A RANDOMIZED DOUBLE-BLIND PLACEBO-CONTROLLED CLINICAL STUDY INVESTIGATING CLINICAL OUTCOME AND GENE EXPRESSION RESPONSES TO INSULIN-ENHANCED CARDIOPLEGIA DURING CARDIAC SURGERY IN INFANTS WITH TETRALOGY OF FALLOT

by

Caterina (Cathy) Boscarino

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Graduate Department of Institute of Medical Sciences

University of Toronto

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A Randomized Double-Blind Placebo-Controlled Clinical Study Investigating Clinical Outcome and Gene Expression Responses To Insulin-Enhanced Cardioplegia During Cardiac Surgery In Infants With Tetralogy of Fallot. Ph.D 2008, Caterina (Cathy) Boscarino, Institute of Medical Sciences, University of Toronto.

Tetralogy of Fallot (TOF) is the most common cyanotic congenital heart defect and infants with TOF incur significant right ventricular (RV) dysfunction due to perioperative injury. Insulin has been shown to reduce perioperative myocardial injury and significantly improve postoperative cardiac function. However, studies are limited to the adult population and the effects in a pediatric heart with a CHD are unknown. To the best of our knowledge, this is the first randomized, double blind, placebo-controlled clinical study designed to investigate insulin’s potential cardioprotective effects postoperatively and mechanisms of action during pediatric cardiac surgery. Thirty infants with TOF were equally randomly allocated to receive either standard cardioplegia (SC) or 10UI insulin-enhanced (IC). Expression profiles of surgery were generated from biopsies extracted from the right ventricular outflow tract (end ischemia and five minutes of reperfusion) and hybridized to Affymetrix HG-U133A GeneChips. Gene expression profiles were generated using two softwares, ArrayAssist V2.6 (paired t-test) and affylmGUI (ANOVA). Survival rate was 100%. Compared to patients in the SC group, patients in the IC group demonstrated a trend toward a 1.8 fold decrease (p = .06) in reperfusion duration (61.93 ± 61.12 vs. 35.20 ± 23.16 hrs., respectively), a significant 2-fold decrease in the length of ICU stay (p = .04) (4.2 ± 3.9 vs. 2.3 ± 1.1 days,
respectively) and a trend toward a 2.5 fold decrease in intubation duration ($p = .06$) (2.5 ± 12.2 vs. 55.0 ± 67 hrs., respectively). Patients in the IC group also demonstrated significantly lower inotropic scores, calculated at 12-hour intervals across a 48-hour ICU period, (ANOVA $p = .01$) and significantly greater urine volume, by 71%, ($p = .02$). IC evoked a cardioprotective gene expression profile aimed at mitigating perioperative myocardial injury, specifically; apoptosis, inflammation, cardiac hypertrophy, arrhythmias and fibrosis. The improved postoperative outcome and cardioprotective gene expression signature with IC suggests that, administration of insulin during cardiac surgery in infants with TOF may prevent cardiac dysfunction as a result of mitigating perioperative myocardial injury. Overall, this exploratory study demonstrated insulin-enhanced cardioplegia to be a potential cardioprotective agent during pediatric heart surgery.
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I hope will always think the world of her aunt. A huge hug and infinite kisses to my greatest fans and supporters, Rocky, Daisy and Theo! And last but never least, God.
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Insulin
A Superior Additive

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-Primary Parameters
-Secondary Parameters
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<th>Description</th>
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<tr>
<td>AA</td>
<td>Array Assist</td>
</tr>
<tr>
<td>affylmGUI</td>
<td>affymetrix linear modeling graphical user interface</td>
</tr>
<tr>
<td>AKT</td>
<td>serine/threonine specific protein kinase family</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ASD</td>
<td>atrial septal defect</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AXCTT</td>
<td>aortic cross-clamp total time</td>
</tr>
<tr>
<td>BSA</td>
<td>body surface area</td>
</tr>
<tr>
<td>CABG</td>
<td>coronary artery bypass grafting</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHD</td>
<td>congenital heart defect</td>
</tr>
<tr>
<td>CPB</td>
<td>cardiopulmonary bypass</td>
</tr>
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<td>CPBTT</td>
<td>cardiopulmonary bypass total time</td>
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<td>cRNA</td>
<td>complimentary ribonucleic acid</td>
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<td>cTnI</td>
<td>cardiac troponin I</td>
</tr>
<tr>
<td>Ctrl</td>
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<td>DE</td>
<td>differential expression</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>FDR</td>
<td>false discovery rate</td>
</tr>
<tr>
<td>GC-RMA</td>
<td>guanine-cytosine robust multivariate analysis</td>
</tr>
<tr>
<td>GIK</td>
<td>glucose-insulin-potassium</td>
</tr>
<tr>
<td>H2O</td>
<td>water</td>
</tr>
<tr>
<td>HFABP</td>
<td>heart fatty acid-binding protein</td>
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<td>HG-U133A</td>
<td>human genome – U133A</td>
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<td>HGL</td>
<td>highest glucose level</td>
</tr>
<tr>
<td>HLL</td>
<td>highest lactate level</td>
</tr>
<tr>
<td>hrs</td>
<td>hours</td>
</tr>
<tr>
<td>IC</td>
<td>insulin-enhanced cardioplegia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>ICU</td>
<td>intensive care unit</td>
</tr>
<tr>
<td>IL-#</td>
<td>interleukin-number</td>
</tr>
<tr>
<td>ILK</td>
<td>integrin-linked kinase</td>
</tr>
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<td>Ins</td>
<td>insulin</td>
</tr>
<tr>
<td>Isch</td>
<td>ischemia</td>
</tr>
<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
</tr>
<tr>
<td>L</td>
<td>left atrium</td>
</tr>
<tr>
<td>LA</td>
<td>left atrium</td>
</tr>
<tr>
<td>LCOS</td>
<td>low cardiac output syndrome</td>
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<tr>
<td>LGL</td>
<td>low glucose levels</td>
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<tr>
<td>LLL</td>
<td>low lactate levels</td>
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<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>M</td>
<td>fold change</td>
</tr>
<tr>
<td>MM</td>
<td>mismatch</td>
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<tr>
<td>min</td>
<td>minutes</td>
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<td>mos</td>
<td>months</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>N</td>
<td>sample size</td>
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<tr>
<td>n</td>
<td>sample size</td>
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<tr>
<td>NF-kB</td>
<td>NF kappa beta</td>
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<tr>
<td>P</td>
<td>probability</td>
</tr>
<tr>
<td>P13</td>
<td>phosphoinositide three</td>
</tr>
<tr>
<td>R</td>
<td>right atrium</td>
</tr>
<tr>
<td>RA</td>
<td>right atrium</td>
</tr>
<tr>
<td>REP</td>
<td>reperfusion</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real time polymerase chain reaction</td>
</tr>
<tr>
<td>RV</td>
<td>right ventricle</td>
</tr>
<tr>
<td>RVOT</td>
<td>right ventricular outflow tract</td>
</tr>
<tr>
<td>S</td>
<td>standard cardioplegia</td>
</tr>
<tr>
<td>SC</td>
<td>standard cardioplegia</td>
</tr>
<tr>
<td>T</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TOF</td>
<td>tetralogy of Fallot</td>
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<tr>
<td>TT</td>
<td>total time</td>
</tr>
<tr>
<td>U</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
</tbody>
</table>
SYMBOLS & UNITS

