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REVIEW

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Beneficial and challenges of exosome application in ischemic heart disease

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Abstract

Cardiovascular diseases are the main cause of death and disability in the clinical setting. Among several pathological conditions, myocardial infarction (MI) is a common clinical finding and happens due to the reduction or complete interruption of blood support. Stem cells and progenitors are valid cell sources with significant potential to alleviate several tissue injuries. Differentiation to mature and functional cells and the release of various growth factors, and cytokines are the main reparative mechanisms by which stem cells mediate their reparative tasks. Exosomes (Exos), a subset of extracellular vesicles (EVs), exhibit great theranostic potential in biomedicine. Along with whole-cell-based therapies, the pre-clinical and clinical application of Exos has been extended in animals and humans with ischemic heart diseases (IHD). Here, in this review article, we aimed to highlight the importance of Exos in IHD and address the mechanism of action by focusing on their regenerative potential.

Keywords Cardiovascular disease, Ischemic changes, Exosomes, Delivery, Regeneration

Introduction

According to a comprehensive population-wide analysis, IHD is the main cause of human death in the twenty-first century [1, 2]. Due to the loss of cardiomyocyte membrane integrity, multiple diagnostic biomarkers such as creatine kinase-MB (CK-MB), cardiac troponin T (cTnT), and cTnI are released into the circulation [3, 4]. Inadequate oxygen levels and nutrient delivery into the myocardium can contribute to an irreversible loss of functional cardiomyocytes and weakening of ventricular contractility, resulting in heart failure [5–7]. Although reperfusion approaches and fibrinolytic therapies are helpful in the restoration of cardiac tissue output, the possibility of bleeding, blood clot formation, wound infection, thromboembolism, pericardial effusion, etc. are life-treating factors in MI patients [8, 9]. Thus, it is mandatory to develop new modalities with more therapeutic outcomes and fewer post-complications in MI patients. While timely diagnosis in the initial stages, long-term follow-up, and prognosis of ischemic changes are mandatory in clinical settings and are beneficial to increase the efficiency of therapeutic protocols [10–13].

Like several tissue types, cardiac homeostasis and the healing process are well-organized intercellular communications and are in part controlled by EVs [14]. EVs are

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heterogeneous and nano-sized vesicles and are involved in the transfer of growth factors, lipids, DNAs, mRNAs, and long non-coding RNAs (lncRNAs) [15–17]. In general, EVs include Exos, microvesicles (MVs or ectosomes), and apoptotic bodies with different sizes, biogenesis systems, cargoes, and functions [18–21]. Among EV types, Exos are produced by the endosomal system and actively participate in intercellular crosstalk [22]. In the acceptor cells, the internalized Exos are guided to the lumen of early endosomes (Fig. 1) [20, 23]. Inside the cells, endogenous Exos are also generated by endosomes and multivesicular bodies (MVBs), leading to the production of numerous intraluminal vesicles (ILVs) [24]. Using multiple effectors such as ESCRTs, SNAREs, RABs, etc. ILVs are released into the extracellular matrix (ECM), hereafter known as Exos (Fig. 2) [25–35].

Recent research has pointed to the valuable role of EVs mainly Exos in the detection and monitoring of IHD (Table 1). Exos can easily reach biofluids, thus making them invaluable diagnostic tools in IHD patients [36]. Exo cargo is a real-time reflection of the metabolic status of original cardiomyocytes. Thus, these nanoparticles (NPs) can be used as valid theranostics after MI occurrence [37, 38]. Cardiomyocyte Exos harbor diverse biomolecules like proteins, lipids, and RNAs [messenger RNAs (mRNAs), microRNAs (miRNAs), circular RNAs (circRNAs), non-coding RNAs (ncRNAs)], and other compounds. The content and cargo types can be varied according to the specific pathophysiological circumstances [39]. Data have confirmed that the release of cardiac tissue EVs is fast compared to free conventional biomarkers like cTnI in various ischemia/reperfusion (I/R) models. Therefore, it is possible to follow cardiac tissue-specific miRNAs in plasma less than 4 h, after the occurrence of MI [40, 41]. Some miRNAs such as miRNA-133a/b, -320, -499, -1, and -208a have relatively diagnostic values for monitoring the function of cardiomyocytes [41]. Exosomal miRNAs and other genetic elements are resistant to degradation processes with key diagnostic values for IHD [42–44]. Most of the exosomal miRNAs are associated with the regulation of cardiomyocyte bioactivity and homeostasis. Thus, elevation/reduction of specific miRNA types can reflect the real-time changes within the heart after being exposed to insulting conditions [39]. For example, p53 signaling cascade-responsive miRNAs such as miRNA-192, -194, and -34a, are abundant in the serum Exos of individuals with acute MI [45]. Hypoxic cardiomyocytes can produce Exos with higher levels of miRNA-222, -143, and matrix metalloproteinases (MMPs) compared to normal cardiomyocytes [46]. There is a close relationship between specific miRNA types and heart tissue function. The levels of exosomal miRNA-152-5p, -204, lncRNA NEAT1, and -3681-5p can indicate the possibility of ST-segment and MI occurrence [47, 48].

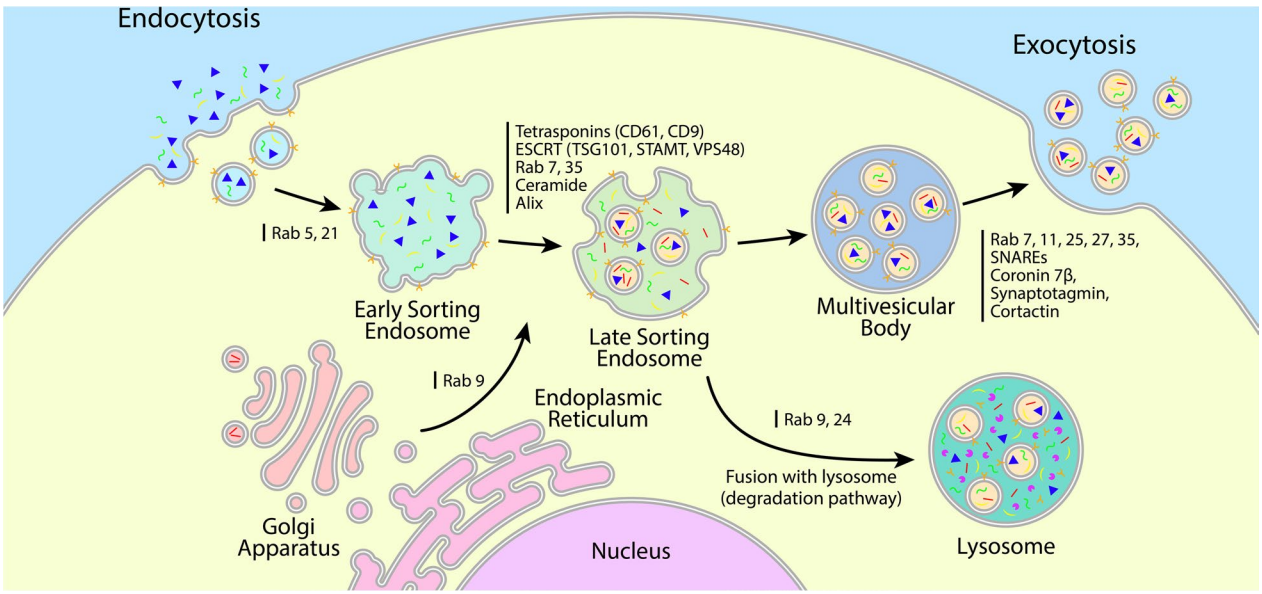

Fig. 1 Exo biogenesis and secretion routes inside the host cells. Internalized Exos are packed inside the intracellular vesicles named early endosomes. Early endosomes mature into later endosomes and MVBs. Along with these changes, several new ILVs are generated via the invagination of the vesicle membrane. Several biomolecules are sequestrated inside the ILVs. MVBs with numerous ILVs are guided toward lysosomal degradation to fuse cell membranes to release their contents (ILVs) into the extracellular matrix where they are called hereafter Exos

