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Cardiac Myocyte Exosome Isolation

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Abstract

Exosomes are cell-derived small extracellular membrane vesicles (50–100 nm in diameter) actively secreted by a number of healthy and diseased cell types. Exosomes can mediate cellular, tissue, and organ level micro communication under normal and pathological conditions by shuttling proteins, mRNA, and microRNAs. Prior to vesicles molecular profiling, these exosomes can be isolated from conditioned cell media or bodily fluids such as urine and plasma in order to explore the contents and functional relevance. Exosomes purification and analyses is a fast growing research field. Regardless of several advances in exosome purification and analyses methods, research still face several challenges. Despite tremendous interest in the role of extracellular vesicles there is no general agreement on dependable isolation protocols. Therefore, there is an urgent need to establish reliable protocol of exosome purification and analysis. Here, we report a simple cost effective isolation and analysis of cardiac myocytes exosomes from conditioned media.

Keywords

exosomes; cardiac myocytes; cell signaling; heart; acetyl choline esterase; protocol

1 Introduction

Exosomes form in the multivesicular body (MVB, fig. 1A) and were originally described as a means to remove unwanted proteins and organelles from the cell (1, 2). However it is now evident that exosomes, which are small lipid vesicles, have a broad range of functions including protein removal and inter-cellular signaling. Exosomes, which are secreted by most cell types, are 50–100 nM in diameter and contain proteins, mRNA, microRNA and DNA, all of which vary depending on the inducing agent and cell source. Electron microscopy of negative staining of fixed exosomes isolated from cardiac myocytes is shown in figure 1C. The trigger(s) for the creation of exosomes, which form through invagination of the membrane of the MVB, which is itself formed from invagination of the cell

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membrane, remain to be defined. As a result of these serial invaginations the outer surface of the exosome is derived from the outer surface of the cell. The MVB can either fuse with a lysosome, leading to destruction, or empty its contents extracellularly through fusion with the cell membrane. The control of this switch point remains unknown.

It is now recognized that exosomes bear proteins, lipids, and RNAs, mediating intercellular communication between different cell types in the body, and thus affecting normal and pathological conditions. In the last few years the role of exosomes in signaling and cancer and their potential as a diagnostic and therapeutic agents have been widely recognized. Exosome research has burgeoned into an exciting field with over 350 articles on exosomes published in the last 3 years alone. Exosomes are found in all biological fluids, including urine, plasma and ascites. Several protein families are commonly present in exosomes, including integrins, metabolic enzymes, cytoskeletal proteins and heat shock proteins, particularly HSP90a and HSP70 (3, 4).

Due to the great potential of exosomes as a diagnostic and therapeutic tool, there is a need to understand their structure and function (5–10). It is imperative to develop efficient reagents, tools, and protocols for exosome isolation, characterization, and analysis of their RNA and protein contents. Commonly used methods for exosome purification involve differential centrifugation, ultrafiltration, size-exclusion chromatography and high-speed ultracentrifugation (11, 12). The most frequently used methods of exosome purification involve differential ultracentrifugation (56%) followed by density gradient or cushion-based ultracentrifugation (27%) and ExoQuick precipitation (13). Beside these traditional isolation techniques, commercially available easy-to-use precipitation solutions, such as ExoQuick™ and Total Exosome Isolation™ (TEI), have also been used in the last few years. Unfortunately, many studies fail to assess the quality and purity of isolated exosome populations before performing functional assays. One important challenge is the lack of standard methods to obtain highly pure and well characterized exosome populations. In this paper, we detail our approach to isolation of exosomes produced by isolated adult rat cardiac myocytes. Furthermore, we enumerate the quality control methods we use for all our preparations to ensure that exosomes were isolated. Finally, we detail our approach to evaluate the reliability and impact of single-step purification of cardiac myocyte exosomes from conditioned media on purity, size, morphology and proteome content.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents to attain sensitivity at 25 °C. Diligently follow all waste disposal regulations when disposing waste materials. It is necessary to wash and autoclave all tools and bottles to be used in isolation. Wash only with Alconox, which is safe for washing equipment used in cell work.