Symbols
α  alpha
β  beta
Σ  sum of
±  plus or minus
>  greater than
°C  degrees celcius
%  percent
2x2  two by two
ΔCt  delta ct
ΔΔCt  delta delta ct
Cl⁻  chloride
χ²  chi square

Units
g  gram
kg  kilograms
ml  millileter
mm  millimeter
mmHg  millimeters of mercury
mmol  millimoles
meq  milliequivalents
mOsm  milliosmolarity
Mg²⁺  magnesium
Na⁺  sodium
pH  hydrogen ion concentration
PO₂  pressure of oxygen
PCO₂  pressure of carbon dioxide
μg  microgram
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17 - ICU total urine volume in infants with TOF randomly assigned to receive either SC or IC during surgery.  

18 - ICU glucose levels at time zero in infants with TOF randomly assigned to receive either SC or IC during surgery.  

19 - ICU PCO₂ over 24-hours in infants with TOF randomly assigned to receive either SC or IC during surgery.  

20 - Sample size of hybridization arrays in each group.
Despite a successful surgical correction, perioperative myocardial injury is the most common cause of mortality and morbidity in pediatric heart surgery (Hirsch et al., 1998, Duke et al., 1997, Hasegawa et al., 2004, Bradley et al., 2001, Allen et al., 2001 and Shen et al., 2003). Ischemia and reperfusion associated with cardiac surgery, are two well established stresses known to contribute to myocardial tissue damage, also known as I/R injury (Bull & Maurer, 2003 and Anselmi et al., 2004). The degree of I/R injury is known to affect the development of postoperative complications (Pavelkova et al., 2006 and Paparella et al., 2002). The most common manifestation of perioperative injury is cardiac dysfunction. In more than 50% of cases, low cardiac output after pediatric cardiac surgery has been attributed to inadequate myocardial protection against I/R injury (Modi et al., 2004 and Goffman et al., 2003). Infants in particular have been shown to fare worse after cardiac surgery compared to children, and thus it is not surprising this age group is an independent risk factor for post-discharge death (Chang et al., 2006 and Stambouly et al., 1996). TOF is not only one of the most severe forms of congenital heart defects (CHD), it is the most common cyanotic CHD with a prevalence of 1/2000 live births (Thom et al., 2006 and Qu, 2004). Patients with TOF require surgery for repair and compared to infants with other CHDs, TOF patients consistently perform worse in the ICU as a result of developing greater perioperative myocardial injury. The composition of cardioplegia has been shown to be an important component of successful operative management in pediatric heart surgery (Doenst et al., 2003, Amark et al., 2005 and Dellgren et al., 2001). The various operative procedures as well as the significant lack of experimental and clinical studies investigating myocardial protection
in pediatric heart surgery have made it difficult to ascertain what type of cardioplegia is best for which type of operative class of patients or procedure (Doenst et al., 2003). For these reasons, myocardial protection during pediatric heart surgery remains suboptimal (Bolli et al., 2004, Allen et al., 2001 and Imura et al., 2001). Insulin-enhanced cardioplegia, as a therapeutic agent during cardiac surgery, has been shown to significantly decrease the degree of perioperative myocardial injury incurred as a result of ischemia/reperfusion injury, as well as significantly improve postoperative cardiac function and thus survival (Bolli et al., 2004 and Das, 2003). A pediatric heart transplant study at the Hospital for Sick Kids demonstrated a significant improvement in patient and graft survival using donor blood cardioplegic solution with added insulin (Dellgren et al., 2001). However, studies investigating insulin-enhanced cardioplegia as a myocardial protective method in pediatric heart surgery for repair of CHD are scarce. In addition, very few studies investigating potential myocardial protective therapies address clinical outcome (Doenst et al., 2003).

Infants with tetralogy of Fallot represent a high-risk patient population for developing perioperative myocardial injury and thus investigation into myocardial protection for this group of patients formed the foundation of this thesis. This study sought to investigate whether insulin-enhanced cardioplegia (IC) during pediatric cardiac surgery provides protection against perioperative myocardial injury in patients with tetralogy of Fallot. To achieve this, the first double-blind, placebo-controlled, randomized clinical study in pediatric heart surgery was designed to investigate the effects of IC on postoperative outcome. Furthermore, due to the complex and interrelated processes that occur between ischemia/reperfusion and insulin, we sought
to investigate the transcript profile using microarray technology as a tool into the global action of insulin-enhanced cardioplegia during surgery.
INTRODUCTION
I – TETRALOGY OF FALLOT
Congenital heart disease (CHD) is the leading cause of all birth defects (Miller-Hance & Tacy, 2004 and Thom et al., 2006). CHD prevalence is the highest among infants, 4 – 10 per 1000 live births (Marelli et al., 2007) and is the most common cause of infant death (Boneva et al., 2001 and Thom et al., 2006). In the US, between 1995 and 1997, approximately 50% of deaths in infants were due to or associated with CHD (Boneva et al., 2001). Severe cardiac abnormalities, characterized as those resulting in fetal loss or death in infancy or childhood (Thom et al., 2006), carries a prevalence of 1.5 per 1000 in infants (Marelli et al., 2007). One of the most common and severe lesions in infants, as well as the major cause of death, is tetralogy of Fallot (TOF) (Thom et al., 2006, Boneva et al., 2001 and Marelli et al., 2007). TOF accounts for 9% to 14% of all congenital heart disease with a prevalence of approximately 1 out of 2000 live births (Thom et al., 2006 and Qu, 2004). Due to its physiological nature it is the most common cause of congenital cyanotic heart disease (Kleinmen, 2004). Since its characterization in 1888, the exact molecular mechanisms responsible for the formation of TOF are unknown (Qu, 2004). It is thought to be due to either environmental factors, genetic factors or both. Unless the disease is surgically repaired, the mortality rate is almost 100% (Anderson & Weinberg, 2007).

A Classical Manifestation

A normal or non-pathological heart consists of 4 chambers; a right and left atrium, separated by an atrial septum and a right and left ventricle, separated by a
ventricular septum. Deoxygenated blood enters the right atrium (RA) via the inferior and superior vena cava and subsequently the right ventricle (RV). Upon contraction, deoxygenated blood in the RV enters the pulmonary artery. The latter is a conduit to the lungs where the deoxygenated blood becomes oxygenated. Blood enters the left atrium (LA) via the pulmonary veins and subsequently the left ventricle (LV). Upon contraction, blood exits the LV and enters the systemic circulation via the aorta (Figure 1A) (Ganong, 1997).

