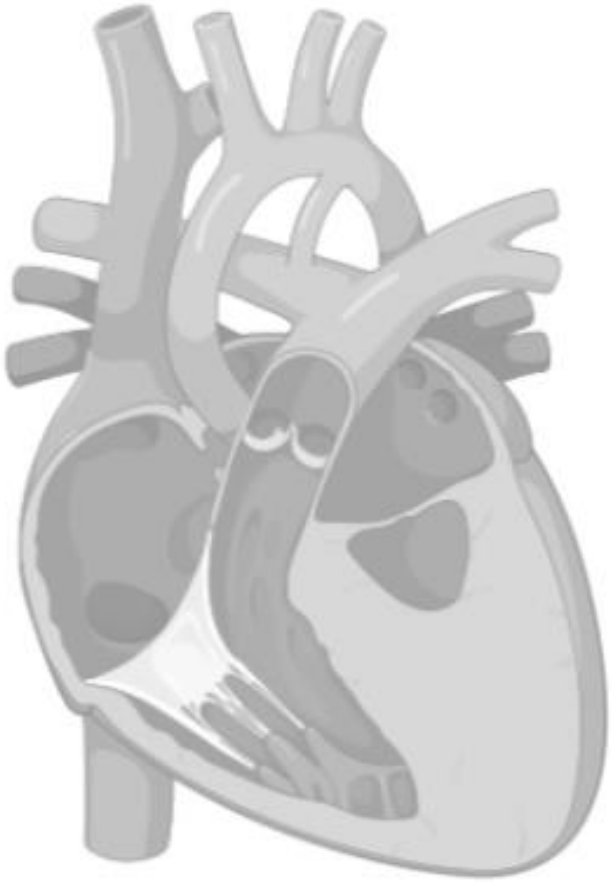
Optimizing the Univentricular Heart: the Potentials of Mesenchymal Stromal Cell Therapy for patients with Hypoplastic Left Heart Syndrome

Literature Review



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Abstract

Hypoplastic left heart syndrome is a congenital heart disease in which the left side of the heart is highly underdeveloped, hindering left-sided systemic circulation after birth. The condition is rare and accounts for only 9% of all congenital heart diseases but is fatal when left untreated. A three-stage univentricular palliative intervention, in which the right ventricle is tuned to sustain systemic circulation, has changed the prognosis of this condition. However, this univentricular circulation cannot be maintained indefinitely, as the right ventricle physiologically is not built to drive systemic circulation, which often leads to cardiovascular complications such as heart failure and, subsequently, complications such as arrhythmias. Potentially, (stem) cell-based therapies could enhance proper ventricular function to a state more suitable for systemic circulation. This review will discuss the potential therapeutic role of mesenchymal stromal cells in patients with hypoplastic left heart syndrome. The morphological differences between the left and right ventricles are significant and must be considered when designing treatments for right ventricle dysfunction. Thus, the intrinsic differences between the two ventricles and the subsequent cellular mechanisms involved in this pathophysiological condition will be a topic of interest in the first part of this review. The paradigm of how mesenchymal stromal cells act in the injured myocardium has been shifted from a mechanism based on cellular engraftment and differentiation towards the cardiac lineage to one primarily based on the paracrine effect. Several preclinical studies will be discussed, including the different disease models for hypoplastic left heart syndrome that laid the groundwork for clinical application. Based on recent literature, we will shed light on the mesenchymal stromal cell secretome setting up cellular pathways that could cause the elucidated therapeutic effect, highlighting reducing reactive oxygen species (-mediated damages) in the myocardium involved in the right ventricular failure. The timing and route of administration are important factors in the effectiveness of therapy and will also be reviewed here. We will conclude with our view on these stem cell-based therapies' most effective application strategies. This review will thus provide an overview of the various ways mesenchymal stromal cells can be a valuable cell source for infants suffering from hypoplastic left heart syndrome to affect right ventricle dysfunction and improve longevity and quality of life.

Laymen's Summary

The heart is a muscular organ that pumps blood through the circulatory system. In humans, the heart is divided into four chambers: the upper left and right atria and the lower right and left ventricles. Deoxygenated blood enters the heart in the right atrium. It is passed to the right ventricle, which sends the blood to the pulmonary circulation into the lungs, where oxygen will be taken up, and carbon dioxide will be given off. The oxygen-rich blood returns to the left atrium and is passed to the left ventricle. Then, the blood is pumped from the left ventricle via the aorta into the systemic circulation. Hypoplastic left heart syndrome (HLHS) is a congenital heart disease in which the left side of the heart is underdeveloped or non-existent. Since the left side of the heart is responsible for the delivery of oxygen to the majority of our organs, HLHS is not compatible with life. Therefore, children must undergo three cardiac surgeries in the early stages of life to completely omit the left side of the heart and use the right ventricle to pump the blood into the systemic circulation. In a healthy heart, the right ventricle has to withstand much lower pressures sending blood into the pulmonary circulation than the left ventricle, which has to overcome more resistance from the systemic arteries. This can also be recognized in the heart, as the left ventricle is much more muscular than the right ventricle. Thus, exposing the right ventricle to systemic pressures can lead to right ventricle failure, significantly reducing the longevity and quality of life of HLHS patients. Therefore, researchers are now trying to find ways to prevent right ventricular failure using cell-based therapies. Right ventricle failure starts with cardiac remodeling and the origin of cellular hypertrophy, an increase in the size of muscular cells in response to various stimuli, including high-pressure overload. This phase of ventricular remodeling can eventually progress into failure and is characterized by the presence of reactive oxygen species (ROS) as a response to oxidative stress, causing harm to the cardiac tissue. ROS are highly reactive chemicals that are a typical product of aerobic dissimilation but can lead to tissue damage when levels are elevated.
Mesenchymal stromal cells (MSCs) can modulate cellular microenvironments by secreting several factors, known as the paracrine effect. For example, MSCs are anti-inflammatory, anti-fibrotic, and anti-apoptotic and, in addition, have been shown to support the formation of new blood vessels. In addition, they have also been shown to reduce ROS and reverse ROS-induced damages. The exact mechanisms of actions of MSCs in right ventricular failure have not extensively been investigated in single ventricle animal models. But recently, a new animal model to recapitulate HLHS has been established. The therapeutic agents can be administered directly into the cardiac tissue, the coronary arteries that supply blood to the heart, or intravenously. Intracoronary administration might be of utmost importance for now because the administration is least invasive, and cell distribution has shown to be most evenly. As the field of cell therapies for HLHS continues to evolve, MSC therapy could be a promising strategy to offer better longevity and quality of life for HLHS patients.