2.1 Rat Cardiac myocyte isolation components

For exosome isolation, adult cardiac myocytes are isolated from 3 month old male Sprague-Dawley rats, as previously detailed (14). For the isolation of myocytes we need following components,

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1. Nalgene beakers (500 ml).
2. Nalgene bottles (500ml, 1L).
3. Nalgene bottle-bottom half.
4. Nalgene bottles-top half with mesh filter inserts.
5. Large pair of scissors.
6. Small pair of scissors.
7. Forceps (Large and small).
8. Bulldog clamp.
9. Suture long enough to tie aorta to cannula.
10. Rubber cell scraper.
11. Sterilizer.
12. Tyrode's Buffer (135 mM NaCl, 5.4 mM KCl, 1mM $MgCl_2$, 0.33 mM NaH_2PO_4, 10 mM HEPES, 10 mM Glucose).
13. Collagenase Enzyme (Worthington).
14. Laminin (10 mg/ml in DMEM).
15. P35 (35 mm) and P100 (100 mm) plates.
16. Ketamine and Xylazine for anesthesia.
17. Heparin.
18. Perfusion pump.
19. Water bath.
20. Human Serum Albumin, as low endotoxin vs. FBS.
21. CaCl2.
22. Butanedione monoxime – only in selected settings where isolation will be difficult, such as for myocytes from aged hearts.
23. 70% Ethanol.

2.2 Sample collection components

1. Cells are grown in Media 199.
2. Media 199 supplemented with:
   a. Penicillin
   b. Streptomycin
   c. Insulin
   d. Human serum albumin

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2.3 Exosome free human serum albumin purification components

1. Ultracentrifugation.
2. Beckman ultracentrifuge with SWTi-30 rotor.
3. Ultracentrifuge tubes.

2.4 Exosomes purification components

1. Ethanol (100% tissue culture grade).
2. RNase/DNase free microtubes and pipette tips
3. Falcon 50mL Conical Centrifuge Tubes (Fisher Scientific).
4. Bentchtop Centrifuge (Thermo Scientific Sorvall).
5. ExoQuick precipitation reagent- EXOTC10A-1 (System Biosciences, Mountain View, CA).
6. Amicon Ultra filter (Millipore, Billerica, MA).

2.5 Exosomes Quality Analysis Equipment and Solutions

1. DPBS (Phosphate Buffered Saline) pH 7.4.
2. Particle sizer (NICOMP 380 zls, PSS, Port Richey, FL).
3. NanoDrop Spectrophometer.
4. Plate reader (SpectraMax M Series Multi-Mode).
5. BCA assay (Pierce, Rockford, IL).
6. BSA 2mg/ml standards (Pierce).

2.6 Exosomes Protein Analysis by SDS-PAGE and Western Immunoblotting

1. Separating buffer (4x solution): 1: 1.5 M Tris (hydroxymethyl) aminomethane (Tris-HCl), pH 8.7, 0.4% SDS.
2. Stacking buffer (4x solution): 0.5 M Tris-HCl, pH 6.8, 0.4% SDS.
3. 30% Acrylamide/Bis solution (37.5:1 with 2.6% C) and *N,N,N,N*-tetramethyl-ethylenediamine.
4. Ammonium persulfate: 10% solution in water prepared immediately prior to use.
5. Running buffer (10x solution): 250mM Tris, 1,920 mM glycine, 1% (w/v) SDS, pH 8.3.
6. SDS lysis buffer (5x): 0.3 M Tris-HCl (pH 6.8), 10% SDS, 25% B-mercaptoethanol, 0.1% bromophenol blue, 45% glycerol, Leave one aliquot at 4 °C for current use and store remaining aliquots at −20 °C.
7. Bromophenol Blue solution: Dissolve 0.1 g in 100 mL water.
8. Page Ruler Prestained Protein Ladder (Thermo Scientific Pierce).

