Impaired Mitochondrial Biogenesis Precedes Heart Failure in Right Ventricular Hypertrophy in Congenital Heart Disease

Georgios Karamanlidis, PhD; Victor Bautista-Hernandez, MD, PhD; Francis Fynn-Thompson, MD; Pedro del Nido, MD; Rong Tian, MD, PhD

Background—The outcome of the surgical repair in congenital heart disease correlates with the degree of myocardial damage. In this study, we determined whether mitochondrial DNA depletion is a sensitive marker of right ventricular (RV) damage and whether impaired mitochondrial DNA (mtDNA) replication contributes to the transition from compensated hypertrophy to failure.

Methods and Results—RV samples obtained from 31 patients undergoing cardiac surgery were compared with 5 RV samples from nonfailing hearts (control). Patients were divided into compensated hypertrophy and failure groups, based on preoperative echocardiography, catheterization, and/or MRI data. Mitochondrial enzyme activities (citrate synthase and succinate dehydrogenase) were maintained during hypertrophy and decreased by \( \approx 40\% \) \( (P<0.05 \) versus control) at the stage of failure. In contrast, mtDNA content was progressively decreased in the hypertrophied RV through failure (by 28±8% and 67±11%, respectively, \( P<0.05 \) for both), whereas mtDNA-encoded gene expression was sustained by increased transcriptional activity during compensated hypertrophy but not in failure. Mitochondrial DNA depletion was attributed to reduced mtDNA replication in both hypertrophied and failing RV, and it was independent of PGC-1 downregulation but was accompanied by reduced expression of proteins constituting the mtDNA replication fork. Decreased mtDNA content in compensated hypertrophy was also associated with pathological changes of mitochondria ultrastructure.

Conclusions—Impaired mtDNA replication causes early and progressive depletion of mtDNA in the RV of the patients with congenital heart disease during the transition from hypertrophy to failure. Decreased mtDNA content probably is a sensitive marker of mitochondrial injury in this patient population. (Circ Heart Fail. 2011;4:707-713.)

Key Words: mtDNA ■ mitochondrial biogenesis ■ PGC-1 ■ congenital heart disease ■ RV failure

Patients with tetralogy of Fallot, pulmonary atresia, truncus arteriosus, hypoplastic left heart syndrome, and other congenital heart defects, frequently have chronic consequences of a volume- and/or pressure-overloaded right ventricle (RV). The adaptive mechanisms of the RV to compensate for the hemodynamic overload may lead to hypertrophy and ultimately, failure.\(^1\) The wide variability in clinical status, extent of RV dilatation, and dysfunction at the time of presentation for surgical intervention has resulted in disparate results after surgical repair.\(^2,3\) Although there is a group of patients that responds favorably to surgical repair, there is also a large subgroup that does not respond favorably. Clinical studies evaluating recovery of RV function after pulmonary valve insertion have indicated that despite elimination of the hemodynamic overload, the RV function, and, moreover, the prognosis of these patients does not necessarily normalize.\(^4,5\) Therefore, it is critical to intervene on those patients before irreversible RV myocardial damage occurs, and the appropriate timing requires further understanding of the cellular and molecular mechanisms underlying the myocardial damage caused by the hemodynamic overload in this population.\(^6,7\)

Clinical Perspective on p 713

Accumulating evidence has suggested that mitochondrial dysfunction plays a critical role in the development of heart failure.\(^8\) We have previously reported that mitochondrial mass and DNA (mtDNA) content was decreased in the left ventricle in end-stage human failing hearts, suggesting impaired mitochondrial biogenesis.\(^9\) In the present study, we took advantage of the unique opportunity to determine the relationship of RV function and the alterations of mitochondrial biogenesis in children with congenital heart disease (CHD) undergoing surgical repair or transplant. Analysis of the RV tissue from these patients allowed us to assess the
mitochondrial mass, enzyme activities, gene expression, mtDNA content, and mtDNA replication in a broad spectrum of cardiac pathologies during the transition from cardiac hypertrophy to failure. We found that impaired mtDNA replication and depletion of mtDNA were early events in RV hypertrophy, preceding the clinical diagnosis of heart failure. Mitochondrial DNA depletion also paralleled the pathologic changes of mitochondrial ultrastructure suggesting that patients with lower mtDNA content are at a greater risk of heart failure, and thus an early surgical relief may improve the outcomes of these patients.