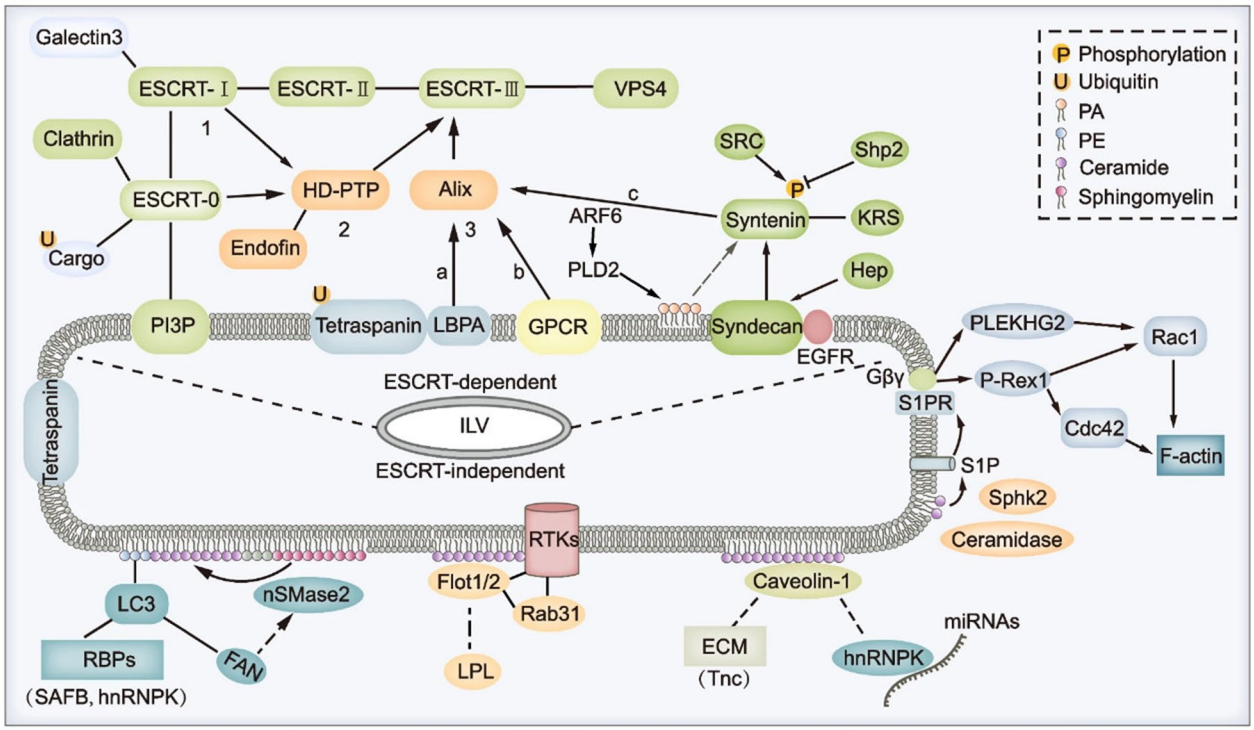

Fig. 2 Different molecular pathways are involved in the generation of ILVs. MVBs with numerous intraluminal ILVs are generated using multiple pathways. To be specific, ESCRT-dependent and ESCRT-independent pathways can control the generation of de novo ILVs. In the canonical ESCRT-dependent pathways, four complexes including ESCRT-0, -I, -II, -III along with ATPase VPS4 regulate the dominant process of ILV formation (1). The complementary non-canonical ESCRT-dependent pathways, effectors such as HD-PTP (2) or Alix (3) in close collaboration with ESCRT-III and VPS4 sequestrate certain biosignaling molecules into the MVBs. In line with these activities, lipid rafts belonging to non-canonical ESCRT-dependent systems are also important. More interestingly, some tetraspanins such as CD63 are involved in the generation of nascent ILVs by both ESCRT and ceramide-independent mechanisms. It is thought that the nSMase2-ceramide pathway is another important molecular axis that regulates the formation of ILVs in MVBs in an ESCRT-independent manner. Factors such as caveolin-1 or flotillins proceed with the formation of ILVs by lipid raft-dependent pathways with simultaneous activation of the nSMase-ceramide pathway in some cell types. [26] Copyright 2022; Molecular Cancer

Exosomal proteins and peptides are also valuable predictive biomarkers for MI. The existence of different proteins in cardiac tissue Exos such as sarcomeric proteins, cardiac-type myosin-binding protein C, myomesin, tropomyosin, and valosin-containing protein can help us to discriminate them from non-cardiac tissue sources [49]. In patients with elevated ST-segment, exosomal platelet-derived GPIIb and vascular endothelial cadherin (VE-cadherin) increased [50]. Other common biomarkers such as TNF-α and hypoxia-inducible factor 1 alpha (HIF-1α) are elevated in cardiomyocyte Exos [51, 52].

Some studies have revealed that the release of Exos from injured cardiomyocytes can promote further cytopathic effects. For instance, cardiomyocyte-derived Exos containing heat shock protein 60 (HSP60), and TNF-α can interact with toll-like receptors (TLR), stimulating apoptotic changes [53]. While other exosomal HSPs like HSP70 can protect the cardiomyocytes against I/R injury via the activation of the TLR4 signaling pathway [54]. The activation of other mechanisms such as autophagy [beclin-1↑, LC3-II/LC3-I ratio↑, and, Atg12↑] by exosomal miRNAs (miRNA-30a) is possible [55]. Local Exos (miRNA-143 and -222) can stimulate the angiogenesis inside the injured myocardium, resulting in the reduction of fibrotic changes. Cardiac progenitor cells can release Exos containing several pro-angiogenesis factors such as vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), transforming growth factor-β, and miRNA-132 to re-establish the blood supplementation, reduce cardiomyocyte apoptosis (PDCD4↓ and miRNA-21↓, and -210), and cardiac fibrosis [miRNA-29b, -323-5p, -455, and -466] [46, 56–59]. These Exos can blunt the oxidative damage of cardiomyocytes and deactivate Caspases 3, and 7 [60].

Table 1 Alteration of some genetic elements and factors in cardiac cell Exos under different conditions

Cytokines and proteins	miRNAs	MI or other stressful conditions compared to the normal status	References
Apolipoprotein D, Apolipoprotein C3, Complement C1q, subcomponent subunit A (C1Q1A), Complement C5 (C5), Glycoprotein Ib Platelet Subunit Alpha (GP1BA), Pro-Platelet Basic Protein, HSp20, HSp60, HSp70, IL-6, Glut1, Glut4, NAD(p)H oxidase, TNF-a, Phosphatase and tensin homolog (PTEN), EGFR, Pregnancy-associated plasma protein-A, Fibronectin, Collagen, Alix, HIF-1α, TGF-β, CD63, MMP, collagen alpha-1 (I) chain precursor, heat shock cognate 71 kDa protein, angiopoietin-related protein 2 precursor, glyceraldehyde-3-phosphate dehydrogenase, serine protease HTRA1 precursor, elongation factor 1-alpha 1-like, sodium/potassium-transporting ATPase subunit alpha-1 precursor, Na, K-ATPase alpha-1 subunit, ATPase, Na + /K + transporting, alpha 1 polypeptide, isoform CRA, apolipoprotein E precursor, L-Lactate dehydrogenase A chain, L-Lactate dehydrogenase B chain, polyubiquitin-C precursor, clathrin heavy chain 1	miR-133a, miR-21, miR-423, miR-328, miR-29b, miR-208, miR-499, miR-1, miR-192, miR-1956, miR-34a, miR-134, miR-194, miR-126, miR-486, miR-3681, miR-152, miR-3681, lncRNA Zinc finger antisense 1 (ZFAS1), Cdr1 antisense (CDR1AS), lncRNA HOX antisense intergenic RNA (HOTAIR), miR-125a-5p, miR-100-5p, miR-365a-3p, miR-193b-5p, miR-99a-5p, miR-193a-5p, miR-361-5p, miR-29a-3p, miR-885-5p, miR-345-5p, miR-1246, miR-3692-5p, miR-193b-3p, miR-654-3p, miR-1260b, miR-22-3p, miR-3168, let-7d-3p, miR-6763-3p, miR-320b, miR-155, miR-206-3p, miR-199a-5p, miR-494-3p, miR-300c, miR-320, miR-17, miR-19a, miR-19b, miR-20a, miR-30v, let-7e, miR-10a, miR-27b, miR-100, miR-126-3p, miR-130a, miR-143, miR-503, miR-222,	Increased	[10, 46, 47, 141–176]
	miR-197, miR-106, miR-223, miR-4520–2-3p, miR-5579-5p, miR-3681-5p, miR-212-3p, let-7f-1-3p, miR-30c-2-3p, miR-126	Reduced	