Introduction

Hypoplastic left heart syndrome (HLHS) is a severe congenital heart disease characterized by anatomical and functional deficiencies on the left side of the heart. The disease affects several structures and can be defined by the stenosis of the mitral and aortic valves, an extreme or even absence of the left ventricle, and aortic hypoplasia (1). Left untreated, HLHS is incompatible with life as the left ventricle fails to sustain systemic perfusion. The anatomical abnormalities in HLHS can be surgically adjusted to one that is more sustainable. This intensive procedure includes three-staged surgical palliations involving Norwood, Glenn, and Fontan operations to commit the right ventricle (RV) to the systemic circulation (2). The first Norwood operation is performed within the first week of life. Its goal is to redirect the RV outflow to the aorta.
The pulmonary blood flow is then supplied via either an RV to pulmonary or a systemic to pulmonary arterial shunt. As the child grows and blood volume increases, this construction will eventually lead to high pulmonary vascular pressures and RV volume overload if left unchanged. Clinicians try to prevent this by performing second palliative surgery. The Glenn surgery, or bidirectional cavopulmonary anastomosis, is performed at around three to six months. Here, the systemic pulmonary shunt is removed and directly replaced by the anastomosis of the superior vena cava to the right pulmonary artery. This lowers pulmonary pressure and reduces RV volume overload by preventing venous blood from returning to the RV. Since this construction still provokes RV pressure overload over time, a third Fontan palliative surgery will be performed at 18-36 months to achieve total cavopulmonary connection by anatomizing the inferior vena cava to the right pulmonary artery. Aside from an overall survival chance of 74% of the Fontan circulation at 20 years, other complications include atrial arrhythmia, right ventricular hypocontractility with a low cardiac output, thromboembolic events, and a risk for protein protein-losing enteropathy and lymphatic malfunction due to high systemic venous pressures (3–5).

Patients with single ventricle circulation remain subject to increased RV systemic afterload, which can transition to systemic right ventricular (SRV) failure (2). Cellular mechanisms underlying SRV failure are similar to left ventricle (LV) failure, but the two have distinct remodeling responses due to non-physiological pressures and volume overload (6). Due to the natural functional morphology, high mechanic stresses are more likely to be translated into oxidative stress, reduction in angiogenic response, and the activation of cell death pathways in the stressed SRV compared to the LV. These mechanisms eventually lead to fibrosis, cardiomyocyte dysfunction, and even cardiomyocyte loss and thus play an active role in SRV failure (7). Eventually, SRV failure will develop in patients with Fontan circulation. The failing systemic ventricle, or other complications mentioned above, nearly always leads to death unless a patient is qualified to get a heart transplant. Heart transplants are scarce, and transplantation after Fontan operation, especially for those with HLHS, is more complex and often is not even the desired option regarding the evolved comorbidities in these patients. Because of this significant risk for mortality, it is vital to develop therapeutical strategies to optimize SRV function in HLHS patients with Fontan circulation. Regenerative and cell-based strategies are of significant interest in tackling this problem.

Mesenchymal stromal cells (MSCs) have gained attention in regenerative medicine due to their potential paracrine effect on surrounding cells (8). Their secretome contains various cytokines, chemokines, and growth factors that might promote immunomodulation, anti-

apoptotic and anti-oxidative effects. Besides, local or systemic MSC administration has been shown to localize to injured regions and improve LV function after myocardial infarction (9,10). They have also been shown to modify their secretome *in situ* by the upregulation of growth factors and cytokines to support the regeneration of the affected tissue (11). This represents the promising potential of MSCs to be used as stem cell therapy to enhance proper SRV function in HLHS patients to avoid the onset of heart failure. This review aims to provide an overview of the cellular disease mechanisms involved in SRV failure, the potential therapeutic effects of MSCs, and their routes of administration to improve SRV dysfunction in HLHS patients with Fontan circulation.

Models for right ventricular failure

Animal models are essential in unraveling the molecular pathways involved in SRV failure, new therapies' mechanisms of action, and their efficacy. Unfortunately, animal models of any disease condition fail to recapitulate the complex clinical pathophysiological spectrum. In addition, disease modeling of SRV failure occurring from the single-ventricle circulation in HLHS animals is rare due to the largely unknown mechanisms of fetal programming leading to LV hypoplasia. Therefore, researchers often rely on models for pulmonary artery hypertension (PAH) for studying (patho)physiology, as this can lead to RV failure, an inevitable result of a single ventricle circulation.

Model for hypoplastic left heart syndrome

Until recently, animal models for HLHS that fully reproduced the cardiac phenotype did not exist. However, a recent study reported the development of a surgically induced mouse model of HLHS that meets the requirements for clinical application (12). Here, the blood flow into the left heart was partially pharmacologically blocked during early development, inducing hypoplasia. Later anatomical analysis revealed that these treated mice exhibit retrograde aortic arch flow non-apex-forming left ventricles and hypoplastic ascending aortas, as seen in HLHS patients. This method is a promising model for finding solutions to previously unanswered questions regarding the mechanisms involved in establishing fetal extraordinaries in HLHS. It can also be of valuable worth in developing (cell)-based therapies targeting hypoplasia or systemic RV failure in HLHS patients.

Surgical model for right ventricular failure

Pulmonary artery banding (PAB) is often used in smaller animals, such as rodents, and larger animals, such as sheep. The technique is characterized by a fixated pulmonary artery constriction which can be regulated in size to induce either compensatory hypertrophy or RV failure (13). Even though this model is only thought to mimic RV adaptive responses to pulmonary stenosis instead of RV systemic circulation, and the LV is still involved in the circulation, unlike in the Fontan circulation, PAB is an effective way to induce symptomatic RV dysfunction. It has been shown to cause maladaptive cardiomyocyte hypertrophy, myocardial fibrosis, decreased capillary density, and reduced diastolic function related to RV failure (14,15). PAB in mice has even been shown to share ventricular mechanisms of pediatric pulmonary hypertrophy (16). This indicates that this model is highly suitable for studying the early onset of RV hypertrophy in children with a single ventricle heart and potential actionable therapies after palliative surgeries for HLHS. This model's primary advantage is avoiding toxic effects, as discussed in other models below.

Monocrotaline and Sugen-hypoxia models

Monocrotaline (MCT) and Sugen-hypoxia (SuHx) are other models commonly used for pulmonary artery hypertension rat models. MCT is a plant seed-derived alkaloid that leads to the onset of dose-dependent pulmonary hypertension and RV hypertrophy (17). This animal model is used often because this model is easily reproducible and inexpensive. MCT rodent models might be of considerable value since the most pronounced response to the alkaloid is an increase in RV afterloads leading to similar RV dysfunctions as in single ventricle hearts. In addition, these animal models mimic other essential pulmonary artery hypertrophy characteristics in terms of hemodynamic and histopathological severity and high mortality (18).

