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Exosomes isolation protocols: facts and artifacts for cardiac regeneration

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1. ABSTRACT

In recent years, exosomes have attracted increasing scientific interest and are no longer considered just as containers for cell waste, but as important mediators of intercellular communication. Among many biomedical research topics, a possible direct role of exosomes in the regenerative medicine field has been underlined in recent studies, including those regarding the so called "paracrine hypothesis". In this perspective, a therapeutic role and/or use of exosomes for tissue regeneration seems to be plausible. However, the majority of the cells isolated and cultured *in vitro* are exposed to an exogenous exosomes source because of the wide use of foetal bovine serum as cell culture supplement. Bovine serum has been gradually considered as a major biological stimulus, but with still unknown outcome. In this review, we present the state of the art about the role of exosomes in regenerative medicine, particularly for the cardiovascular system. We also analyse the most commonly used exosome isolation techniques that, since their discovery, have undergone continuous development to reach the highest degree of scalability for future clinical translation.

2. INTRODUCTION

2.1. Exosome overview

Exosomes are bi-lipid membrane vesicles that belong to the extracellular vesicles (EV) class, together with microvesicles and apoptotic bodies. Among these, exosomes are the only ones with endocytic origin and homogeneous shape and size (40-100nm). Their biogenesis starts from the inward budding of the membranes of late endosomes, also called multi-vesicular bodies (MVB), resulting in the formation of intraluminal vesicles (ILVs) (1). During ILVs formation, transmembrane proteins are incorporated into the folding membrane while the cytosolic components are engulfed within the vesicles. The MVBs move up to the cell surface, fuse with the plasma membrane and, finally, release ILVs outside the cell. Exosomes are released *in vitro* by several cell types, and have been isolated *in vivo* from a variety of body fluids (such as urine, saliva and plasma). Even if their protein composition varies depending on the cell type of origin, a conserved set of membrane markers has been identified, such as Tetraspanins (CD63, CD81 and CD9). Molecules like Alix, TSG101 (Tumor susceptibility gene 101) and clathrin are highly associated with exosomes. One class of cytosolic proteins, the largest small GTPase family, the Rabs, interact with proteins involved in vesicular transport and fusion, regulating exosome docking and membrane fusion, supported by the Annexins' family. Concerning their content, exosomes are enriched with a wide variety of proteins, such as: heat-shock proteins, metabolic enzymes, ribosomal proteins, signal transduction proteins, adhesion

molecules, ATPases, cytoskeletal proteins and ubiquitin molecules (2). Exosomes content is also enriched with specific nucleic acids, in particular RNAs and miRNAs, which exist within protein complexes. Exosomes have been originally attributed the function of protein excess removal. They are attracting increasing scientific interest, though, since they are no longer considered as simple containers for cell waste, but as cellular structures involved in cell-cell communication. Once in the extracellular space, they are able to interact with target cells inducing, according to the delivered molecules, a modulation of the phenotype towards a differentiated or activated state. They have been extensively studied in cancer research and immunology, because they can affect cancer stem cell niches and tumour progression (3), mediate antigen presentation, responses to infections and autoimmunity. These features suggested novel approaches involving them as biomarkers or immunotherapeutic agents (4, 5). More recently, exosomes have entered the scenario of the neurodegenerative disorders (6) and cardiovascular diseases (7) pathophysiology, potentially playing both diagnostic and therapeutic roles.