Exos as drug delivery platforms in the cardiovascular system

In terms of cardiovascular disease, it is possible to deliver naïve or engineered Exos using a variety of administration methods (Fig. 3). For instance, intravenous, intracardial, intradermal, epicardial, and intraperitoneal routes have been proposed to deliver the Exos to target sites [61–65]. Among different administration methods, intravenous injection is a non-invasive approach. However, off-target distribution and rapid elimination from circulation are the main drawbacks. The sequestration of Exos by hepatic, splenic tissues, and pulmonary vascular system after intravenous administration requires repeated and high doses of Exos [66–68]. Notably, data have indicated that intravenously infused Exos accelerated the healing of ischemic myocardium in several animal models [69–71]. In contrast to intravenous infusion, the direct intracoronary artery and intramyocardial injection can circumvent several limitations such as off-target delivery and non-specific sequestration in the liver, spleen, and other tissues [8]. Based on previous data, the direct introduction of Exos via myocardium and intracoronary route improved efficiently the healing of ischemic myocardium in animal models [72, 73]. For example, the intramyocardial injection of cardiosphere-derived cells in pig models led to improved ventricular function compared to intracoronary infusion groups [74]. It seems that intramyocardial injection is more effective in the alleviation of pathological conditions compared to the intracoronary administration method because this approach provides a suitable platform for the efficient delivery of higher exosomal cargo into the injured myocardium with enhanced retention time, and prominent Exo-to-cardiac cell interaction [75]. Besides, this approach avoids the direct contact of Exos with the blood components and specific biological barriers. However, catheter-based delivery or open-chest surgery is available for the intramyocardial injection of Exos [76, 77]. It has been indicated that the intramyocardial injection can increase left ventricular ejection fraction in both acute and chronic MI models via the reduction of abnormal cardiac remodeling, and fibrosis [72]. Despite these advantages, the invasive entity of intramyocardial injection makes it relatively problematic in clinical cardiology [72]. The direct injection of Exos into the injured myocardium can contribute to rapid elimination, and loss of exosomal integrity before obtaining regenerative benefits [75]. Due to poor negative charge, Exos form microaggregates which affect their distribution, and even delivery properties [78]. Of note, repeated high doses of Exos can provoke immune cell reactivity in allogeneic models, leading to the stimulation of inflammation at the site of injury and a postponed healing process [79]. The therapeutic effects of Exos at the site of injury are associated with the promotion of phenotype acquisition in macrophages, and the reduction of inflammation and aberrant ECM remodeling [49,

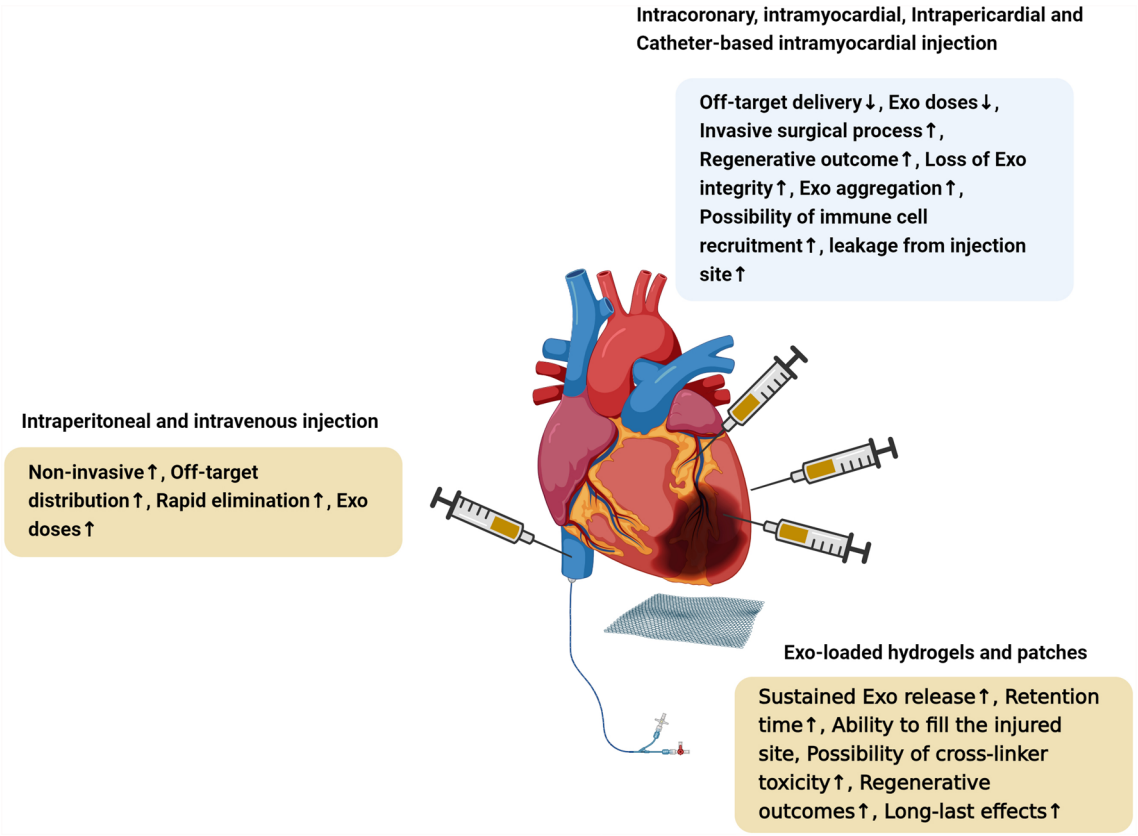

Fig. 3 Several possible Exo applications for the alleviation of ischemic myocardium. Each approach has its advantages and limitations. The systemic administration of Exos can yield low on-target effects while increasing the rapid elimination of circulating Exos via the activity of immune cells. Local administration of free Exos (direct intracoronary artery, intramyocardial, intrapericardial, and catheter-based intracardial injections) or injection via supporting substrates and hydrogels can increase the on-target delivery efficiency into the ischemic area. Created with BioRender online software

80]. For instance, rat bone marrow MSC Exos can blunt nuclear translocation of NF-κB p65 and NLRP3 inflammasome with the potential to control the inflammatory response in oxygen/glucose-deprived H9c2 cardiomyocytes [81]. Certain molecular complexes such as miR-5p/TRAF axis can regulate inflammatory response via engaging the NF-κB signaling pathway [82]. Cardiac phenotypic plasticity after treatment with stem cell Exos is another mechanism by which these cells can resist harsh microenvironments. To be specific, cardiomyocytes can differentiate into the myofibroblasts to escape from the apoptotic changes, and inflammatory conditions [83]. The reduction of fibrosis is related to the reduction of collagen fiber deposition and conversion of dormant fibroblasts into reparative phenotype [84]. In situations associated with an extensive myocardial infarction, or epicardial ischemia, Exos can be administrated from the epicardial surface via open heart surgery and/or video-assisted cardiac surgery [85, 86]. It is postulated that this approach enables us to more focused conveyance of Exos into the injured sites especially in patients without the possibility of open-heart surgery [85, 86].

