Review Article

Cell-Free Treatments: A New Generation of Targeted Therapies for Treatment of Ischemic Heart Diseases

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Abstract

Although recent progress in medicine has substantially reduced cardiovascular diseases (CVDs)-related mortalities, current therapeutics have failed miserably to be beneficial for all patients with CVDs. A wide array of evidence suggests that newly-introduced cell-free treatments (CFTs) have more reliable results in the improvement of cardiac function. The main regeneration activity of CFTs protocols is based on bypassing cells and using paracrine factors. In this article, we aim to compare various stem cell secretomes, a part of a CFTs strategy, to generalize their effective clinical outcomes for patients with CVDs. Data for this review article were collected from 70 published articles (original, review, randomized clinical trials (RCTs), and case reports/series studies done on human and animals) obtained from Cochrane, Science Direct, PubMed, Scopus, Elsevier, and Google Scholar) from 2015 to April 2020 using six keywords. Full-text/full-length articles, abstract, section of book, chapter, and conference papers in English language were included. Studies with irrelevant/insufficient/data, or undefined practical methods were excluded. CFTs approaches involved in growth factors (GFs); gene-based therapies; microRNAs (miRNAs); extracellular vesicles (EVs) [exosomes (EXs) and microvesicles (MVs)]; and conditioned media (CM). EXs and CM have shown more remarkable results than stem cell therapy (SCT). GF-based therapies have useful results as well as side effects like pathologic angiogenesis. Cell source, cell's aging and CM affect secretomes. Genetic manipulation of stem cells can change the secretome's components. Growing progression to end stage heart failure (HF), propounds CFTs as an advantageous method with practical and clinical values for replacement of injured myocardium, and induction of neovascularization. To elucidate the secrets behind amplifying the expansion rate of cells, increasing life-expectancy, and improving quality of life (QOL) for patients with ischemic heart diseases (IHDs), collaboration among cell biologist, basic medical scientists, and cardiologists is highly recommended.

Keywords: Cardiovascular Diseases, Exosomes, Extracellular Vesicles, Gene Therapy, microRNAs

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Introduction

According to the reports of World Health Organization (WHO), cardiovascular diseases (CVDs) have been among the leading causes of death worldwide for the last 15 years. In 2017, they accounted for a combined total of 17.8 million recorded deaths; being expected to exceed 22.2 million deaths by 2030 (1). Current approaches for treatment of ischemic heart diseases (IHDs) include procedures restoring blood flow, pharmacological treatments lessening cardiac remodeling, cardiovascular surgical interventions, and various immunopathophysiological strategies inducing revascularization (2). Unfortunately, current therapeutic strategies only postpone the progression from IHDs to heart failure (HF). In other words, those aforementioned therapeutic approaches lack the ability to completely reverse clinical manifestations in patients with CVDs. Therefore, loss of myocardial tissues with progression to HF remains as a problematic challenge in IHDs treatment (3). Those challenges have encouraged basic medical scientists to make a borderless and integrative collaboration by the usage of interdisciplinary frameworks aimed at seeking for more efficient approaches that are based on cellular and molecular immunopathophysiology which play an indispensable role in the microenvironment of CVDs. Accordingly, stem cell therapy (SCT) is considered as a recent, and promising strategy in myocyte replacement after a myocardial infarction (MI) (4).

In heart tissue regeneration through SCT, wide array of stem cells are used aimed at improving clinical outcomes to increase angiogenesis, decrease pathological cardiac remodeling, increase left ventricular ejection fraction (LVEF), amplify regional contractility, decrease scar tissue and infarct size, improve New York Heart Association (NYHA) classification, and improve quality of life (QOL) for patients with CVDs. However, for improving regenerative functions of an infarcted heart through SCT, there are many unresolved challenges like: selection of an optimal cell

type; possible ambiguities with specification of the cell dose, route and frequency of administration (5, 6); possibility of arrythmogenicity, complex cellular mechanisms of action, and low number of survived stem cells after transplantation in the infarct zone (7). Thus, SCT remains to be determined as the only responsible source for improvement of cardiac function (8). Various cellular and molecular mechanisms have been proposed to justify the immunobiological properties-derived clinical applications of SCT, including: i. differentiation of stem cells into heart muscle cells, ii. differentiation of stem cells into blood cells, iii. paracrine effects; and, iv. cell fusion (7). It has been demonstrated that paracrine mechanisms play indisputable roles for exacerbating the function of stem cells in regenerative medicine for treatment of MI. Paracrine effects, as cell-free treatments (CFTs), have attracted the attention of researchers because of their clinical potentials in heart tissue regeneration (9). In other words, CFTs have shown advantages through paracrine effects in heart regenerative medicine than SCT (10). Not only CFTs-based secretome and paracrine mechanisms can mimic the beneficial effects of SCT, but they also can reduce some of limitations and drawbacks regarding the clinical usages of SCT (8, 11).