TOF is described by four discrete morphological abnormalities, which present together: 1. A ventricular septal defect (VSD): a hole between the right and left ventricle (Figure 1B) 2. Pulmonic stenosis: an obstruction in the right ventricular outflow tract (RVOT) (Figure 1B). The degree of stenosis varies among TOF patients and is the primary determinant of symptoms and severity. 3. Right ventricular hypertrophy: in order to compensate for obstruction in the RVOT, the muscle in the RV thickens and is thus a secondary anomaly in TOF (Figure 1B). 4. Overriding aorta: the aorta is positioned directly over the VSD rather than the LV. (Figure 1B) (Qu, 2004; Kleinman, 2004 and Anderson & Weinberg, 2005).
Figure 1: A. Morphology of a non-pathological heart. B. Morphology of tetralogy of Fallot. TOF is comprised of four common anatomic abnormalities: 1-VSD (ventricular septal defect), 2-right ventricular outflow tract obstruction (pulmonary stenosis) 3-right ventricular hypertrophy, and 4-overriding of the aorta. (www.nhlbi.nih.gov)

The anatomic spectrum of TOF may include other variants such as pulmonary atresia and atrioventricular septal defect (Alexiou et al., 2001 and Qu, 2004).

Clinical Presentations

Due to the mixing of oxygenated and deoxygenated blood in the left ventricle via the VSD and the preferential flow of deoxygenated blood entering the aorta due to RVOT obstruction, this right-to-left shunt results in low arterial blood oxygen saturation. Depending on the ratio of pulmonary to systemic blood flow, the primary sign is low blood oxygen saturation with anywhere from no cyanosis to severe cyanosis; an arterial oxygen saturation below 95% with possible presentation as a bluish coloration of the skin (Qu, 2004 & Kleinman, 2004). Other symptoms include, failure to
gain weight, difficulty feeding, clubbing of the fingers and toes, polycythemia, dyspnea on exertion and overall poor growth development (Anderson & Weinberg, 2007).
Cardiac Surgery = Ischemia/Reperfusion Injury

It is estimated that 2.3 per 1000 live births or one third of 300,000 infants with CHD born in the United States each year will require surgical treatment within the first year of life (Thom et al., 2006 and Clancy et al., 2000). Cardiac surgery often subjects the heart to a period of ischemia (I); a reduction or complete blockade of blood flow and thus loss of oxygen supply to a tissue or organ, followed by a period of reperfusion (R); restoration of oxygenated blood flow (Marczin et al., 2003 and Bull & Maurer, 2003). Although surgery is necessary in order to repair the defect, both ischemia and reperfusion are known to induce perioperative myocardial injury (Podgoreanu et al., 2005, Ungerleider, 2005 and Shen et al., 2003). The pathogenesis is I/R injury is complex and involves the activation, coordination and amplification of several pathways, many of which have yet to be completely defined (Toledo-Pereyra et al., 2004). Two events, apoptosis and inflammation, although not fully elucidated, are initiated during I/R and are well known to contribute to the development of postoperative complications (Shernan, 2003, Bull & Maurer, 2003 and Paparella et al., 2002).

Limiting perioperative myocardial injury is a major research goal in cardiac surgery, since it has been shown to be a major source of serious postoperative complications such as cardiac, renal, neurological and pulmonary dysfunction in pediatrics with CHD (Modi et al., 2003, Allen, 2003 and Stafford-Smith, 2005).

Perioperative myocardial injury is greater in patients with TOF

Studies have shown that infants develop greater perioperative myocardial injury after cardiac surgery compared to children (Modi et al., 2003). Furthermore,
perioperative myocardial injury is greater in patients with TOF compared to patients with other CHDs. Cardiac troponin-I (cTnI) is found exclusively in myocardial cells and it acts as a potent inhibitor of actin-myosin cross-bridge formation. Numerous studies have confirmed cTnI as a specific and sensitive biochemical marker of myocardial damage. Studies have shown significant correlations between postoperative cTnI serum levels and length of ICU and hospital stay, suggesting that elevated cTnI levels reflect difficulty of recovery after congenital heart surgery (Hirsch et al., 1998 and Lipshultz et al., 1997, Immer et al., 1999). In a study of 37 patients (2 days to 116 months) with various CHDs including TOF undergoing elective repair requiring CPB, Hammer et al. (2001) found serum cTnI levels to peak immediately after surgery as well as increase 4-fold when aortic cross-clamp times exceeded 80 minutes. Taggart et al. (1997) found significant correlation with an increased release of cTnI in patients less than 12 months of age. In 21 patients with elective ventricular septal defect (VSD) repair, (3 – 21 months), who underwent an average ischemic time of 35 minutes (range 23 – 56), Caputo et al. (2002) found serum troponin I levels in infants to be almost two fold greater compared to children. ATP, the main energy source for myocardial contraction, is strongly correlated to cardiac function (Ozeki et al., 2007, Woo et al., 2005 and Taylor et al., 2005). In Taggart et al. (1997) study, infants also demonstrated a significant drop in ATP (adenosine triphosphate) levels after ischemia compared to children, 40% vs. 10%, respectively. Hasegawa et al. (2005 & 2004) monitored serum levels of heart fatty acid-binding protein (HFABP), a rapid indicator for assessment of myocardial damage after cardiac repair in infants and children (n = 98) with VSD (2005) and in a separate study consisting of infants and children with CHDs, including TOF (n = 220) (2004). In both
studies the authors observed a significantly greater release of HFABP in infants, compared to children, both peri and postoperatively, as well as a worse postoperative outcome; an increase in inotropic support, ICU stay and duration of intubation.

Thus, cardiac surgery places the heart at risk of incurring myocardial injury. Infants with tetralogy of Fallot develop greater myocardial injury during surgery, compared to infants and children with other CHDs, and thus represent a unique high-risk patient population.
Postoperative Outcome

It is well documented that perioperative myocardial injury is a major
determinant of postoperative complications after pediatric heart surgery (Modi et al.,
2003, Modi et al., 2004, Hirsch et al., 1998, Duke et al., 1997, Hasegawa et al., 2004 and
Bradley et al., 2001). Although operative mortality for patients with TOF is less than 1%,
it is the postoperative period, which remains a concern for these patients (Alexiou et al.,
Sahn, 2004). Clinical post-operative parameters, such as length of ICU stay and
hemodynamic indices are excellent markers for evaluating the degree of damage
incurred during surgery (Sachdev et al., 2006).