In less invasive approaches, hydrogel-bearing Exos can be used as cardiac patches on the pericardial surface or directly injected into the deep layers of the myocardium for sustained release of Exos into the target sites [87]. The porosity, swelling rate, and biodegradability of these compounds make them release loaded Exos in a controllable manner [2]. Based on data from different studies, Exos can be loaded into the hydrogel using different strategies. In one approach, Exos, polymers, and cross-linkers are mixed simultaneously and injected into the site of injury. The gelation in in vivo conditions enables the sustained release of Exos [88]. To help the gelation of hydrogels, ion change, UV irradiation, pH, and temperature regulation are also suggested based on the chemical structure and type of substances [89, 90]. Hydrogels acquire the shape and geometries of injured sites to fill and adhere the inured site to surrounding neighboring tissue [90]. It is also possible to mix polymers and cross-linkers before

the addition of Exos. This approach promotes hydrogel gelation and Exos are incorporated into the hydrogels. In another strategy, the dehydrated hydrogels can be incubated in Exo-containing solutions. The porous structure allows the breathing of Exos into deep layers of hydrogel [91]. Therefore, hydrogels should possess a pore size larger than Exo diameter to facilitate exosomal breathing while a much bigger pore size increases uncontrolled Exo leakage before and after transplantation [88, 92]. In the last protocol, Exos and polymeric solution are mixed followed by the addition of cross-linkers to accelerate the gelation [93]. The cross-linked hydrogels possess suitable mechanical strength and degradation rates. However, the existence of unreacted cross-linkers can lead to cytotoxic effects. Even, some cross-linker types are toxic even after being reacted with the polymeric backbone. Thus, the use of nontoxic compounds for hydrogel polymerization should be lessened [94]. In an experiment, growth hormone-releasing peptides in combination with amphiphilic peptides C16-GTAGLIGQ were used to increase the mesenchymal stem cell (MSC) Exo retention time in a rat model of MI [95]. Data indicated the reduction of inflammation, apoptosis, fibrosis, and stimulation of angiogenesis [95]. In another study, tyramine-functionalized hyaluronic acid (6%) was blended with cardiomyocyte Exos (~ equals 10–100 exosomal protein) and used for the differentiation of human MSCs into cardiomyocytes [96]. This platform stimulated the viability of human MSCs and differentiation toward GATA4↑, Nkx2.5↑, and Tbx5↑ cardiomyocytes [96]. It seems that the covalent attachment of Exos into the polymeric structure increases the retention time after transplantation into the target sites. In an experiment, thiolated Exos anchoring a CP05 peptide was cross-linked to thiolated hyaluronic acid using epoxy macromere and aniline tetramer. The developed hydrogel can promote the migration of human endothelial cells (ECs) and MSCs. The injection of Exo-bearing hydrogel was shown to accelerate angiogenesis potential [PECAM↑, VEGF↑ (isoforms A and B), α-SMA↑, vWF↑) and cardiogenesis (Cx43↑, SERCA2a↑, Ki-67↑) [97].

It seems that pre-treated cell sources can produce specific Exo types with improved regenerative outcomes. The incorporation of HIF-1α-expressing MSC Exos into RGD hydrogels efficiently reduced the fibrosis rate and promoted the function of the injured myocardium with a reduction of apoptotic cardiomyocytes (Caspases 3↓, and 7↓) [98].

Engineered exos in cardiovascular disease

It is mandatory to use sophisticated strategies to maximize Exo distribution into the myocardium via direct interaction with cardiomyocytes or cardiac tissue vascular system [6, 7]. To date, two main modification strategies have been used for exosomal surface and internal engineering (Fig. 4). For this purpose, Exos can be modified before isolation (indirect method) by manipulation of host cells or Exos are directly subjected to engineering protocols after enrichment (direct method) (Table 2) [1]. These approaches have led to enhanced healing capacity and targeting efficiency (Table 3). In recent years, the application of gene editing tools such as CRISPR-Cas9 systems has been extended to biological systems. EVs are valid bioshuttles for transferring the CRISPR-Cas9 products into the recipient cells [99]. This system is eligible to efficiently modify the surface of EVs for various therapeutic purposes (Fig. 5) [100]. In a recent work conducted by Mun et al., they used CRISPR-Cas9 ribonucleoprotein (RNP)-loaded EVs decorated with cardiac-targeting peptide (T) for the edition of ischemic cardiomyocyte miR-34a [101]. Data revealed the successful delivery (~ twofold) of EVs into cardiac tissue, regulation of target miR-34a, and reduction of apoptotic changes (Cleaved caspase 3↓, and Bax↓) in infarcted mice. Schary and co-workers found that the disruption of the TLR4 gene using CRISPR-Cas9 in MSCs led to an increase in viability, suitable cardiac tissue remodeling, and function in infarcted mice [102]. Data indicated that the parent cell manipulation via CRISPR-Cas9 can affect the levels of inflammatory factors inside released MSC EVs [102]. Despite these adamantanes, genetic materials have relatively short lives inside EVs which can lead to a lack of appropriate gene editing therapy, and regenerative benefits [99]. Besides, its immunogenicity, and non-desired immune responses should be precisely monitored [103].

To the best of our knowledge, there are few reports related to the application of engineered Exos in the cardiovascular system [8]. In this scenario, Exo modification has been done for different purposes as follows; a) to track fluorophore-, luminescence reporter-, and radiotracer-labelled Exos after being injected into in vivo systems. Both Exo surface and lumen can be modified with these tracers, b) to increase Exo bioactivity and therapeutic benefit, c) to improve on-target delivery efficiency by using various peptides, proteins, and lipids on the exosomal surface, and d) to stimulate internalization rate and endo-lysosomal escape using certain cell-penetrating and pH-sensitive peptides, and cationic lipids [104]. The indirect Exo modification can provide a continuous source for biomedical purposes [20, 105]. To modify the parent cells to produce certain Exos, direct genetic manipulations, incubation with certain compounds, and changes in culture conditions are applicable options [104, 106–111]. Tetraspanins, Lamp-2b, lactadherin, glycosyl-phosphatidyl-inositol (GPI), and PDGFR are target proteins for the production of engineered Exos [112, 113]. The treatment of *Lamp2b* expressing