Methods

Study Population

Thirty-one RV samples were collected from discarded myocardial tissue of patients undergoing cardiac surgery at Children’s Hospital Boston. Cardiac tissue was obtained intraoperatively and stored immediately in liquid nitrogen or fixed for electron microscopy examination. Five nonfailing samples were obtained from the RV wall of donor hearts with no history or macroscopic or laboratory signs of cardiac diseases. This study was approved by Boston Children’s Hospital Institutional Review Board.

Clinical information as well as preoperative and postoperative echocardiography, MRIs, and the clinical diagnosis of heart failure were retrospectively reviewed. RV ejection fraction (EF) calculated from the echocardiography and MRI was within the normal range in the hypertrophy group (n=25), whereas RV failure group showed diminished RV EF (<30%; n=6). In addition, the presence of an increased RV mass, exuberant muscle bundles, and a thick RV wall were considered indicators of hypertrophy. The presence of RV dilatation and the clinical diagnosis of RV failure were used to include patients in the heart failure group. Patients with (suspected) mitochondrial disease were excluded to focus the study the mitochondrial pathology caused by the cardiac stress due to congenital heart malformation.

Biochemical Assays

Citrate synthase (CS) and succinate dehydrogenase (SDH) enzyme activities were measured in tissue homogenate as previously described.10,11 Total RNA was isolated from frozen RV tissue, using the RNeasy Kit (Qiagen). Real-time PCR was performed using SYBR green (Bio-Rad), and the results of each gene were normalized to 18S rRNA levels. The primers are described in online-only Data Supplement Table I. The mtDNA content was measured in a total cell lysate or isolated DNA using the DNeasy kit (Qiagen), by real-time PCR, using primers amplifying the Cytochrome C Oxidase subunit I (COI) region, and it was normalized to nDNA (18S) or the TCA cycle, SDH, was also decreased only in RV failure by 35% (Figure 1B). The mtDNA copy number has been previously used as a widely marker of mitochondrial mass.14,15 The CS enzyme activity in whole tissue extracts was preserved in RVH, whereas in the RV failure it was reduced by 46% (Figure 1A). Similarly, the activity of another key enzyme of the TCA cycle, SDH, was also decreased only in RV failure by 35% (Figure 1B). The mtDNA copy number has been previously used as an indicator of mitochondrial biogenesis.16,17

Electron Microscopy

Mitochondrial ultrastructure was studied in freshly collected RV specimens by electron microscopy. Samples were dissected in 1- to 2-mm3 sections and immediately fixed with 2% glutaraldehyde, postfixed with 1% osmium tetroxide, and embedded in epon resin. For each sample, 10 randomly chosen fields at the magnification of ×5000 were used for the quantification of the mitochondrial number. To estimate the mitochondrial cristae density, a computerized point grid was digitally layered over the micrographic images at ×25 000 magnification. The densities of dots on the point grid, as well as the spacing of selected sections, were designed to result in 960 total point probes overlying each image. To estimate the mitochondria size distribution, the longest mitochondrial dimension was measured in randomly chosen micrographs at ×25 000 magnification. At least 10 images were analyzed for each sample in a blinded fashion.

Table. Patient Characteristics and Clinical Data

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<th>Failure</th>
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<td>6</td>
</tr>
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<td>2</td>
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<tr>
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</tr>
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<td>57.9 (2.1)</td>
<td>20 (2.6)</td>
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</table>

RV indicates right ventricle; EF, ejection fraction; TOF, tetralogy of Fallot; TA, truncus arteriosus; DORV, double-outlet RV; DCRV, double-chambered RV; PS/PA, pulmonary stenosis/atrial; HLHS, hypoplastic left heart syndrome; and AVC/VSD, atriointerventricular canal/ventricular septal defect.

Statistical Analysis

Citrates synthase and succinate dehydrogenase data were expressed as medians in scatterplots, whereas the age of the patients is given as median and minimum to maximum (min-max). The rest of the data are shown as mean of the fold changes ±SEM. Comparisons among groups were analyzed by Mann-Whitney test and regressions analysis by Spearman rank correlation. A value of P≤0.05 was considered significant (2-tailed). All analyses were performed using GraphPad Prism 5.0.

Results

General Clinical Characteristics

The diagnoses of the patients in the study population are described in the Table. Among the 31 patients undergoing surgery, 25 were classified in the RV hypertrophy (RVH) group and 6 in the RV failure group. The group with RVH consists of pressure- or volume-overload patients as primary diagnosis, whereas the RV-failure group consists of patients with hypoplastic left heart syndrome (HLHS), which causes RV pressure overload. Because of the severity of this disease, this is the only group of patients in our study that was presented with heart failure at such an early stage of life.

