Reversible Pulmonary Trunk Banding - VI:
Glucose-6-Phosphate Dehydrogenase Activity in
Rapid Ventricular Hypertrophy in Young Goats

Renato S. Assad, MD, PhD¹; Fernando A. Atik, MD²; Fernanda S. Oliveira, MD¹;
Miriam H. Fonseca-Alaniz, BPh, PhD¹; Maria C. D. Abduch¹, VMD, PhD¹;
Gustavo J. J. Silva, PE, PhD¹; Jose E. Krieger, MD, PhD¹; Noedir A. G. Stolf, MD, PhD¹

1- Heart Institute, University of São Paulo Medical School, São Paulo, Brazil;
2- Instituto de Cardiologia do Distrito Federal, Brasilia, Brazil

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Corresponding Author: Renato S. Assad, MD, PhD
Heart Institute University of São Paulo Medical School
Division of Surgical Research
Ave. Dr. Eneas C. Aguiar, 44
São Paulo, SP – Brazil 05403-000
E-mail: rsassad@cardiol.br
ABSTRACT

Objective: Increased myocardial Glucose-6-Phosphate Dehydrogenase (G6PD) activity occurs in heart failure. This study compared G6PD activity in 2 protocols of right ventricle (RV) systolic overload in young goats.

Methods: 27 goats were separated into 3 groups: Sham (no overload), Continuous (continuous systolic overload), Intermittent (four 12-hour periods of systolic overload paired with a 12-hour resting period). During a 96-hour protocol, systolic overload was adjusted to achieve a 0.7 RV/aortic pressure ratio. Echocardiographic and hemodynamic evaluations were performed before and after systolic overload every day postoperatively. After the study period, the animals were humanely killed for morphological and G6PD tissue activity assessment.

Results: A 92.1% and 46.5% increase occurred in RV and septal mass in the Intermittent group, respectively, compared to the Sham group; Continuous systolic overload resulted in a 37.2% increase in septal mass. A worsening RV myocardial performance index occurred in the Continuous group at 72 hours and 96 hours, compared to Sham (p<0.039) and Intermittent groups at the end of the protocol (p<0.001). Compared to Sham, RV G6PD activity was elevated 130.1% in the Continuous group (p=0.012) and 39.8% in the Intermittent group (p=0.764).

Conclusions: Continuous systolic overload for ventricle retraining causes upregulation of myocardial G6PD activity, which can elevate levels of free radicals by NADPH oxidase, an important mechanism in the pathophysiology of heart failure. It suggests that intermittent systolic overload may provide better
results for 2-stage Jatene surgery compared to the continuous protocol, with better preservation of myocardial performance and less exposure to hypertrophic triggers.

**Key words:** Hypertrophy, physiopathology; Right ventricular hypertrophy; Transposition of great vessels; Goats.
UltraMini-Abstract: In a young goat model, continuous systolic overload caused upregulation of myocardial G6PD, which can elevate levels of free radicals, an important mechanism in the pathophysiology of heart failure, while intermittent systolic overload has promoted more efficient RV hypertrophy, with better preservation of myocardial performance and less G6PD activity.
INTRODUCTION

Traditional pulmonary artery banding (PAB) aimed at ventricular retraining causes an abrupt and fixed systolic overload. Although clinical studies have proved that PAB induces myocardial hypertrophy, it is frequently preceded by ventricular dysfunction. Therefore, an adaptation period with inotropic support is generally required. Most importantly, previous studies have demonstrated myocardial edema and necrosis in hearts that experience abrupt systolic overload, followed by late ventricular failure.¹

However, it is essential to understand the molecular mechanisms involved in PAB-induced myocardial hypertrophy to establish training protocols that lead to a desirable “physiologic hypertrophy” versus a deleterious “pathologic hypertrophy”. Because a known shift occurs in energy substrate utilization in favor of glucose in pathologic conditions, energy metabolism might be altered in PAB ventricular retraining protocols.² In addition, recent experimental studies have linked an unbalanced oxidative and reductive process to a variety of diseases, such as atherosclerosis and heart failure.³

Glucose 6-Phosphate Dehydrogenase (G6PD), the rate-limiting enzyme that commits glucose to the pentose phosphate pathway, is mainly responsible for the generation of nicotinamide adenine dinucleotide phosphate (NADPH) and ribose 5-phosphate, an essential precursor of the de novo synthesis of RNA and DNA. G6PD-derived NADPH, a cofactor for glutathione and thioredoxin reductase, preserves reducing potentials and protects the cell from oxidative stress in normal conditions.⁴ In human diseases, G6PD can be either activated or
inhibited; however, evidence has emerged that the overexpression and activation of G6PD enhances NADPH oxidase-derived superoxide generation and increases oxidative stress in diseases like diabetes, heart failure, and hypertension.5

In regard to rapid ventricular training, it would be of great interest to study myocardial energy metabolism in response to different cardiac hypertrophy models, and its relationship to heart function.6 The main objective of this study was to compare the G6PD activity in 2 right ventricle (RV) training protocols through an adjustable pulmonary artery banding system.

