Differences in extra-cellular matrix and myocyte homeostasis between
the neonatal right ventricle in hypoplastic left heart syndrome
and truncus arteriosus

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Abstract

Objective: The right ventricle in hypoplastic left heart syndrome (HLHS) works at systemic pressure and large volume loading before and after first stage palliation. There is a paucity of information regarding the intrinsic characteristics of the right ventricle in HLHS. We studied extra-cellular matrix composition, myocyte homeostasis and gene expression in right ventricular biopsies obtained from patients with HLHS undergoing neonatal first stage palliation and from patients undergoing neonatal truncus arteriosus repair.

Methods: Tissue was evaluated using histological and real-time PCR techniques using the truncus group as a comparative group. Mean difference in outcomes between the HLHS and truncus groups was estimated using linear regression models in unadjusted and age-adjusted analyses.

Results: Markers of cell proliferation, apoptosis and fibronectin were significantly higher in the right ventricular myocardium of patients with hypoplastic left heart syndrome compared to truncus arteriosus. Type I collagen content and NKX2.5 expression were significantly lower in HLHS than the truncus group.

Conclusion: The neonatal right ventricle in HLHS demonstrates a number of intrinsic differences compared to the right ventricle in truncus arteriosus including relative immaturity of the extra-cellular matrix, inappropriately low transcription factor expression and increased myocyte apoptosis.

Keywords: Hypoplastic left heart syndrome; Right ventricle; Myocardium; Extra-cellular matrix

1. Introduction

The right ventricle (RV) in hypoplastic left heart syndrome (HLHS) works at systemic pressure and increased volume loading before and after stage I palliation. There is growing evidence that the right ventricle of palliated HLHS patients has reduced function compared to other univentricular hearts with similar parallel circulations [1–3]. Despite innovations in surgical techniques and intensive care [4–6], HLHS patients experience a more precarious course and persistent risk of early death than other palliated complex cardiac anomalies.

Little is known regarding the intrinsic structure of the right ventricular myocardium in HLHS. Analysis of archived HLHS specimens has identified reduced cardiac extra-cellular matrix collagen content that might contribute to myocyte slippage and suboptimal ventricular function [7]. Changes in myocyte homeostasis characterised by increased apoptosis and progressive net myocyte loss are known to contribute to ventricular dysfunction in both ischaemic and non-ischaemic heart disease [8,9]. Differing myocardial composition and homeostasis in HLHS might affect the right ventricle’s ability to adapt to the obligatory volume loading, increased wall stress and changes in coronary perfusion that accompany stage I palliation.

The modification of the originally described Norwood procedure to include a restrictive RV to PA conduit has also allowed access to right ventricular biopsies. The right ventricle in neonates with truncus arteriosus works under similar workloads with parallel circulations and large volume loading. Neonatal repair of truncus arteriosus is the only
procedure that provides access to RV samples both of similar age and where the loading conditions of the RV are similar to the un-palliated HLHS.

The purpose of this cross-sectional comparative study was to ascertain whether the intrinsic properties of the right ventricular myocardium differ between patients with HLHS and truncus arteriosus (TA) in order to improve understanding of the response of the right ventricle to surgery and increased load.

2. Methods

2.1. Patient data

Right ventricular myocardial biopsies were obtained from infants undergoing stage I Norwood procedure for typical HLHS (n = 14) or neonatal repair of truncus arteriosus (n = 7). Diagnosis of hypoplastic left heart syndrome was made by two-dimensional echocardiography and required underdevelopment of the left heart with significant hypoplasia of the left ventricle including atresia, stenosis or hypoplasia of the aortic or mitral valves, hypoplasia of the ascending aorta and aortic arch with duct-dependent systemic circulation and retrograde flow in the aortic arch. Patients with unbalanced atroventricular septal defect or univentricular hearts with subaortic stenosis requiring Damus—Kaye—Stansel connection and shunt palliation in the neonatal period were excluded. Six patients with truncus arteriosus were of type 1 variant and one type 4. Median age at operation for HLHS and TA patients was 3.0 days (range, 2—8) and 14.0 days (range, 5—44), respectively. All patients were born after 38 weeks of gestation. One patient in the HLHS group had gut malrotation, which required later correction. Two truncus arteriosus patients had 22q deletion and another had DiGeorge syndrome. The study was approved by the Royal Children’s Hospital Ethics in Human Research Committee and parents gave informed consent (Table 1).