In order to design paracrine mechanisms-based therapeutic approaches, properties of different stem cell-derived secretomes and agents influencing components of the secretome are of utmost importance for cell biologists. Optimization of cells obtained from cell culture medium and preparation of clinically-effective cells, are prime examples that should be specified for designation of the most therapeutic strategies in heart regeneration. So, comprehensive knowledge and outstanding understanding on molecular and immunobiological mechanisms of action related to CFTs-based regenerative activities [particularly genetically-remodeling exosomes (EXs)], as well as physiological cardiac remodeling can definitely open promising windows into recovery of the infarcted area, improvement of QOL, and increasing life-expectancy for patients with IHDs. Hence, through more collaborations between physicians, cellular cellular/molecular biologists, and laboratory scientists, preclinical and basic medical studies can simplify translation of laboratory-based data into the clinical settings, decipher secrets of more accurate medical decisions, make optimal clinical outcomes, and increase life-expectancy and QOL for patients with CVDs.

Here, we aim to summarize different mechanisms of action and functional roles of CFTs in research laboratories, highlight the role of data acquired from basic medical sciences, focus on their therapeutic applications for treatment of CVDs, and investigate the proficiency and clinical efficiency of these strategies to improve clinical outcomes for patients with CVDs.

Types of cell-free treatment

The hypothesis, "main regeneration activities of stem cells are done indirectly through a paracrine manner" is traced back to the proposal for bypassing cells and simultaneous usage of supposedly-paracrine factors (12). Stem cell secretomes are considered as off-the-shelf therapeutic approaches that mitigate safety risks, overcome the risks of occlusion in microvasculature, and refrain from unregulated growth (particularly for administration of large amounts of viable cells). In clinical settings, the promising results acquired from mesenchymal stem cells (MSCs)-derived secretomes are similar to the results from transplantation of MSCs (13, 14).

In the CFTs method, heart tissues are regenerated by collaborative functions of growth factors (GFs), cytokines, microRNAs (miRNAs), involved genes, EXs, microvesicles (MVs), and conditioned media (CM)-derived from stem cells. Their presence in the surrounding tissues leads to the activation of intrinsic repairing mechanisms (15). This secretion regulates several procedures like: myocardial protection, neovascularization, cardiac remodeling, and induction of endogenic cardiac stem cells (CSCs) differentiation (16). Figure 1 lists the regulatory effects of MSCs secretomes in heart regeneration (17, 18).

Conclusively, since heart is not a post-mitotic organ, it seems that there is an imperative need to a comprehensive perception from secretomes and paracrine mechanisms to design new and practical therapeutic approaches aimed at heart tissue regeneration.

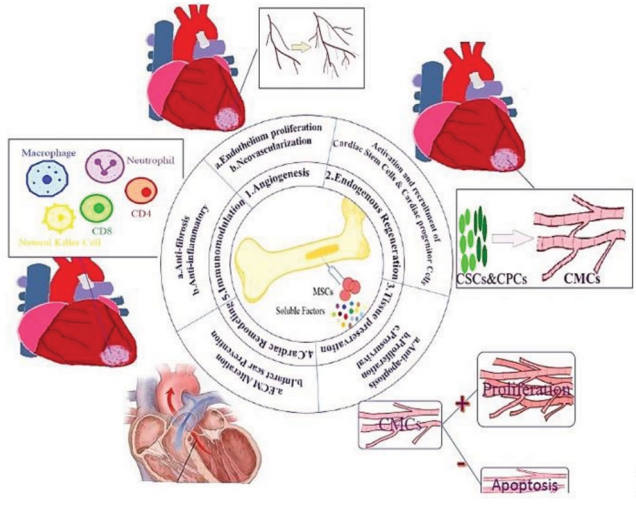

Fig.1: The effect of MSCs secretomes in heart tissue regeneration. MSCs secretomes show their regulatory properties through various paracrine processes. CMCs; Cardiomyocytes, CPCs; Cardiac progenitor cells, CSCs; Cardiac stem cells, and MSCs; Mesenchymal stem cells.

Extracellular vesicles in ischemic heart diseases

Extracellular vesicles (EVs) include EXs and MVs. The components of MVs are 0.1-1 μm in size and they are shed from the cell surface of normal or damaged cells during membrane blebbing. EVs can 'hijack' both membrane components and engulfed cytoplasmic contents. They contain several structural proteins and lipids that are

similar to those in the membranes of the cells from which they are originated. Those EVs also contain intracellular proteins, messenger RNAs (mRNAs), regulatory miRNAs, and intact organelles such as mitochondria (10).

EXs have a diameter of 30-100 nm which are secreted by different cells through the assimilation of multi-vesicular bodies within the plasma membrane. They carry complex of proteins, RNAs and miRNAs, mediating cell signals such as immune responses, cell survival mechanisms, and intercellular communications (19). EXs can be obtained from three groups of cells, including stem cells [MSCs, CSCs, and embryonic stem cells (ESCs)]; mature cells present in heart tissues [cardiomyocytes (CMCs) and fibroblasts]; and from cells exposed to pathological conditions or genetically-modified cells (8). EXs are considered as acceptable therapeutic tools for heart regenerative medicine which are categorized into more functional members compared to MVs and apoptotic bodies. Structurally, the myocardial origin of CMCs-derived EXs is characterized by their specific protein cargo patterns which are affected by the cellular microenvironment. EXs released from cardiosphere derived cells (CDCs) show anti-apoptotic and proliferative properties on CMCs (20). The therapeutic potency of EXs is usually functionalized in the presence of a biologically-relevant protein or RNA in the EXs (21). In a study on a mouse model of ischemia/reperfusion (I/R), intravenous injection of MVs on ischemia had led to reduced myocardial injury, diminished infarct size, and lessened number of apoptotic CMCs which totally improved cardiac function (22).