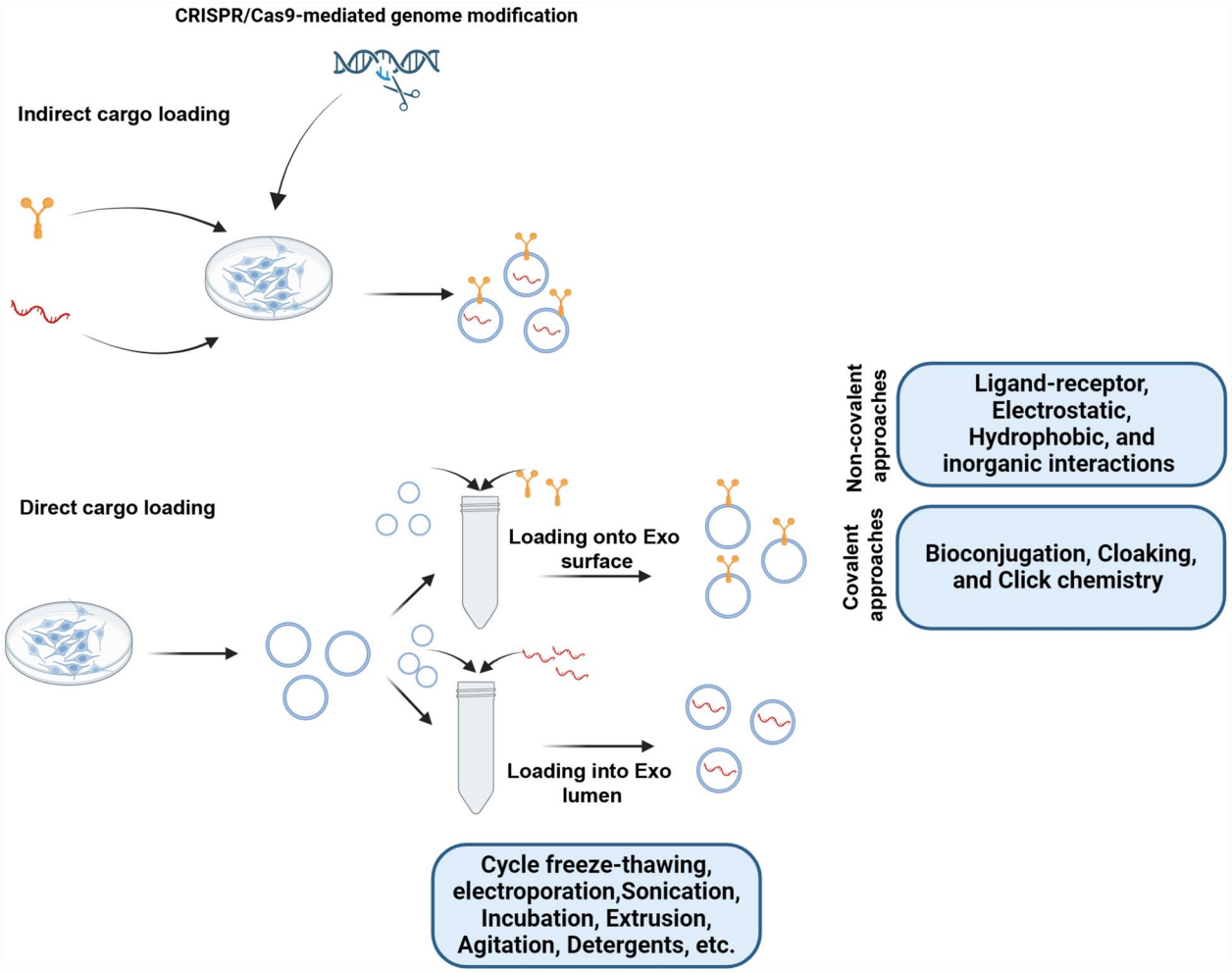

Fig. 4 Different strategies are used for cargo loading and increase of on-target delivery of Exos into the ischemic myocardium. The sophisticated manipulations can be done on the parent cells or directly on the purified Exos before injection into the target sites. These approaches increase the delivery efficiency and therapeutic outcomes of administrated Exos in the ischemic myocardium. Created with BioRender online software

cardiosphere derived cell Exos with cardiac-specific peptide, WLSEAGPVVTVRALRGTGSW, produced engineered Exos with enhanced internalization capacity into the cardiomyocytes and higher retention time compared to non-engineered Exos (Fig. 6) [114]. In a similar study, transfection of HEK293 cells with cardiac-targeting peptide-*Lamp2b* expressing vector generated engineered Exos with higher delivery efficiency in in vitro and in vivo conditions [115]. Other researchers produced engineered Exos with recombinant Lamp2b-ischemic myocardial targeting peptide [CSTSMLKAC] or Lamp2b-heart homing peptide with a higher internalization rate in hypoxic cardiomyocytes [116, 117]. In addition to the targeting approach, some authorities used modified Exos for the alteration of metabolic profile. For instance, *βARKct* expressing cardiosphere cell Exos was used in a mouse model of catecholamine toxicity. Data indicated that βARKct-bearing Exos efficiently inhibits GRK2, resulting in the reduction of heart failure [118]. Despite the advantages related to indirect Exo modification, genetic manipulation of host cells may alter the biogenesis of Exos and the levels of target molecules can be less in the released Exos [104]. Therefore, direct modification approaches can be used for improving loading and targeting efficiencies irrespective of host cell origin [25].

To encapsulate exogenous compounds into the Exo lumen, various techniques including cycle freeze–thaw, electroporation, sonication, incubation, extrusion, agitation, and treatment with detergents have been used in different studies [20]. Among these approaches, freeze–thaw and incubation are inactive encapsulation methods. Using freeze–thaw methods, hydrophobic curcumin was loaded in Exos for the alleviation of MI [119]. Bheri and colleagues used electroporated

Table 2 Commonly used technique for the production of engineered Exos

Modification Type	Methodology	Description	Advantages	Limitations
Interior modifications	Pre-isolation approaches	Modifications are done on donor cells before Exo isolation	Simple, and direct loading	Loading efficiency↓
	Post-isolation approaches	Incorporating of target molecules after Exo isolation	Loading efficiency↑	Injury to Exo integrity↑
	(a) Passive loading [177]	Isolated Exos are incubated with target compounds and these compounds are loaded into the Exos after membrane diffusion	Easy, and protect the Exo structure	Loading efficiency↓
	(a1) Active loading [178]	The Exo membrane is temporarily permeabilized to permit the cargo loading	Loading efficiency↑	Needs complex protocols and instruments
	(a2) Electroporation [179]	Induces pores in the Exo membrane	Loading efficiency↑	Aggregation and possible injury to Exo integrity↑
	(a3) Extrusion [180]	The combination of Exos and target compounds is extruded through a membrane	Loading efficiency↑	Needs extrusion instrument
	(a4) Freeze–thaw cycles [132, 181]	Repeated freezing and thawing help the loading of target molecules into Exos	Applicable for sensitive and fragile compounds	Time-consuming, and the possibility of endogenous cargo loss, reduces the Exo number, and increases the Exo size
	(a5) Chemical transfection [182]	Using chemicals such as surfactants, pores are induced in the Exo membrane for cargo entry	Simultaneously various compounds can be loaded	Injury to Exo integrity↑
Surface Modifications	Genetic engineering of donor cells [183]	Genetic modification of donor cells is done to up-/down-regulate specific molecules on the Exo surface	Applicable for enhanced delivery purposes	Needs complex and sophisticated gene editing approaches
	Direct manipulation	Direct modification is done on isolated Exos	Targeting delivery↑	Injury to Exo integrity↑
	(a1) Covalent binding via click chemistry [184]	Covalent bonds are generated between target molecules and Exo surface	Specificity↑	Needs specific chemical reactions and alters Exo integrity
	(a2) Non-covalent approaches [185]	Approaches such as receptor-ligand binding or electrostatic interactions are applied	Simple	Stability of reaction↓
	(a3) Hybridization with fusogenic liposomes [186]	Exos and liposomes combination are used to increase delivery efficiency	Internalization rate↑	Complexity↑ and requires two vesicle types
	(a4) Direct hydrophobic component loading [187]	Incorporation of hydrophobic drugs onto Exos	Targeting delivery↑	Only hydrophobic compounds can be used

Table 3 Some studies related to the application of engineered Exos in cardiovascular diseases