9. **9.** Transfer buffer (Tris-Glycine): 25 mM Tris, 190 mM glycine, plus 0.05% (w/v) SDS.
10. **10.** Nitrocellulose membrane (0.45 um, #162-0117, Bio-Rad Laboratories) and thick filter paper (#170-5040, Bio-Rad laboratories).
11. **11.** Tris-buffered saline, as a 10x solution (#170-6435, Bio-Rad Laboratories) with 0.5% Tween-20 (TBS-T).
12. **12.** Blocking buffer: 5% (w/v) nonfat dry milk (#170-6404, Bio-Rad Laboratories).
13. **13.** Mini PROTEAN 3 system glass plates (# 1653311, Bio-Rad Laboratories)
14. **14.** Medium binder clips (1¼ in).
15. **15.** Plastic container.
16. **16.** Primary antibody: Mouse anti HSP60 (Enzo Life Sciences, Farmingdale, NY).
17. **17.** Secondary antibody: Anti mouse IgG conjugated to horseradish peroxidase (GE Health Care Life Sciences/Amersham, #931A).
18. **18.** Chemiluminescent substrates for horseradish peroxidase (HRP # 32106 from Pierce).
19. **19.** Autoradiography film, two-sided (# 34088, Thermo scientific Pierce).

3 Methods

To isolate exosomes, adult cardiac myocytes are prepared from the hearts of 3-months old male rats, as previously described (14). Adult cardiac myocytes are cultured in media containing human serum albumin. As serum samples contain exosomes, the human serum albumin is first centrifuged at 164,000 *g* for 2 h to remove all exosomes, as previously described (15).

3.1 Myocyte Isolation

Adult rat cardiac myocytes are isolated with the Langendorff method, where the heart is perfused retrograde with Type II Collagenase (Worthington, Lakewood, NJ) through the aortic cuff. It is important to not advance the cannula into the ventricle, as the coronaries will then not be perfused. Sterile, cell culture grade water is needed for the isolation (*see* Note 1). Make sure water bath and pump are working so that solutions are maintained at 37°C. PH of the solution needs to be 7.4 at all times for optimal digestion of the heart and good cell quality. Heparin is given as an IP injection 30 minutes before removing heart for myocyte isolation. (*see* Note 2). As the heart is perfused through the aortic cuff, the height of the column of fluid determines the pressure. For optimal isolation, pressure should be in

[1]The water used for the cardiac myocytes isolation is very important. Small amounts of calcium or endotoxin in the water can damage the cardiac myocytes. Water must be sterile and cell culture grade. The water source cannot be casually changed without careful thought and testing. For example, a malfunctioning deionizing/water purification in one lab led to poor isolations, until someone made the connection between the sudden change in water quality secondary to equipment malfunction coinciding with problems with cardiac myocyte isolations. We find it best to purchase cell culture grade water for preparation of isolated cells.

[2]Use 400–450 IU heparin for a 250–300 gm rat given as an IP injection 30 min. before the heart is to be removed. This gives heparin time to circulate and inhibit clotting. Adjust heparin dose for body weight.

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physiologic range. A bubble trap is in place at the top of the system, as a bubble in the perfusion system acts like an embolus in the coronary artery, preventing perfusion and leading to poor digestion and thus poor quality cardiac myocytes. The heart is rinsed in ice cold media before hanging it. At this point excess tissue can be trimmed off. A critical step is to rapidly hang the heart on the perfusion apparatus to minimize time heart is not perfused with oxygen (*see* Note 3). All tubing used in the procedure needs to be clinical grade so as to not leach chemicals into the perfusate. Maintaining the cleanliness of the system without using harsh/toxic chemicals is essential to having good cell isolations. If kept clean, tubing needs only to be changed every 6 months or longer, depending on frequency of use (*see* Note 4). This procedure yields 80% rod-shaped cardiac myocytes and 8–10 million cells with quality isolation. Figure 2 shows a rat heart failure heart perfused on the isolation apparatus and the cardiac myocytes obtained from this heart. Yield of cells from a diseased heart is somewhat less than from a healthy heart.

