

Extracellular Vesicles in Regenerative Medicine, a Brief Review**Mehdi Soleymani-Goloujeh¹, Samaneh Saberi² and Faezeh Shekari^{3, 4*}***1. Dept. of Stem Cells and Developmental Biology at Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran**2. HPGC Research Group, Department of Medical Biotechnology, Pasteur Institute of Iran, Tehran, Iran**3. Dept. of Molecular Systems Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran**4. Dept. of Developmental Biology, University of Science and Culture, Tehran, Iran***KEYWORDS**Extracellular vesicles,
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ABSTRACT

Extracellular vesicles were initially known as cellular waste carriers, while recent studies demonstrate that extracellular vesicles play important biological roles in all aspects of life—from single cells to mammals. Their pathophysiological roles in some diseases like cancer are being decoded. Extracellular vesicles are divided into some classes and there are different strategies to isolate them. Regenerative medicine is a collective term which comprised of different approaches to heal and repair damaged tissues and organs. A wide spectrum of options in regenerative medicine, makes this more dynamic field, which is appealing prospect for cell therapists and tissue engineers. EVs derived from mesenchymal stem/stromal cells and other probable sources are one of the options on the table to regenerate damaged tissues with lower risks, but their potential roles have not been fully elucidated. This cell-free based approach inspires cell therapist and tissue engineers in order to control immune reactions as well as regeneration at the same time.

Corresponding Information: Faezeh Shekari Ph.D., Royan Institute, Banihashem Sq., Banihashem St., Ressalat Highway, Tehran, Iran
E-mail: Faezeshshekari@gmail.com

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Introduction

Nowadays, it has been demonstrated that cells from different organisms, from eukaryotes to prokaryotes can release vesicular bodies into the extracellular environment (1, 2). Extracellular vesicles (EVs) are considered as significant factors in inflammation and immune responses, antigen presentation, cancer progression and metastasis, immunomodulation, coagulation, tissue regeneration, organ repair, cell-cell communication, senescence, proliferation and differentiation, etc. in the body (2-5). Since they were discovered in the last decades of twentieth century, always there have been different opinions about their functions. Though EVs were initially known as cellular waste carriers,

introducing fundamental features revealed that these nano/micro-sized particles play important biological roles in all aspects of life—from single cells to mammals (6, 7). EV is a collective term which refers to heterogeneous cell-secreted structures comprised of a bilayer phospholipid membrane surrounding a wide range of macromolecules including proteins, lipids, and nucleic acids (e.g., cell specific antigens, surface markers, adhesion molecules, ligands, receptors, enzymes, miRNAs, lncRNAs, mRNAs, growth factors, etc.) (5, 8). According to parameters like their various biochemical, morphological, and biogenesis, EVs are classified into two groups;

exosomes and ectosomes (9). The term 'exosome' was initially used for vesicles with sizes about 30-200 nm which were released from a wide range of cultured cells (i.e., their exact origin was not determined) and carrying 5'-nucleotidase activity (5, 10). Currently, the term exosome is adopted to refer to intraluminal vesicles (ILVs) formed by reverse budding of endosomal membrane and finally secreted upon fusion of multivesicular endosomes (MVE) or late endosomes with plasma membrane (2, 9). On the other hand, ectosomes or microvesicles with sizes about 50-5000 nm, are directly shed from plasma membrane and released into intercellular space (11).

To date, five different isolation methods of EVs including; ultracentrifugation-based methods, size-based methods, Immunoaffinity capture-based methods, precipitation-based methods, and microfluidic-based methods have been developed (12) (An schematic illustration of the most prevalent methods for isolation of EVs, is shown in Figure 1). There are potential advantages and disadvantages for each method, which researchers should be considered in choosing an appropriate method based on their needs and possibilities. The ultracentrifugation-based method (UC) is a gold standard method in isolation of extracellular vesicles. In this method, a heterogeneous mixture is subjected to a centrifugal force and particles in the mixture can be separated according to their physical properties as well as density and viscosity of the solvent (12). There are more additional steps in order to obtain desired particles. In the first step, a low speed spin ($300 \times g$) is needed to eliminate dead cells and bulky debris. After depletion of larger particles, $2,000 \times g$ centrifugal force is applied to eliminate remaining cells and debris. In the next step, larger EVs like microvesicles are pelleted in the forces which varies among laboratories ($10,000$ - $20,000 \times g$). EVs are then pelleted at high speed spin ($100,000$ - $120,000 \times g$) (13, 14). One drawback of ultracentrifugation is co-precipitation of protein aggregates, nucleosomal fragments and apoptotic bodies, which results in less purity of final extracts.