METHODS

Twenty-seven young goats, ages between 30 and 60 days and comparable weight (p=0.38) were split into 3 groups: Sham (n=7; weight: 11.93kg ± 2.67kg), Continuous (n=9; weight: 10.74kg ± 2.62kg), and Intermittent (n=11; weight: 10.25kg ± 2.20kg). All animals received humane care in compliance with the guidelines established by the Brazilian regulations for animal experimentation. The protocol was reviewed and approved by the Ethics Committee for Research Protocols at the University of São Paulo Medical School.

Surgical Procedure

All operations were performed with the goats under general anesthesia (pentobarbital sodium 5 mg/kg I.V. and ketamine 20mg/kg I.M.) and through a left lateral thoracotomy. The lung was retracted laterally to allow exposure of the
right ventricular outflow tract, pulmonary trunk, and descending aorta. A 17-gauge heparinized catheter was inserted into each of these structures, and its corresponding pressures were measured at specific time intervals during the entire study. The adjustable PAB system (SILIMED, Silicone e Instrumental Médico-Cirúrgico e Hospitalar Ltda., Rio de Janeiro, Brazil) was implanted just beyond the pulmonary valve and sutured at the adventitia of the pulmonary trunk, as previously described. Antibiotics (cefazolin 500 mg and gentamicin 40 mg) were administered daily during the study, as were digoxin (0.04 mg/kg) and subcutaneous heparin (5000 U).

Training Protocol
RV systolic overload was initiated 72 hours postoperatively. Baseline hemodynamic data (right ventricle, pulmonary artery, and aortic pressures) were collected in a conscious, immobilized animal with the adjustable banding system deflated. Blood pressure measurements were obtained through computer software (ACQknowledge 3.01, Biopac Systems, Inc, Goleta, CA). Then, the banding system was adjusted to achieve an RV/aortic pressure ratio of 0.7, limited by a 10% drop in systolic blood pressure. That rule was violated in case of the latter occurring or if there were agitation, dyspnea, arrhythmia, or a combination of these. The banding system was then deflated up to a tolerable point.
Continuous Group Protocol

The animals remained with continuous systolic overload for 96 hours, with daily assessment to keep the RV/Aortic pressure ratio at 0.7. Hemodynamic data were collected once a day (mornings) during PAB readjustments.

Intermittent Group Protocol

The animals underwent 4 daytime periods of RV 12-hour systolic overload, alternating with a 12-hour nighttime resting period. Hemodynamic data were collected twice a day (every 12 hours), during PAB readjustments.

Sham Group Protocol

The PAB system was maintained deflated during the entire protocol. Hemodynamic data were collected daily (mornings).

Echocardiography

A single experienced observer conducted the echocardiographic examination with the animals under light sedation (ketamine 15 mg I.M.) approximately 120 hours before the beginning of the protocol and daily thereafter until the end of the protocol. Image acquisition was obtained through a 7.5-MHz and 2.5-MHz multi-frequency transducers (Acuson Cypress Echocardiology System, Siemens, Erlagen, Germany). The following echocardiographic parameters were studied: left ventricle (LV), RV, and septal wall thicknesses, RV end-diastolic volume, and myocardial performance index.
Morphology

Animals were humanely killed after 96 hours of the study protocol. Cardiac samples were drawn from the RV, LV, and ventricular septum just before cardiac arrest. These samples were immediately frozen at -80º Celsius (Forma Scientific Inc., Marietta, OH), to be subsequently analyzed for G6PD activity. The pericardial fat, both atria, and semilunar valves were dissected from the heart; RV, LV, and ventricular septum were separated by the Fulton technique, individually weighed, (METTLER AE-200, Mettler-Toledo AG, Greifensee, Switzerland) and indexed to each animal’s body weight.¹⁸

Water content was obtained individually in each cardiac chamber by subtracting the collected sample weight at autopsy from the weight of the dehydrated chamber (70 hours at 60º Celsius). Values were obtained as a percentage of weight change.