Nevertheless, the establishment of lung edema with a decreased endothelial barrier function and increased inflammatory adventitial proliferation is a substantial difference between PAB and MCT-induced pathophysiology. These adverse effects may make MCT models less suitable for studying RV failure than PAB for disease modeling. SuHx models combine a vascular endothelial growth factor antagonist (Su) with exposure to 9-10% hypoxia (Hx) for three weeks. The two components can also be used independently. Still, the combination leads to higher and more long-term RV systolic pressure and hypertrophy, more abundant vascular remodeling, and lower contractile function (19,20). On the downside, SuHx models can cause angio-obliterative lesions in the pulmonary artery, which develop progressively. However, its severity can be diminished by weekly Su administration instead of one (19). Recent studies suggest experimental variations on the SuHx to circumvent hypoxia and the establishment of lesions by, for example, combining morphine with Su. This has also been shown to significantly induce vascular remodeling and development of PAH without the pathophysiological role of angio-obliterative lesions in the animal model (21). On the other hand, hypoxia is a characteristic phenotype of the failing SRV and, therefore, may thus provide an extra dimension to HLHS animal models. However, the data gathered from traditional SuHx models with the interplay of angio-obliterative lesions likely cannot give an unambiguously answer to research questions regarding heart functionality.

Mechanisms involved in right ventricular failure

Crucial differences between the left and right ventricle

The RV differs from the LV in macroscopic, ultrastructural, and biochemical properties and is not just a weaker version of the LV. These differences are at the baseline of developing SRV failure. Macroscopically, a healthy RV has a wall thickness of 2-3 mm at the end of diastole, while its significantly thicker left equivalent has an end-diastolic wall diameter of about 8-11 mm, able to reach forces to measure up to those needed for systemic circulation. In addition, there is a fundamental morphologic difference in ventricle ultrastructure; the RV is more coarsely trabeculated than the LV, which is more compactly organized (22). Trabeculation is linked to ejection fraction, as hypertrabeculation is typically associated with reduced LV ejection fraction, as it lowers ventricular compliance and restricts the ventricle filling (23). From a biochemical view, the RV composes a more considerable proportion of α-myosin associated with more rapid but less energy-efficient contraction than the β-myosin-rich LV (24). Concluding these differences, the RV is naturally built to withstand lower workload than the LV.
A healthy RV is exposed to the relatively low resistance of the pulmonary vascular bed. Exposing the RV to systemic pressure from the systemic vasculature makes the ventricle susceptible to ventricular failure, a gradual process characterized by different cellular pathways depending on the specific phase of failure. These phases include RV hypertrophy in the early stages, fibrotic tissue formation, and cover switches in energy metabolism when progressing to RV failure. The pathological conditions of RV remodeling are often associated with increased deposition of extracellular matrix (ECM) proteins. The type of fibrotic tissue deposition differs during LV and RV remodeling and hypertrophy, and consists mainly of fibrillar collagens type I and III (25). Differences between fibrotic tissue in the two ventricles mainly concern the deposition of collagen I, collagen IV, laminin, and fibronectin (26). There is wide heterogeneity in the progression of cellular hypertrophy to RV failure. Some patients develop RV failure relatively soon after RV hypertrophy, while others can live with hypertrophy their whole lives with limited comorbidities. Interestingly, this heterogeneity is independent of pressure overload and thus highlights the importance of cellular responses to the RV pressure overload (27). Many pathways are involved in this pathophysiological process, including the activation of apoptotic pathways, inflammation, and the production of reactive oxygen species (ROS) in the early stages of afterload-induced SRV failure (28,29).

A shift in energy metabolism

In healthy conditions, the energy demand of both ventricles relies on the mitochondrial oxidation of available free fatty acids within the myocardium. When pressure overloads arise, the SRV depends more on the glycolic ATP production (6). Potential mechanisms contributing to the shift toward this less-oxygen-demanding state of metabolism are reduced coronary perfusion and the inability for angiogenic upregulation, leading to hypoxia. SRV coronary perfusion occurs, unlike LV perfusion, during systole. Increasing SRV systolic pressures decrease SRV coronary blood supply, potentially aiding in the relatively early onset of right ventricular ischemia. A general way to increase myocardial vascularization during hypoxia is via the secretion of hypoxia-induced factor-1α (Hif-1α) and the subsequent activation of vascular endothelial growth factor (VEGF) expression. Myocardial capillary density

assessments were done on rat models of pulmonary hypertension accompanied by RV failure. The progression into RV failure was characterized by a decrease in VEGF mRNA expression, while, interestingly enough, an increase in nuclear Hif-1α was seen (30). This suggests that other mechanisms regulating VEGF expression might be involved. A decrease in VEGF expression puts angiogenesis on hold and might evoke the metabolic switch toward glycolysis. An increase in the glycolic enzymes hexokinase and lactate dehydrogenase in monocrotaline-induced pulmonary hypertension models further supports this (31). Long-term reliance on glycolysis for ATP production is detrimental to healthy myocardial function. It steers cardiomyocytes to starvation, playing a critical role in heart failure and the rise of oxidative stress (6).

Cardiomyocyte hypertrophy

The onset of cardiac hypertrophy is essential in the early stages of SRV failure. One of the first potential mediators in cardiomyocyte hypertrophy in response to SRV overload is inflammation, driven by inflammatory mediators such as tumor necrosis factor TNF-α (28). These cytokines could subsequently play a role in activating apoptotic pathways and producing reactive oxygen species (ROS). ROS are highly reactive chemicals that are a typical product of aerobic dissimilation but can lead to tissue damage when levels are elevated. It remains elusive whether RV inflammation is determinative for cardiomyocyte hypertrophy or a natural protective response to the metabolic stresses at the onset of RV malfunction. It can also be a protective response at first but later worsen, leading to hypertrophy. Inflammation is, however, a result of ROS production in many chronic diseases, including heart failure. ROS causes unfavorable changes in DNA and RNA composition, leading to the secretion of inflammatory molecules (32). It, therefore, has a crucial role in the pathophysiology and may be a therapeutic target in later phases of ventricle malfunction.

As mentioned, the metabolic shift in cardiomyocytes evokes oxidative stress. Oxidative stress is a determinator in the onset of maladaptive SRV hypertrophy. It is suggested to play a crucial role in the transition to right ventricular failure. It can produce ROS in cardiomyocytes and surrounding inflammatory cells, endothelial cells, and fibroblasts (33–36). ROS directly impact cardiomyocytes by acting on cellular growth, sarcomere proteins, myofibrils, mitochondrial function, and cell death (37). In addition, downstream signaling pathways affected by increased ROS result in cardiac and endothelial dysfunction. Mechanisms activated in cardiomyocytes by increased ROS include p38 mitogen-activated protein (MAP) activation, defunctionalization of tropomyosin, desensitization of β-adrenoreceptors and induction of matrix metalloproteases 2, 9, and 13 (37).