Therefore, often UC is used in combination with density gradient (DG) methods like sucrose density gradients or sucrose cushions which separate EVs according to their floatation densities (15). Using volume-excluding polymers like polyethylene glycol (PEG) is another option for isolation EVs from diverse biofluids. In this method, the precipitate can be isolated using low speed centrifugation or filtration. Polymer-based precipitation is an easy to use method, which does not need any requirements or specialized equipment (16), but there are some concerns about the purity of final extracts (14). In this short review, the intent is not to provide an extensive review about the isolation methods of EVs. Herein, promising breakthroughs about utilization of EVs in regenerative medicine are highlighted.

Regenerative medicine

Regenerative medicine is defined as various approaches and actions to replace lost tissue(s) with new tissues/cells or enhance regeneration of damaged tissues in a broad spectrum of indications (e.g., myocardial infraction, osteoarthritis, lung diseases, acute kidney injuries, chronic wounds, muscular dystrophies, bone and cartilage defects, etc.) (4, 5, 17-19). There are different strategies towards tissue/organ regeneration, from cell transplantation to utilizing biomaterials alongside stem cell therapy, which are called tissue engineering (20). Despite stem cell transplantation is adopted as one of the major approaches in regenerative medicine, it exhibits some limitations. Transplanted cells might be tumorigenic in some cases or injected stem cells trapped in some organs (e.g., inside the lungs) (4). In the recent years, a promising strategy has been emerged, which facilitates regeneration process and hampers side effects of other approaches when used in combination with them (5). This cell-free based approach utilizes EVs to inspire cell therapist and tissue engineers to control immune reactions as well as regeneration at the same time. Regenerative properties of EVs are, at least in part, attributed to their protein and miRNA contents (20).