G6PD Activity

Tissue samples were homogenized in extraction buffer (proportion 1:5 weight/volume). The material was stored in ice and homogenized for 30 seconds, using Polytron (PT 3100, Kinematica AG, Littau-Lucerne, Switzerland) at maximum speed and centrifuging (15 kg, 15 minutes, 4º Celsius) to separate from cell remnants. Enzymatic activity analysis was performed using the supernatant of the last centrifugation. Proteins were quantified with the protein assay kit BCA (PIERCE Biotechnology, Rockford, IL). Results are expressed as
nmol.min\(^{-1}\).mg\(^{-1}\) of protein. The extraction buffer for G6PD contained Tris-HCl (50 mM) and EDTA (1 mM), with a pH of 8. The assay buffer (270 \(\mu\)L/sample) was Tris-HCl (8.6 mM), MgCl\(_2\) (6.9 mM), NADP\(^+\) (0.4 mM), and Triton X-100 0.05\%(volume/volume), with pH of 7.6. The reaction was initiated by adding 15 \(\mu\)L of Glucose-6-phosphate (1.2 mM) to the enzymatic extract (15 \(\mu\)L of sample) for 10 minutes at 25\(^\circ\) Celsius. The absorbance was monitored at 340 nm, the extinction coefficient being 6.22 for that particular wavelength. The biochemical reaction is based on the glucose phosphorylation into glucose 6-phosphate, and posterior formation of 6-phosphogluconate by the action of G6PD. The G6PD activity was indirectly determined by the total production of NADPH through the pentose phosphate pathway.\(^9\) RV and ventricular septum values were indexed to each animal LV value.

**Statistical Analysis**

Values are expressed as means and standard deviation. Comparison of variables was performed with 2-way analysis of variance (ANOVA), except for body weight, G6PD activity, wall masses, and water content, which were performed with 1-way ANOVA. Both analyses were followed by the Bonferroni post-hoc test. The level of significance utilized was 5%. Statistical analysis was performed using GraphPad Prism v.4 software (La Jolla, CA) and SigmaStat 3.11.0 for Windows (Systat Software, Inc., Chicago, IL).
RESULTS

Hemodynamic Variables

Systolic RV to PA gradient increased in both study groups (p<0.001) and in every time period (p<0.001). There were peak systolic gradients in the Intermittent group, alternating with 12-hour rest periods when they were similar to those in the Sham group (Figure 1, panel A). To quantify the exposure to systolic overload, the area under the RV to PA gradient curve was determined (Figure 1, panel B). The Continuous group was the group most exposed to systolic overload (p<0.05).

Echocardiographic Variables

RV wall thickness was thinner than septal and LV thicknesses in all animals preoperatively (group factor: p=0.663; heart wall factor: p<0.001). There was a marked increase in RV wall thickness after 48 hours in the Intermittent group and after 72 hours in the Continuous group, compared to the baseline values (Table 1). The Intermittent group developed a thicker RV wall (5.85mm ± 1.32mm) than the Continuous group did (4.54mm ± 0.51mm) at 96 hours (p<0.001). At the end of the training protocol, RV wall thickness increased 103.8% in the Intermittent group and only 38.4% in the Continuous group. No significant differences occurred in septal and LV wall thicknesses among groups (p=0.491) and across time (p=0.865). The graph with the percentage of changes in RV wall thickness is available online (figure E-1).
Regarding RV end-diastolic volume, there was a significant difference among groups (p<0.001) and throughout the protocol (p=0.024). RV volume was significantly dilated in the Continuous group at 24 hours and thereafter, compared to that in the Intermittent and Sham groups (p<0.001). The graph with the percentage of changes in RV end-diastolic volume is available online (figure E-2). Worsening of RV myocardial performance index was observed in the continuous group at 72 hours and 96 hours of the protocol, compared to that in the Sham group (p<0.039) and to the Intermittent group (p<0.001) at the end of the protocol (Figure 2).