Altogether, the production of ROS appears to be a crucial determinator for the onset of cardiomyocyte hypertrophy and therefore increases the chances of developing SRV failure. MSCs regulate microenvironments with excessive ROS production by their high levels of antioxidant secretion and may assist in reducing ROS-induced damage (38,39). Preventing the accumulation of ROS in SRV cardiomyocytes might be a target of interest in hampering the progression into heart failure. Therefore, in the next section, we will shed extra light on the ROS pathway leading to SRV cardiomyocyte hypertrophy.

Reactive oxygen species lead up to right ventricular hypertrophy

The production of ROS plays an essential role in the effects of cardiac pressure overload. Understanding the mechanisms of pathophysiological ROS production in the SRV is necessary. It can serve as a potential therapeutic target to affect SRV failure in the early stages. The presence of ROS can either be categorized as cytosolic or mitochondrial. In addition, a decrease in ROS defense mechanisms is also a driver of increased ROS formation. Ultimately, an imbalance in ROS levels accelerates an increase in TNF-α, Angiotensin II, and norepinephrine in cardiac tissue, which are associated with the onset of cardiac diseases, including heart failure (38).

Mechanisms increasing systolic ROS production include the increased expression of NADPH oxidase enzymes, including NADPH oxidase (NOX)-enzymes and uncoupled nitric oxide synthetase (NOS). NOX-enzymes are ROS-producing NADPH oxidases that fulfill a broad spectrum of physiological functions, but excessive activation may lead to pathophysiological mechanisms. One molecule implicated in RV hypertrophy via the promotion of NADPH oxidase-dependent ROS generation is serotonin (5-hydroxytryptamine; 5-HT) (40). A recent study proposes that serotonin levels increase parallel with cardiac myofibroblast populations, which might happen in early RV remodeling (41,42). A rat model of pulmonary hypertension suggests that serotonin mediates a right ventricular increase in protein carbonylation, a ROS oxidation process. This is thought to result from low concentrations of the serotonin-degrading enzyme monoamine oxidase A, triggering NOX mediated O_2^- production (40,43). In addition, NADPH-dependent superoxide formation has been shown to double in the presence of angiotensin II, a vasoactive hormone with a broad profile in the development of heart failure (44). RV pressure overload increases O_2^- production by activating the NOX membrane subunit gp91, likely caused by activated upstream interactions involving protein kinase C (37,45). In addition, the establishment of RV hypertrophy in monocrotaline-induced pulmonary hypertension models shows an increase in the NOX4 expression (46). Increased NOX4 levels may be necessary for the pathophysiology of cardiac fibrosis leading to heart failure (47).
On the contrary, NOX4 expression is downregulated by the activation of the α_{1A}-adrenoreceptor as a potential mechanism for cardiomyocyte protection (48). Uncoupled NOS is mainly involved in accelerating the transition from hypertrophy into failure. NOS switches its nitric oxide production to synthesize superoxide anion O_2^-. A hypoxic knock-out model indicates a strong relationship between uncoupled NOS and the progression to RV failure, as genetic rescue experiments and NOS inhibition prevented RV failure significantly (49). A metabolic shift in arginine metabolism from NOS to arginase is one mechanism of NOS uncoupling associated with increased cardiovascular risk (50).

Mitochondrial ROS production is also affected by an increased afterload and has opposite effects in the beginning stages of hypertrophy compared to the progression into RV failure. Mitochondrial ROS production is lower during RV hypertrophy but rises during the transition to RV failure. This can be explained by analyzing the mitochondrial membrane polarization, which is increased during RV hypertrophy, indicating a reduction in oxidative phosphorylation activity and, thus, ROS production (51). This effect can be linked to the inhibition of pyruvate dehydrogenase kinases (PDK). These kinases are essential gatekeepers in the progression of glycolysis into oxidative phosphorylation and, when inactivated, will hinder oxidative phosphorylation. As mentioned, the energy source in hypertrophic RV cardiomyocytes switch their metabolism from one primarily based on oxidative phosphorylation to one that relies more

on glycolysis. The inhibition of pyruvate PDK activity might contribute to this, which is also regulated by hypoxia upstream of the HIF-1α signaling (52). A decrease in mitochondrial oxidative phosphorylation decreases ROS formation (53). Thus, overall mitochondrial activity during RV hypertrophy is lower due to the reduced dependency on free fatty acid oxidation and decreased PDK activity, leading to lower mitochondrial ROS levels. Eventually, when hypertrophy progresses into failure, mitochondrial ROS play a significant role in the increased intracellular ROS levels.

Mitochondrial ROS production is increased upon the transition to RV failure. This is due to a downregulation of the mitochondrial ROS defense system superoxide dismutase 2 (SOD-2), indicating a less effective free radical clearance (54). When RV failure arises, the SOD2 gene is epigenetically silenced by DNA methylation and has downstream effects on mitochondrial metabolism, which then switches back to the fatty acid oxidation (55). Mitochondrial hyperpolarization is then lost, and mitochondrial ROS production rises again. Compared to LV failing, the antioxidant superoxide dismutase was only activated in later stages of RV failure, and glutathione peroxidase activities remained non-existent throughout (56). Treatment with the superoxide dismutase-like antioxidant EUK-134 showed attenuation of right ventricular cardiomyocyte hypertrophy, oxidative stress, proapoptotic signaling, and increased systolic function by a dramatic reduction of end-systolic volume of 42% (54). The molecular pathways involved in this ROS-mediated pathogenesis are summarized in figure 1.