2.2. Exosomes and stem cells: the paradigm of cardiovascular regenerative medicine

The fields of stem cells and regenerative medicine are paying increasing attention to the effects mediated by exosomes, particularly in the cardiovascular system. In fact, exosomes derived from mesenchymal, cardiac and embryonic stem cells (8-16) have been shown to exert angiogenic and cardioprotective effects in ischaemic heart failure models, mediating survival and cell-cycle re-entry of cardiomyocytes as well as activation of endogenous cardiac repair by resident progenitor cells. Mesenchymal stem cell-derived exosomes have been shown to produce vascular remodelling and tissue protective effects in a stroke model, as well (17). Such strong paracrine regenerative approach may overcome several hurdles occurring in the clinical translation of heterotopic stem cell therapy protocols, such as those related to immunologic (i.e. allogeneic mesenchymal cell therapy) or oncologic (i.e. embryonic stem cell-derived teratomas) concerns, since such approach would include only a non-cellular regenerative product, that is EVs. Concerning autologous orthotopic cell products for cell therapies, such as resident Cardiac Progenitor Cells (CPCs) (18-20) where cardiovascular commitment is intrinsic, paracrine effects are nonetheless important co-factors in the overall therapeutic outcome, together with direct regeneration. In fact, cell sources are needed with unequivocal cardiomyogenic commitment to achieve successful regeneration, and resident CPCs, which have been tested in many animal models and recently in clinical settings, seem to have a very promising potential (21, 22). Initially, the idea was that transplanted cells, once injected in the infarcted tissue, could directly regenerate new cardiomyocytes. However, pre-clinical studies in different animal models have shown that many of the transplanted cells are lost within few hours after injection, so that only about 5-10% of them can be detected after 1 day (23, 24). Furthermore, a large number of cells, although initially retained, die because of the unsuitable microenvironment of the damaged tissue. Moreover, the cells that still survive in the heart, only partially differentiate into new cardiomyocytes or vessels (25-27). However based on the evidence that cell injection has a positive outcome on heart function, even without significant long-term cell engraftment, and that even concentrated stem cell-conditioned media could sustain regenerative effects (28), a new idea has been formulated: the paracrine hypothesis. The rationale of this idea is based on the increasing evidence showing that the observed therapeutic effects, even with cardiovascular-committed resident CPCs, are partly, but significantly, mediated by secretion of humoral factors (26, 29). For example, it has been shown that mesenchymal stem cell-conditioned medium (MSC-CM) enhances cardiomyocytes survival after hypoxia induced injury, promotes angiogenesis and reduces infarct size (30, 31). Paracrine effects have been shown to be responsible also for intercellular communications among different stem cell types, mediating for example the enhancement of cardiovascular commitment of MSCs by CPCs (32). Moreover a study from Arslan *et al.* (10), demonstrated that exosomes isolated from MSC-CM are able to increase ATP levels, decrease oxidative stress, activate survival pathways, reduce infarct size and prevent adverse remodelling after myocardial ischemic injury (Figure 1). These evidences allowed exosomes to enter, for the first time, the scenario of paracrine mechanisms of cardiac regeneration. Thereafter several studies have demonstrated that exosomes, independently from the cell type of origin, exert an *in vitro* pro-survival action in hypoxia conditions (as a post HF damage simulation), protect from oxidative stress, promote proliferation, migration and formation of tubes in HUVEC (10-16, 33, 34). It has also been demonstrated that cardiomyocyte progenitor cell (CMPC)-derived exosomes are able to stimulate endothelial cells migration *in vitro*, increasing capillary density, through mechanisms involving matrix metalloproteinases (MMP) and extracellular MMP inducers (35) (Figure 1). CPCs themselves release a wide panel of humoral factors or vesicles (growth factors, cytokines, chemokines, regulatory binding proteins), defining a specific functional "secretome", which exerts proangiogenic and anti-apoptotic effects (26, 36), and mediates the activation of endogenous repair mechanisms *in vivo* (27). Furthermore, it has been demonstrated that CPCs are able to release exosomes (37), whose regenerative effect has been investigated in two recent papers (13, 38). The results evidenced that CPC-derived exosomes are able to stimulate angiogenesis *in vitro*, promote cardiomyocytes proliferation and inhibit apoptosis. Similar beneficial outcomes have been observed *in vivo* using a myocardial infarction (MI) mouse model with injection

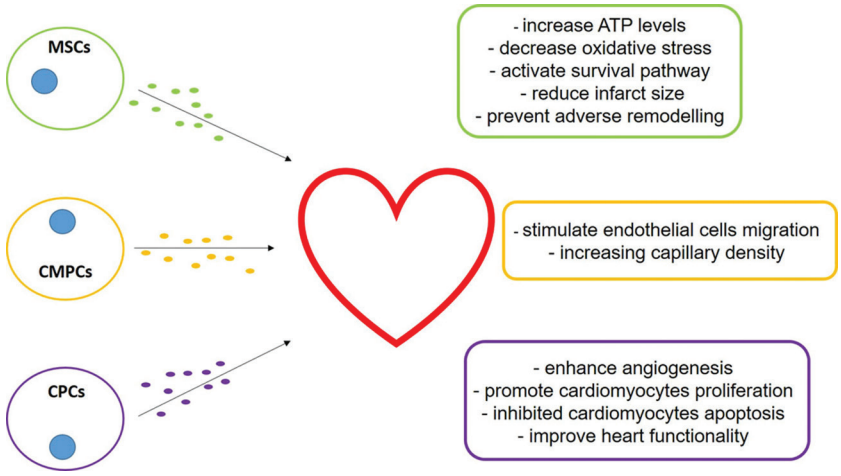

Figure 1. Therapeutic effects of exosomes derived from MSCs, MPCs and CPCs (10,13,35,38).

