Review Article on Pediatric Heart

Cardiac tissue engineering for the treatment of hypoplastic left heart syndrome (HLHS)

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Abstract: Hypoplastic left heart syndrome (HLHS) is a deadly congenital heart disease that arises when the left ventricle and outflow tract fail to develop appropriately, inhibiting the adequate perfusion of the rest of the body. Historically, this disease has been treated via a series of surgeries that allows the heart to use a single ventricle. These surgeries are often a palliative measure, and heart transplantation is the only definitive therapy that exists for this condition. It has been hypothesized that stem cell-based regenerative therapies could have a role in promoting cardiac tissue regeneration in HLHS patients who are undergoing palliative surgery. Several clinical trials have demonstrated that introducing pluripotent cells into the heart is safe, feasible, and capable of improving right ventricular ejection fraction (RVEF). However, while these approaches show great promise, there is still room for development. There is a substantial body of pre-clinical work that is focused on generating increasingly large and complex pieces of cardiac tissue in the form of cardiac patches, with the idea that these could be used to rebuild and strengthen the heart in a robust and long-lasting manner. In total, stem cell-based therapies have much to offer when it comes to improving the treatment of HLHS.

Keywords: Tissue engineering; hypoplastic left heart syndrome (HLHS); stem cell clinical trial; congenital heart disease; heart failure

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Introduction

Congenital heart defects are defined as malformations of the heart or great vessels that occur *in utero*. Moderate to severe congenital heart defects occur in roughly 1.5 out of every 1,000 live births and impose a huge morbidity and mortality (1). Among these severe defects is hypoplastic left heart syndrome (HLHS), in which the left ventricle and corresponding outflow tract fail to develop appropriately. This condition is fatal if untreated and is typically palliated with a series of surgeries that utilize the single ventricle physiology to provide adequate tissue perfusion (2).

Palliative surgery typically occurs in three stages. Stage 1 surgery is a Norwood procedure typically completed in the neonatal period that establishes systemic blood flow from the right ventricle (RV) to the aorta and shunts blood to

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the pulmonary arteries. Stage 2 is partial cavo-pulmonary bypass in which the superior vena cava (SVC) is connected with the pulmonary arteries at 4–6 months of age. In stage 3, the inferior vena cava (IVC) is connected with the pulmonary arteries at age 18 months to 4 years, creating Fontan circulation (3,4).

Though the advent of these procedures has made survival possible at all, the long-term prognosis for these patients remains poor. One study of 244 HLHS patients born between 1998 and 2012 estimated that 63.5% of patients survived to 1 year of age, 58.6% to 5 years, 54.6% to 10 years, and 32.6% to 15 years (3). In some cases, patients are unable to tolerate one of the stages of the palliation process, and are reverted back to the prior stage and listed for heart transplant (5). It has been observed that outcomes for HLHS patient that require heart transplant are poor, with one study observing that 53% of these patients had survived at the 10-year time point (5). Patients who achieve full Fontan circulation often experience complications, including protein-losing enteropathy, arrhythmia, and pulmonary hypertension (6). Thus, there is a need to improve on the benefit conferred by surgical palliation procedures for HLHS.

It has been hypothesized that using stem cell-based regenerative techniques could help strengthen and remodel the under-developed heart. The principle underlying this approach is that introduced pluripotent cells can induce the self-regenerating capacity of the damaged/hypoplastic tissue via paracrine signaling rather than integrating within the tissue themselves (7,8). It should be noted that there has been some controversy around the regenerative capacity of adult cardiac tissue, such that the original studies showing that bone marrow-derived pluripotent cells could integrate into cardiac tissue and drive regeneration have been called into question (9). Nonetheless, there is preclinical and clinical data that suggest that pediatric cardiac tissue maintains some regenerative capacity through unknown mechanisms, such that stem cell-based approaches might be tractable in these settings (10). Here we will review the various clinical trials that have tested the hypothesis that pediatric heart tissue can be induced to regenerate, the limitations of these trials, and a brief overview of the novel approaches that are being developed in the pre-clinical setting.