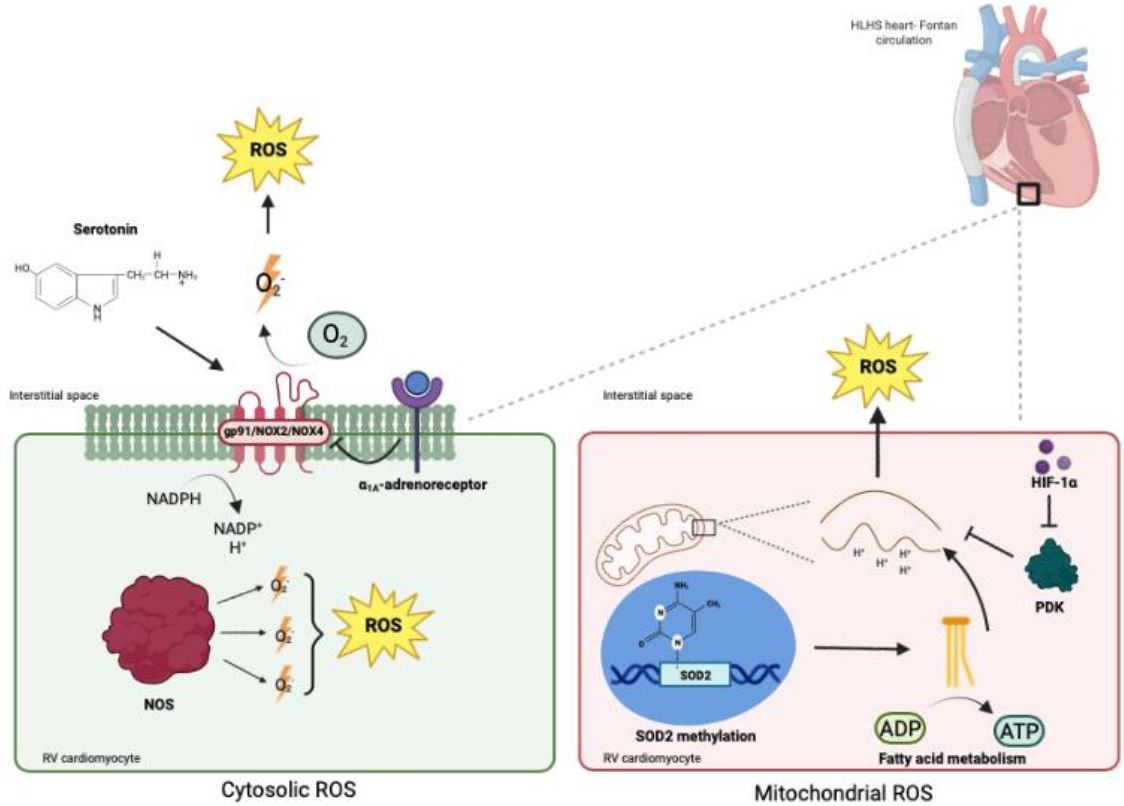

Figure 1 **The generation of reactive oxygen species in hypertrophic and failing RV cardiomyocytes.** Cellular hypertrophy is a key mechanism in the development of RV failure. Multiple factors affect cardiomyocyte hypertrophy, of which the production of ROS is of crucial importance. ROS are produced in the cytosol during early hypertrophy (left cell) and in the mitochondria during later progression toward RV failure (right cell). Cytosolically increased levels of systemic serotonin due to cardiac remodeling, which stimulates NOX, a switch of NOS from NO to O_2^- synthesis, and the activation of the α_{1A}-adrenoreceptor are involved in the regulation of ROS production. Oxidative phosphorylation in the mitochondria is responsible for ROS production during the progression of hypertrophy to RV failure. During early hypertrophy, in response to HIF-1α, PDK activation is inhibited, leading to hyperpolarization of the mitochondrial membrane, disrupting oxidative phosphorylation and ROS production. In later stages, the SOD2 gene is methylated, leading to an oxidative phosphorylation-dependent metabolic switch in fatty acid metabolism. Hyperpolarization is then lost. *ROS = Reactive oxygen species, NOX = NADPH oxidase, NOS = Nitric oxide synthase, HIF = Hypoxia-inducible factor, PDK = Pyruvate dehydrogenase, SOD = Superoxide dismutase.* Image created with BioRender.

Mesenchymal stromal cells and heart failure

Mesenchymal stromal cells (MSCs) are currently being investigated for their therapeutic effects in a variety of diseased conditions in general. This cell type is also considered a promising and effective therapeutic tool for treating heart failure. Mainly because of their favorable intrinsic characteristics as these cells are nonhematopoietic cells originating from the mesoderm, and they possess the ability to self-renew and contribute to multilineage mesodermal, as well as ectodermal and endodermal differentiation (57). MSCs can be isolated and expanded *in vitro* from multiple autologous or allogeneic tissue sources; the bone marrow, adipose tissue, the umbilical cord, fetal liver, and lung. Generally, two primary mechanisms in which cardiac function could benefit MSC treatment. MSCs could either differentiate themselves into cardiomyocytes or repair the myocardium by increasing cell density. Alternatively, a mechanism of action that gained much interest recently is the paracrine effect of MSCs, in which these stem cells contribute to tissue repair and regeneration via the release of various cytokines, chemokines, and growth factors.

The paracrine effect

Of the strategies mentioned above in which MSCs could potentially be of therapeutical relevance, the underlying beneficial effects are most likely a result of the MSC paracrine effect (58). MSCs are known for their anti-inflammatory, anti-fibrotic, and anti-apoptotic effects and, in addition, have been shown to support neovascularization. MSCs exert these functions via many different pathways, of which the most abundant will be discussed briefly. Inflammation can be defined as a protective response by immune cells, such as macrophages, to fight pathogens. Treatment of pro-inflammatory macrophages with a bone marrow-derived MSC-conditioned medium revealed a decrease in mRNA transcription of the pro-inflammatory cytokines IL1β and IL6, leading to the subsequent inhibition of macrophage activation *in vitro* (59). In addition, bone-marrow-derived MSC-conditioned medium has also been shown to inhibit the production of TNF-α in activated macrophages by producing interleukin-1 receptor antagonists (54). Even though the exact relation between the cycle of inflammation, ROS production and the onset of cardiomyocyte hypertrophy remains unclear, this indicates the potential anti-inflammatory therapeutic effects of MSCs in combatting the beginning of RV failure.

Collagen deposition and fibroblast proliferation in the myocardial interstitium are fundamental events during the remodeling phase leading to heart failure and physically limiting cardiomyocyte contractility (56). Regarding the anti-fibrotic effects MSCs, culturing cardiac fibroblasts in an MSC-conditioned medium has also been shown to attenuate proliferation and, in turn, collagen synthesis *in vitro* (55). After 48h culture of cardiac fibroblast in the MSC-conditioned medium, a significant downregulation of collagen type I and type III expression was accompanied by an upregulation of antiproliferation-related genes, such as myocardin (55). Besides, MSCs might also affect extracellular matrix (ECM) remodeling and fibrosis via the secretion of matrix-mediating factors such as matrix metalloproteinase (MMP)-2, tissue inhibitors of matrix metalloproteinases (TIMPs)-1 and -2, and the matricellular proteins, known for their capability of modulating many cellular processes within the ECM (57).
Concerning potential cardioprotective effects concerning hyperproliferation and anti-apoptotic exosomes isolated from bone marrow-derived, MSCs have been shown to secrete

hyperproliferative and apoptosis-resistant mediators, ameliorating RV hypertrophy *in vitro* (60). These effects were suggested to be a result of the repression of signal transducer and activator of transcription 3 (STAT3) and the subsequent inhibition of proliferative miR-17 microRNA and the upregulation of anti-apoptotic miR-204 microRNA. Another study found that MSC-derived exosomes activate PI3K/Akt, a pathway involved in cellular survival, and reduced pro-apoptotic phosphorylation of c-JNK in cardiomyocytes (61).