A common postoperative complication observed in TOF patients is a decrease in
cardiac output as a result of right ventricular dysfunction (Shekerdemian et al., 2000,
Gaynor, 2005 and Chowdhury et al., 2006). A decrease in cardiac output, otherwise
known as low cardiac output syndrome (LCOS), is a major contributor to morbidity and
mortality after pediatric heart surgery (Modi et al., 2004 and Hoffman et al., 2003).
Studies have demonstrated a correlation between LCOS and an increase in intensive
care unit (ICU) stay and inotropic support (Modi et al., 2004 and Chittithavorn et al.,
2006). Although Alexiou et al. (2001) reported an operative mortality of 1.1% in 89
infants (15 days to 12 months) with simple TOF, almost 15% displayed early
postoperative complications. The majority of patients exhibited cardiorespiratory
failure and thus required significantly prolonged ventilation and/or inotropic support.

As mentioned earlier, cTnI is a specific marker of perioperative myocardial
injury. In infants, serum cTnI levels have been shown to significantly correlate with the
following postoperative parameters; duration of intubation, inotrope duration and ICU stay (Modi et al., 2003). Hirsch et al. (1998) measured and correlated cTnI levels with intra and postoperative parameters in 55 patients with ASD, VSD and TOF (n = 16). TOF patients demonstrated the greatest cTnI release immediately postoperatively and the levels significantly correlated with duration of CPB, aortic cross-clamp time, ICU stay and hospital stay. Compared to ASD and VSD patients, TOF patients remained in the ICU and hospital significantly longer. Inotrope use was also greatest in the TOF group. In a multiple regression analysis involving 64 infants and children with various CHDs including TOF (n = 6), TOF as a pathology was an independent and significant variable for cTnI release (Taggart et al., 1997). In a study involving 133 patients with ASD (n = 41), VSD (n = 46) and TOF (n = 46), Modi et al. (2003) monitored cTnI release during a 48- hour postoperative period. Compared to ASD and VSD patients, TOF patients exhibited a significantly greater cTnI release over the entire 48-hour period. TOF patients also exhibited longer durations of inotropic support, ventilation, ICU and hospital stay.

Thus, postoperative outcome is a reflection of the degree of perioperative injury incurred. Patients with tetralogy of Fallot appear to demonstrate a worse postoperative outcome and thus, management of I/R injury is of great importance in this particular pediatric patient population.
II – Insulin Enhanced Cardioplegia
Insulin

Insulin is a peptide hormone secreted by the β cells of the pancreatic islets of Langerhans in response to increased circulating levels of glucose. Most notable actions of insulin include glucose homeostasis, lipid synthesis in liver and fat cells and attenuating fatty acid release from triglycerides in fat and muscle. In addition to the above, insulin mediates activation of gene transcription, regulation of ion channels and transporters, cellular growth and survival, and many more (Pessin & Saltiel, 2000 and Summers et al, 1999).

Insulin’s actions are mediated via the insulin receptor, a transmembrane glycoprotein with protein kinase activity. The receptor is an α2/β2 tetramer with the α-subunits located on the extracellular surface (Figure 2). Insulin binds to the α-subunit of the receptor and induces autophosphorylation and activation of the protein kinase domain on the cytoplasmic portion of the β-subunit. Once activated, several key intracellular proteins become phosphorylated, which in turn ignite a cascade of diverse and complex signaling pathways downstream, many of which remain elusive (Hiraoka, 2003, Pessin & Saltiel, 2000 and Summers et al, 1999).

One of the most important and well-documented effectors is PI3-Kinase (phosphatidylinositide 3′-kinase). PI3-kinase stimulates signaling pathways ultimately leading to the phosphorylation of the serine/threonine kinase Akt/PKB. Once activated, Akt/PKB ignites insulin signaling pathways downstream involved in glycogen synthesis, protein synthesis and the inhibition of apoptosis. Evidence supporting Akt/PKB’s role in mediating insulin’s regulation of cell survival is substantial (Summers et al., 1999 and Pessin & Saltiel, 2000).
Figure 2: The insulin receptor is a $\alpha_2/\beta_2$ tetramer. Insulin binds to the $\alpha$ subunits located on the extracellular surface, inducing phosphorylation on the $\beta$ subunits (Bowen, 2004).
Cardioplegia composition is considered the most important instrument in mitigating perioperative myocardial injury, as well as, in the successful operative management in pediatric heart surgery (Åmark et al., 2005, Allen et al., 2001, Doenst et al., 2003, Dellgren et al., 2001 and Mou et al., 2004). Although it is accepted as the gold standard in myocardial protection, protection during pediatric heart surgery remains suboptimal. The various operative procedures as well as the significant lack of experimental and clinical studies in pediatric heart surgery have made it difficult to ascertain what type of cardioplegia is best for which type of operative class of patients or procedure (Doenst et al., 2003). Nevertheless, limiting perioperative myocardial injury remains a priority in these patients as it significantly dictates postoperative outcome (Allen, 2004).

A recent meta-analysis of eleven randomized studies employing GIK during cardiac surgery (n = 468 patients with CABG or heart valve replacement), concluded that insulin significantly improved postoperative cardiac function and reduced the risk of arrhythmias after surgery (Bothe et al., 2004). Insulin’s beneficial effects are believed to operate via two major cellular pathways responsible for I/R injury; apoptosis and inflammation (Eefting et al., 2004 and Yellon and Baxter, 1999). A decrease in apoptosis and inflammation are associated with a significant improvement in postoperative hemodynamic performance. In the few studies failing to demonstrate a benefit with insulin, adverse events were not observed (Das, 2003 and Doenst et al., 2003).
Attenuates I/R Injury

Apoptosis, programmed cell death, occurs after I/R and contributes to the loss of cardiomyocytes resulting in cardiac dysfunction (Aikawa et al., 2000). There is substantial experimental evidence demonstrating insulin’s ability to attenuate I/R induced apoptosis. In isolated rat heart models of I/R, insulin administered at the onset of reperfusion was associated with a significant decrease in apoptosis, specifically via the prosurvival target of AKT, BAD, and thus myocardial cell death (Jonassen et al., 2001, Sack & Yellon, 2003 and Jonassen et al., 2000). This reduction in I/R apoptosis was associated with an improvement in cardiac function. In an acute myocardial ischemia/reperfusion canine model, insulin administered during reperfusion significantly inhibited apoptosis in coronary arterial endothelial cells. The latter was associated with a significant improvement in cardiac functional recovery compared to the control group (Ma et al., 2006).

In addition to apoptosis, persistent lactate production following ischemia has been associated with a delay in recovery of aerobic metabolism and subsequently postoperative ventricular dysfunction (Cohen et al., 1999). Cardiomyocyte cultures from patients with tetralogy of Fallot subjected to insulin prior to simulated ischemia/reperfusion demonstrated an increase in cell viability, ATP and a reduction in extracellular lactate release, compared to control (Rao et al., 1998). Similar findings were noted in CABG patients infused with insulin by Kjellman et al. (2000), that is, lactate production was significantly decreased in the insulin group (n = 13) during ischemia compared to control (n = 15).