Modification method	Approaches	Animal model or in vitro study	Outcome	References
Direct (post-isolation)	Cardiac progenitor cell Exos were decorated with cardiac homing peptide using a DOPE-NHS linker	Rat model of infarction	Targeting efficiency↑, Fibrosis↓, Infarct and scar size↓, Cell proliferation↑, angiogenesis↑	[125]
	The surface of Exos was decorated with cardiac targeting peptide and loaded with NOX4 siRNA	Mouse model of angiotensin II-induced atrial fibrillation	Targeting efficiency↑, Cardiac function↑, Fibrosis↓, Arrhythmia inducibility↓	[188]
	The surface of Exos was decorated with cardiac targeting peptide using DBCO Sulfo-NHS and miR-34a were elevated inside Exos using CRISPR-Cas9-	Mouse model of myocardial infarction	Targeting efficiency↑, hydrogen peroxide-induced apoptosis↓,	[101]
	The surface of MSC Exos was decorated with fluorinated peptide dendrimers by incubation	In vitro incubation with HUVECs	HUVEC uptake↑, angiogenesis↑, migration↑	[129]
	Mycophenolic acid was loaded into RAW 264.7 macrophage Exos using passive incubation and in TritonX-100	In vitro rat cardiomyoblasts,	Uptake in rat cardiomyoblasts↑, Inflammation↓, Intracellular oxidative stress↓	[124]
	Using Sonication melatonin was loaded into MSC Exos	Hypoxia/serum-deprived rat cardiomyoblasts, and and mouse model of infarction	Uptake levels↑, Apoptosis↓, Oxidative stress↓, Fibrosis↓, and microvessel formation (CD31) ↑	[189]
	Using electroporation, miR-126 was loaded onto the cardiac progenitor cell Exos	rat model of ischemia–reperfusion, and in vitro cardiac endothelial cells	Uptake levels↑, Angiogenesis↑, Fibrosis↓, Myocardial function↑, Hypertrophy↓, Microvascular density (isolectin $B4^+$, α-SMA^+ cells↑)	[120]
	The surface of MSC Exos was cloaked with platelet membrane using extrusion	In vitro incubation with human coronary artery ECs, and mouse model of myocardial infarction	Uptake levels↑, Tubulogenesis↑, Proliferation↑, Mitochondrial activity↑, Inflammation↑, Targeting efficiency↑, infarct size and fibrosis↓,	[190]
In-direct (pre-isolation)	Cardiosphere-derived Exos were coated with Lamp2b- and cardiomyocyte-specific peptide using expressing plasmids in parent cells	In vitro incubation with cardiomyocytes, HUVECs, and cardiac fibroblasts, and mice	Uptake by cardiomyocytes↑, Retention time↑, Apoptosis↓, Hypertrophy↓	[114]
	HEK 293 cell Exos were decorated with cardiac-targeting peptide-Lamp2b using expressing plasmid	H9C2 cells, and mice	Delivery efficiency↑,	[115]

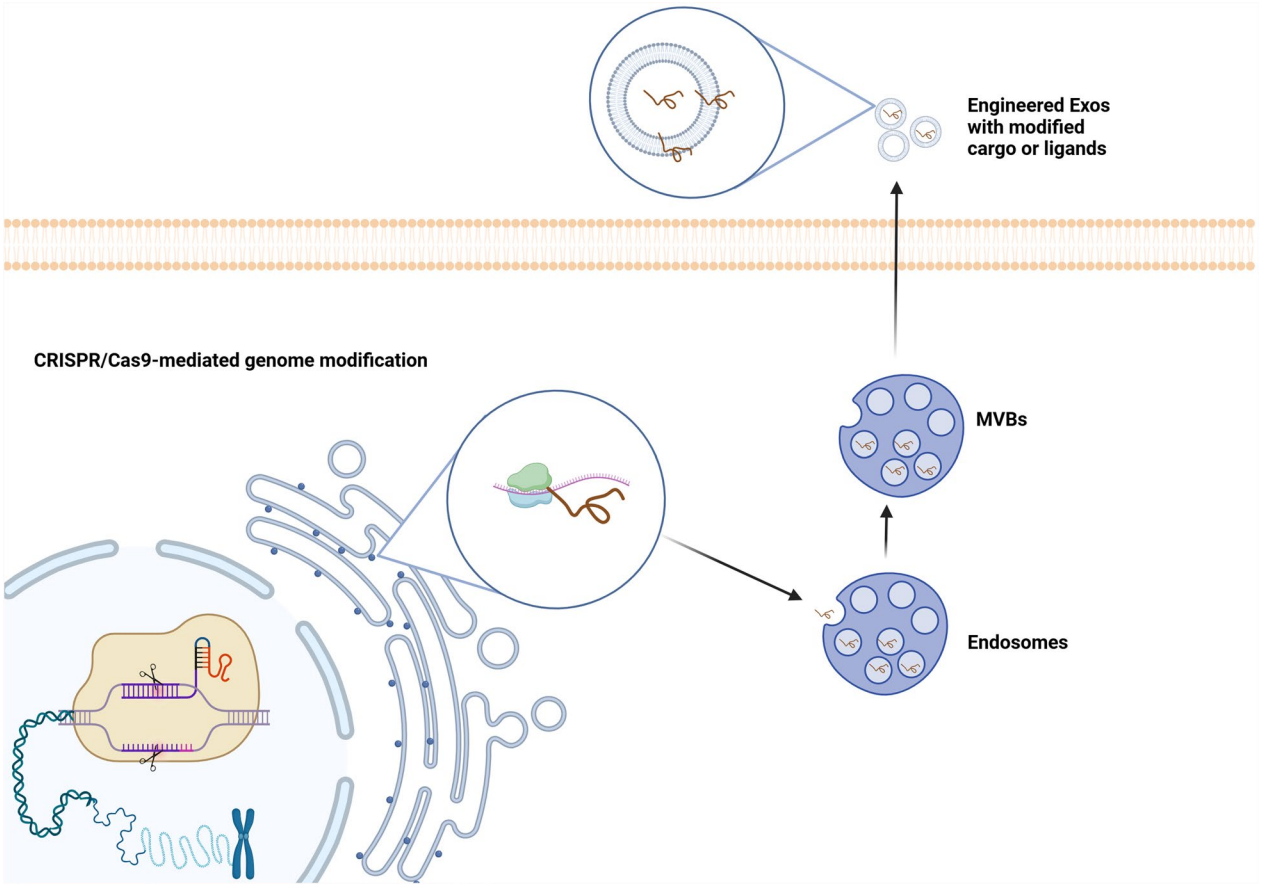

Fig. 5 CRISPR/Cas9-mediated genome modification for the increase of therapeutic efficiency of Exos. Up-regulation, down-regulation, deletion, and or addition of certain factors, or improvement of delivery ligands and delivery molecules can be done in the genetic pool using CRISPR/Cas9. Created with BioRender online software

EC-specific miRNA-126-loaded CPC EVs for the reduction of fibrosis and promotion of cardiac repair in a rat model of I/R injury [120]. Data exhibited reduced fibrotic region and cardiomyocyte diameter in the infarct zone after 28 days. Along with these changes, the number of Isolectin-B4$^+$ capillaries and α-SMA$^+$ arterioles was increased compared to the sham groups and rats that received naïve EVs (Fig. 7) [120]. Sonication or ultrasound waves can produce several microspores in the lipid membrane and help the internalization of compounds into the Exo lumen [121]. Lamichhane and co-workers loaded HER2 siRNA into EVs for targeting human breast cancer cells MCF-7 [122]. In another experiment, melatonin was loaded into adipose tissue stem cell EVs via sonication and administrated in a mouse model of MI. Data confirmed improved myocardial regeneration via angiogenesis (CD31$^+$ vessels), and reduction of oxidative stress (dihydroethidium↓) [123]. Additionally, detergents such as Triton-X100 (0.01%) were used for loading different compounds such as mycophenolic acid in immune cell Exos (i.e. macrophages) to reduce inflammatory response [124].