**Morphologic Variables**

As demonstrated in Figure 3, the Intermittent group had a 92.1% increase in RV mass (1.46 g/kg ± 0.53 g/kg) and a 46.5% increase in ventricular septal mass (1.26 g/kg ± 0.29 g/kg), compared to RV (0.76 g/kg ± 0.12 g/kg) and ventricular septal (0.86 g/kg ± 0.10 g/kg) masses in the Sham group (p<0.05). On the other hand, the Continuous group had an increase only in the ventricular septal mass (1.18 g/kg ± 0.14 g/kg; p<0.05). LV mass was not altered by RV training protocols (p=0.217). Regarding water content, both RV (81.59% ± 1.07%) and ventricular septum (79.69% ± 0.62%) in the Continuous and both RV (81.84% ± 1.11%) and ventricular septum (79.45% ± 0.62%) in the Intermittent groups had a mild, however significant, increase compared to that in the Sham group (RV: 78.84% ± 2.41% and ventricular septum: 77.11% ± 2.08%; p<0.01). No significant changes were observed in LV water content in the 3 groups.
G6PD Activity

G6PD activity ratio of RV to LV was significantly elevated by 130.1% in the Continuous group \((p=0.012)\), while the Intermittent group showed a nonsignificant smaller increase of 39.8% in the G6PD activity ratio of RV to LV \((p=0.764)\), compared to that in the Sham group (figure 4). There was no significant difference in the ventricular septum to LV G6PD activity ratio among groups \((p=0.198)\).

DISCUSSION

The present experimental study aimed to compare pulmonary artery banding-induced RV hypertrophy in continuous versus intermittent systolic overload in young goats, with the emphasis on G6PD activity analysis. Both study groups were capable of promoting different degrees of myocardial hypertrophy compared to the Sham group. However, the Intermittent group had greater RV and ventricular septal masses than the Sham group had, despite less exposure to systolic overload. The mild increase in RV and ventricular septum water content in both trained groups would not by itself justify the previous findings, suggesting that it was probably related to the enhanced protein synthesis and cell proliferation, as previously documented by Abduch and colleagues.\(^\text{10}\) The Continuous group had a series of deleterious effects at the end of the protocol. Persistent RV dilation was followed by impaired RV function and increased G6PD activity, G6PD being the rate-limiting enzyme in the oxidative
branch of the pentose phosphate pathway. Because the pentose phosphate pathway is a major source of NADPH in cardiac myocytes, this is an important finding, and it may indicate an unbalanced redox, with the occurrence of oxidative stress and generation of reactive oxygen species related to NADPH oxidase.\textsuperscript{11} In pathologic conditions, the excessive production of NADPH via G6PD overexpression is a result of multiple factors, such as angiotensin II, thrombin, and tumor necrosis factor alpha. The final consequence of this cascade of events would be the cardiomyopathy related to protein aggregation due to reductive stress.\textsuperscript{12,13}

It has been demonstrated that either G6PD activation or inhibition are associated with diseases. However, growing evidence has emerged that G6PD overexpression correlates with oxidative and reductive stress, and new investigational drugs are currently under development to suppress its action.\textsuperscript{14} For instance, diabetic patients had upregulation of G6PD with high NADPH levels, and that was linked to inhibition of nitric oxide synthesis and endothelial dysfunction.\textsuperscript{15} Although the mechanisms underlying the increased production of reactive oxygen species in the heart is not completely understood, it has been proposed that the high rate of glucose oxidation increases mitochondrial membrane potential, which enhances production of superoxide anion.\textsuperscript{16,17} The latter would be a modulator in diabetic vasculopathy and precede the development of diabetic cardiomyopathy.\textsuperscript{18,19}

Furthermore, in pacing-induced heart failure, an established model of dilated cardiomyopathy in dogs, it has been demonstrated that a 10-fold
overexpression of G6PD occurs compared to that in controls.\textsuperscript{20} Recent studies demonstrated that G6PD upregulation in mice adipocytes has been implicated as one of the causes of altered hormonal release observed in obesity and insulin resistance.\textsuperscript{21}

Although the present work has not evaluated the generation of reactive oxygen species related to NADPH oxidase, it is tempting to speculate that, in case of persistent systolic overload, upregulation and hyperactivity of myocardial G6PD observed in the Continuous group strongly suggest that the pentose phosphate pathway enhances cytosolic NADPH availability, thus fueling free radical production by NADPH oxidase and uncoupled NO synthase. Therefore, it may induce superoxide anion myocardial injury, as well as protein aggregation, and subsequently heart failure. However, other unknown mechanisms of G6PD in heart failure could not be ruled out. This issue has been investigated worldwide.