Cardiac surgery is associated with systemic inflammation, which has been shown to significantly contribute to adverse postoperative complications (Pavelkova et
al., 2006). One of the most potent mediators of inflammation is TNF-α, a cytokine released by cardiac tissue. TNF-α directly decreases myocardial contractility in a dose-dependent manner (Das, 2003). Numerous studies have shown insulin to inhibit the production of TNF-α, as well as increase the production of anti-inflammatory cytokines, IL-4 and IL-10 (Das, 2003). Insulin has also been shown to decrease NF-κB, a key mediator in the expression of pro-inflammatory cytokines (Dandona et al., 2002). In a prospective, randomized, controlled study involving adults admitted to the surgical ICU after an acute MI, those receiving intensive insulin therapy demonstrated a significant reduction in inflammation (CRP levels), as well as a 50% decrease in mortality (Das, 2002).

**And Thus Improves Cardiac Function & Postoperative Outcome**

In cardiac surgery, insulin has been shown to improve cardiac function and reduce inotrope requirement after coronary artery bypass surgery (CABG) (Ranasinghe et al., 2006). Bothe et al. (2004) conducted a meta-analysis of randomized studies examining the effect of insulin during cardiac surgery. Data from 11 studies, which included two groups of patients: CABG and heart valve replacement, noted a significant improvement in postoperative cardiac function as well as a reduction in the incidence of atrial arrhythmias in patients receiving insulin. The majority of the studies also reported lower inotropic requirements in the insulin group. A prospective randomized, double blind placebo-controlled trial, conducted by Quinn et al. (2006), was designed to investigate insulin’s role in providing myocardial protection to patients undergoing first time CABG. Patients in the insulin group (n = 138) demonstrated higher cardiac indices, a decreased incidence in low cardiac output
episodes, and a decrease in inotropic support. Furthermore, myocardial injury, indicated by serum troponin I (cTnI) levels, was significantly less in the insulin group at 6 hours postoperatively compared to placebo. These findings have been repeated by the same authors in an additional 240 patients undergoing first time isolated on-pump CABG (Ranasinghe et al., 2006). In 24 adult patients undergoing isolated (CABG) with tepid (29°C) cardioplegia, patients randomized to receive insulin-enhanced (10IU/L Humulin R) cardioplegia (n = 13) displayed significantly greater lactate extraction during early reperfusion and significantly greater cardiac output compared to patients receiving standard cardioplegia (n = 11) (Rao et al., 1996). In a porcine model of I/R, pigs receiving insulin demonstrated a significant improvement in systolic, diastolic and global LV function with greater lactate extraction (Zhu et al., 2000). The latter two studies suggest insulin administration during surgery may attenuate cardiac dysfunction as a result of ischemia/reperfusion injury. In another porcine model of I/R, pigs receiving insulin during ischemia and reperfusion demonstrated significantly less tissue acidosis, reduced cell death and improved cardiac function (Lazar et al., 1995). In pediatric heart transplant patients, donor heart organs reperfused with insulin demonstrated a significant improvement in patient and graft survival compared to the standard cardioplegic solution (Dellgren et al., 2001). Thus, insulin administered during ischemia and/or reperfusion attenuates I/R injury, specifically, apoptosis, lactate production as well as, significantly improves postoperative outcome.

Insulin has also been shown to significantly improve hemodynamic performance in patients with preoperative cardiac impairment. Thirty patients demonstrating impaired preoperative cardiac function scheduled for mitral valve replacement were randomly assigned to receive either saline (n = 15) or insulin (n = 15) 12 hours preop.
Those who received insulin required less inotropic support, had fewer ventricular arrhythmias and improved hemodynamic indices; increased cardiac output and decreased systemic vascular resistance (Besogul et al., 1999). Girard et al. (1992) demonstrated an improvement in cardiac index, right ventricular workload index and a decrease in systemic vascular resistance in patients who received insulin one hour before CPB compared to those who received placebo. The authors demonstrated, although it did not achieve statistical significance, a 2-fold lower requirement in inotropic support and arrhythmias in patients with a cardiac index of 2.5 L/min.m-2. In a double blind study, similar improvement in hemodynamic indices as well as postoperative outcome were found in CABG patients with unstable angina and/or compromised left ventricular function (Wistbacka et al., 1995 and Wistbacka et al., 1994).

Insulin has been shown to improve postoperative impaired contractile function when applied as a metabolic therapy. In patients with left ventricular failure postoperatively, Gradinac et al. (1989) randomly assigned twenty-two patients to receive either insulin therapy or placebo for up to 48 hours postoperatively. Patients in the insulin group (n = 11) demonstrated a significant decrease in inotropic support and a significant increase in cardiac index compared to the placebo group. Similar studies have repeated the latter findings in the treatment of cardiac failure after cardiac surgery (Coleman et al., 1989 and Svedjeholm et al., 1995). The above data highlight insulin’s cardioprotective role in a functionally compromised heart.
Microarray Technology

Microarray is a powerful tool for studying the expression of thousands of genes simultaneously, otherwise known as the expression profile. The profound implications of this technology has placed it at the heart of a wide variety of medical and biological research studies such as; classifying diseases, understanding complex biological processes and functional disorders, as well as identifying new drug targets (Chen et al., 2004, Quackenbush, 2006 and Napoli et al., 2002).

**Microarray Technique**

The experiment is a multi-step process (Chen et al., 2004). In general, thousands of oligonucleotides (probes) are fixed to a slide surface (also known as the gene chip). The target mRNA is extracted from the tissue of interest, fluorescently labeled and allowed to hybridize to complimentary gene specific probes on the array (Figure 3). Once hybridization is complete, the level of fluorescence intensity represents an estimated level of gene expression (Quackenbush, 2006 and Allison et al., 2006).

**Two Types of Microarrays**

The two most basic types of microarrays are the two-color arrays and the Affymetrix GeneChips®.

In the two-color arrays, two samples of RNA, usually a test and a control, are each labeled with different dyes and simultaneously hybridized to the array (Figure 4A). This approach compares paired samples and reports expression as ratio of RNA in the test sample relative to the control sample (Quackenbush, 2006).

Affymetrix GeneChip® use a set of 11-20 oligonucleotide probes, each consisting of 25 bases in length (25-mer), to represent a gene. While the 25-mer probe length
confers high specificity, multiple probes provide high sensitivity and reproducibility. Each probe pair is designed to have a perfect match (PM) to its target sequence, and a mismatch (MM), whose sequence is identical to the PM probe except for a single base in the center. The MM serves as an internal control for its PM partner by hybridizing to non-specific sequences. This PM/MM probe pair maximizes sensitivity and specificity.