Finally, the MSC secretome also aids in stimulating neovascularization and might therefore limit the consequences of hypoxia in the failing ventricle. MSCs, in particular, are known for secreting high levels of proangiogenic and proatherogenic factors such as VEGF, basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) (62). VEGF seems to have a central role as intravenous administration of MSCs enhanced angiogenesis in the ischemic myocardium upon myocardial infarction (63). Supporting this, direct intramyocardial injection of MSCs resulted in higher vascularization in chronic ischemia myocardial tissue (64). In addition, endothelial progenitor cells, involved in microvessel formation, were found to improve myocardial angiogenesis in response to thymosin β4 secreted by MSCs both *in vitro* and *in vivo* (65). The factors present in the MSC secretome affecting RV function and ROS secretion are summarized in figure 2.

Reducing reactive oxygen species

ROS production is a hallmark of early RV failure and is responsible for TNF-α, Angiotensin II, and norepinephrine production. It is, therefore, an exciting target in preventing the development of this chronic disease. Stem cells are favorable for therapeutical use in tissues with high ROS levels. These cells prefer anaerobic glycolysis over energy production by oxidative phosphorylation and maintain low intracellular ROS levels (38). As discussed earlier, ROS synthesis is accelerated via activating various signaling molecules such as c-Jun N-terminal kinases (JNKs), p38, and mitogen-activated protein (MAP) kinases. Stem cells can regulate these mechanisms via the ataxia telangiectasia mutated (ATM) serine/threonine gene. ATM-knockout mice are associated with increased ROS levels and impaired stem cell function (66).

MSCs specifically has also been shown to play a role in microenvironmental ROS modulation. *In vitro* intramyocardial injection of MSC hydrogels in infarcted heart rats substantially decreased ROS levels, enhanced cardiomyocyte survival and angiogenesis, and inhibited apoptosis and fibrosis under oxidative stress and hypoxic conditions (67). Furthermore, human allogeneic MSCs have been shown to secrete low levels of stromal cell-derived (SDF)-1α *in vitro*, which subsequently decreases the generation of mitochondrial ROS by lower TNF-α expression (68). In contrast to autologous MSCs, which negatively correlated with TNF-α mRNA and ROS levels.

Besides direct microenvironmental modulation of ROS, MSCs may also exert their function in reducing ROS-induced damage. Extracellular vesicles derived from infant MSCs have been shown to neutralize ROS and reverse ROS-induced tissue damage by activating SOD-1 and SOD-3 in a necrotic skin flap model by targeting the MAP kinase pathway (69). In addition, the expression of Prostaglandin E2 (PGE2), Interleukin 1 receptor antagonist (IL1RN), Hepatocyte growth factor (HGF), and soluble transforming growth factor receptors (sTβR) proteins stimulate the secretion of anti-inflammatory and anti-fibrotic molecules such as TNF-

α, TGF-β, interferon- γ (IFN-γ), and IL-10, helping the regulation of oxidative damages induced by ROS (38).

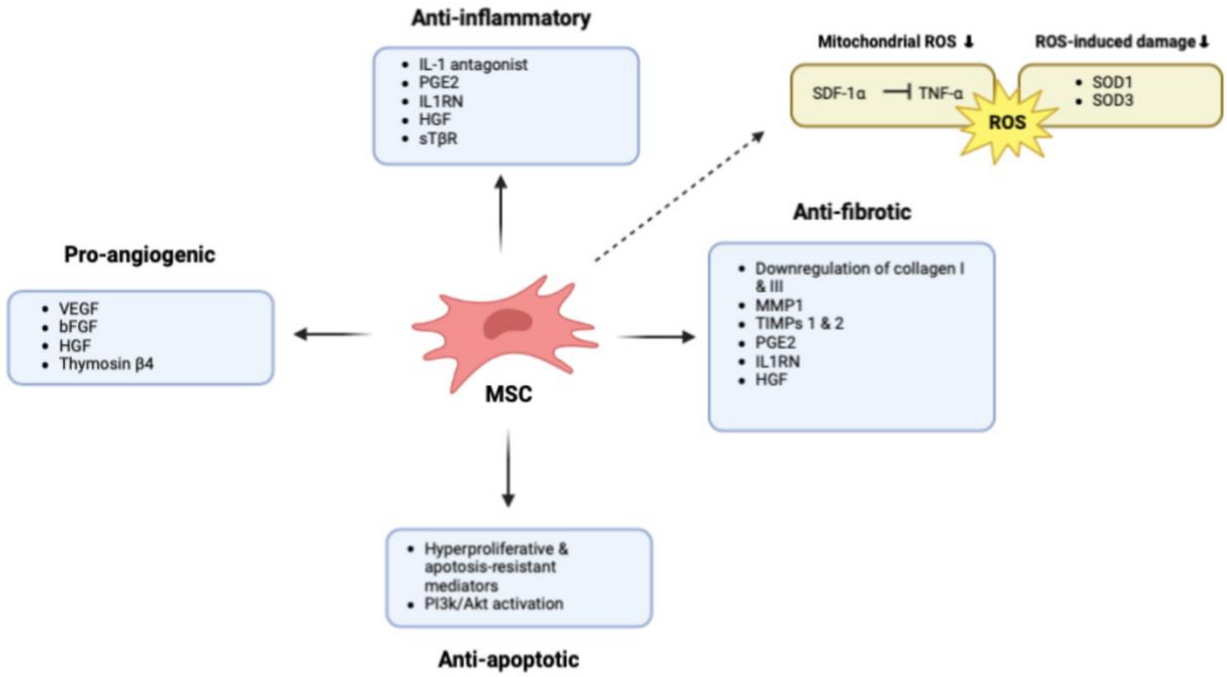

Figure 2 **The paracrine effect of mesenchymal stromal cells.** MSCs extent their therapeutic effects by paracrine mechanisms., via the secretion of several cytokines, chemokines, and growth factors. They mediate anti-inflammatory, anti-fibrotic, anti-apoptotic and pro-angiogenic effects. They also regulate microenvironmental ROS production by the secretion of SDF-1α and antioxidants SOD1 and SOD3. *IL = Interleukin, PGE = Prostaglandin, ILRN = Interleukin receptor antagonist, HGF = Hepatocyte growth factor, sTβR = Soluble transforming growth factor β receptor, SDF = Stromal cell-derived factor, SOD = Superoxide dismutase, MMP = Matrix metalloproteinase, TIMPs = Tissue inhibitors of metalloproteinases, VEGF = Vascular endothelial growth factor, bFGF = Basic fibroblast growth factor.* Image created with BioRender.