Clinical trials for stem cell-based therapies for HLHS

The clinical trials that have been successfully completed in pediatric cardiac regeneration have used the basic method of introducing pluripotent cells into the tissue, through injection either directly into the heart wall or into the coronary arteries. The pluripotent cells have come from a variety of sources, including cardiac tissue, umbilical cord blood, and bone marrow-derived mesenchymal cells (11). These cellular sources are different in terms of ease of accessibility and constituent cell populations. The generation of "cardiosphere-derived cells" (CDCs) requires intraoperative sampling of cardiac tissue, and subsequent expansion of stem cell-like ($CD90^+CD105^+$vimentin$^+$) (12,13) cardiac cells. In contrast, umbilical cord blood-based therapy requires a prenatal diagnosis of HLHS but is more readily accessible than cardiac tissue at the time of birth. The umbilical cord blood is enriched for mononuclear cells, a heterogeneous population that includes stem cells (14). Bone marrow-derived mesenchymal cells are by far the easiest to acquire, especially as they can come from an allogeneic source and be used "off the shelf" in the case of products like Lomecel-B (15).

There are several safety concerns that are particular to pluripotent cells that needed to be addressed by the clinical trials. Because pluripotent cells maintain their reproductive functionality, there is the theoretical risk that they could start replicating in an uncontrolled manner and form tumors (16). Similarly, poor integration of new electrically conductive tissue into the existing structure has the potential to be a nidus for arrhythmia formation. Therefore, the clinical trials observed these particular adverse events as part of their safety profile.

The first phase I trial of this kind, "Transcoronary Infusion of Cardiac Progenitor Cells in Patients With Single Ventricle Physiology" (TICAP), utilized cultured cardiac tissue-derived cells (13). The stem cells were obtained by intraoperative right atrial myocardial biopsy during one of the palliation procedures, from which "cardiospheres" were expanded (12). Seven patients were assigned to receive intracoronary injection of CDCs via cardiac catheterization 4–5 weeks after the palliation surgery, while 7 received the standard palliation procedures. Inclusion in this trial required a diagnosis of HLHS with a plan for stages 2 and 3 palliation surgery within a month after the initial screening echocardiogram. Patients were excluded if they had concomitant renal or hepatic disease, cardiogenic shock, intractable arrhythmia, cancer, or an inability to complete the study protocol. The primary safety endpoint observed was cardiac death from arrhythmia or ischemia after infusion of the stem cells, while the secondary safety endpoint was

hospitalization for complications related to the procedure. The preliminary efficacy end points observed were right ventricular ejection fraction (RVEF) and heart failure status as defined by the New York University Pediatric Heart Failure Index (NYUPHFI). Overall, they found that the procedure was safe, with no participants exhibiting signs of ischemia, arrhythmia, or cardiac tumor formation as a result of the stem cell injection. There was an increase in RVEF of 8.0%±4.7% in the experimental group at 36 months, compared to 2.2%±4.3% in the controls (P=0.03) (17). The CDC recipients also had lower NYUPHFI scores at the 36-month time point, suggesting that the effects of the treatment were durable over time.

The phase II follow-up study to TICAP was "Cardiac Progenitor Cell Infusion to Treat Univentricular Heart Disease" (PERSEUS) (18). Patients were eligible for enrollment if they were <20 years old, had a diagnosis of HLHS, planning to undergo stages 2 and 3 palliation procedures, and had a starting RVEF <60%. Of 41 eligible patients, 23 patients were randomized to receive CDCs, while 18 controls received only the typical palliation procedures. Heart function was evaluated at 3 months, before which 1 patient from the control group was excluded for an ejection fraction (EF) >60%, and 6 patients from the experimental group were excluded for EF >60% (n=3), infective endocarditis (n=1), and withdrawal of consent (n=2). In total, 17 patients from each group were included in the analysis. At the 3-month time point, the controls were given the option to receive the CDCs, and all of them elected to do this. In the end, 34 patients received the treatment (17 in the initial experimental group, and 17 in the control group that was allowed to cross over) and were followed for 12 months with evaluation of heart function via cardiac MRI. As in the TICAP trial, none of the patients developed cardiac tumors. At the 3-month time point the CDC-treated group had a 6.4%±5.5% increase in RVEF, compared to 1.3%±3.7% in the controls (P=0.003). However, once the control group was allowed to have the "late" CDC infusion they too exhibited an increase in RVEF from 34.8%±7.4% at baseline to 38.8%±7.7%. At the 12-month mark, this increase in RVEF was durable, with an average increase of 6.4%±4.5% between the two treated groups. Notably, the effect size was diminished in the control recipients who received the infusion later, suggesting that the timing of the procedure is significant.