Progressive Depletion of mtDNA During the Transition From Cardiac Hypertrophy to Failure

CS is a key enzyme in the Krebs cycle, and its activity is a widely used marker of mitochondrial mass.14,15 The CS enzyme activity in whole tissue extracts was preserved in RVH, whereas in the RV failure it was reduced by 46% (Figure 1A). Similarly, the activity of another key enzyme of the TCA cycle, SDH, was also decreased only in RV failure by 35% (Figure 1B). The mtDNA copy number has been previously used as an indicator of mitochondrial biogenesis.16,17

Interestingly, in our study, the mtDNA content normalized to
total tissue protein was progressively decreased in RVH and RV failure (Figure 1C). Similar significant decrease was also observed when mtDNA was normalized to nDNA (nDNA, Figure 1D) and there was no change in the nDNA copy number (Figure 1E). Despite the decrease in mtDNA, the expression of the mtDNA encoded mRNA, ND6, CYTB, COI, and 16S rRNA was maintained in RVH but not in RV failure (Figure 1F).

Similarly, the protein levels of ND6 were unaltered in RVH but significantly reduced in RV failure (Figure 1G). These results suggest that mtDNA depletion precedes the decreases in mitochondrial enzyme activities or protein levels as well as the clinical manifestation of heart failure.

Decreases in the CS activity and mtDNA content were also observed in the left ventricle (LV) of children with advanced stage of heart failure (online-only Data Supplement Figure I).

However, we were unable to obtain samples at the stage of LV hypertrophy to assess the progression of mtDNA depletion in the LV.

Defective mtDNA Replication Leads to mtDNA Depletion

We subsequently tested the hypothesis that mtDNA replication was impaired in RVH and RV failure by measuring the mtDNA replication intermediates in the tissue samples. We found that the extension of single-strand DNA beyond the D-Loop and normalized to the mtDNA content (A) in nonfailing right ventricle (RV) (dashed line; n=5), RV hypertrophy (RVH) (n=25), and RV failure (n=6). Data are shown as the mean of the fold changes ± SEM over the nonfailing RV (*P≤0.05 versus nonfailing).

Transcriptional Regulation of Mitochondrial Biogenesis

The evidence of impaired mtDNA replication in RVH prompted us to examine the expression and activity of the PGC-1 pathway, the master regulators of many genes involved mitochondrial biogenesis. Interestingly, both PGC-1α and
PGC-1α mRNA were increased at the stage of hypertrophy and returned to normal levels at the stage of RV failure (Figure 3). There were no changes in the expression of the PGC-1 interacting partner NRF1, whereas there was a switch in the expression of the NRF2 isoforms; NRF2A expression was increased, whereas NRF2B2 was decreased. Among the genes regulated by NRF1/2, TFAM, an important regulator of mtDNA transcription as well as mtDNA maintenance, was modestly decreased; TFB1M expression was increased, whereas TFB2M was decreased. Despite the changes at the mRNA levels, the protein levels of these genes were not reduced in RVH (Figure 4). Similarly, the expression of several other nDNA-encoded mitochondrial proteins not related to mtDNA maintenance was also unchanged in RVH (online-only Data Supplement Figure III). These findings suggest that mtDNA depletion in RVH is not due to the downregulation of the PGC-1 levels, instead, the higher PGC-1 expression probably contributes to the compensatory increases in the transcription of mtDNA, resulting in a sustained level of the mtDNA-encoded proteins at the stage of compensated RV hypertrophy despite the decreased mtDNA content (Figure 1F).

Changes in Mitochondrial Ultrastructure Are Associated With Lower mtDNA Content

We compared electron microscopic images of cardiac tissues from patients with compensated hypertrophy with different mtDNA content. There were no differences in the size and density of mitochondria in the hearts of patients with normal or low mtDNA levels (Figure 5). However, there were striking differences in the mitochondrial ultrastructure. In the tissue samples with low mtDNA content, the mitochondria were swollen; the cristae density was reduced, disorganized, and oriented in varying oblong and oblique directions in the matrix (Figure 5). The parallel changes of mtDNA content and the mitochondrial ultrastructure suggest that mtDNA depletion in RVH is a meaningful indicator of mitochondrial injury.