3.2 Exosomes Isolation by ExoQuick Precipitation

1. For the isolation of exosomes by precipitation, cardiac myocytes are cultured in 199 supplemented with penicillin, streptomycin, insulin and exosomes free human serum albumin media in ten P100 plates for two hours. The diagram in figure 3 summarizes initial steps for exosome isolation.

2. The supplemented Media 199 is then replaced with media 199 which contains all the above components, except albumin. This step is necessary because large amounts of protein, which is sticky, will make it difficult to purify exosomes. Myocytes and many other cells can be cultured without albumin for several hours without deleterious effects.

3. We have found that both hypoxia/reoxygenation and ethanol, at levels found with intoxication, induce exosome production by cardiac myocytes. Cardiac myocytes are treated with 65.1 mM cell culture grade ethanol (*see* Note 5) and incubated for 2 hours at 37 °C or with 2 h of hypoxia in an anaerobic chamber (Forma) and 1 h of reoxygenation, as previously described (16). Both of these are mild injuries, and do not cause significant cell damage. Ethanol treatment leads to the production of ROS, which stimulates exosome production, as previously reported (16).

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[3]Check the condition of the cells at every step by examining a drop under the microscope. If right after mincing there aren't mostly living cells (rods), then the problem is with the solutions or the perfusion. It is also very important to hang heart as quickly as possible and run fluid rapidly while hanging heart to avoid air emboli. Alternatively one can cannulate the aorta with the heart immersed in media.

[4]New tubing that is not cell culture or medical grade can lead to the leaching of chemicals into the perfusate, even with a small section of tubing. This can be lethal for the cardiac myocytes. We use Tygon tubing for our work. Tubing can last 6 mo. or more depending on frequency of isolations and care of equipment. A decline in cell yield or quality can be tip-off that it is time to change the tubing. After an isolation we flush the system with 200 ml of 70% ethanol (mixed from a 100% pure ethanol stock), drain the system to remove all ethanol, and then flush with 200 ml sterile cell culture grade water. We drain system to remove all fluid.

[5]Untreated adult cardiac myocytes in culture produce a very minimal amount of exosomes. Exosome production by cardiac myocytes is increased by ethanol treatment. Treatment with cell culture grade ethanol, at concentrations found in humans consuming alcoholic beverages, is done for 2 hours and greatly increases exosome production. The ethanol concentration corresponds to legally intoxicated levels and levels found with the consumption of multiple alcoholic drinks (18). Similar concentrations have been studied using *in vivo* rodent models of ethanol ingestion (19).

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4. At conclusion of the treatment times outlined above, the media is collected and centrifuged at 300 x g for 10 min at 4 °C to remove any non- adherent cells.
5. The media is then centrifuged a second time at 4700 x g for 30min at 4 °C to remove cellular debris and extra cellular DNA.
6. The resulting supernatant, which contains the exosomes, is then collected and concentrated at 4 °C from 50ml to 140ul with 100,000 molecular weight cut-off concentrating filters (Amicon Ultra Centrifugal filters), as per directions of the manufacturer.
7. ExoQuick is then added to the concentrated media to complete isolation of the exosomes. Exoquick, which essentially precipitates the exosomes (*see* Note 6) is added at a 1:1 ratio in a screw capped tube, mixed gently with a pipette and incubated overnight (at least 12–15 hrs) at 4 °C.
8. The next day the mixture is centrifuged at 1800 g for 30 min at 4° C.
9. The supernatant is then aspirated and the pellet is centrifuged at 1800 g for 5 min at 4° C to remove all the residual solution.
10. The supernatant is aspirated and the exosome pellet is dissolved in DPBS (Phosphate Buffered Saline) pH 7.4 for exosome quality analysis and study.