The origin of EXs affects their biological behaviours in the microenvironment. CDCs-derived EXs with miR-146a in a MI model could improve cardiac function and decrease scar mass (11). Administration of MSCs-derived EXs increased the amount of miR-19a with anti-apoptotic properties in a rat MI model, which played indisputable roles in activation and differentiation of endogenous CSCs in the infarcted heart (8). Table 1 lists the functions of different particles in stem cell-derived Exs and their role in the treatment of IHDs. Table 2 lists a number of EXs that have been recently used in clinical studies on MI models. In addition, the age of the donor cells appears to be effective on the composition of secretomes. Sharma et al. (23) have reported that neonatal cardiac progenitor cells-derived conditioned medium (nCPCs-CM) were more effective than adult CPC-CM (aCPCs-CM). Surprisingly, nCPCs-CM were more effective than neonatal CPCs (nCPCs) and nCPC-EX.

Compared to other cell-based therapies used for heart regeneration, EXs are very stable and preservable. There is no aneuploidy risk, and also the possibility of immune rejections after *in vivo* allogeneic administrations is low (24). Adamiak et al. (25) compared the cardiac reparative capabilities of induced pluripotent stem cells (iPSCs)-derived EXs with iPSCs in a mouse model of reperfused MI. Their results showed that iPSCs-derived EVs induced superior cardiac repair in vivo compared to iPSCs. In their study, injections of iPSCs resulted in teratoma formation, whereas injections of iPSCs-derived EVs were safe. They concluded that iPSCs-EVs represented a feasible, and safer alternative for potential therapeutic applications of EVs in patients with ischemic myocardial damages.

Table 1: The function of different EXs and their effective particles in IHDs

EX-derived cells	EX enriched particles	Function	References
Cardiac endothelial cells	miR-126 and miR-210	Proangiogenic	(26)
CPCs	miR-144	Cardioprotective	(26)
	miR-132, miR-146a,	Proangiogenic	
	miR-210		
	miR-29a	Anti-fibrotic	
	miR-132, miR-146a,miR-451, miR-210	Cardioprotective, post-MI neovascularization, and healing of damaged heart tissues	
CDCs	miR-146a	Survival, angiogenesis, and CMCs proliferation	(15)
	miR-210, miR-132, miR-146a-3p	Survival	
		Angiogenesis	
Sca-1^{+}, mouse	miR-451	Survival	(15)
Cardiac myocytes	Hsp60	Induction of CMCs apoptosis	(27)
Hypoxic CMCs	TNF-α	Triggers cell death in other CMCs	(27)
iPSCs	miR-21, miR-210	Cardioprotective	(27)
MSCs	miR22	Anti-apoptotic	(17)
		Improvement of ischemic CMCs injuries	
	20S proteasome subunits (PMSA 1-7)	Cardioprotective	
	miR-21	Anti-apoptotic	(27)

EX; Exosomes, IHD; Ischemic heart disease, CPCs; Cardiac progenitor cells, CMCs; Cardiomyocytes, MSCs; Mesenchymal stem cells, iPSCs; Induced pluripotent stem cells, and MI; Myocardial infarction.

Table 2: Comparison of different EXs used as therapeutic tools for different models of IHDs

Type of EX	MI model	Outcome	Reference
CPCs-derived EX	Rat	Improved cardiac function, Less profound cardiac apoptosis, ↑ Intracardiac angiogenesis	(26)
SHH-containing EVs	Murine	Proangiogenic, anti-apoptotic, and vasculoprotective effects ↓ Infarct size	(26)
MSCs-derived EX	MIRI mouse model	Improved CMCs survival ↓ Cardiac fibrosis and apoptosis compared to hearts treated with control EX	(27)
MSCs-IPC EX	Mouse		(27)
CDCs EX	Porcine AMI and CMI	↓Scarring, halted adverse remodeling, improved LVEF	(28)
Akt-hucMSC derived EX	Acute MI rat model	Improved cardiac function, Promoted angiogenesis	(29)
ESCs derived EX	Acute MI mouse model	Enhanced neovascularization, augmented cardiac function after MI, reduced fibrosis, promoted CPC and myocyte survival and proliferation	(30)
iPSCs-derived EX	Reperfused MI in mice	Improved LV function, reduced apoptosis, promoted angiogenesis, attenuated LV hypertrophy, and iPSCs-EX injection was safe.	(25)

EX; Exosomes, IHD; Ischemic heart disease, CPCs; Cardiac progenitor cells, SHH; Sonic hedgehog, EV; Extracellular vesicles, MSCs; Mesenchymal stem cells, IPC; Ischemic preconditioning, CDC; Cardiosphere derived cells, hucMSCs; Umbilical cord mesenchymal stem cells, iPSC; Induced pluripotent stem cells, ESC; Embryonic stem cells, iPSCs; Induced pluripotent stem cells, MIRI; Myocardial ischemia-reperfusion injury, AMI; Acute myocardial infarction, CMI; Chronic myocardial infarction, MI; Myocardial infarction, CMCs; Cardiomyocytes, and LVEF; Left ventricular ejection fraction.