Another trial evaluated the safety of intramyocardial injection of harvested autologous umbilical cord blood-derived mononuclear cells (UCB-MNCs) (NCT01883076) (14). Preclinical studies had shown that this was a safe procedure (19) with potential to improve RV function (20). In this protocol, UCB-MNCs were harvested at birth from patients with antenatal diagnoses of HLHS. For inclusion, patients had to have appropriate collection and processing of umbilical cord blood, they had to be a candidate for the stage 2 surgical procedure, and the maternal serologies had to be negative for HIV, hepatitis B, and hepatitis C. The patients also had to be negative for an extensive set of exclusion criteria including cancer, infection, and significant cardiac or extracardiac comorbidities. 10 HLHS patients received intramyocardial injections of UCB-MNCs during the stage 2 palliation surgery. In this trial, it was demonstrated that the technique was feasible, with successful preparation of the umbilical cord mononuclear cells and minimal intraoperative complications (only one patient had an epicardial bleed that was addressed by over-sewing). There were also minimal cardiac-related adverse events, with no patients exhibiting ischemia, arrhythmia, or hemodynamic instability after receiving the cellular therapy. However, one patient had a peri-operative complication of necrotizing enterocolitis during the 30-day post-operative window, leading the study to be placed on clinical hold. There was no association found between this complication and the cellular therapy, and no protocol changes were recommended. In terms of preliminary findings around efficacy, there was no detectable change in RVEF at 6 months. This set of patients was later compared to a retrospective control cohort of patients who only received palliation surgery, and it was demonstrated that the surgery-only group had declines in RVEF at discharge and smaller body weights, relative to the UCB-MNC recipients, who remained stable over time (21). Though this is an imperfect comparison, it suggests that the UCB-MNC treatment conferred some clinical benefit relative to the surgical intervention alone.

"Allogeneic Human MEsenchymal Stem Cell Injection in Patients with Hypoplastic Left Heart Syndrome: An Open Label Pilot Study" (ELPIS) was a phase I trial that evaluated intramyocardial injections of allogeneic bone marrow-derived mesenchymal stem cells (MSCs) during the stage 2 palliation procedure (15,22). Patients were enrolled if they had a diagnosis of HLHS and were going to undergo a stage 2 palliation procedure. Exclusion criteria included: a concomitant restrictive/intact atrial septum, significant coronary artery sinusoids, arrhythmia, a requirement for mechanical circulatory support prior to surgery, penicillin or streptomycin allergy, serum positivity for HIV, hepatitis

B or hepatitis C, and inability to participate in follow-up. 10 patients who met these criteria were given Lomecel-B, a proprietary preparation of MSCs. Nine patients were evaluated at the 6-month time point, and 8 at the 12-month time point. None of the patients who were evaluated at the 1-year time point required transplantation, and there were no episodes of myocardial infarction, arrhythmia, stroke, or allergic reaction. RVEF was stable over the study period, with no increases or significant declines. This trial was distinct from the others that have been completed in that it utilized allogeneic stem cells. The advantage of this approach is that it does not rely on the foresight to save umbilical cord blood or require an invasive cardiac biopsy. The disadvantage is the potential that the donor cells will be immunogenic to the recipient and induce a pathologic inflammatory response.

There are several other pending trials in this field. "Cardiac Stem/Progenitor Cell Infusion in Univentricular Physiology" or APOLLON (NCT02781922), is the phase III trial for the methodology used in the TICAP and PERSEUS trials and is currently recruiting. The phase II follow-up to NCT01883076 evaluating the efficacy of intramyocardial injection of autologous UCB-MNCs has finished recruitment and is in progress. "Autologous Cardiac Stem Cell Injection in Patients with Hypoplastic Left Heart Syndrome" (CHILD) (NCT03406884) is a phase I/II study that proposes intramyocardial injection of c-kit + cardiac-derived progenitors. Another phase I trial (NCT03079401) is evaluating the efficacy of intraendocardial injection of allogeneic mesenchymal precursor cells (MPCs) in patients with HLHS, unbalanced atrioventricular canal, or borderline left heart. In addition to these trials, it is important to acknowledge the studies that have not come to fruition in addition to the positive studies in the field. There are two studies that have been terminated or completed with no results posted for reasons that have not been publicized. All identified studies are summarized in *Table 1*.