Besides the load of several therapeutics into the Exo lumen, modification strategies can be done on the Exo surface to increase the cardiomyocyte targeting properties using physical and chemical approaches. The chemical strategy of Exo surface includes covalent (bioconjugation, cloaking, and click chemistry) and non-covalent (ligand-receptor, electrostatic, hydrophobic, interactions, and inorganic interaction) modifications [20]. In a recent study done by Shiqi et al., they used platelet membranes to cloak the Exos and increase the internalization rate into cardiomyocytes and ECs [44]. Vandergriff and co-workers used the DOPE-NHS linker to attach cardiac homing peptide on the external surface of stem cell Exos. The application of these Exos reduces the number of apoptotic cardiomyocytes via the induction of cellular uptake. These features resulted in the reduction of fibrosis, infarct area, and vascularization [125]. In another study, NOX4 siRNA-loaded Exos were

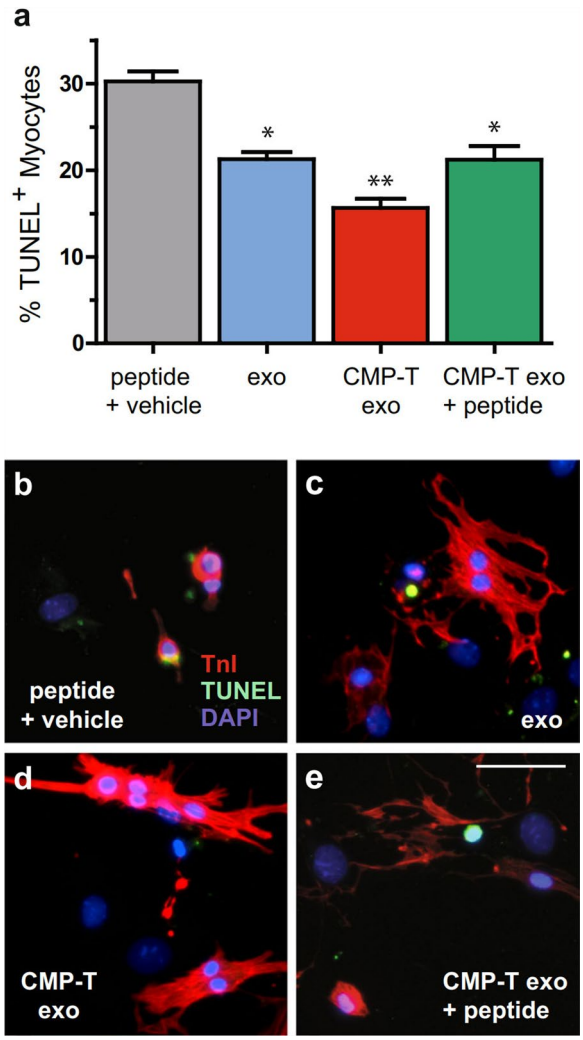

Fig. 6 Cardiomyocyte-specific binding peptide (CMP)-targeted Exos reduced mouse cardiomyocyte programmed cell death in vitro after 7 days (**a**–**e**; Scale bar: 100 µm). Cardiomyocytes were incubated with peptide (WLSEAGPVVTVRALRGTGSW) plus vehicle (10 µM DMSO) (**b**), naïve Exos (**c**), CMP-targeted Exos (**d**), or CMP-targeted Exos + synthetic peptide (**e**) for 24 h for controlling the apoptosis. Data indicated that cells co-treated with CMP-targeted Exos or CMP-targeted Exos plus synthetic peptide had fewer apoptotic changes. *$p < 0.05$ (compared with peptide plus vehicle control), and **$p < 0.05$ compared with Exos and CMP-targeted Exo + peptide. One-way ANOVA followed by Tukey's Multiple Comparison post-hoc test. [114]. Copyright 2019; Scientific Reports

chemically decorated with cardiac targeting peptide. Data confirmed the modified Exos are valid delivery platform for the regulation of cardiac tissue fibrillation via the angiotensin system [126].

Some fractions of internalized Exos are directed toward lysosomal degradation. It is thought that the application of specific modification strategies can help us to produce Exos with the potential to promote micropinocytosis, cytoplasmic release, and lysosomal degradation escape [104, 127]. Fabrication of arginine-rich peptides-decorated Exos increases the possibility of macropinocytosis [128]. For instance, decorating Exos with artificial leucine-zipper peptide (also known as K4) and E3 ubiquitin ligase induces cell uptake via micropinocytosis [128]. The application of fluorinated peptide dendrimers in the Exo structure increases the lysosomal escape and simultaneously increases the cytosolic release [129]. Besides, cationic lipids, pH-sensitive peptides can also be beneficial in Exo uptake and cytosolic release [130].

General limitation related to exo application in the clinical setting

Irrespective of the reparative properties of Exos in the alleviation of varied pathological conditions, several limitations restrict unhindered use of them in their human counterparts [131]. For instance, the development of ready-to-use and off-the-shelf Exo sources is mandatory for the application in the clinical setting [131]. Recent data have proved that storage temperature and pH changes can influence the integrity of Exos and EVs [132]. It has been shown that multiple freeze–thaw cycles can distort the physiochemical properties of Exos [132]. The metabolic status of parent (donor) cells is a crucial factor in the cargo composition of isolated Exos. Even though, stressful conditions and pro-inflammatory status change the cargo sorting and density of cytokines inside the Exos [133]. Another issue is related to the lack of standard protocols for the expansion of parent cells and large-sized isolation of Exos from biofluids with minimum damages and higher-rate purities [134]. Besides, the sterility protocols should be respected in the laboratory setting before the isolation of Exos. Due to the similarities in Exos and viral particle size, emerging data have shown that Exos shared common production pathways with viruses which per se can lead to the horizontal spreading of viral particles [25]. The possibility of unwanted side effects such as thrombosis with concomitant hemostatic perturbations remains challenging in allograft recipients [135]. The activation of allo-reactive T cell responses and rapid elimination by reticuloendothelial system cells can be disadvantages of Exo application [25]. Notably, the major hurdles that limit and slow down Exo-based therapies in human medicine are mainly associated with the non-developed GMP-grade isolation, purification, and preparation protocols for different regenerative purposes [131].

Another question is whether Exos can exhibit independent bioactivities such as growth, and/or division with the right action under specific biological conditions remained unanswered. Along with the generation of Exos via the ESCRT-related axis, the exact mechanisms that orchestrated the production of Exos via