From cellular aspects, it has been demonstrated that several cellular procedures indicate potential properties in EVs-based therapies aimed at regeneration of an infarcted heart. These aforesaid cellular processes mainly prevent from apoptosis by inducing autophagy via modulation of AMPK/mTOR, Akt/mTOR, and Wnt/β-catenin pathways. Hence, clinical usages of EVs lead to a wide range of improved clinical outcomes like increased survival rate of CMCs in ischemic lesions, neovascularization in the peri-infarcted myocardial zone, restrained pathological remodeling, and regulation of CMCs function (14). All in all, it sounds that cells used in SCT-based approaches now can be genetically altered by GFs, cytokines, and hypoxia-exposed stem cells under pathologic conditions. Eventually, secretomes derived from these genetically-altered stem cells can be used according to the pathophysiological stage of the patients with CVDs and optimized to treat them (10).

Heart tissues-target therapy with exosomes

Most systemically-injected EXs are accumulated in organs such as the liver, kidneys, lungs, and bone marrow (BM). Despite the high efficacy observed in concentrated and pure doses of infused EXs, the therapeutic potentials of EXs mostly depend on their bio-distribution. Mostly-secreted EXs tend to be less specific to a particular cell type in the cellular microenvironment. EXs are mostly cleared by macrophages within the reticulonodular system, limiting their therapeutic applications in clinical trials. Therefore, the cardioprotective effects and specific/efficient delivery of administered EXs are not well-established (31, 32). To reduce systemic clearance of EXs and increase their numbers, as well as increase tropism of EXs in target tissues, altering the surface of these particles by creating ligands is highly recommended. Those ligands bind EXs to specific molecules or antibodies in target tissues. In an *in vivo* MI study done by Mentkowski and Lang (31), engineered CDCs expressing LAMP2-cardyomyocytes targeting peptide (LAMP2-CMP) on the membranes of secreted Exs, could increase the CMCs endocytosis potential of CDCs (which are originated from EXs). LAMP2, a membrane protein on the EXs surface, was fused to CMP (a CMC specific peptide). Intramyocardial injection of these biologically-engineered EXs showed that they were more likely to be absorbed by CMCs than the non-engineered types. In comparison with the non-engineered types, these engineered CDCs could significantly reduce apoptosis, increase the survival rate of CMCs and induce more cardiac retention.

Tian et al. (33) used cyclo (Arg-Gly-Asp-D-Tyr-Lys) peptide [c(RGDyK)] conjugated EXs, which were called EXs-c(RGDyK) to treat a mouse model of cerebral ischemia. Biologically, c(RGDyK) is a peptide that has a high affinity for binding to integrin $\alpha_v\beta_3$ of cerebrovascular endothelial cells after ischemia. The results of their study showed that intravenous infusion of this engineered EXs strongly suppressed the inflammatory responses, and lowered cell apoptosis in the lesion area of the cerebral ischemia.

As a result, engineered EXs provide a convenient and effective way to transfer therapeutic materials into different tissues. These engineered EXs could be enriched with angiogenic or protective substances to be delivered to specific target organs and tissues such as the heart in order to produce effective angiogenesis and increase CMCs survival for patients with IHDs.

microRNAs in ischemic heart diseases

As non-coding RNAs, miRNAs are approximately 22 nucleotides in length, mediating regulatory effects on post-transcriptional gene expression (34). They were initially discovered in *Caenorhabditis elegans*

(*C. elegans*) two decades ago (35). miRNAs regulate various biological and physiologic pathways such as differentiation, proliferation, growth, and apoptosis, as well as pathological processes such as cancer, Alzheimer's and CVDs. miRNAs are found in tissues and body fluids such as the blood, urine, saliva, plasma and serum (36). Recently, circulating miRNAs can be used for diagnosis, prognosis, and therapeutic applications for a wide range of disorders. In case of MI, it has been speculated that heart tissue undergoes various pathological and physiological processes such as apoptosis, angiogenesis, tissue perfusion, and fibrosis. Generally, inhibition or activation of several families of miRNAs, including miRNAs-15, miRNAs-21, miRNAs-24, miRNAs-29, miRNAs-34, miRNAs-92a, miRNAs-101, miRNAs-133a, and miRNAs-320 leads to cardiac tissue repair and improved heart function (37, 38).

In terms of CVDs, Wang et al. (39) showed that knockdown of miR-16-5p increased cell viability and angiogenesis in human microvascular endothelial cells (HMVEC), and inhibited cell apoptosis by increasing insulin receptor substrate 1 (IRS1). Zhao et al. (40) tried to clarify the regulatory mechanisms of miR-132 in MI-induced myocardial remodeling. They reported that up-regulation of miR-132 increased LVEF and LV fractional shortening, and inhibited CMCs apoptosis to ameliorate myocardial remodeling through down-regulation of IL-1β.

Growth factor therapy in ischemic heart diseases

Several GFs and cytokines have been used to treat CVDs in clinical and preclinical studies because of their direct and distinct effects on several cellular functions such as adhesion, proliferation, and migration. GFs can induce regenerative mechanisms that include anti-apoptotic pathways, angiogenic properties, positive remodeling of the ECM, CMCs proliferation, and CSC recruitment. The results of most studies indicate that daily subcutaneous injections of GF might have cardioprotective effects through up-regulation of the Akt pathway, and CPC differentiation (41, 42).