2.2. Operative details

All stage I reconstruction procedures for HLHS were performed using right ventricle to pulmonary artery conduits as the source of pulmonary blood flow. Norwood operations were conducted using continuous cardiopulmonary bypass at a systemic temperature of 25 °C. A 3.5 mm diameter polytetrafluoroethylene graft was anastomosed end-to-side to the innominate artery and a cannula inserted at this level to maintain systemic perfusion during this phase was maintained via separate 2 mm and 4 mm diameter olive-tipped cannulae snared within the ascending and descending aorta, respectively to permit arch reconstruction with a beating heart. Repair of truncus arteriosus was carried out in the first month of life in keeping with our institutional practice, using aortobicaval cardiopulmonary bypass at 32 °C. Intermittent antegrade cold blood cardioplegia was used in both groups; after giving half of the induction dose of cardioplegia at 32 °C, the remainder together with further maintenance doses at 20 min intervals was given at 24 °C. Thus the myocardium in both groups was unloaded and arrested at equivalent temperatures.

Transmural cylindrical cores of tissue were obtained from equivalent regions of the right ventricular free wall selected for ventriculotomy, below the pulmonary valve and adjacent to the left anterior descending artery. Samples were assigned a code to blind their origin and snap frozen in liquid nitrogen to optimally preserve immunogenicity until analysis.

2.3. Immunohistochemistry

Sections of 5 μm from HLHS and TA tissue were mounted side-by-side on silane slides and fixed with 4% paraformaldehyde. The sections underwent treatment for antigen retrieval and were then incubated in a blocking solution to reduce non-specific binding. For each investigational target, a further 10 sections were prepared from the mesocardial layer of each sample. In all cases sections were double-stained with antibodies of different species origin using the second stain as an internal control to verify adequate preparation and staining. If the accompanying target did not stain correctly the run was repeated.

Primary antibodies were: ACAM/N-cadherin, α-sarcomeric actin, connexin-43 (all Sigma—Aldrich, Castle Hill, Australia), caspase-3 active (R&D Systems, Minneapolis, USA), collagen types I and III (Southern Biotech, Birmingham, USA), desmin, fibronectin (both DakoCytomation, UK), NKX2.5 (Santa Cruz Biotech, Santa Cruz, USA), phosphorylated histone H3 (Upstate, Charlottesville, USA) and human troponin T (Chemicon, Temecula, USA). Secondary antibodies conjugated to the fluorophores Alexa Fluor 488 or 594 (Becton Dickinson) were used. Co-localisation of cardiac heavy chain myosin or troponin T with other markers of interest was used to verify their myocytic disposition.

2.4. DNA-strand breaks in myocytes

Myocyte apoptosis was also investigated by labelling nuclear DNA-strand breaks with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) and counterstaining nuclei with 4’,6-diamidino-2-phenylindole. The total number of cardiomyocyte nuclei in 10 random high-power fields was identified, and the percentage of these containing TUNEL-labelled DNA breaks calculated.
2.5. Image capture and analysis

Slides were evaluated using fluorescent inverted (Olympus IX70, Olympus Corp., Tokyo, Japan) and laser scanning confocal microscopy (Leica TCS SP2 SE, Leica Microsystems GmbH, Wetzlar, Germany) with proprietary software (Spot version 3.4.2, Diagnostic Instruments Inc., Sterling Heights, USA) without gamma correction. Background levels of autofluorescence on unstained sections and those incubated with secondary antibodies only were used as controls. Once established for each target of investigation, microscope settings were retained throughout the series. Ten random fields were recorded from each section for later analysis. The microscope operator was blinded to the origin of the sections.