The mRNA sample is converted to biotinylated cRNA and one array is used for each sample (Figure 4B). Expression levels per each gene in each sample are generated as CEL files and used in subsequent data analysis (Gautier et al., 2004, Quackenbush, 2006, Allison et al., 2006 and Affymetrix, 2001).

Figure 3: The underlying concept of microarray technology. Target RNA is fluorescently labeled and the amount of fluorescence upon hybridization is a reflection of gene expression level. (The above figure was excerpted from Quackenbush, NEJM, 2006;354:2463-72).
Figure 4: Two basic types of microarray systems. A) Two-colored. A target sample is labeled with one dye and a control sample is labeled with another dye. Both samples hybridize to the same array. Gene expression is a ratio of target to control. B) Affymetrix GeneChip®. The target sample is labeled and hybridizes to one array. Gene expression values are based on fluorescence intensity. (The above figure was excerpted from Quackenbush, NEJM, 2006;354:2463-72).
Microarray expression data analysis remains a challenging and daunting task and to date, a universal analytic procedure does not exist (Gautier et al., 2004). Data analysis involves 3 key steps: 1) Preprocessing, 2) Inference Testing and 3) Validation of findings (Allison et al., 2006).

1) **Normalization**

In order to compare gene expression results, it is essential to normalize the data. Normalization involves adjusting microarray probe intensities in order to take into account experimental variability. There are several methods available and each one attempts to detect and correct systematic differences by compensating for differences in labeling, hybridization and RNA quantity between chips, thus allowing data from different chips to be directly compared (Gautier et al., 2004, Allison et al., 2006 and Quackenbush, 2006). The optimal normalization procedure continues to be an area of active research and debate (Quackenbush, 2006).

2) **Inference Testing**

This step involves statistical analyses of the hypotheses. Unfortunately a universal statistical package does not exist for this daunting task. Thus one must choose a software package tailored to the experimental design and specific aims (Wettenhall and Smyth, 2004). Once the appropriate statistical tests are conducted, generating a gene list or potential biomarkers is a balance between biological relevance and statistical significance (Allison et al., 2006).

In dealing with thousands of genes, multiple testing becomes an issue. The probability of producing false-positives (Type I error), increases sharply (Reiner et al.,
Benjamini and Hochberg (1995) devised the False Discovery Rate (FDR) in order to control the Type I error rate. FDR is the expected proportion of erroneously rejected null hypotheses among the rejected ones. FDR control is considered sufficient when the purpose of the microarray experiment is to extract genes as potential candidates for further investigation (Reiner et al., 2003).

3) Validation

This step involves confirming the veracity of the microarray data results. Real time reverse transcriptase (RT) polymerase chain reaction (real-time RT-PCR) is currently the most powerful technique to quantify gene expression (Larionov et al., 2005 and Livak & Schmittgen, 2001). Upon gene selection, real-time RT-PCR simultaneously measures the gene expression levels as the reaction is proceeding in many different samples (Vandesompele et al., 2002 and Ginzinger, 2002).

RT-PCR involves two steps: synthesis of complimentary DNA (cDNA) from RNA by reverse transcription (RT) and amplification of cDNA by PCR. The PCR reaction consists of 3 phases: 1) Exponential, 2) Linear and 3) Plateau (Figure 5). In the Exponential Phase, the product is doubling at every cycle. During the Linear Phase the reaction components are being consumed and hence the reaction begins to slow down. Depletion occurs at different rates for each sample, and thus this phase is highly variable. The Plateau Phase reflects reaction termination. Due to the variability in reaction progress in the linear and plateau phases, the exponential phase is the optimal point for analyzing data (Ginzinger, 2002). In real-time PCR experiments, a fluorescence signal threshold is determined during the exponential phase. The threshold is the point at which the reaction reaches a fluorescent intensity above background. The cycle at which the sample reaches this threshold is the cycle threshold (Ct) value. These values are
directly proportional to the amount of starting material and are the basis for calculating mRNA expression levels (Ginzinger, 2002).

**Figure 5:** The above diagram depicts the 3 phases of a PCR reaction for one sample. At the start of the reaction, the samples begin to amplify exponentially (Exponential Phase). Reporter dyes are fluorescing during this phase. As the reaction progresses, reagent depletion occurs and doubling no longer occurs (Linear Phase). Upon reaction completion, the sample reaches the Plateau Phase.
**SPECIFIC AIM**

The primary aim of this randomized, double-blind, placebo-controlled clinical exploratory trial is to provide clinically and molecular informative data toward improving myocardial protection during cardiac surgery in infants with tetralogy of Fallot, a patient population highly susceptible to developing cardiac dysfunction.

**GOALS**

Thus, the following two intimately linked goals formed the foundation of this thesis:

1. To assess the effect of insulin-enhanced cardioplegia on clinical outcome.

2. To determine the potential mechanisms of action of insulin-enhanced cardioplegia at the gene expression level.
HYPOTHESES & OBJECTIVES
**HYPOTHESES**

Compared to patients randomly assigned to receive Standard Cardioplegia (SC), patients randomly assigned to receive Insulin-enhanced Cardioplegia (IC) will exhibit:

1. Improved postoperative outcome, reflective of a decrease in perioperative myocardial injury.

2. Improved cardiac function.

3. A cardioprotective gene expression signature during surgery.

**OBJECTIVES**

The following objectives will assess the above hypotheses. In SC and IC patients, to determine:

1. A) Length of ICU stay
   B) Length of HSC stay
   C) Chest tube volume and duration
   D) The following every 4-hours during a 48-hour period in the ICU: lactate, base excess and deficit, PO$_2$, PCO$_2$, pH and bicarbonate.

2. A) Duration of ventilatory support.
   B) Inotropic support (duration, amount and total).
   C) Urine output every 24 hours for 2 days.
   D) Mixed venous saturation (%).

3. In myocardial tissue biopsies from SC and IC patients, to determine and compare:
   A) The gene expression profile during ischemia.
   B) The gene expression profile during reperfusion.
STUDY DESIGN

The study was approved by the Hospital for Sick Children’s Research Ethics Board (REB 1998/060). The study’s rationale and design was thoroughly explained by the Research Coordinator to the parent(s)/guardian(s) of the patient (Appendix 1). Those in agreement to participate signed the respective consent form (Appendix 1).

PATIENT POPULATION

Thirty infants (1 mo. – 12 mos.) with tetralogy of Fallot (TOF) were enrolled based on the criteria below.

Enrollment Criteria

Inclusion Criteria:

1. Elective cases requiring cardiopulmonary bypass.
2. Myocardial resection.
3. Weight greater than 2.2 kg. (With weights less than 2.2 kg there is generally a higher incidence of pre-maturity as well as a significant greater risk of mortality).
4. Mortality of repair estimated to be less than 15%. (Simple TOF have an associated mortality less than 15% and TOF with the addition of other complex lesions not only increase the mortality risk but would obscure the heterogeneity of the study).