Induction of angiogenesis by GFs during coronary artery occlusion is an important mechanism that protects heart tissues against hypoxemic conditions. The process of angiogenesis is regulated by a various GFs, such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), and platelet-derived growth factor (PDGF). VEGF is a major initiator of angiogenesis and recruitment factor for endothelial cells; FGF-2 is a mitogen of various cell types, recruitment factor for pericytes, and a producer of the ECM. PDGF is a recruitment factor for smooth muscle cells and a maturation factor for new vessels, playing an important role in angiogenic processes. As a result, these processes lead the microenvironment toward increased tissue perfusion, reduced inflammation and fibrosis, and ultimate improvement in heart muscle performance (41, 42).

Several GFs have been used to treat CVDs. Thavapalachandran et al. (43) in their experimental study evaluated the therapeutic effects of recombinant human platelet-derived growth factor-AB (rhPDGF-AB) protein in a clinically-relevant porcine model of myocardial ischemia-reperfusion injury (MIRI). They demonstrated that infusion of rhPDGF-AB promoted post-MI cardiac repair by altering the mechanics of the infarcted scar, being resulted in improved LVEF, myocardial contractility, and increased survival rate, as well as decreased ventricular arrhythmias. Furthermore, injection of stromal-derived factor-1 (SDF-1/CXCR4) in the myocardia of patients with IHDs was reported to be a safe and experimentally-feasible approach. Patients who received the highest doses (15 mg and 30 mg) of SDF-1 had shown improvement in QOL, the six-minute walking distance test, and NYHA class (44).

According to the reported side effects due to administration of high or inappropriate dosages of certain cytokine/GFs (like organization of aberrant and perforated vessels, hypotension, and tumour angiogenesis), there is an imperative need for further investigations on the proper clinical usage of GFs and cytokines for treatment of patients with CVDs (18).

Conditioned media in ischemic heart diseases

As described earlier, secretomes consist of proteins, miRNAs, GFs, anti-oxidants, proteasomes, and EXs secreted by stem cells. Literally, those culture media which are conditioned by secretomes are called CM. A variety of paracrine factors, including VEGF, hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), IGF-2 and SDF-1, are secreted into the cell culture medium during the cell culture processes. These factors are associated with physiological and pathophysiological processes like cell proliferation, apoptosis, inflammation, neovascularization, MI, angiogenesis, and fibrosis. It is presumed that addition of secretomes to the cell culture media of the damaged organs increases metabolic activities, oxygen supply, and remodeling the ECM in order to ultimately prevent from increased organ damage (45, 46).

In case of CVDs, CM can be used to induce clinically-advantageous effects on target tissues. For example, either CDC-derived CM or MSC-derived CM have the capability to increase the survival potential of CMCs against hypoxia (16, 19). Secretomes are the most functional unit of stem cells and EXs play an indispensable role in improving their therapeutic manifestation; however, Sharma et al. (23) reported that sole administration of EXs does not suffice and CM is needed for maximum clinical benefit. In addition, they reported that division of CM into EXs and EX-free fractions diminishes its capacity to recover myocardial function. MVs and CM derived from stem cells have the same pro-regenerative potentials as infusion of intact cells (10) which can replace the cells in SCT. Fortunately, the allogeneic CM method lacks ethical controversies and immune rejection. Then, it appears to be a perfect, and promising solution for immediate clinical applications with the ability to minimize the amount of surgeries invasiveness (47). Table 3 shows the effects of CMs that are originated from different stem cells in animal models of IHDs.

Table 3: Comparison of different CMs for treatment of IHD models

Source of CM	Animal model	Outcome	Reference
hADSCs	SCID and C57BL/6 mice model of MI	Improved cardiac function ↓ Infarct size ↑ Reparative angiogenesis ↓ CMCs apoptosis (The observed effects of ADSCs application on the first three mentioned outcomes were superior to those reported from ADSC-CM.)	(48)
Human STRO-3-mesenchymal precursor cells	Athymic nude rat model of MI	↑ Ventricular function ↓ Ventricular dilatation and infarct size ↑ Neovascularization	(48)
Human embryonic stem cell-derived MSCs	Porcine model of MI	↑ Capillary density ↓ Infarct size ↑ Myocardial performance	(48)
Porcine peripheral blood endothelial progenitor cells	Porcine model of MI	↑ Angiogenesis ↑ CMCs remodeling and contractility	(48)
Human MSCs	Porcine model of MI	↑ Myocardial capillary density ↓ MI size, and preserved systolic and diastolic performance	(45)
SHED-CM	Mouse model of I/R	↓ MI size ↓ Apoptosis ↓Inflammatory cytokine levels of TNF-α, IL-6, and IL-β ↑ Cardiac function ↑Survival of cardiac myocytes in response to hypoxia	(47)
nCPCs	Rodent model of MI	↑ Recovering cardiac function ↑ Stimulation of neovascularization ↑ Myocardial remodeling	(23)
Hypoxic-ADMSCs	Rat model of MI	↓ Infarct size, apoptosis index, and apoptosis related proteins	(49)

HD; Ischemic heart disease, CM; Conditioned media, hADSCs; Human adipose tissue-derived stem cells, MSCs; Mesenchymal stem cells, ADMSCs: Adipose tissue-derived mesenchymal stem cells; SHED; Stem cells from human exfoliated deciduous teeth, CPCs; Cardiac progenitor cells, MI; Myocardial infarction, CMCs; Cardiomyocytes, I/R; Ischemia/reperfusion, ESCs; Embryonic stem cells, and nCPCs; Neonatal CPCs.