Overall, the completed clinical trials have demonstrated that using injected stem cells to promote cardiac repair is safe and can improve cardiac function. The biggest predicted risks—arrhythmia, ischemic events, cardiac tumor formation—have not come to pass, which suggests that the overall technique has promise. Furthermore, the TICAP and PERSEUS trials demonstrated reproducible increases in right ventricular functional status, and corresponding improvement in quality of life.

One purpose of these various trials is to establish which methodology is optimal for this application. There are two main points of variation between the trials: cell source and method of delivery. Between cardiac-derived cells, bone marrow-derived cells, and umbilical cord blood cells, the CDCs are the only ones that have clearly demonstrated improvement in RVEF, while others have demonstrated only stabilization/lack of decline to date. However, this analysis is complicated by the fact that the trials that used CDCs were also the only trials that administered the cells via catheterization 4–5 weeks after completion of palliation surgeries, while the UCB-MNC (NCT01883076) and MSCs (ELPIS) were administered intramyocardially during the second stage surgery. This makes it difficult to parse whether the efficacy observed in the TICAP and PERSEUS trials is primarily driven by the cellular source or the route. Notably, in the PERSEUS trial it was observed that the second group of patients that received the CDCs "late" had diminished improvement relative to those that got it "early"—suggesting that the timing of the cell administration relative to the palliation surgery does have some impact (18). Another insight that arose from the TICAP (17) trial is that there appears to be a relationship between the age of the recipient and the success of the procedure, such that younger patients are more likely to benefit. The outcomes of the pending trials will help clarify the "best practices" with regard to the cellular source, route of administration, and the timing of the procedure.

Limitations of the clinical trials

Despite the preliminary success of these approaches, the limitations must be acknowledged. The trials that have been completed have demonstrated improved function of the single ventricle in HLHS patients, but the underlying structural defect is still substantial. Long-term follow-up with patients who have received this therapy is required to determine if the degree of improvement is sufficient to stave off the need for transplantation indefinitely, or if this is another palliative method to stabilize patients for a transplant later in life. In addition, further follow-up needs to be done to determine whether these therapies have an impact on the downstream development of common complications such as pulmonary hypertension or protein-losing enteropathy.

It is unclear to what degree these injected cells either engraft in the tissue or maintain their ability to enhance

Table 1 Summary of stem cell based clinical trials for HLHS

Study (NIH identifier)	Cell type	Delivery method	Patient numbers	RVEF improvement (time point)	Phase (status)
TICAP (NCT01273857)	CDC	Intracoronary	Treatment: 7; control: 7; total: 14	Treatment: 8.0%±2.2%; control: 2.2%±4.3%; P=0.03 (36 months)	I (completed)
PERSEUS (NCT01829750)	CDC	Intracoronary	Treatment: 17; control (later crossover): 17; total: 34	Treatment: 6.4%±5.5%; control (pre-crossover): 1.3%±3.7%; P=0.003 (3 months)	II (completed)
APOLLON (NCT02781922)	CDC	Intracoronary	–	–	III (recruiting)
(NCT01883076)	UCB-MNC	Intramyocardial	Treatment: 10; total: 10	Treatment: 0%	I (completed)
(NCT03779711)	UCB-MNC	Intramyocardial	–	–	II (active, not recruiting)
(NCT04907526)	UCB-MNC	Intramyocardial	–	–	I (recruiting)*
CHILD (NCT03406884)	CDC	Intramyocardial	–	–	I/II (recruiting)
(NCT03079401)	MPC	Intraendocardial	–	–	I/II (active, not recruiting)*
ELPIS (NCT03525418, formerly NCT02398604)	MSC	Intramyocardial	Treatment: 8; total: 8	Treatment: 0%	I/II (completed)
(NCT03431480)	UCB-MNC	Intracoronary	–	–	I (completed, results not posted)
(NCT01445041)	UCB	–	–	–	I (terminated, results not posted)