Routes of administration

During the development of any new therapeutic agent, it is crucial to consider the various possible ways to administer the agent, the most fortuitous timing, and the optimal dose for the therapy. Research in the most optimal dosage and routes of administration for cell therapies in cardiovascular diseases is an ongoing research topic, but the most optimal ones are not yet established. Cell-based therapies can generally be administered in four ways: intramyocardial, which includes epicardial injection; intracoronary; transvenous coronary sinus; and intravenous. Studies comparing these different routes of administration with each other for the treatment of HLHS have not yet been reported. Therefore, a general overview of the different ways of MSC administration and clinical outcomes in HLHS will be given in this section.

Intramyocardial injection

Intramyocardial injection involves the injection of active agents directly into the myocardium. The significant drawbacks of this method involve its invasive nature and the risk of perforation at the injection sites causing serious inflammation (70). The ELPIS phase I trial was recently conducted to assess the safety and feasibility of intramyocardial injection of allogeneic MSCs into the SRV during the second stage Glenn operation in pediatrics with HLHS (71). Ten patients received eight intramyocardial allogenic MSC injections during second-stage palliation and were followed for one-year post-administration. Target cell doses of 2.5 x 10^5 cells/kg were used.

No significant adverse cardiac events or other safety concerns were noted, indicating that intramyocardial allogeneic MSC administration is safe and feasible. This trial also revealed promising results concerning improved tricuspid regurgitant fraction, while global longitudinal strain and SRV ejection fraction remained unchanged. A non-invasive exosome miRNA analysis was performed on patients after 6 and 12 months of MSC administration. Cluster analysis revealed the presence of three main clusters whose pathways indicate enrichment of terms such as positive regulation of cell migration, response to growth factor, VEGFA-VEGFR2 signaling, and apoptotic signaling. Even though these data have not been peer-reviewed yet, it suggests a promising beneficial therapeutic effect of MSCs on disease progression. Another phase I clinical trial evaluated the safety and feasibility of intramyocardial injection of autologous umbilical cord blood-derived mononuclear cells during Glenn surgery (72). This cell population contains MSCs, hematopoietic stem cells, endothelial progenitor cells, and other mono- and lymphocytes usually found in the blood. Here, a dose of 3 x 10^6 cells/kg was administered slowly at one place of the SRV. One adverse effect was directly correlated to the cell product delivery and included an epicardial bleed. Besides this minor event, there was no associated arrhythmia, hemodynamic instability, or evidence of myocardial ischemia in the treated patients. A 6-month follow-up analysis revealed promising results, as it showed preservation of the baseline RV function. The epical injection method is considered the most reliable route of administration, as several studies have shown that epicardial injection yields better myocardial retention (70).

Nonetheless, it has not been used in a clinical setting, and limited pre-clinical research has been done on this route of administration for RV failure. A dose of 4.7 x 10^6 human cord blood stem cells were injected epicardial in a PAB sheep model (73). Right ventricular function was measured one month post-injection. Notably, the cord blood stem cells were found to have migrated to the myocardium and spleen, kidney, and bone marrow six weeks after transplantation and adopted hematopoietic cell fates. Functional improvements in the RV were seen in the treated group: the end-systolic elastance was increased, and pre-and afterload values improved. The cord blood cells had no significant effect on RV in sheep without PAB.

Intracoronary injection

The intracoronary injection is a less invasive route of administration, as it can be performed via heart catheterization and thus does not require high-risk surgery as during myocardial intramyocardial injection. Therefore, the timing of administration does not have to be simultaneous with one of the three palliative surgeries. This might increase the efficacy of the cell therapy since open-heart surgery triggers an inflammatory response due to surgical trauma, which might negatively impact the therapeutical effects of the cell therapy. Stem cells can mobilize and proliferate in response to inflammation (74). Previously the anti-inflammatory effects of MSCs were discussed, but it may be that the acute inflammatory cardiac environments have an inhibitory effect on the regenerative potential and proliferation (75). A potential advantage of this route of administration may include the homogenous distribution of the cells in the myocardium (70). Clinical trials in which MSCs were administered intracoronary in HLHS patients have not been reported yet. However, its therapeutical effects are investigated in animal models. A rat model of LV ventricular pressure overload hypertrophy with biventricular failure was treated with intracoronary delivery of 1×10^6 MSCs to assess the SRV effects of MSC transplantation (76). The impact on the SRV was measured in the treated animals after 21 and 28 days and showed stabilization of SRV hemodynamics, including SRV end-systolic pressure.
On the contrary, non-treated animals showed progressive worsening of SRV function. This indicates that MSC could provide SRV protective effects. In addition, MSC treatment resulted in a downregulation of pro-inflammatory, apoptotic, and ECM-remodeling markers. The intracoronary administration strategy has been used in clinical trials to protect the SRV from the consequences of high-pressure overloads with the use of cardiosphere-derived cells (CDCs) and showed improved RV ejection fraction, improved somatic growth, reduced heart failure status, and lowered incidence of coil occlusion for collaterals (77). The timing of administration was different from those seen during intramyocardial injection, as the intracoronary infusion was performed one month after Glenn's operation via cardiac catheterization.

Intravenous administration

Systemic intravenous administration is accompanied by a shallow risk and is non-invasive. A significant disadvantage of this route of administration is that the injected stem cells rely heavily on migration toward the injured myocardium. Systemic administration of bone marrow-derived MSCs after myocardial infarction has shown to be feasible and safe, but homing toward the myocardium has shown to be difficult as the labeled MSCs appear to be entrapped in the lungs (78). However, in a pre-clinical report, allogeneic human MSCs were administered intravenously in patients with acute myocardial infarction (79). The dose ranged from 0.5, 1.6 to 5 million cells/kg. The global symptom score and ejection fraction improved significantly compared to placebo subjects. Besides, the MSC treatments led to reverse LV remodeling and thus improved LV function. No adverse events were reported. Notably, considering the high likability for the MSCs to be trapped in the lungs, the lung function of the MSC-treated patients was compared to baseline functionalities. This data revealed a beneficial effect on the pulmonary airways shortly after administration, confirming pulmonary migration with a potential clinical benefit. This could be of serious interest to HLHS patients where significant pulmonary hypertension is the main component in inducing SRV failure. A recent study

showed that MSC exosomes could improve pulmonary hypertension by attenuating pulmonary remodeling via the upregulation of Wnt5a (80).

Discussion

Inconveniences associated with high SRV pressure overloads in children with HLHS that have undergone univentricular palliative interventions remain of increased burden. The non-physiological high pressures applied to the SRV can cause serious cardiac remodeling leading to severe SRV failure. This has led to extensive research on the potential beneficial effects of cell-based therapies on cardiac dysfunction. The underlying mechanism of SRV failure differs from those underlying LV failure. It is primarily a result of cardiomyocyte hypertrophy, resulting from a switch in energy metabolism and the rise of ROS. The delivery of MSCs happens to be feasible and safe. Pre-clinical studies show promising results concerning improved RV function upon MSC transplantation. MSCs exert their effects via a paracrine route rather than cellular engraftment and cardiac differentiation and secrete various cytokines, chemokines, and growth factors modulating inflammation, fibrosis, proliferation, and angiogenesis. In addition, increasing scientific evidence has shown that MSCs might modulate and decrease ROS in cardiac microenvironments. Therefore, MSCs might be a promising agent in resolving ROS-induced cardiomyocyte hypertrophy and reducing its effects on SRV failure.