3.3 Exosomes Quality Analysis

1. The quantity of protein is determined by the BCA microassay method using BSA as a standard. Total protein is measured as an index of the amount of exosomes present.
2. Exosomes are too small to readily visualize. The quality of exosome preparations is confirmed by measuring the hydrodynamic radius and the particle size distribution in terms of intensity, number, and volume by dynamic light scattering (DLS) on a Nicomp 380 DLS Submicron Particle Sizer (Santa Barbara, CA). Particle sizing by Dynamic light scattering (DLS) is the most versatile and useful set of techniques for measuring sizes, size distributions, and (in some cases) shapes of nanoparticles in liquid. DLS measurements are conducted at 25 °C by adding 200ul of 0.5–1.0 mg/ml of total protein (based on BCA assay measurement of total protein in an aliquot of exosomes) in a glass tube (figure 2B). There are 1,024 correlation channels equipped with a 15 mW He-Ne laser diode at 632.8 nm and a photodiode detector set at a 90°angle. Light scattering is recorded for 900 s with two replicate measurements and each exosome preparation is representative of five technical replicates. This step is important as it is the only confirmation that one has isolated vesicles of appropriate size.
3. Acetylcholine esterase activity, which reflects the amount of cell membrane present, is used to indirectly assess the quantity of exosomes isolated along with

[6]We prefer to prepare exosomes by the ExoQuick method approach that meets our daily needs of experiments and also involves less labor than the serial centrifugation we have used previously (13).

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the measurement of total protein by BCA assay (Pierce, Rockford, IL), as previously described (13, 14). Acetylcholine esterase activity is measured by adding 5-5′-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylcholine iodide (ATC) in a 1:1 ratio with 10ug of exosomes (total protein) in a cuvette and measuring absorbance at 412 nm (17). Acetylcholinesterase (AchE) is an enzyme present in many tissues incluing nervous tissue, muscle, and red blood cells, which catalyzes the hydrolysis of acetylcholine and acetic acid. AChE has a very high catalytic activity, with each molecule of AChE degrading about 25,000 molecules of acetylcholine (ACh) per second. DTNB allows quantification of the thiocholine produced from the hydrolysis of acetylthiocholine by AChE in solutions. The absorption intensity of the DTNB adduct is used to measure the amount of thiocholine formed, which is proportional to the AChE activity. AChE activity is one way of confirming that one has highly consistent preparations of exosomes (15, 16). The total reaction volume per cuvette is 1ml, consisting of 10 μl (1μg/μl) isolated exosomes, 450 μl of ATC (2.8 mM) and DTNB (0.3mM), and 90 μl DPBS pH 7.4. The enzyme is pre-incubated at room temperature for a few min. The absorbency is measured continuously every 5 min for 30–60 min at 412 nm at room temperature using a NanoDrop 2000C spectrophometer. The values are plotted using Graph Pad Prism5, and different exosome preparations compared. We have found that AChE activity correlates with exosome counts.

4. SDS-PAGE is performed by the method of Laemmli (18). Proteins from each exosome isolate are standardized to the original sample volume and equal volumes are applied per lane of 12% SDS-PAGE gel.
5. Western immunoblotting is performed to analyze the presence of the specific protein HSP60, as previously described, as well as other proteins of interest (16).
6. The SDS-PAGE gel is transferred to a nitrocellulose membrane and blocked for 1 h at room temperature with nonfat dried milk. We have found that the powdered nonfat dried milk marketed by Biorad (Richmond, CA) gives much cleaner blots than the cheaper powdered milk products available in the grocery store. After blocking, the membrane is probed for 1½ hours at 4 °C with primary antibody, washed and then developed with secondary antibody linked to HRP(horse radish peroxidase).
7. The bound immune complexes are visualized using chemiluminescence (ECL, *see* Note 7) which leads to the development of bands on radiographic film. The resulting bands are qualified by digitizing the X-ray film image.

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[7]Chemiluminescent substrates available in the market are not exactly fit for the detection and reflection of all the protein bands density to measure equally, either they are very strong or weak ECL's. To get precise amount of band intensity in western blots that should reflect the amount of protein loaded on SDS-PAGE, we mixed right proportion of two different chemiluminescent substrates (Pico and Femto-ECL) for the detection of horseradish peroxidase (HRP) activity from antibodies and other Western blot probes.