Gene therapy in ischemic heart diseases

Although several GFs have been used to stimulate new vessel formation after MI, none of them could achieve significant results in phase I/II clinical trials of heart regeneration. A clinically-acceptable solution to overcome this problematic challenge is gene therapy that shows desired clinical outcomes. To date, plasmids with adenovirus vectors delivering VEGF, FGF and HGF for treatment of patients with severe coronary artery diseases (CADs) and MI have been the focus of investigation in several randomized controlled trials (RCTs). Researchers proposed that the combination

of gene-based therapies with SCT could significantly improve clinical outcomes in heart regeneration medicine (50).

Gene-based therapies are techniques that use biological carriers to simplify the insertion and expression of a therapeutic gene in target cells (51), in order to induce or inhibit synthesis of specific proteins and alter the structure and function of the cells in target tissues (52).

Gene-based therapies mainly refer to the utilization of genetically-altered cells as carriers for the genes and plasmids aimed at transferring these target genes to damaged heart tissues for treatment of patients with IHDs. These genetic changes in cells go back to the fact that they are measured out to increase the expressions of specific genes, GFs, and cytokines, affecting immunobiological processes such as angiogenesis, proliferation and differentiation of CSCs, apoptosis, remodeling, and ventricular function (51, 52).

Various carriers used to transfer the desired genes to the target cells include: i. Plasmid carriers for transportation of naked DNA, ii. Viral vectors such as Adenoviruses, Adeno-associated viruses, Retroviruses, and Lentiviruses (51); and iii. Gene-modified cells (52). Gene-modified cells with the ability to overexpress the transgenes act as transgene carriers, leading to reinforced/desired levels of therapeutic proteins in target tissues (52).

Despite substantial progress in novel therapeutics, there are still several types of disorders especially those which have not an efficient and successful treatment, leading basic medical scientists and clinical specialists toward novel therapeutic strategies like gene-based therapies. For instance, modified BM-MSCs that overexpress *IL-35* gene in an Imiquimod-induced psoriasis-like mouse model will most likely become an effective therapeutic approach for the worldwide problem of psoriasis (53). Lin et al. (54) investigated whether overexpression of the *IGF-1* gene could enhance BM-derived stem cell (BMSC) viability, migration, anti-apoptotic, and protective effects of CMCs in an acute MI (AMI) rat model. They reported that BMSCs that overexpressed *IGF-1* gene called BMSCs-IGF-1 could significantly rescue cardiomyoblasts from hypoxia-induced apoptosis, preserve cell viability under hypoxic conditions and reduce the infarct volume. Su et al. (55) in their study on an AMI rat model, loaded Adenoviruses that carried the *SDF-1α* gene onto microbubble carriers by ultrasound targeted destruction of these microbubbles. The results of their study suggested that ultrasound-mediated transduction of the exogenous *SDF-1α* genes into the AMI rats could effectively promote the homing of endogenous BMSCs into the infarcted heart.

Figure 2 shows a schematic presentation of novel strategies recruited by CFTs aimed at treatment of patients with CVDs.

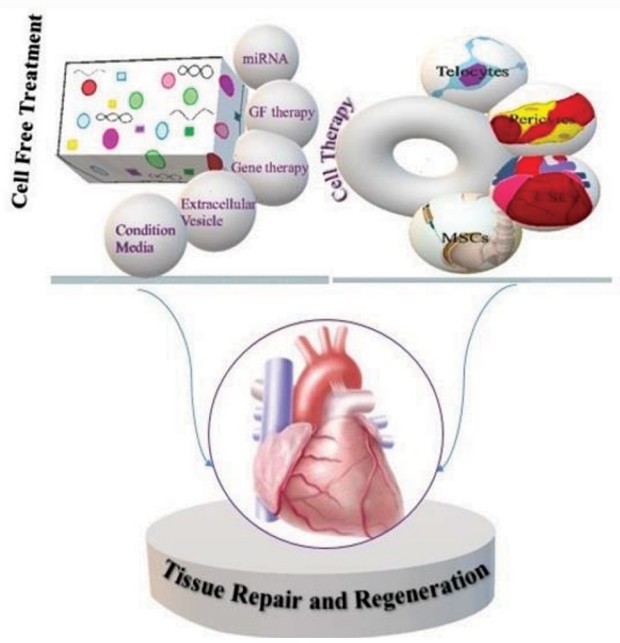

Fig.2: Schematic presentation of novel therapeutic strategies for CVDs. CFTs includes GFs, gene-based therapies, miRNAs, EVs, and CM. EX and CM usually have more remarkable results than stem cell therapy. CVDs; Cardiovascular diseases, CFTs; Cell-free treatments, CSCs; Cardiac stem cells, GF; Growth factor, miRNAs; microRNAs, EVs; Extracellular vesicles, EX; Exosomes, CM; Conditioned media, and MSCs; Mesenchymal stem cells.