Other cell-based therapies for HLHS patients other than MSCs could be C-kit$^+$ Cardiac Progenitor Cells (CPCs), Cardiosphere-Derived Cells (CDCs), or Umbilical Cord Blood (UCB)-derived MSCs. The therapeutic effects of these cells also seem to rely on the paracrine effects instead of differentiation and integration with the myocardium (2). CPCs are resident cardiac progenitor cells characterized by the expression of the c-kit receptor tyrosine kinase. These progenitor cells have been shown to improve LV function in animal models for acute and chronic myocardial ischemia (81). Their therapeutic efficiency does seem to be highly dependent on donor age and environmental factors, such as hypoxia; neonatal and hypoxia-conditioned CPCs might be the most promising CPC type. Regarding the pathophysiological conditions of cardiac cells in the HLHS heart, autologous CPCs might be an interesting cell source for HLHS eminently. The effectiveness of autologous CPC injection in HLHS patients is tested for safety and feasibility in the CHILD phase I/II trial (71). CDCs are heterogenous, self-assembling cell clusters from myocardial tissue cultured on a poly-D-lysine (82). Clinical studies using CDCs to improve LV function after myocardial infarction show mild functional improvements (83,84). Although this review primarily focused on bone-marrow-derived MSCs, UBC-derived MSCs have also been shown to increase LV ejection fraction and reduce heart failure symptoms in patients with stable heart failure (28). Ideally, the cellular origin of such therapies is a pre-screened allogeneic source since not every cell population has similar therapeutic effects. Having an allogeneic cell source evokes questions concerning immunological rejection.
Regarding rejection, allogeneic MSCs are a promising option because of their low immunogenicity *in vivo* and a higher potential of reducing ROS compared to autologous MSCs (68,85). There might be a higher risk of cell rejection upon allogeneic CPCs and CDCs transplantation. While the immunogenicity of CDCs and CPCs is less studied, animal studies with these cell therapies have shown a slight transient immunogenic response in response to allogeneic CDCs and no signs of immune rejection upon CPC transplantation (86). However, these studies were performed in animal models, which could have other human results. For example, humans injected intramyocardial with CDCs show the presence of donor-specific

antibodies against human leukocyte antigens, suggesting the rise of an immunogenic response (81).
Future trials using MSCs for RV functional improvements in HLHS patients could be optimized with hypoxic, pharmacological, or chemical pre-conditioning of the MSCs. Pre-conditioning of MSCs has been shown to improve regeneration in multiple tissues, such as the liver and intervertebral discs (87,88). Culturing MSCs in a glucose-depleted medium has been shown to augment their ability to repair an infarcted myocardium by increasing the expression of paracrine factors (89). Glucose depletion increased the expression of AKT, together with Insulin-like growth factor-1, to enhance cellular survival. In addition, these pre-conditioned MSCs also increased FGF-2, VEGF, and SDF-1α levels, which are associated with improved cardiac performance.
On the other hand, increased SDF-1α levels seem to upregulate the mitochondrial ROS (68). Also, treatment with High Density Lipoprotein (LDL) has been shown to protect MSCs from ROS-mediated oxidative stress, which is present in the diseased SRV in HLHS patients (90). Other ways to pre-condition MSCs include hypoxic preconditioning to prolong the survival time of MSCs in the ischemic area, mainly by activating HIF-1α-mediated pathways (91). Pharmacological MSC preconditioning can be targeted to modulate the inflammatory environment further and improve cardiac function. For example, IGF-1 preconditioning significantly improved inflammation by the upregulated secretion of pro-inflammatory cytokines and attenuated cardiac dysfunction (92). Other effective preconditioning techniques are outlined by Matta *et al.,* (91), and highlight the promising potential of cellular preconditioning to improve SRV function in HLHS patients. Finally, novel approaches regarding the genetic modification of MSCs could increase the secretion of the most beneficial cytokines, chemokines, and growth factors for SRV failure and ROS clearance. Since the transplantation of genetically modified cells cannot be clinically used, MSC-derived exosomes might have more clinical relevance. Pre-clinical models of myocardial infarction treated with preconditioned and enriched MSC-derived exosomes have demonstrated therapeutic effects for the treatment of cardiac diseases (93).

Regarding the timing and route of MSC administration, it may be essential to consider the potential harmful acute inflammatory environment upon cardiac surgery since this might influence the MSC regenerative potential (75). Therefore, intracoronary cell administration via intravenous catheterization would be preferred as this is less invasive and can be performed between Glenn and Fontan surgery when acute inflammation has diminished. Glenn and Fontan's surgeries are performed at around 6 and 18-36 months, respectively. The optimal timing of administration would be at the onset of developing cardiac remodeling events, which can appear within weeks to months, depending on the patient. MSC retention, and thus their paracrine therapeutic effect, may be transient due to poor retention in large animals (94). Therefore, multiple injections interspaced over several months might create more therapeutic potential. Another strategy could be engineered cellularized cardiac patches made of (biological) polymers, such as collagen, to improve cellular retention and therapeutic effects (91). The use of these cardiac patches in patients presenting LV post-ischemic myocardial scars showed significant improvement in heart functionality compared to cellular treatment alone (95).

In conclusion, this review summarized the mechanisms leading to SRV failure and the crucial roles of ROS. MSC therapy could potentially be of valuable interest in increasing SRV functionality and decreasing disease progression.

Conclusion

Increasing evidence supports the potential for MSC administration to combat SRV failure in pediatrics with HLHS. Pre-clinical studies in animal models for pulmonary artery hypertension have revealed that the paracrine effect of MSCs responsible for the therapeutic effects. The secreted molecules fulfill their function by secreting cardioprotective cytokines, chemokines, and growth factors. ROS secretion is thought to be crucial in the early stages of RV failure via stimulating cardiomyocyte hypertrophy, which is a promising target to prohibit RV failure. MSCs have shown also shown to modulate ROS levels by decreasing ROS secretion directly or reversing ROS-induced cellular damage. Recently established single-ventricle heart animal models might help confirm and identify the molecular mechanism of HLHS pathophysiology and the pathways targeted by the MSC secretome. As the field continues to evolve, the latter could potentially aid in improving stem cell therapy by targeting the signal cascades primarily involved in SRV failure, for example, via MSC-preconditioning. MSC therapy is a promising strategy to offer better longevity and quality of life for HLHS patients.

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