*, signifies that the study population includes but is not exclusively HLHS patients. Trials were identified via NIH registry (https://www.clinicaltrials.gov/), with the identifiers listed in the first column. The type of cells, method of delivery, number of subjects who made it to the end of the follow-up period (regardless of initial enrollment), improvement in RVEF, and phase/status are listed for each study, as available. HLHS, hypoplastic left heart syndrome; NIH, National Institutes of Health; RVEF, right ventricular ejection fraction; TICAP, Transcoronary Infusion of Cardiac Progenitor Cells in Patients With Single Ventricle Physiology; CDC, cardiosphere-derived cell; PERSEUS, Cardiac Progenitor Cell Infusion to Treat Univentricular Heart Disease; APOLLON, Cardiac Stem/Progenitor Cell Infusion in Univentricular Physiology; UCB-MNC, umbilical cord blood-derived mononuclear cell; CHILD, Autologous Cardiac Stem Cell Injection in Patients with Hypoplastic Left Heart Syndrome; MPC, mesenchymal precursor cell; MSC, mesenchymal stem cell; ELPIS, Allogeneic Human Mesenchymal Stem Cell Injection in Patients with Hypoplastic Left Heart Syndrome: An Open-Label Pilot Study; UCB, umbilical cord blood.

growth and remodeling. Because the strain on the ventricle is constant in HLHS, the regenerative capacity would also have to be maintained long-term in order to preserve heart function, such that a steady state is reached between cardiac damage and regeneration. This raises the possibility that patients could require multiple doses of stem cell-based therapy throughout their lifetime. In the TICAP trial responses to therapy were observed up to 36 months after the treatment (17), which suggests that the response can be durable over time. However, data beyond this time point is not available. Further work would have to determine how many doses and over what timeline would be sufficient to preserve function.

Finally, it should be noted that the patient population is selected for HLHS patients who largely do not have other significant cardiac, hepatic, or renal comorbidities. This was presumably done to reduce confounding variables. Yet, this makes it difficult to generalize to those patients who have associated developmental disorders, such as those with heterotaxy, Turner syndrome, or Jacobsen syndrome (23).

Preclinical studies of cellular therapies for cardiac regeneration

To date, clinical trials have focused on the injection of suspensions of pluripotent cells into the cardiac tissue. A more "definitive" regenerative medicine solution to HLHS would involve driving the proper development of the hypoplastic left ventricle. However, the formation of such a large and complex structure is not within the realistic scope

of injections of pluripotent cells. Other approaches are currently being developed that move towards promoting the synthesis of more complex structures. Towards this end, the focus has been on developing cardiac patches, three-dimensional (3D) arrays of cells that can be implanted into damaged heart tissue. Though these studies are not explicitly geared towards the treatment of HLHS, there is great potential for the application of these technologies to this pathology.

The concept of "patching" damaged cardiac tissue with a composite of synthetic materials and pluripotent cells has been moved forward in the setting of adults with localized ischemic damage from myocardial infarction (24,25). In this approach, larger sections of material can be grafted on top of a region of cardiac tissue that needs support, in order to both provide mechanical support and promote regeneration in the local tissue.

The patch "material" can take the form of a scaffold seeded with cardiac cells that would provide the soluble factors necessary to drive regeneration from the endogenous cardiac cells. There is a wide variety of substrates that have been used for these scaffolds, ranging from purely synthetic polymers to decellularized biological tissue (26,27). Several pre-clinical models have looked at the utility of these type of cardiac patches in strengthening the RV. Sugiura *et al.* tested the utility of such patches for the repair of a defect in the RV outflow tract in athymic rats (28). The patches were made of polyglycolic acid (PGA) and poly (l-lactic-co-ε-caprolactone) copolymer (PCL) seeded with induced pluripotent cell-derived cardiac myocytes (iPS-CM). Notably, the patches were found to not contain the seeded cells at 4- and 8-week time points but were found to contain "nests" of alpha-actin + cardiac myocytes at 16 weeks, suggesting that the seeded cells had extravasated and promoted the growth of endogenous cells. A useful follow-up study would be to genetically mark and track the fate of the seeded cells to identify where (if anywhere) they traffic to from the patch, in order to gain insight into the mechanism of patch re-population.