Regarding the training protocol, previous studies have demonstrated that ventricular training protocols induce myocardial hypertrophy 96 hours after progressive systolic overload.\textsuperscript{22} The search for a physiologic hypertrophy has been the focus of our laboratory for over 2 decades, and we postulate that intermittent systolic overload promotes the desirable effects of myocardial hypertrophy without its adverse effects.\textsuperscript{7} RV to PA gradients were more pronounced in the Intermittent group after 48 hours of training. We would argue that the 12-hour resting period allowed the myocardium to replenish energy substrates and reestablish subendocardial perfusion due to a lower ventricular
wall tension. That would probably provide better hemodynamic performance at periods of systolic overload.\textsuperscript{23}

**Study Limitations**

First, inferences based on animal findings do not necessarily translate into the same conclusions in humans. Second, right ventricular hypertrophy was studied here, as opposed to human hearts with transposition of the great arteries. However, experimental models of aortic banding are associated with prohibited mortality rates.\textsuperscript{24} It is difficult to make definitive conclusions about a hypertrophic process based on a single enzyme activity. However, it is essential to correlate these biochemical findings with production of superoxide anions and apoptosis to better understand the role of oxidative stress in hypertrophy training protocols.

**Conclusions**

This study demonstrates that continuous systolic overload for ventricle retraining causes hyperactivity in myocardial G6PD, together with RV dilation and dysfunction. That enzyme hyperactivity may be related to an unbalanced redox determined by a constant and pathologic systolic overload. Given that pentose phosphate pathway enhances cytosolic NADPH availability, this altered energy substrate metabolism can elevate levels of free radicals by NADPH oxidase, an important mechanism in the pathophysiology of heart failure. On the other hand, intermittent systolic overload has promoted a more efficient RV hypertrophy than the continuous one, with better preservation of myocardial performance and smaller G6PD activity. It suggests that intermittent systolic overload may provide
better results for 2-stage Jatene surgery compared to a continuous protocol. Clinical studies comparing adjustable and traditional pulmonary trunk banding should be welcomed to translate those findings to the practice of ventricular retraining.
**Table 1.** RV wall thickness of the 3 groups measured by echocardiography in each time period of the protocol.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>RV wall thickness (mm)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Sham (n=7)</td>
<td>Continuous (n=9)</td>
</tr>
<tr>
<td>Preop</td>
<td>3.24 ± 0.14</td>
<td>3.28 ± 0.35</td>
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<tr>
<td>0</td>
<td>3.3 ± 0.15</td>
<td>3.31 ± 0.33</td>
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<tr>
<td>24</td>
<td>3.36 ± 0.11</td>
<td>3.37 ± 0.33</td>
</tr>
<tr>
<td>48</td>
<td>3.39 ± 0.09</td>
<td>3.63 ± 0.68</td>
</tr>
<tr>
<td>72</td>
<td>3.37 ± 0.14</td>
<td>4.4 ± 0.64†</td>
</tr>
<tr>
<td>96</td>
<td>3.36 ± 0.08</td>
<td>4.54 ± 0.51†</td>
</tr>
</tbody>
</table>

Values (mm)= means ± SD

* p<0.001 compared to its respective baseline value;
† p<0.001 compared to that in the Sham group at the same time;
∥ p<0.001 compared to that in the Continuous group at the same time.
RV to PA gradient (mm Hg) temporal pattern of Sham, Continuous, and Intermittent groups (Panel A). According to variations in RV to PA gradients in relation to time, the area under the curve (mm Hg.h) was calculated to determine the systolic overload imposed on each group (Panel B).

* p<0.001 compared to that of the Sham group.

# p<0.001 compared to the Intermittent group.
Figure 2 Legend

Right ventricle myocardial performance index during the 96 hour protocol in Sham, Continuous and Intermittent groups.

- p<0.04 compared to that in the Sham group at the same time.

- # p<0.001 compared to that in the Intermittent group at the same time.
RV, ventricular septum and LV weights in the 3 groups, indexed to body weight. Values (g/kg)= Mean ± Standard deviation.

* p<0.05 compared to that in the Sham group.
Figure 4 Legend

RV to LV ratio of glucose 6-phosphate dehydrogenase activity in Sham, Continuous and Intermittent groups.

Values= Mean ± standard deviation.

Sham: n= 5; Continuous: n= 5; Intermittent: n= 10.

* p<0.05 compared to that in the Sham and Intermittent groups.
Figure E-1 Legend

Δ% changes of RV wall thickness of Sham, Continuous and Intermittent groups throughout the protocol.

* p<0.001 compared to the respective baseline value.

# p<0.001 compared to Sham group at the same time.

++ p<0.02 compared to Continuous group at the same time.
Δ% changes of RV end-diastolic volume of Sham, Continuous and Intermittent groups throughout the protocol.

* p<0.001 compared to the respective baseline value;
# p<0.001 compared to Sham group at the same time.
REFERENCES