**RV Pressure Is a Determinant of mtDNA Gene Expression**

Within the RVH group, the expression of the mtDNA-encoded genes, ND6, CYTB, COI, and 16S rRNA, was significantly lower in patients with higher than average RV pressure (RVp >70 mm Hg) compared with those with RVp <70 mm Hg (Figure 6A). Furthermore, the mtDNA copy number was inversely correlated with the RVp in the RVH group (Figure 6B), whereas the CS activity did not correlate with RVp (Figure 6C). These results collectively suggest that as the RV pressure overload develops, there is progressive mtDNA depletion, leading to the eventual failure of maintaining the mtDNA-encoded gene expression. Taken together with the parallel changes of ultrastructure and mtDNA content, our observations suggest that pathological stresses associated with the pressure overload have progressively affected the mitochondrial reserve predisposing the heart to failure.

**Discussion**

In the present study, we have assessed different aspects of mitochondrial biogenesis in the RV of children and young adults with CHD who were presented with a varying degree of hemodynamic overload, with and without RV failure. An important finding is that mtDNA replication was impaired and mitochondrial ultrastructure becomes abnormal in the hypertrophied RV before the patients meet the clinical and functional criteria of heart failure. These observations indicate that mtDNA depletion is a sensitive and meaningful marker of mitochondrial injury in CHD presented with RV hemodynamic overload. We have also shown that mtDNA depletion occurs in the absence of downregulation of the PGC-1 family genes but is coupled to defective mtDNA replication mechanism, and mtDNA-encoded gene expression decreases in an RV pressure-overload–dependent manner. These findings provide novel information for

![Figure 3](image-url)  
*Figure 3.* Mitochondrial RNA levels of the PGC-1 pathway in overloaded right ventricle (RV). Mean of the fold changes ±SEM in gene expressions for the PGC-1 pathway in the RV hypertrophy (RVH) (n=25) and RV failure (n=6) relative to nonfailing controls (*dashed line*; n=5; *P*≤0.05 versus nonfailing).

![Figure 4](image-url)  
*Figure 4.* Protein levels of the PGC-1 downstream targets in overloaded right ventricle (RV). Representative Western blots and mean of the fold changes ±SEM in gene expressions for the PGC-1 pathway in the hypertrophy (RVH) (n=5) and RV failure (n=5) relative to nonfailing controls (*dashed line*; n=5; *P*≤0.05 versus nonfailing).
understanding the pathogenesis of mitochondrial dysfunction in human heart failure.

Role of Mitochondrial Biogenesis in the Transition From Cardiac Hypertrophy To Failure

It has been well documented that marked changes of energy metabolism have occurred in cardiac hypertrophy and failure, including glycolysis, fatty acid oxidation, and overall ATP synthesis and turnover. Although animal studies strongly indicate a role of mitochondrial dysfunction in the development of heart failure, mitochondrial studies in human heart failure have been challenging because of inherent difficulties in obtaining cardiac tissue samples from patients at different stages of the disease. In the present study, we were able to examine the mitochondrial biogenesis in patients with CHD with a broad spectrum of severity at the time of surgery. Our findings suggest that mtDNA replication and maintenance is impaired in the hemodynamically overloaded RV, even though the standard functional assessment considers the heart at the stage of compensated hypertrophy. It is noteworthy that not only does the mtDNA level track with the development of heart failure, it also parallels the pathological changes in mitochondrial ultrastructure in hypertrophied hearts, thus reliably reflecting the severity of the disease. The importance of mtDNA depletion or mutation in the development of mitochondrial dysfunction and cardiomyopathy has been well documented in mitochondrial diseases or drug toxicity. Preservation of the mtDNA copy number in mouse hearts significantly delays the development of heart failure post myocardial infarction. These pieces of evidence collectively show that the maintenance of mtDNA is critical for the normal heart function although unlikely to be the sole mechanism of heart failure.

Mitochondrial dysfunction and depletion of mtDNA has been associated with downregulation of the PGC-1α gene and its downstream targets in animal models of heart failure. In contrast to rodent models, we have previously shown in end-stage human heart failure that PGC-1 gene expression is not decreased in the failing LV. In support of this, it has been previously reported that in dogs with tachycardia-induced heart failure there was no change in the PGC-1 protein levels. We show that the expression of the PGC-1 genes is increased at the stage of hypertrophy and it returns to normal at the stage of failure. As discussed above, the increased expression of the PGC-1 genes might play a very important role in sustaining the transcriptional activities of the mtDNA in RVH, although it does not prevent the impairment of mtDNA replication.