of CPCs conditioned medium (CPCs-CM). In fact, the direct injection of the injured tissue with the exosomes fraction isolated from CPCs-CM, inhibited cardiomyocyte programmed cell death, enhanced angiogenesis, and improved hearth functionality. These effects were similar to what observed with CPCs transplantation in the CADUCEUS clinical trial (21). Through a qualitative analysis of the CPCs secreted exosomes content, these studies identified several microRNAs (miRs) as new and important mediators of the beneficial effects of regenerative therapy approaches. In general, miR-210 and miR-132 inhibit apoptosis in cardiomyocytes and enhance tube formation in endothelial cells, respectively, by the ephrin A3, PTP1b and RasGAP-p120 down-regulation (13). Furthermore miR-146a, in synergy with miR-22 and miR-24, interfering with Toll-like receptor and pro-fibrotic TGF-beta signalling pathways, modulates cardiac fibrosis decreasing scar formation and increasing, at the same time, the viable tissue portion (38). Overall, these results underline the hypothesis of considering CPC-derived exosomes as a potential therapeutic agents or, at least, as important mediators of regenerative mechanisms for cardiac cell therapy (Figure 1).

2.3. Exosomes: targets or contaminants

EVs are present in the mammalian plasma, which means that standard FBS, used as cell culture supplement, contains abundant exogenous EVs. Thus, exosome-free media recipes are necessarily required for the *in vitro* collection of cell-type specific exosomes released in the supernatant, for both their isolation and use as therapeutic tools, as well as for characterization purposes. Nevertheless, bovine exosomes represent an active biological component of FBS, contributing to the overall trophic and proliferative stimuli it grants to cell culture media. A major emerging issue, in any exosome-related research field, is the conflict between the desired cell-specific purity of any exosome isolation protocol, and the not negligible technical requirements for efficient FBS biological activity on cell cultures. Two recent papers (39, 40) have underlined how the effects of bovine exosomes on cell culture yields and outcomes are important factors to take into account. It has been demonstrated that, the main effect of bovine exosome-depleted media is indeed a reduction of cell growth and proliferation rate. This aspect could highlight the need to analyse, in a critical way, the *in vitro* results obtained until today, in order to understand if and how much the exosomes present in serum might have influenced them. Furthermore, it has been shown that exosomes yield, characteristics and subsequently their effects on cell cultures, are influenced by the isolation protocol used. For this reason, it is important to choose an exosome isolation method according to the requirements needed for downstream applications. In this perspective, we provide here an overview to compare the most used exosome isolation protocols, in order to evidence their advantages and disadvantages.

3. EXOSOME ISOLATION PROTOCOLS

3.1. Ultracentrifugation

The most commonly used protocol for exosome purification involves several centrifugation and ultracentrifugation (UC) steps (41). Briefly, the first three steps are designed to eliminate cells (300 rcf for 10 minutes), large dead cells (2,000 rcf at 4°C for 10 minutes) and large cell debris (10,000 rcf at 4°C for 30 minutes). After each step, pellets are discarded and the supernatant is kept for the next. At the end, the supernatant is ultracentrifugated two times at 100,000 rcf at 4°C for 70 minutes to pellet exosomes and discard contaminating proteins. Starting from this protocol, in 2014 Cvjetkovic *et al*. (42) analysed if different rotors, such as fixed angle (FA) or swinging bucket (SW) rotors, and variation in length of ultracentrifugation were able to affect exosomes yield and purity. Both rotor types could be used to isolate vesicles with similar characteristics,

in terms of exosomal features, but the composition of the pellet generated by each rotor is slightly different. In particular, the ratio between the protein and RNA fraction within the pellet is higher in the SW than the FA rotor. These results suggest that the choice of the rotor has an impact on the quality of the isolated material and, as the authors suggest, it would be advisable and important to report in any protocol the RCF as well as the *k*-factor, to ensure accurate replication of any exosomal isolation procedure. Concerning the centrifugation time, they demonstrated that by extending it, a higher vesicle yield could be obtained. Therefore, the common 70 minutes ultracentrifugation protocol would not be sufficient for the isolation of exosomes, but, on the other side, prolonged time is to be avoided to reduce soluble proteins contamination. The authors then suggest a 4 hours centrifugation time, underlying that longer centrifugation programs could be good for exosome isolation only when followed by purification steps. For that reason, in most of the cases, the exosome pellet, including contaminants, normally undergoes further centrifugation procedures, such as gradient of sucrose or sucrose cushions to separate vesicles and particles based on their density. However, as Jeppesen *et al*. (43) suggest, the use of cushions and gradients increases the time required for purification, and results in loss of sample material. Furthermore, it is also still unclear if the floatation in gradients may affect the biological and functional characteristics of exosomes. The authors examined the impact of differential ultracentrifugation *g*-forces (ranging from 33,000 to 200,000 rcf) on the exosome isolation outcome from two different cell types. A higher purity of the samples was found in both cell lines after different ultracentrifugation rates. Furthermore, rising centrifugal *g*-force leads to increased quantity of contaminating proteins in pellets, while there is a tendency to reach plateau for the number of exosomes recovered. Based on their results, it seemed that contamination from mitochondria and ER (microsome) could efficiently be eliminated by pre-clearing centrifugation steps at 2,000 rcf and 33,000 rcf, respectively, even if these additional steps reduced the final yield of the isolation process. They also observed, especially in the higher *g*-force fractions, different expression patterns for two exosomal markers (TSG101 and syntenin). Their conclusion was that, probably, there were some sub-populations of exosomes expressing different markers and with different sedimentation profiles.