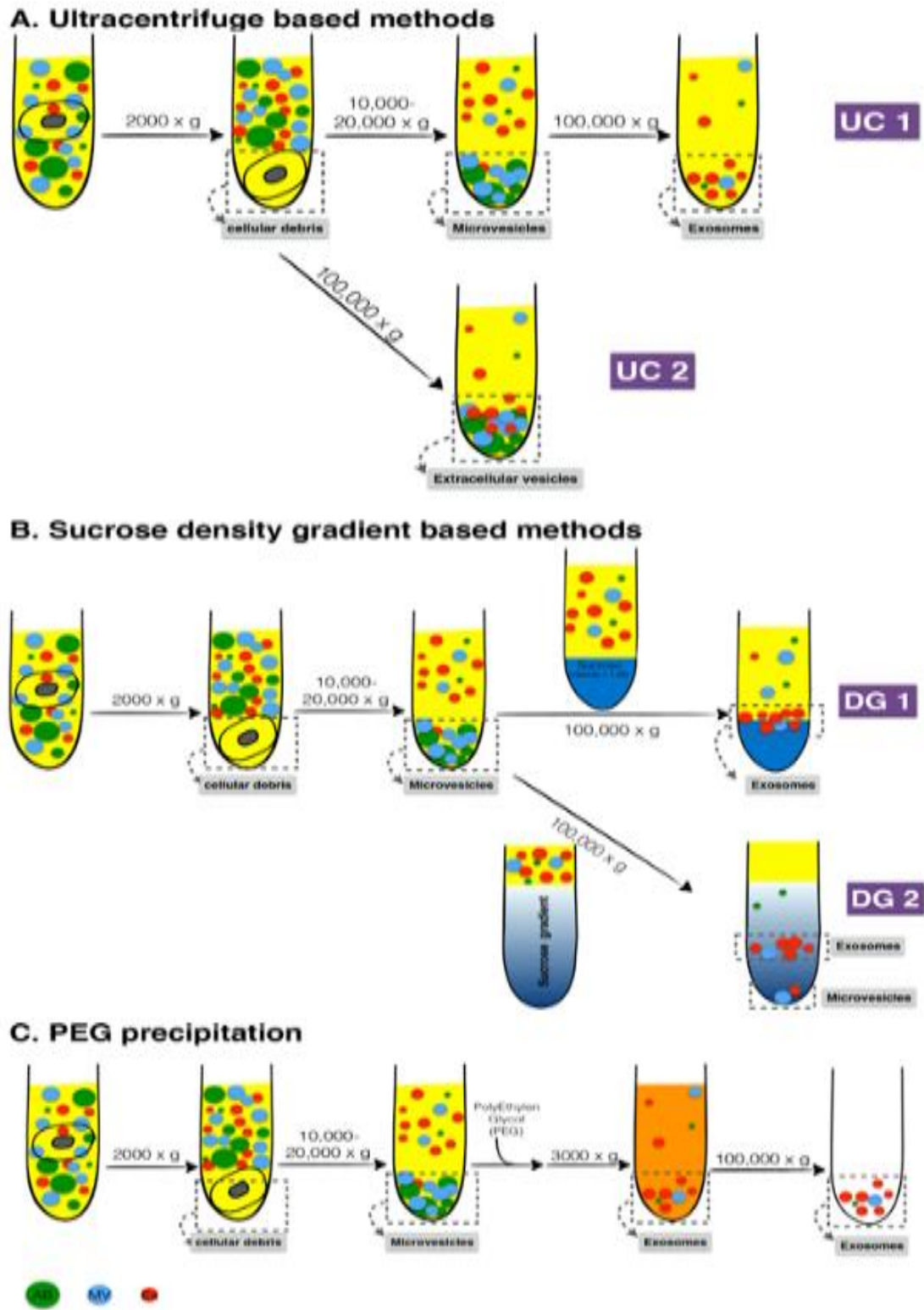


Fig1. Schematic illustration of the most prevalent methods for isolation of extracellular vesicles; A: Ultracentrifuge-based methods, B: Sucrose density gradient-based methods, C: PEG precipitation-based methods (AB: Apoptotic Bodies, Ex: Exosomes, DG: Density Gradient, MV: Microvesicles, UC: Ultracentrifuge, PEG: Poly-Ethylene Glycol).

Extracellular vesicles in regenerative medicine

EVs derived from mesenchymal stem/stromal cells (MSCs) and other probable sources are one of the options on the table to regenerate and repair damaged or diseased tissues with lower risks and limitations (21). The investigations span a broad usage of EVs along with other materials (i.e., cells, tissues, biomaterials, etc.) or alone (20, 22). In the last few years, numerous studies have been carried out in order to reveal the potential roles of EVs, especially exosomes, in tissue repair and regeneration, but their potential roles have not been fully elucidated (21).

Zhang et al. proposed an approach to increase cutaneous wound healing due to collagen synthesis promotion based on exosomes secretion of human-induced Pluripotent Stem Cell-derived MSCs (hiPSC-MSC-Exos). They found that proliferation and migration of human dermal fibroblasts as well as angiogenesis could be affected by concentration of exosomes secreted by hiPSC-MSC (23). Balbi et al. showed that c-KIT⁺ human amniotic fluid stem cells dynamically released EVs (hAFS-EVs) while the cells were under hypoxic conditions. hAFS-EVs represented remarkable regenerative and immunomodulatory effects on a model of skeletal muscle atrophy (HSA-Cre, Smn^{F7/F7} mice) (24). Despite the fact that significant improvements have been made in regeneration of bone defects in recent years, there are still controversial issues, which shall be addressed. Innovative strategies using EVs have paved the way for new options in bone and cartilage regeneration. In a study carried out by Qi et al., in vitro experiments revealed that hiPSC-MSC-Exos could enhance cell proliferation, and alkaline phosphatase (ALP) activity. Also, mRNA upregulation besides increased protein expression of osteoblast-related genes were observed. On the other hand, in vivo studies showed that hiPSC-MSC-Exos dramatically altered bone regeneration and