Monochromatic image content was evaluated using an automated counting program and pre-specified morphometric criteria (ImagePro Plus, Media Cybernetics, Silver-spring, USA). Mean intensity per microscope field was also recorded as in some circumstances this measurement better represented tissue staining. To avoid overestimation, areas immediately adjacent to blood vessels were excluded from assessment. False colours were later assigned to aid recognition upon merging images of the multiply-stained sections.

2.6. Real-time polymerase chain reaction

Total RNA was extracted from samples of right ventricular myocardium from both patient groups and reverse transcribed into cDNA. Intron-spanning primer sequences were selected for β2 microglobulin and NKX2.5; primer sequences are available in the data supplement. Real-time polymerase chain reaction (PCR) assays were carried out in triplicate using SYBR Green Master Mix (Sigma–Aldrich). Melting curve analysis was performed after the final PCR cycle to check for the presence of non-specific PCR products and primer dimers. We analysed relative differences in 2^−ΔΔCT values after normalising data using β2 microglobulin as a housekeeping gene.

### Table 2

Table 2 shows the results of image analysis for each of the variables, displaying mean count, mean intensity or percentage TUNEL positive nuclei per random microscope field.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Unadjusted</th>
<th>Adjusted</th>
<th>95% confidence intervals</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HLHS mean (SD)</td>
<td>Truncus mean (SD)</td>
<td>Mean difference</td>
<td>Mean difference</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>C: 65.8 (24.4)</td>
<td>34.8 (19.3)</td>
<td>30.9</td>
<td>19.0</td>
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<tr>
<td></td>
<td>I: 36.3 (6.8)</td>
<td>23.4 (3.1)</td>
<td>12.9</td>
<td>11.8</td>
</tr>
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<td>TUNEL %</td>
<td>C: 1.43 (0.36)</td>
<td>0.67 (0.19)</td>
<td>0.76</td>
<td>0.70</td>
</tr>
<tr>
<td>PH3</td>
<td>C: 103.3 (33.6)</td>
<td>77.8 (26.5)</td>
<td>25.4</td>
<td>46.6</td>
</tr>
<tr>
<td></td>
<td>I: 39.3 (13.1)</td>
<td>30.7 (7.3)</td>
<td>8.7</td>
<td>6.2</td>
</tr>
<tr>
<td>Collagen I</td>
<td>C: 165.1 (21.2)</td>
<td>218.7 (15.5)</td>
<td>−53.7</td>
<td>−45.5</td>
</tr>
<tr>
<td></td>
<td>I: 40.7 (3.0)</td>
<td>40.8 (4.4)</td>
<td>−0.1</td>
<td>−2.3</td>
</tr>
<tr>
<td>Collagen III</td>
<td>C: 41.3 (15.0)</td>
<td>70.8 (32.6)</td>
<td>−29.4</td>
<td>−19.3</td>
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<tr>
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<td>I: 31.6 (3.4)</td>
<td>34.4 (4.9)</td>
<td>−2.8</td>
<td>0.7</td>
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<tr>
<td>Fibronectin</td>
<td>C: 417.8 (58.6)</td>
<td>303.5 (120.5)</td>
<td>114.3</td>
<td>159.5</td>
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<tr>
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<td>I: 50.8 (3.2)</td>
<td>35.3 (6.6)</td>
<td>15.56</td>
<td>17.2</td>
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<td>NKX2.5</td>
<td>C: 118.2 (14.9)</td>
<td>204.7 (13.8)</td>
<td>−86.5</td>
<td>−62.5</td>
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<td></td>
<td>I: 26.8 (4.3)</td>
<td>35.2 (4.7)</td>
<td>−9.4</td>
<td>−7.9</td>
</tr>
</tbody>
</table>

I: microscope field’s mean intensity; C: automated count for discrete/nuclear activity; PH3: phospho-histone H3; *: where bootstrap method used, p value not shown; 95% confidence intervals not containing zero imply p < 0.05.