Route, time, and dosage of cell-free treatment administration

CFTs-based therapy for heart regeneration is administered by intravenous, intra-arterial, intramuscular, or intramyocardial injections of several recombinant angio-peptides such as VEGF, FGF, and HGF (56). To be more precise, for having an ideal route of administration of CFTs (GFs)-based therapies, time- and dose-dependent function/efficacy of recruited genes can be considered as a major hindrance for heart regeneration. Bauzá et al. (57) investigated whether optimized doses of high mobility group box 1 (HMGB1) would promote angiogenesis signaling, and expression of specific regenerative genes in sheep with acute MI. They reported that intramyocardial injection of high-dose (250 μg) HMGB1 had induced angio/arteriogenesis, reduced the infarct size, and improved LV function two months post-treatment.

Although delivery of angiogenic factors has the potential abilities to stimulate formation of new blood vessels both *in vivo* and *in vitro*, there are some limitations that should not be underestimated. Those limitations include the explosive or uncontrolled release of GFs that leads to high toxicity; decreased half-life of GFs; and elevated cost for purified GFs. One strategy for overcoming these challenges is delivery of the gene of the same factor in the cells for producing the required angiogenic GFs (58). Gene delivery strategies will be discussed in the following paragraphs.

Direct surgical injection, catheter-based intracardiac or intracoronary injections, pericardial delivery, the V-focus system, and surgical delivery strategies are used for

gene transferring and gene delivery to the myocardium. Although there is no unique delivery system or a virus serotype specifically-optimized for cardiac orientation that lacks simultaneous expression in other tissues. The molecular cardiac surgery with recirculating delivery (MCARD) vector-mediated gene is undoubtedly considered as one of the most promising gene transfer systems, providing very high expression levels with low morbidity, diminished immune responses, and minimal simultaneous expression (59).

Selection of the most proper transgenes, the most efficient routes of gene delivery, the type and quality of these recruited vectors for gene-based therapies, and dosage are all determining factors for optimal therapeutic outcomes in gene-based therapies of CVDs (60).

A meta-analysis conducted by Yang et al. (61) for a systematic review of the efficacy of EVs on MI in both small and large animals did not show any significant associations between efficacy and type of stem cells, ligation-to-injection interval, route of delivery, dosage, and follow-up period. However, Liu et al. (62) performed a meta-analysis to systematically review the efficacy of EVs in preclinical acute kidney injury (AKI) rodent models. They reported that the route of delivery, dosage, and cell origin of EVs were independent factors that influenced clinical effects of the EVs. For achieving maximum efficacy without adverse effects in EVs-based therapies, determining the optimal dosage, appropriate time window for administration of EXs, number of repeated dosages, and route of administration are considered as the most important issues to be resolved (63).

Clinical trials of cell-free treatment in ischemic heart diseases

Effective results acquired from preclinical studies have encouraged scientists to continue experimental studies toward clinical trials. In recent decades, many clinical trials have been conducted aimed at CFTs-based therapies for patients with IHDs. These studies focused on the clinical usages of angiogenic genes such as *VEGF, FGF*, *HGF*, developmental endothelial locus-1 (*DEL-1*), and *SDF*. Meng et al. (64) performed intracoronary transplantation of Adenoviruses that carried the *HGF* gene (Ad-*HGF*) in patients with CADs. They concluded that long-term administration of Ad-*HGF* was safe that did not cause any adverse reactions or unwanted clinical manifestations (like tumors, prolonged fever, arrhythmia, and retinal vessel anomalies). The results of their study showed that intracoronary transplantation of Ad-*HGF* efficiently improved echocardiographic EF at the 36-month follow-up compared to the results acquired from baseline levels.

Table 4: The most important clinical trials with different GFs and gene therapies in patients with IHDs

Author and year of publication	Vector or GF	Patients	Outcome measurements	Results
Anttila et al. (67), 2020	Epicardial injection of AZD8601 (VEGF-A165 mRNA formulated in biocompatible citrate-buffered saline and optimized for high-efficiency VEGF-A expression with minimal innate immune response)	24 patients with stable CADs and moderately decreased LVEF (30%-50%) who were undergoing coronary artery bypass graft surgery	The safety and tolerability of AZ8601, effect of AZD8601 on regional and global stress myocardial blood flow, LV end-diastolic volume, LV end-systolic volume, and LVEF, regional myocardial wall motion, NYHA class, change in troponin T and NT-proBNP levels in six months	Ongoing clinical trial
Greenberg et al. (68), 2016	Intracoronary adeno-associated virus 1 / sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase	250 patients who had NYHA class II-IV HF and LVEF≤35%	Time to recurrent events, defined as hospital admission because of HF or ambulatory treatment for worsening HF in 12 months	No evidence of improvement in the clinical course and outcome
Chung et al. (69), 2015	Endomyocardial injection of plasmid SDF-1	93 subjects with IHF on stable guideline-based medical therapy and LVEF ≤40%	Safety, efficacy, LV functional and structural measures were assessed	Attenuating LV remodeling and improving EF. Demonstrated safety
Penn et al. (70), 2013	Endomyocardial injection of JVS-100 (a DNA plasmid encoding human SDF-1)	17 subjects with ischemic cardiomyopathy, NYHA class III HF, and EF ≤40% on stable medical therapy	Major adverse cardiac events, QOL, NYHA class, six-minute walking distance, single photon emission computed tomography, NT-proBNP and safety over 12 months	All of the cohorts demonstrated improvements in six-minute walking distance, QOL, and NYHA class. The primary safety end point was met