3.2. Commercial exosome precipitation solutions

In the last years, several polymer-based exosome isolation kits have become commercially available. Concerning the most used in the literature, the Exo-Quick TC from System-Bio is useful for a quantitative isolation of exosomes from low sample volumes, it is compatible with any bio-fluid, and is an effective and proven alternative to ultracentrifugation. According to the literature and the instruction manual, the culture medium is centrifuged at 3,000 rcf for 15 minutes and the supernatant sieved through 0.22 um filtering units. The appropriate volume of precipitation solution (according to the manufacture's suggestions) is added to the medium, mixed by inverting and placed at 4°C, ranging from 30 minutes to overnight for serum and other bio-fluids, or culture media, respectively. After refrigeration, the mixture is centrifuged at 1,500 rcf for 30 minutes to remove all traces of fluids, and the exosome pellet is ready to be used. With these solutions, high quality exosomes can be quickly and easily isolated from most biofluids, using a protocol that can be easily performed on multiple samples and requires very low volumes of input. Furthermore, isolated exosomes retain biological activity and can be used in functional assays.

3.3. Heparin affinity purification

In 2015 Balaj *et al*. (44) have shown that a three day heparin-based affinity chromatography protocol can be used to purify intact EVs to study their functional activities, or to simply use them as isolated biomarkers from bio-fluids. Briefly, the 24-hour conditioned media (DMEM with 5% overnight-UC EV-depleted FBS) was first centrifuged at 300 rcf for 10 minutes to remove cells, then the supernatant was centrifuged at 2,000 rcf for 15 minutes to remove other debris, and finally filtered. At this point, media was concentrated by centrifuging at 1,000 rcf for 10 minutes using a 100 kDa Molecular Weight Cutoff (MWCO) ultra-filtration device. For heparin purification, the concentrated conditioned media was added to heparin coated beads, incubated overnight on a tube rotator at +4°C to allow binding of EVs to the beads. Heparin beads were spun at 500 rcf for 5 minutes and the unbound fraction (supernatant) was collected. Heparin-coated beads were washed several times with PBS, each wash supernatant was saved, and a solution of NaCl in PBS was added to the beads and incubated overnight at +4°C on a tube rotator. Finally, heparin-coated beads were centrifuged at 500 rcf for 5 minutes, and the supernatant, corresponding to the eluate, was collected and stored at −80°C. In the same work, they also compared, in terms of yield and scalability, the chromatography protocol with ultracentrifugation and commercial kit. They found that, even if starting from the same input volume of media, the yield of RNA was similar in all methods, and the affinity purification is a more scalable method than centrifugation-based purification.

3.4. Sequential filtration

As discussed in section 3.1 UC protocols are incompatible with future high-throughput automation of exosome isolation and characterization processes, and the development of clinically implementable diagnostics or therapies. Starting from these considerations, in 2014 Heinemann *et al*. (45) proposed sequential filtration as a simplified, clinically applicable method for robust and specific exosomes isolation from biofluids. Their protocol

Table 1. Comparison of different exosome isolation methods based on analysed papers (41-48)