Exclusion Criteria:

1. True emergent operation.
2. Acute infection.
3. Disorders of the pituitary adrenal axis.
4. Use of other investigational drugs except nitric oxide or phenoxybenzamine.

**Sample Size**

As a clinical observational study, a meaningful power calculation was not possible. The expected number of qualified patients in a one-year span was fifty and the expected number to enroll was thirty, resulting in a 60% participation rate and thus the sample size chosen.

**Randomization**

We attempted to limit the degree of heterogeneity in a CHD patient population by selecting subjects who underwent the same anesthetic, surgical and perfusion techniques, and who were as phenotypically similar as possible with the exception of gender and ethnic background. To achieve balanced study arms, the effect of the variation in patient population was accounted for by randomization. Using random sized blocks using a random number generator, patients were randomly assigned in a double-blind manner to one of the following cardioplegia groups: Standard cardioplegia (SC, n = 15) or Insulin-enhanced cardioplegia (IC, n = 15), in which participants, doctors and nurses were blinded to which protocol the patients was receiving.

**CARDIOPLEGIA COCKTAIL & PROTOCOL**

**Cardioplegia (CP) Cocktail**

Both groups had blood-based CP (2 parts blood: 1 part crystalloid).

**Standard Cardioplegia (SC)**
Potassium, glucose and magnesium were added to a base solution of 1000 ml of Plasma-Lyte 148 base solution (Baxter Healthcare Corporation, Deerfield Ill) to achieve concentrations as follows:

**Initial Infusion:**

Na⁺ 134 mmol, K⁺ 45 mmol, Glucose 25 mmol, Cl⁻ 134 mmol, Mg²⁺ 41.5 mmol, pH 5.8-6.3, osmolality 405 mmol/kg H₂O approx. Just prior to use, 10 meq of NaHCO₃ and 25 mOsm of 50% dextrose were added per litre.

**Reinfusion – to maintain cardiac arrest:**

Na⁺ (136 mmol), K⁺ (25 mmol), Glucose (12.5 mmol), Cl⁻ (116 mmol), Mg²⁺ (21.5 mmol), pH 5.76-5.91, osmolality (334 – 340 mmol/Kg H₂O).

**Insulin-Enhanced Cardioplegia (IC)**

Regular Humulin insulin (Eli Lilly and Company, Indianapolis, Ind), biosynthetic insulin, was added to the standard cardioplegia cocktail to achieve a final concentration of 10IU/L.

**Cardioplegia Protocol**

Once the appropriate level of sedation was achieved with Anagen, a sternotomy and thymectomy were performed. Cardiopulmonary bypass was initiated through aortic and bicaval cannulation at normothermia. An aortic crossclamp was applied and 30 ml/kg cold cardioplegic solution was delivered to the heart via the aortic root. The defect was exposed and repaired, during which cardiac arrest was maintained by subsequent doses, 10-20 ml/kg, of the respective cardioplegic solution every 20 minutes. Upon repair and chest closure, adequate rewarming and reperfusion was
initiated and the patient was weaned off cardiopulmonary bypass. A bolus (50 - 100 ml) of FFP (with citrate anticoagulant) and 25% Mannitol (0.5 g/kg) was given prior to cross-clamp removal. The FFP serves to reduce the circulating calcium level and the mannitol acts as an osmotic diuretic and free radical scavenger.

**Biopsies**

Biopsies were obtained from resected myocardial tissue as part of the repair. Using biopsy forceps or a scalpel, myocardial tissue (about 2x2 mm in size) was resected from the right ventricular outflow tract just prior to crossclamp removal (end-ischemia) and at five minutes during the rewarming phase (reperfusion). Upon resection, the tissue was immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

**CLINICAL PARAMETERS**

Various clinical outcome variables were collected during the Operative period and the Intensive Care Unit (ICU).

**Operative**

**Preoperative**

The following time measurements and parameters were recorded prior to CPB:

- CPB start time.
- Time aortic cross-clamp on.
- Pre-CPB coronary sinus serum lactate level (mmol/L).
- Pre-CPB arterial serum lactate level (mmol/L).
**Perioperative**

The following measurements were recorded during CPB:

- Highest serum glucose level ($ORHGL$) and lowest serum glucose level ($ORLGL$) (mmol/L).
- Highest serum lactate level ($ORHLL$) and lowest serum lactate level ($ORLLL$) (mmol/L).

**Post-CPB/Postoperatively**

The following time measurements and parameters were recorded immediately following surgery:

- CPB Time-off and CPB total time (TT)
- Aortic Cross Clamp TT
- Coronary Sinus Serum Lactate (mmol/L) at 1 and 5 minutes.
- Arterial Serum Lactate (mmol/L) at 1 and 5 minutes.

**Intensive Care Unit (ICU)**

The following clinical variables were recorded and/or measured during the ICU period. Variables are divided into primary (cardinal markers of outcome assessment) and secondary.

**Primary**

- Length of ICU stay (days)
- Length of hospital (HSC) stay (days)
Duration of ventilatory support (hrs.)

Inotropic Support (1- Dopamine, 2-Nipride, 3-Milrinone, 4-Epinephrine): dose required at 4-hour intervals for the first two days.

Secondary

- Every 4 hours for a 24-hour period in the ICU:
  - $PO_2$
  - $PCO_2$
  - $pH$
  - Bicarbonate
  - Serum Glucose
  - Serum Lactate
  - Base excess and deficit
- Urine output (ml): every 24 hours for 2 days.
- Chest Tube: Volume (ml) and duration (hours).
- Mixed Venous Saturation (%) was recorded at no specific time during the patient’s ICU duration.

STATISTICS

The overall objective was to determine if insulin had a positive effect on infants undergoing heart surgery. Any predictors that may influence the response variables were adjusted for in the analysis. These predictors included age, sex, weight and in some instances time.

Single Measurements
General Linear Models (GLM) were used (SAS : proc GLM) to assess the effect of insulin on the outcome variables listed above. All models included the following predictors: age, sex and weight, to attenuate potential sampling biases.

The following outcome variables were not normally distributed: ICU (intensive care unit) DAYS, HSC (hospital stay) DAYS, REP (reperfusion duration), CPBTT (cardiopulmonary bypass total time) and AXCTT (aortic cross-clamp total time). To normalize the distribution of the data, the log of the variables was used as the response rather than the raw data values. Initial models included all predictors and interactions between group*age, group*sex and group*weight. Factors (including interactions) were sequentially removed from the preliminary models until the final model contained only significant factors or the main factor of interest at the 0.05 \( \alpha \) level. All p-values generated were 2-sided except if the hypothesis was 1-sided, i.e.: insulin improves the infant’s outcome, in which case a 1-sided p-value was generated.