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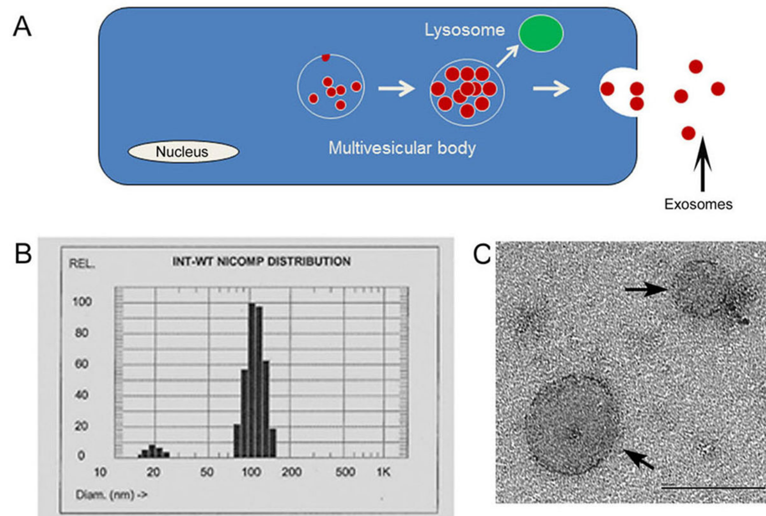

Figure 1.

A) Cartoon summarizes exosome formation in the multivesicular body (MVB), which forms from an invagination of the plasma membrane. The exosomes then form via invagination of the MVB membrane. The MVB can either fuse with a lysosome, leading to degradation of contents, or fuse with the plasma membrane, releasing the exosomes into the extracellular space. B) Nanotrak particle sizing profile. Exosomes peak at 100 nm. C) EM of exosomes from plasma sample. Bar is 100 nm.

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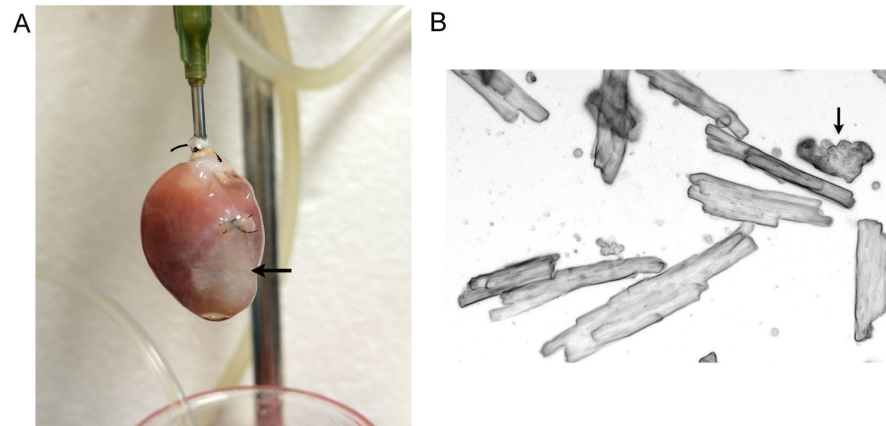

Figure 2.

A) Isolated rat heart perfusion in preparation for isolation of cardiac myocytes. This heart has heart failure secondary to large infarct (arrow). B) Ischemic heart failure cells isolated from heart in A. Most of cells are rods. Arrow points to dying cell that has folded up into a rounded shape. We are able to maintain these cells in culture for up to 24 h, vs. the 48 h duration of culture with cardiac myocytes from a normal rat heart.

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Cardiac myocytes + Media 199 (Exosomes free human serum albumin)

Cardiac myocytes + Media 199 (No human serum albumin)

ETOH

Cell culture Supenatant

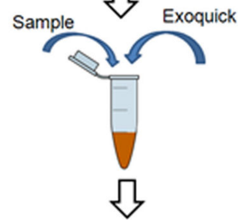
300g-10min

→ **Live and non adherent cells**

4700g-30min

→ **Cell debries, Various vesicles and extra-cellular DNA**

Supenatant CC: from 50ml to 150ul



Exosomes Pellet

Figure 3.

Schematic provides overview of steps from cell culture plate to purified exosomes.