	Time	Exosome recovery (%)	Exosome specificity	Scalability	Pros and cons	Ref.
Ultracentrifugation	2-96 h	2-80	YES/NO	NOT YET (too much variability)	Standard gold method but very high variability depending on physical parameters and equipments	(41,42,43)
Commercial exosome precipitation kit	2-20 h	~80	YES	YES	Easily accessible, does not require expensive equipments or protocol optimization	(44)
Heparin affinity purification	~33 h	~80	YES	YES	More scalable than ultracentrifugation but requires multiple steps based on different methods	(44)
Sequential filtration	Dependent on filtering rate	>80	YES	YES	Highest exosomes recovery but requires accurate monitoring of flux and pressure parameters	(45,46)
SEC	~10'-20'	43 (without the concentration step)	NO	YES/NO (depending on the column)	Still needs accurate optimization for contaminants removal	(47,48)

derived from a previous method proposed by Lamparski *et al*. (46), based on tangential flow filtration (TFF) and ultracentrifugation on a deuterium-sucrose cushion, but in their case, they tried to avoid the second part. Sequential filtration consists of three steps: 1) Dead-End filtration, 2) Tangential Flow filtration, and 3) Track-etch filtration. In the first part, the 48-hour conditioned media (with 0.2% of TFF exosome-depleted FBS) was filtered at 22°C using a 0.1 um membrane to eliminate large and rigid media components. In the second step, the filtrate was continuously aspirated from a conical bottle, pumped through a fiber system, with a very low trans-membrane pressure (1.5 and 2.5 PSI), and recycled into the conical bottle. With this mechanism, large molecules, such as free proteins, were discarded. The filtrate went through five rounds of dia-filtration to deplete the sample from contaminants smaller than 500-kDa. Finally, the sample was loaded into a syringe pump and attached to a disposable pressure transducer with a 100 nm filter. The filtration took place at 22°C with a pressure below 3.5 PSI. Compared to a sequential ultracentrifugation pellet, in which they obtained 11% more particles, sequential filtration yielded a more exosome-enriched sample with >80% of the particles in the exosomes size range.

3.5. Size-exclusion chromatography

Size-exclusion chromatography (SEC) is commonly used to isolate platelets from platelet-rich plasma, and recently Böing *et al*. (47) have investigated the efficacy of single-step SEC for isolation of extracellular vesicles from human platelet-free supernatant. Platelets were isolated with 3 cycles of centrifugation for 20 minutes at 1,550 rcf and 20°C. According to the authors, SEC has several major advantages compared to the most used protocols for vesicles isolation, such as UC. For example, there is no risk of protein complex formation and vesicle aggregation, and the high viscosity of plasma does not affect the recovery of vesicles. The chromatographic column consists of Sepharose CL-2B in a 10mL plastic syringe with a diameter of 1.6 cm and height of 6.2 cm. The supernatant was loaded on the column, followed by elution with PBS/0.32% citrate (pH 7.4). Briefly, they showed that vesicles of a diameter larger than 75nm can be isolated from complex body fluids, such as plasma, by single-step SEC. Compared to ultracentrifugation, SEC results in a good recovery of vesicles (43% vs 2-80%) with almost complete removal of contaminants, taking less than 20 minutes (much less time, if compared to 2-96 hours for ultracentrifugation). Thus, in general, isolation of vesicles by SEC is quick, easy and cheap. However, for Welton *et al.* (48) "homemade" columns create problems related to reproducibility, such as variations from column to column, and the time needed to allow columns to settle without the formation of bubbles. In this perspective, they analysed the potential utility of a commercially available size-exclusion chromatography column for rapid purification of vesicle, exhibiting exosome characteristics. Seven days CM (10% 18-hour UC exosome-depleted FBS) was centrifuged (400 rcf for 10 min and 2,000 rcf for 15 min) and filtered through a 0.22 um membrane to remove cell debris. Conditioned medium was added to the commercial column prior to elution with EDTA-PBS buffer. However, even if serial fractions revealed a peak for typical exosomal proteins (such as CD9, CD81), and the columns showed good reproducibility with the exosome-relevant material being collected in less than 10 minutes, nonetheless the post-column vesicle concentration steps lead to low nanoparticles recovery (a loss of over 94%). In conclusion, even if this commercially available column provides a convenient, reproducible and highly effective means of eliminating approximately 95% of non-vesicular proteins from biological fluid samples, several optimizations are certainly still required to minimize vesicle loss.

4. CONCLUSIONS

In the last decade, the role of exosomes as one of the key factor for tissue regeneration has emerged, considering the tremendous impetus given to their biotechnological and clinical translation. Therefore, for reliable research data collection, underlying any possible future clinical perspective, many isolation methods will have to be developed to reach a high degree of exosome recovery and specificity, to reduce procedure time and to use easily accessible equipment (Table 1). Up to date, every method presents pros and cons that, in different ways, block a possible scalability for a future clinical translation. Starting from the significant effects of FBS exosome-depletion from cell culture media, it could be important, at least in some cell culture protocols, to consider the best suitable isolation method while planning a characterization of cell specific exosomes, and to verify how much the depletion of bovine exosomes from FBS could modify the specific cell phenotype under investigation. The acquired experience may therefore improve the results quality and reliability, making them real facts and not artefacts.

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