angiogenesis in critical-sized calvarial defects in ovariectomized rats (25). Liu et al. (26) investigated the effects of iPSC-MSC-Exos on osteonecrosis of the femoral head (ONFH). Their results revealed an increase in proliferation, migration and tube-forming capacities of endothelial cells, whereas PI3K/Akt signaling pathway was activated by iPSC-MSC-Exos. As a matter of fact, considering newly proposed methods is an inevitable parameter in tissue/organ repair and regeneration. Therefore, a deep search is prerequisite for handling future studies. Table 1 illustrates some translational and in vivo studies employing cell/stem cell-derived microvesicles and exosomes in the case of regeneration and tissue repair in recent years.

Concluding remarks and future prospects

Regenerative medicine seeks the most applicable approaches to overcome some limitations in tissue remodeling, tissue regeneration and organ repair. The future medicine will be attributed with the most facile recovery methods in personalized medicine, which are compatible, economic and possess better regenerative effects. A major hurdle in the case of cell-based approaches toward regeneration is entrapment of injected cells inside some organs like lungs. These cells are somehow high-risked due to their tumorigenesis. Accordingly, EVs have been approved to be reliable substitutions instead of direct cell administration. It is still unclear which contents/properties of exosomes are capable of promoting tissue regeneration. At last but not least, by virtue of exosomal engineering, we might be able to modify the contents of exosomes by adding therapeutic drugs or compounds to enhance their regenerative potential in regenerative medicine. Moreover, seeking newly proposed strategies like combination therapies (i.e., Cell-therapy/EVs, small molecule treatments/EVs and Cell Therapy/small molecule treatments), will pave the way of a new era in the future regenerative medicine.

Table 1. *In vivo* studies employing cell/stem cell-derived microvesicles and exosomes in the case of regeneration and tissue repair (Some parts are adopted from Ref. (18))

