system of the cell observed under a fluorescence microscope was altered from a uniform

filamentous structure to a condensed clump; at the same time, the measured exosome uptake capacity was gradually reduced.

Previous studies confirmed that FTY720 can lead to cell apoptosis through various signaling pathways (Sönmez *et al.*, 2017; Mingri *et al.*, 2021). For example, Zhong *et al.* showed that FTY720-induced apoptosis and autophagy through the PP2A/AMPK pathway can cause cell death in multiple myeloma cells (Yuan *et al.*, 2020; Marie-Nicole *et al.*, 2021). In this study, with the increase in the FTY720 concentration, the apoptosis rate of ST cells increased correspondingly, and the absorption of exosomes by ST cells decreased significantly. This suggests that cell viability directly affects the ability to take up exosomes.

Exosomes can be used as information carriers to transmit regulatory signals or other signaling substances between cells. By understanding the factors that affect the ability of exosome absorption of recipient cells, the efficiency of cells receiving exogenous information can be improved by correspondingly adjustment of the conditions for the recipient cells.

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IBR approval

Approval from Institutional Review Board of Heilongjiang Academy of Agriculture Science was obtained for this study.

Ethical statement

We have followed EU standards for the protection of animals used for scientific purposes. The experimental protocols used in this study were reviewed and approved by the Animal Care and Use Committee of Heilongjiang Academy of Agricultural Sciences, People's Republic of China.

Statement of conflict of interest

The authors have declared no conflict of interest.

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