3. Data analysis

Mean scores of intensity and count derived from histological data were calculated for each patient. These mean scores were used as the observations in our analyses to circumvent issues with analysing correlated data [10]. The distribution of the outcome variables was examined using normal probability plots. Mean and standard deviations are presented for the outcomes separately for each group. Linear regression was used to compare the mean outcome between the HLHS and truncus groups in unadjusted analyses and analyses that were adjusted for age as a linear effect. Partial residual plots did not suggest that the effect of age was non-linear for any of the 14 outcomes. There was little overlap in age between the comparison groups and the appropriateness of using linear regression to adjust for age relies on the relationship between outcome and age being the same in each comparison group. In order to assess this, scatter plots were drawn. Because some of the outcomes were not normally distributed and the sample size was small, bias corrected accelerated (BCA) bootstrap confidence intervals [11] were constructed to validate the assumptions underlying the confidence intervals from the linear regressions. The non-parametric bootstrap algorithm was used to construct 2000 bootstrap datasets for each analysis. Where the bootstrap intervals were similar to those from the main analyses the latter results are presented. Where they were considered to be different the bootstrap intervals are presented. All data were analysed using Stata 9.0 (StataCorp, College Station, Texas, USA).

4. Results

Table 2 shows the results of image analysis for each of the variables, displaying mean count, mean intensity or percentage TUNEL positive nuclei per random microscope field.
4.1. Cell replication and apoptosis

Cell replication, as indicated by the presence of phosphohistone-H3, was significantly higher in the HLHS group compared to TA (mean count 103.3 vs 77.8; age-adjusted \( p = 0.03 \)) as depicted in Fig. 1A–C. To investigate myocyte apoptosis in HLHS, separate heart sections were stained for caspase-3 active or TUNEL. Apoptosis was assessed by staining for caspase-3 active, a key apoptotic mediator and member of a multigene family of cysteine proteases that cleave key cellular proteins, leading to the apoptotic demise of cells. Mean intensity of caspase-3 active staining in the HLHS group was significantly higher compared to TA (mean intensity 36.3 vs 23.4; age-adjusted \( p = 0.007 \), Fig. 2). The frequency of myocyte apoptosis in the truncus...
arteriosus group as demonstrated by TUNEL staining was 0.67% of cardiac nuclei. In contrast, the frequency of myocyte apoptosis in HLHS tissue was 1.43%, twice as high as in TA (age-adjusted \( p = 0.004 \)) (Fig. 3A and B). These data demonstrate that myocyte apoptosis is higher in HLHS than in TA.

4.2. Extra-cellular matrix

Type I collagen content was significantly lower in HLHS compared to TA, (mean count 165.1 vs 218.7; BCA 95% confidence intervals –76.82 to –27.77, Figs. 1A, 1B and 4). This was accompanied by increased fibronectin content in HLHS (mean intensity 50.8 vs 35.3; age-adjusted \( p < 0.001 \)) as shown in Fig. 5. There were no significant differences in type III collagen or laminin.

4.3. Cardiac transcription factors

Semi-quantitative indirect immunofluorescent staining for the cellular expression of NKX2.5 was significantly lower in HLHS compared to TA (mean intensity 26.8 vs 35.2; age-adjusted \( p = 0.02 \)) as shown in Fig. 6A. Real-time PCR showed that NKX2.5 expression was significantly lower in HLHS compared to TA (mean relative concentration 0.62 vs 0.98; age-adjusted \( p = 0.04 \)) Fig. 6B and C.