Target tissue/Model	Cellular Source	Species-exosome (Origin into Target)	Cell-derived agent	Method	Dose	Reference
Heart/IR	MSC	Human into Mouse	Exosomes	HPLC	0.4 mg	(27)
Heart/infarct	MSC	Rat into Rat	Exosomes (w/GATA4)	ExoQuick	(4×10 ⁶ MSC)	(28)
Heart/IR	MSC	Human into Mouse	Exosomes, ATP	HPLC	0.1-0.4 mg	(29)
Heart/infarct	MSC	Human into Rat	EVs	100 K × g	80 mg	(30)
Heart/infarct	MSC	Rat into Rat	Exosomes	ExoQuick	80 mg	(31)
Kidney/gentamycin	MSC	Rat into Rat	Exosomes	100 K × g	100 mg	(32)
Kidney/cisplatin	MSC	Human into Rat	Exosomes	100 K × g	250 mg	(33)
Brain/TBI	MSC	Human into Rat	Exosomes	ExoQuick	100 µg	(34)
Brain/TBI	MSC	Human into Mouse	Exosomes	An Chrom	30 mg	(35)
Brain/stroke	MSC	Rat into Rat	Exosomes	100 K × g	100 mg	(36)
Brain/ischemia	MSC	Human into Ovine	EVs	PEG	(1 × 2 × 10 ⁷ MSC)	(37)
Brain/TBI	MSC	Rat into Rat	Exosomes	ExoQuick	100 mg	(38)
Brain/stroke	MSC	Human into Mouse	Exosomes	110 K × g	(2 × 10 ⁶ MSCs)	(39)
Liver/fibrosis	MSC	Human into Rat	Exosomes	100 K × g	250 mg	(40)
Liver/drug injury	MSC	Human into Mouse	Exosomes	100 K × g	0.4 mg	(41)
Lung/hypoxia	MSC	Mouse into Mouse	Conditioned Medium, Exosomes	PEG-S200	0.1–10 mg	(42)
Lung/drug	MSC	Mouse into Mouse	Exosomes	100 K × g	25mg	(43)
Lung/silicosis	MSC	Human into Mouse	Microvesicles	ExoQuick	10 mg	(44)
Hypertension	MSC	Human into Mouse	Microvesicles	100 K × g	(3 × 10 ⁶ MSCs)	(45)
Lung/fluid filled	MSC	Human into Human	Microvesicles	100 K × g	160 mg	(46)
Lung/E.coli endotoxin	MSC	Human into Mouse	Microvesicles	100 K × g	(9 × 10 ⁶ MSCs)	(47)
Intestine/enterocolitis	MSC	Human into Rat	Exosomes	PureExo	50 ml IP	(48)
Intestine/enterocolitis	MSC	Rat into Rat	Microvesicles	100 K × g	50-200 mg	(49)
Skin/wound	MSC	Human into Rat	Exosomes, Wnt4	100 K × g	200 mg	(50)
Skin/wound	MSC	Human into Rat	Exosomes	100 K × g	160 mg	(23)
Skin/wound	MSC	Human into Mouse	Exosomes, miRNA	120 K × g	100 mg	(51)
Limb ischemia	MSC	Human into Mouse	Exosomes	100 K × g	200 mg	(52)
Skeletal Muscle/cardiotoxin	MSC	Human into Mouse	Exosomes, miR-494	110 K × g	50 µl	(53)
Skeletal Muscle/ALS	MSC	Mouse into Mouse	Exosomes, SOD1	PureExo	0.2 mg/ml	(54)
Wound healing in T2DM	nAT-MSCs	Human into Mouse	Microvesicles	100 K × g	(3 × 10 ⁵ MSCs)	(55)
Cisplatin and Glycerol induced AKI	hWJMSC	Human into Mouse	Microvesicles	100 K × g	100 µg	(56)
Glycerol induced AKI	MSC	Human into Mouse	Microvesicles	100 K × g	15 µg	(57)
Liver/Model of 70% hepatectomy	HLSCs	Human into Rat	Microvesicles	100 K × g	15 µg	(58)
Eye/ONC model	MSCs	Human into Rat	Exosomes	100 K × g	(1 × 2 × 10 ⁶ MSC)	(59)
SCI/ model of rat cervical avulsion	Cortical neurons treated with the RARβ agonist CD2019	Human into Rat	Exosomes	100 K × g	10 µg	(60)
Heart/MI	CDCs	Human into Mouse	Exosomes	ExoQuick	3.5 × 10 ⁸ or 2 × 10 ⁸	(61)
ischemia/reperfusion injury and partial hepatectomy	hepatocytes	Mouse into Mouse	Exosomes	ExoQuick	200 µg	(62)
Chronic cutaneous wounds	platelet-rich plasma	Human into Rat	Exosomes	100 K × g + 30% sucrose-D ₂ O cushion	50 µg/ml	(63)
Osteoarthritis	human synovial membrane MSCs / iPSC-MSCs	Human into Mouse	Exosomes	Ultrafiltration	10 ¹⁰ /ml	(64)
Dental Pulp Tissue Regeneration	naïve human dental pulp stem cells (DPSCs) and human bone marrow derived stromal cells (HMSCs)	Human into Mouse	Exosomes	ExoQuick	1.25 × 10 ⁶ HMSCs + 250 × 10 ³ DPSCs	(65)

Abbreviations

AKI: Acute Kidney Injury, ALS: Amyotrophic lateral sclerosis, CDCs: cardiosphere-derived cells, DPSCs: naïve human dental pulp stem cells, EVs: Extracellular Vesicles, HLSCs: Human Liver Stem Cells, hWJMSC-MVs: human Wharton-Jelly MSCs derived microvesicles, iPSC: induced Pluripotent Stem Cells, IR: myocardial ischemia/ reperfusion injury, HPLC: High Pressured Liquid Chromatography, PEG: Polyethylene Glycol, MI: Myocardial Infraction, miR: microRNA, MSC: Mesenchymal Stem/Stromal Cell, nAT-MSCs: non-diabetic healthy donor adipose tissue derived mesenchymal stem/stromal cells, ONC: Optic Nerve Crush, SCI: Spinal Cord Injuries, TBI: Traumatic Brain Injury, T2DM: Type II Diabetes Mellitus.

Conflict of Interest:

Authors declared no conflict of interest.

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