Mitochondrial Injury as a Marker for the Timing of Surgery in Children With CHD

Results of RV surgery in patients with CHD are controversial, with some patients not improving RV size or function despite a technically successful operation. The long-term outcomes of the surgery varies highly, with some centers reporting...
significant improvement in RV end-diastolic volume and systolic function, as measured by EF, and other centers not seeing any improvement in RV function. There is increasing evidence suggesting that the timing of surgery rather than the removal of associated defects is critical for the reversibility of RV dysfunction. For example, it has been shown that myocardial fibrosis or the development of pulmonary regurgitation after surgery, both indicative of irreversible pathological remodeling of the RV, correlate closely with the adverse clinical status and the poor recovery of the repaired hearts. In the present study, we have shown that impaired replication and depletion of mtDNA are initiated at the stage of clinically compensated hypertrophy and progressively affect mitochondrial protein expression and enzyme activity in a pressure-dependent manner in RVH and during the transition to failure. Furthermore, the depletion of mtDNA is accompanied by significant morphological changes in the mitochondria ultrastructure, suggesting that it is a sensitive and early marker of mitochondrial damage. Given the vital roles of mitochondria as the powerhouse as well as the key regulator of cell death in cardiac myocytes, these findings may assist to identify a group of patients who should benefit from early surgery.

Limitations
Because of the differences in the cardiac developmental defect and its severity, we are able to examine cardiac tissue from the RV with varying degrees of dysfunction in this pediatric population. This unique opportunity also presents a challenge in finding age-matched control RV tissue. As a result, the median age of the control group in this study is older than the patient group and the age range of the control subjects only partially overlap with the age range of the patients. There is a possibility that the content and the regulation mtDNA transcription and replication of the infant hearts are different from older children. However, the main focus of this study is the mitochondrial biogenesis in RVH without clinical failure. The RVH patients have considerable age overlap with the control group, and we did not observe age-dependent changes of mtDNA content and replication in control hearts (nonfailing) between 4 to 18 years old.

Because of limited number of samples used for electron microscopic examination, we were quite limited in the power of the statistical analysis. Because our study population is small and consists of a mixture of diagnosis and stages of the disease, future studies with a larger number of patients in each subcategory are needed to identify strong clinical surrogates for mitochondrial damage, which may serve as a useful guide to establish appropriate timing for surgery.

Conclusions
Our findings suggest that impaired mtDNA replication and maintenance is integral to the development and transition from RVH to heart failure. It provides an important basis for the future development of “mitochondrial oriented” clinical and surgical treatment of patients with overloaded RV caused by CHD. Specifically, the mtDNA depletion pattern could help to establish adequate timing for intervention. To this end, large clinical studies determining the link of mitochondrial impairment at the time of surgery and the outcome in this patient population are warranted.

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We thank Dr Jeffrey Saffitz for advice regarding the sample preparations for the electron microscopy and for critical evaluation of the quality of the electron micrographs. We would also like to express our special gratitude to Dr Federica del Monte for valuable input as well as for providing nonfailing heart samples. Last but not least, thank Dr Pantelis Xofis and William Jen Hoe Koh for assisting with the statistical analysis.

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Disclosures
None.
REFERENCES


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### SUPPLEMENTAL MATERIAL

**Supplemental Table 1. Primer Sequence for Real-Time PCR**

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Supplemental Figure 1. Assessment of mitochondrial mass and mtDNA content in the LV. (A) Citrate Synthase activity and (B) mtDNA content normalized to nDNA in LV failure (n=8) compared to the Non-Failing LV controls (n=5). Data are given as the mean of the fold changes ± SEM relative to the Non-Failing RV (* p≤0.05 vs. Non-Failing).
Supplemental Figure 2. Impaired mtDNA replication in LV failure. MtDNA replication was assessed by measuring the extension of 7S DNA beyond the D-Loop and normalized to mtDNA in LV failure (n=8) compared to the Non-Failing LV controls (n=5). Data are given as the mean of the fold changes ± SEM relative to the Non-Failing RV (* p≤0.05 vs. Non-Failing).
Supplemental Figure 3. mRNA expression of nDNA encoded mitochondrial proteins. Mean of the fold changes ± SEM in gene expressions for nDNA genes encoding mitochondrial proteins in the RVH (n=25) and RV-failure (n=6) relative to Non-Failing controls (indicated by the dashed line; n=5; * p≤0.05 vs. Non-Failing).