Fig. 4. Scatter plot of type 1 collagen count in the right ventricle of patients with hypoplastic left heart syndrome and truncus arteriosus against age in days. HLHS outcomes are displayed as round dots and solid lines and TA with triangles and dashed lines, respectively.

![Fig. 4](image)

Fig. 5. Scatter plot of fibronectin staining intensity in the right ventricle of patients with hypoplastic left heart syndrome and truncus arteriosus against age in days. HLHS outcomes are displayed as round dots and solid lines and TA with triangles and dashed lines, respectively.

![Fig. 5](image)

Fig. 6. Results of NKX2.5 studies. (A) Scatter plot of NKX2.5 immunofluorescent staining intensity in the right ventricle of patients with hypoplastic left heart syndrome and truncus arteriosus against age in days. HLHS outcomes are displayed as round dots and solid lines and TA with triangles and dashed lines, respectively. (B) NKX2.5 expression as determined by real-time PCR (mean ± SEM) by group, unadjusted for age. *\( p = 0.001 \). (C) NKX2.5 expression determined by real-time PCR against age, with linear regression lines (truncus group boxes with dashed line, HLHS circles and solid line). Regression analysis shows significant inter-group differences for both unadjusted (\( p = 0.001, \) B) and age-adjusted analyses (\( p = 0.04, \) C).
increased expression of phosphohistone-H3 and caspase-3 replication and apoptosis in the HLHS group, as manifest by indicative of a chain of appropriate adaptive responses [20].

5. Discussion

Outcomes for staged surgical reconstruction for HLHS have improved [12,13], however patients continue to experience impaired systemic cardiac output, RV dysfunction and tricuspid regurgitation, leading to increased risks of morbidity and mortality. Whilst there is a paucity of knowledge concerning the myocardial composition of the right ventricle in HLHS, imaging studies have demonstrated impaired right ventricular function in patients with HLHS compared to other single-ventricle physiologies [2,3,14]. In this study, we have shown the composition of the right ventricular myocardium in HLHS to differ significantly compared to truncus arteriosus despite similar loading conditions even when mitigating for age.

5.1. Cardiac development and transcription factors

During fetal development, myocardial growth is coordinated by transcription factors including NKX2.5 which orchestrate developmental gene expression, cardiac-lineage determination and the formation of the multi-chambered heart [15]. Myocardial NKX2.5 expression normally undergoes a temporal decline from embryogenesis onwards, remaining at low levels postnatally where it assists in maintaining cardiac phenotype and facilitates responses to changing functional demands such as pressure overload [16,17].

Knock-out murine models have highlighted the importance of NKX2.5 in normal structural development [18], however similar mutations are relatively uncommon in clinically-pertinent structural phenotypes [19], which are likely to originate from interactions between multiple genetic and environmental influences. In this study, we found NKX2.5 expression to be significantly lower in the right ventricular myocardium of HLHS patients compared to those with TA, even when adjusting for age and despite similar right ventricular loading conditions. These differences are contrary to predicted chronological changes where higher levels of NKX2.5 would be expected in the younger HLHS group. It is therefore plausible that NKX2.5 expression either makes a positive contribution to a dynamic ventricular phenotype including adaptation to loading conditions, or that it is widely indicative of a chain of appropriate adaptive responses [20].

5.2. Cardiomyocyte homeostasis

The transition from late intra-uterine to neonatal life is normally accompanied by an increase in myocardial mass achieved principally by cellular hypertrophy with simultaneous cardiomyocyte cycling and renewal to aid adaptation to changes in hemodynamic workload [21]. Increased cell replication and apoptosis in the HLHS group, as manifest by increased expression of phosphohistone-H3 and caspase-3 active together with TUNEL staining may reflect attempts at adaptation in response to increased loading conditions simultaneous to ongoing myocyte loss. These findings, when considered with the reduced expression of NKX2.5 observed in the HLHS group suggests a different myocardial phenotype compared to the truncus arteriosus group. As alluded to above, reduced levels of NKX2.5 may diminish recruitment and proliferation of new, and maintenance of existing cardiomyocytes or inhibit rescue from apoptosis, a phenomenon previously observed in the development of the cardiac conduction system of NKX2.5 knock-out mice [22].