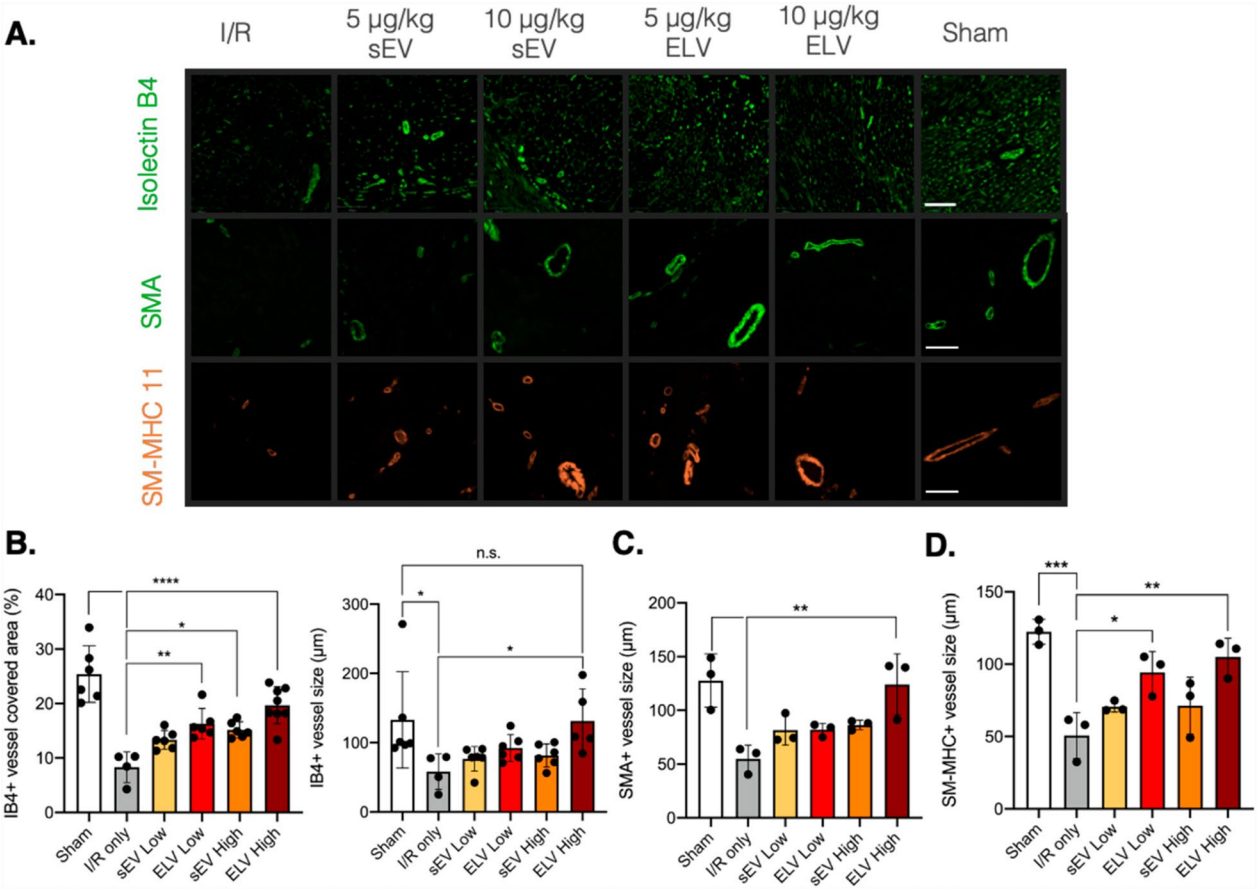

Fig. 7 Administration of miR-126-loaded CPC EVs (miR-126 + ELVs) in rats with I/R injury led to angiogenesis after 28 days (**A**–**D**). Immunofluorescence staining for detection of isolectin-B4$^+$ capillaries, SMA$^+$ arterioles, and MHC$^+$ large vessel (**A**). Measuring the local density of the isolectin-B4$^+$ area and vessel size (**B**), SMA$^+$ arterioles (**C**), and MHC$^+$ large vessel (**D**) in the myocardium. sEV-like vehicle: ELVs; SMA = smooth muscle actin; Small extracellular vesicles; sEVs; SM-MHC = smooth muscle-myosin heavy chain. Scale bar = 100 µm; One-way ANOVA with Tukey post hoc analysis. n.s. = not significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. [120]. Copyright 2023; ACS Nano

ESCRT-independent pathways, i.e. ceramide-mediated activities have not been addressed and discovered [136]. Thus, further studies should focus on the addressing of exact Exo behavior under different situations. The story might be hard to interpret when because each Exo harbors both endogenous and exogenous genetic and protein contents [137]. Whether the time and scale dependence of different exosomal cargo types differ should be also answered in this regard. In a common belief, Exos can release their contents directly into the cytosol, endoplasmic reticulum, and nucleus or back-fusion with the endosomes or other intracellular compartments in the recipient cells [138, 139]. The surface molecular signature of Exos can stimulate specific plasma membrane proteins in the recipient cells which can pre-determine the fate and activity of Exos upon internalization [140]. Due to the complexity of the involved mechanisms and lack of enough knowledge, future studies should elucidate the underlying mechanisms in the direction of exosomal cargo into different subcellular components in the targeted cells.

Conclusion

Although the number of clinical trials is low in humans it is thought that the application of Exos will be extended in the clinical setting due to several benefits compared to whole-cell-based therapies. By February 2025, the public clinical trial database https://clinicaltrials.gov presented 6 studies related to EVs in humans (Table 4). Based on several pre-clinical studies, the administration of naïve Exos can lead to the alleviation of pathological conditions after ischemic changes. The promotion of angiogenesis, cardiomyocyte proliferation, and the reduction of apoptotic changes can accelerate the healing of injured myocardium. Despite these advantages, off-target delivery is limited to the bulk application of Exos in MI patients especially via systemic routes. Thus, the naïve Exos can be engineered to increase the on-target delivery rate into

Table 4 List of EV studies in humans related to the cardiovascular system adapted from https://clinicaltrials.gov up to February 2025

Study title	Study Status	Study Results	Conditions	Interventions	Phases	Study type
Treatment of non-ischemic cardiomyopathies by intravenous EVs of cardiovascular progenitor cells	Recruiting	NO	Heart failure with reduced ejection fraction	Biological: EV-enriched secretome of cardiovascular progenitor cells differentiated from induced pluripotent stem cells	Phase1	Interventional
Salivary EV-associated lncRNAs in heart failure (seal-hf)	Recruiting	NO	CHF/ADHF/Control		–	Observational
EV microRNA profiling in congenital heart disease: fetal-maternal regulation in neonatal thrombosis	Recruiting	NO	Congenital Heart Disease/Single-ventricle/Thrombosis	OTHER: Collecting discarded blood samples	–	Observational
Safety evaluation of intracoronary infusion of EV in patients following coronary stent implantation	Enrolling by invitation	NO	Percutaneous Coronary Intervention	DRUG: PEP	Phase1	Interventional
Antiplatelet therapy effect on EVs in acute myocardial infarction	Completed	YES	Myocardial Infarction	DRUG: Ticagrelor/DRUG: Clopidogrel	Phase4	Interventional
Silent myocardial ischemia in patients undergoing non-oncological abdominal surgeries	Recruiting	NO	Silent Myocardial Ischemia/Acute Myocardial Infarction	GENETIC: Silent myocardial ischemia, STEMI		Observational

the injured myocardium. It seems that with the progress in engineering methods, new engineered Exo products can be introduced for patients with ischemic changes and MI. Due to the lack of enough data related to the application of naïve and engineered Exos in clinical settings, any interpretation should be done with caution. Further clinical trials associated with different parent cells, purification methods, doses, preparation steps, loading techniques, etc. are mandatory to be applied to the MI patients according to the standard guidelines.

Abbreviations

cTnT	Cardiac troponin T
circRNAs	Circular RNAs
CK-MB	Creatine kinase-MB
ESCRT	Endosomal sorting complex required for transport
ECs	Endothelial cells
ESCRT	Endosomal sorting complex required for transport
Exos	Exosomes
ECM	Extracellular matrix
EVs	Extracellular vesicles
HSP60	Heat shock protein 60
HIF-1α	Hypoxia-inducible factor 1 alpha
ILVs	Intraluminal vesicles
I/R	Ischemia/reperfusion
IHD	Ischemic heart disease
I/R	Ischemic/reperfusion
lncRNAs	Long non-coding RNAs
MMPs	Matrix metalloproteinases
MSCs	Mesenchymal stem cells
mRNAs	Messenger RNAs
miRNAs	MicroRNAs
MVs	Microvesicles
MVBs	Multivesicular bodies
MI	Myocardial infarction
NPs	Nanoparticles
RAB	RAS-related protein
sncRNAs	Small ncRNAs
TLR	Toll-like receptors
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor

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Author contributions
N. M., P. K., Z. A-M., S. S., N. D. K., S. A. C., F. J., M. A., L.S., and A. R. collected data and wrote the draft. S.Z.M. drew the illustrations. R.R. supervised the study, edited the final draft, and acquired funding.

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Data availability
No new data were generated or analyzed in the current article.

Declarations

Competing interests
No potential conflict of interest was reported by the authors.

Ethics approval and consent to participate
The study was registered as titled "Title of proposal: Roll of Toll-like receptors in differentiation and maturation of murine cardiac stem cells into cardiomyocyte-like cells." under an approval code of IR.TBZMED.VCR.REC.1398.035 from Research Ethics Committees of Vice-Chancellor in Research Affairs—Tabriz University of Medical Sciences on 2019-04-15.

Consent for publication
Not applicable.

Human and animal participate
No human and/or animal samples were used in this study.

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