IHD; Ischemic heart disease, GFs; Growth factors, CADs; Coronary artery diseases, NYHA: New York Heart Association, IHF; Ischemic heart failure, NT-proBNP; N-terminal pro b-type natriuretic peptide, HF; Heart failure, QOL; Quality of life, EF; Ejection fraction, LV; Left ventricle, SDF-1; Stromal-derived factor-1, LVEF; Left ventricular ejection fraction, and VEGF-A; Vascular endothelial growth factor A.

Gross et al. (65) conducted a randomized, prospective, double-blind, and placebo-controlled clinical trial (SITAGRAMI) that included 174 patients with acute MI. Participants were treated with placebo (control group) or a 1:1 ratio of granulocyte-colony stimulating factor (G-CSF) plus sitagliptin (case group). The results of their study that included a 4.5 year follow-up, showed no significant differences in the incidence rate of major adverse cardiac and cerebrovascular events (MACCE) between both control and case groups. Treatment with sitagliptin did not have any significant effects on the clinical outcomes of patients with MI.

Kukuła et al. (66) conducted a double-blind clinical trial (NCT00620217) on 52 patients with CADs, who received intramyocardial injections of VEGF-A165/bFGF plasmid into the ischemic regions of the heart tissues. The control group received a placebo plasmid. The results of the 10 year follow-up showed that this vector was also safe and there was no evidence of increase in the prevalence of death or malignancies. They indicated that the incidence of stroke, MI, and cardiovascular-related mortalities were similar between both control and case groups.

Table 4 summarizes the most important clinical trials that used various GFs and gene-based therapies for patients with IHDs.

However, our search results could not find any reasonable clinical usage of EXs, CM, and miRNAs among current clinical trials of EVs for patients with IHDs. Although CFTs-based strategies are promising approaches for treatment of patients with CVDs, more preclinical studies are needed to evaluate the efficacy of CFTs as effective therapeutic approaches for IHDs. These studies should particularly be conducted with large animal models of IHDs, enabling progress toward more efficient and productive clinical trials.

Conclusion

Despite substantial progress in the prognosis, diagnosis, and treatment of diseases, CVDs-related morbidities and mortalities still remain high. Hence, scientists are encouraged toward more practical methods to obtain better clinical outcomes. In this context, clinical applications of stem cell-derived secretomes as a missing piece in the puzzle of CVDs target therapy can be considered as off-the-shelf methods with controlled and predictable outcomes, decreased adverse effects, and reduced constrains of SCT. Neovascularization, differentiation, and proliferation of new CSCs are significant features that should not be underestimated for heart regenerative medicine, which may provide effective outcomes for treatment of patients with IHDs, and increase LV contractility in patients with HF.

According to the results acquired from current studies, EXs and CM have shown more practical effects among the various types of CFTs, as well as when compared to SCT. The usage of genetically-modified cell culture medium through neonatal cell-derived CM, and the components present in Exs, and CM allow us to expect cost-effective and optimized results for treatment of various clinical stages in CVDs. Despite the successful results acquired from clinical studies done on CVDs models, adverse effects such as hypotension, tumor angiogenesis, severe inflammatory reactions, arrhythmia, off-target responses, and poor gene transfer efficiency have been reported, complicating clinical applications of GF-based therapies in patients with CVDs. Although a myriad of preclinical and laboratory studies have used GFs and gene-based therapies for improvement of cardiovascular performance in patients with IHDs, they have failed to achieve much success in improving cardiac function and halting the MI process.

Totally, due to less adverse effects, CFTs can hopefully pave the way for providing more promising therapeutic approaches for patients with CVDs, reducing IHDs-related mortalities and morbidities rates, as well as diminishing hospitalization expenses for patients and the health system.

CFT, with a broad spectrum of profound regulatory effects on clinical outcomes of patients with CVDs is still in its infancy. Further investigations aimed at elimination of the ambiguities related to the clinical applications of this recently-introduced method are highly recommended for achievement of beneficial effects of CFTs. In order to reach this purpose, additional preclinical and clinical studies, and collaboration among cellular and molecular biologists, clinical laboratory scientists, immunobiologists, gene and cell therapists, clinical specialists, diseases-specific biomarker scientists, cardiologists, medical biotechnologists, and health science coordinators are unquestionably needed.

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Authors' Contributions

A.E.; Supervision, project corresponding, and verification of the last version before submission. A.E., N.B.; Contribution to conceptualization, validation, and formal analysis. N.B.; Designation of main methodology, search strategy, academic/scientific/grammatical revision for important intellectual content, preparation, and main designation of the final draft of the manuscript. N.D., N.B.; Major conception, data extraction, and data interpretation. N.D.; Data gathering and preparation of first draft of manuscript, visualization, image/table designation. All of the authors attest to the validity and legitimacy of data, receiving an electronic copy of the final version, and published version of the manuscript.

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