5.3. Extra-cellular matrix

The extra-cellular matrix provides structural support to the myocardial interstitium, maintaining myocyte alignment, minimising slippage and permitting interdigitation throughout the cardiac cycle. The heart’s principal extra-cellular matrix components are collagen types I and III and fibronectin. Typically extra-cellular matrix in the late embryonic period exhibits a shift in the fibronectin:collagen ratio in favour of collagen which serves to increase tissue robustness [23], a phenomenon which also usually occurs in right ventricular hypertrophy induced by pressure overload [24]. In this work, type I collagen content was found to be significantly lower in the mesocardium of HLHS compared to TA and accompanied by increased expression of fibronectin in HLHS. The reduced content of type I collagen and increased fibronectin in the HLHS group may represent an inability to make such a switch or relative immaturity and may lead to greater ventricular compliance.

5.4. Implications

The findings of this novel work support the notion that the neonatal systemic right ventricle in HLHS may be less able to adapt to increased loading conditions than the one in truncus arteriosus. In patients undergoing surgical reconstruction for HLHS, suboptimal right ventricular geometric and myocardial characteristics are compounded by a 40% increase in the hydraulic cost of work associated with the loss of a biventricular heart [25]. This may contribute to the more precarious course of these infants and supports the rationale for pharmacological or mechanical off-loading following stage I procedures.

5.5. Limitations

In view of the limited quantities of tissue available, we elected to use immunofluorescent histological techniques and real-time PCR to investigate several targets of interest. With immunofluorescence, quantification of the various markers does not necessarily infer activity or efficacy of the gene products themselves where applicable nor their possible post-translational modification. We did not attempt to identify and correlate preoperative risk factors nor outcomes to histological findings between groups.

An important limitation in this study is the differing maturity of the right ventricular myocardium in the two patient groups. We chose neonatal truncus arteriosus patients as a group for comparison because the right
ventricle in neonatal truncus patients also works under increased pressure and volume loading conditions that would be comparable to the right ventricle in hypoplastic left heart syndrome. In a study of this type, it is ideal to have age-matched subjects. However, in our institution we do not perform any other surgery in the first week of life that involves the routine removal of right ventricular myocardium. Similarly it is not possible to obtain myocardium from fresh, structurally-normal neonatal hearts in Australia for ethical reasons and TA was used in preference to other neonates with right to left shunts who lack significant volume loading.

The precise intra-operative timing of the biopsies between the two groups differed slightly but significant structural changes are unlikely within this timeframe. All study patients were born at or after 38 weeks gestation so prematurity should not be a confounder. There were fewer truncus samples than HLHS. Differences in age between the two groups were unavoidable. Scatter plots of outcome against age did not indicate a similar relationship for the HLHS and truncus groups for caspase-3 intensity, NKX2.5 intensity and type I collagen count and the effectiveness of age adjustment may be questioned here. The scatter plots for PH3 count, TUNEL and fibronectin intensity, however, did suggest the use of linear regression for age adjustment was appropriate and, overall, the pattern of results indicates morphological trends not explained by expected age-related changes alone.

6. Conclusion

This study has demonstrated a number of intrinsic differences between right ventricular myocardium in patients with HLHS and truncus arteriosus. Differing myocardial composition and myocyte homeostasis might affect the ability of the right ventricle to adapt to the obligatory volume loading, increased wall stress and changes in coronary perfusion that accompany stage I operations and resulting parallel circulation. Improved understanding of the composition of the right ventricle in hypoplastic left heart syndrome may help inform future treatment strategies in this group of patients.

References