Another group derived a composite polyurethane/small intestine submucosa patch that they seeded with urine-derived stem cells to repair a RV outflow tract defect in a rabbit model (29). Streeter *et al.* generated "personalized" patches—i.e., patches that are designed to leverage the specific characteristics of the patient's cardiac-derived progenitors (30). This group identified a key gene that was expressed by a subset of patient stem cells that allowed for enhanced growth on fibronectin (FN)-coated electrospun PCL patches that were then used to improve cardiac function in a rat model of right ventricular heart failure. These and other studies (31-33) demonstrate that composites of stem cells and synthetic scaffolds can be used to repair defects in the cardiac wall and restore some functionality.

Another approach to cardiac patching is to generate constructs made of fully-differentiated cardiac tissue, both with and without scaffolds. It has been demonstrated that the inclusion of a vascular component in these types of patches is critical for scaling up graft size and enhancing longevity (34). This is a more complex construction, as it requires *in vitro* differentiation of both electrically active myocardial tissue and the vasculature necessary to support it, without the precise mechanical and chemical cues that the cells would receive in normal development. In one method, human induced pluripotent stem cells (hiPSCs) are differentiated into cardiomyocytes, smooth muscle cells, and endothelial cells (35,36). These three cell lineages are then combined with a fibrin scaffold, enabling them to form an electrically active, contractile cardiac patch that can engraft into the heart in models of ischemia. Alternatively, others have combined fibroblasts, cardiomyocytes, and endothelial cells into discrete cardiac "spheroids", and then assembled these spheroids into cardiac patches (37). This has the advantage of more control over the distribution of the three cell types throughout the tissue.

Recent developments in 3D bioprinting have enhanced the construction of fully differentiated patches, as this technology allows for the precise placement of multiple cell types in relation to each other. In one study by Noor *et al.* (38), patient omental tissue was decellularized into a "personalized hydrogel", while the extracted cells were made into hiPSCs, then differentiated into cardiomyocytes and endothelial cells. These individual components were formulated into "bio-inks" that were then printed into pre-vascularized cardiac patches that were able to recapitulate some vascular and cardiac function *in vitro*. A similar approach was taken by Jang *et al.*, such that they constructed bio-inks out of decellularized porcine cardiac extracellular matrix (ECM), cardiac progenitor cells, and MSCs, and used patterned printing to generate pre-vascularized stem cell patches (39). Bioprinting techniques have even been applied to complex spheroids that contain multiple cell lineages. In one study, spheroids were generated from hiPSCs that had been differentiated into cardiomyocytes, a fibroblast cell line, and HUVECs (human umbilical vein endothelial cell line). The spheroids were then 3D-printed

into scaffold-free patches that were able to enhance tissue healing in a rat model of myocardial ischemia (40). Bioprinting allows for the precise construction of structures within patches, therein increasing the efficiency with which pre-vascularized cardiac patches can be made.

Conclusions

Stem-cell based therapies have great potential for the treatment of congenital heart diseases like HLHS. Clinical trials have demonstrated the feasibility and safety of introducing stem cells directly into the heart tissue. They have also demonstrated the potential to preserve and even improve the function of the single-ventricle physiology. However, it remains to be seen to what degree that these injections are a long-term solution to the problem of increased cardiac strain, as opposed to a stop-gap measure that improves quality of life and lengthens the time to transplant.

Ongoing work in the field of cardiac regeneration is focused on producing larger cellular constructs that can be incorporated into a failing heart. Towards this end, many kinds of cardiac patches are under development, utilizing a wide range of synthetic and biologic materials, and pluripotent cells from a variety of sources. The simplest cardiac patches consist of stem cells seeded onto a scaffold, which have the ability to promote regeneration in the endogenous tissue. More complex cardiac patches have been designed that have fully functional cardiac tissue, complete with vasculature. These patches are able to integrate into the damaged areas, and provide functional support, in addition to promoting growth in the surrounding areas. The construction of these fully differentiated cardiac patches is greatly aided by the advent of cellular 3D printing technologies. These emerging technologies have the potential to revolutionize the treatment of HLHS and other similar congenital heart defects.

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