Paired data was statistically analyzed using a paired \( t \)-test (SPSS 12.0), with significance set at \( p \leq .05 \).

Inotropic support was measured in terms of duration (hrs.), frequency (# patients on a particular inotrope), number of inotropes (# inotropes each patient received per group) and score. Inotropic score was calculated using two separate methods, that of Moise et al. (1995) defined as the \( \Sigma \) of the rate (\( \mu \text{g/kg/min} \)) x number of hours delivered during the first 48 hours and the second method, by Besogul et al. (1999), which divides score across the 48-hour period at 4-hour intervals summed at 12-hour intervals. An independent \( t \)-test, with significance set at \( p \leq .05 \), was performed to test the following null hypotheses: 1. The mean difference in duration between the two study groups is zero. 2. The mean difference in frequency between the two study groups is zero. 3. The
mean difference in score between the two study groups is zero and 4. The mean
difference in number of inotropes between the two study groups is zero. To test the null
hypothesis that the frequency of inotropic support is independent of cardioplegia
treatment a $\chi^2$ test was performed.

Repeated Measurement Analysis

Repeated measurement analysis was performed using a mixed model (SAS; proc
mixed) for variables collected on the same patient over time (i.e.: ICU data collected
every 4 hours for a 24 hour period). Models were constructed and reduced in the same
way as previously described. Interpretation follows that of the GLM.

All the above statistical permutations have been approved by the Statistical
Consulting Department at the University of Toronto (Prof. L. Duquette).

MICROARRAY

Total RNA Extraction

Each tissue biopsy was placed in a collection tube (Qiagen - 19560) containing 1
ml of Trizol (Invitrogen - 15596-02) and four 2.5 mm stainless steel beads (Glen Mills
Inc.) and was homogenized using a Mini-Bead Beater (Glen Mills Inc., 3110BX). Tissue
homogenate was centrifuged (Beckman Allegra 64R Centrifuge - F3602) and the
supernatant removed. The addition of chloroform (Sigma - C-2432), followed by
centrifugation, separated the supernatant solution into the aqueous RNA phase and the
organic phase. The RNA phase was precipitated at 4°C overnight using isopropanol
(Sigma - I-9516) and linear acrylamide (AMBION Inc. - 9520). The solution was
centrifuged to achieve an RNA pellet. The RNA pellet was carefully washed using 75% ethanol, briefly air-dried and dissolved in RNAse-free water (Sigma - W4502).

RNA quantity, by A260 measurement, was measured using UV/Visible Spectrophotometer (Pharmacia Biotech - Ultrospec 3000) and RNA integrity was analyzed using the Agilent BioAnalyzer (Agilent Technologies - 2100). The RNA pellet was stored at -80°C until further analysis.

**GeneChip Hybridization**

Only RNA samples with an OD ratio of 1.8-2.0 at 260/280 were used for microarray analysis. A total of 27 samples: (SC Group: n = 8 Ischemia, n = 8 Reperfusion and IC Group: n = 5 Ischemia, n = 6 Reperfusion) (Figure 6), were hybridized on the Human HG-U133A GeneChip Set (Affymetrix, Santa Clara, CA). All Samples were prepared according to standard Affymetrix instructions and performed by the same facility staff member at the Microarray Facility, The Centre for Applied Genomics at the Hospital for Sick Children.

Briefly, a primer encoding the T7 RNA polymerase promoter linked to oligo-dT_{17} was used to prime double-stranded cDNA synthesis from each mRNA sample using Superscript II RNase H- reverse transcriptase (Life Technologies, Rockville, MD). Each purified (Qiaquick kit, Qiagen) double-stranded cDNA was in vitro transcribed using T7 RNA polymerase (Enzo IVT Kit, Biochemicals, New York, NY), incorporating biotin-UTP into the cRNAs followed by purification using RNEasy (Qiagen), and quantitated
by measuring absorption at 260 nm/280 nm. Samples were fragmented and hybridized to the HG U133A chip for 16 hr. at 45°C and scanned (Agilent GeneArray 2500 Scanner).

Figure 6: In the SC group, a total of 12 patients were arrayed, of which 4 are paired. In the IC group 6 patients were arrayed, of which 5 are paired. In total 27 samples were arrayed. (n denotes the number of samples arrayed.)

**Data Analysis**

The study design is a 2x2 factorial design (Figure 7). The two factors are Cardioplegia Composition (with levels standard and insulin) and Surgical Period (with levels ischemia and reperfusion). The main effect is ‘The effect of insulin during surgery’ and the arrows in Figure 7 denote the four interactions. Gene expression profiles were statistically analyzed using two independent softwares; affylmGUI (affymetrix linear modeling Graphical User Interface, Bioconductor & Insightful) and ArrayAssist V2.6 (Iobion Informatics). affylmGUI was chosen for it’s robust ability to accurately analyze gene expression data with small number of arrays as well as its capability to run an ANOVA (Smyth, 2004). In analyzing microarray data ANOVA increases the sensitivity and specificity and when combined with a t-test the confidence in data interpretation increases as well (Pavlidis, 2003). Paired data was analyzed using a paired t-test in ArrayAssist. In both softwares, gene chip data were normalized using CG-Robust Multichip Analysis (GC-RMA) (Wu et al., 2004).
**Figure 7**: 2x2 Factorial Design. Factor 1 = Cardioplegic Composition; Control: standard and Insulin: insulin supplemented, Factor 2 = Period of Surgery; ischemia or reperfusion. Arrows denote contrast groups or interactions. (Ctrl = control, Isch = Ins = insulin, Isch = ischemia and Rep = reperfusion)

In determining a differential expression (DE) and p-value cut-off several techniques were integrated and employed. In ArrayAssist the DE cut-off and p value were varied and various gene lists were generated. A gene list was deemed relevant if a great majority of the genes had a biological association with insulin. RT-PCR was used to verify the DE cut-off and associated p value. The DE cut-off was found to be 0.6 (1.2 fold change) and genes were considered significant at a p value \( \leq 0.05 \). In affylmGUI, a gene list was generated for all four contrasts, with and without Benjamini-Hochberg/FDR as the correction method for multiple testing.

Paired data was analyzed using Array Assist using a paired t-test and the DE and p value were kept the same.

**Quantitative Real Time RT-PCR**

Quantitative real time reverse-transcriptase (RT) polymerase chain reaction (PCR) was used to confirm mRNA expression levels yielded by microarray. Quantitative RT-PCR was performed using pre-designed FAM-labeled TaqMan primer sets (Applied Biosystems, Branchburg, NJ) and expression was determined using the ABI PRISM 7700 sequence detection system.

Total cDNA was synthesized in a two-step RT-PCR protocol using Superscript First-Strand Synthesis for RT-PCR. Quantitative RT-PCR was performed on 3.6ng of total cDNA per well. For each gene of interest, each sample was run in triplicate and only samples demonstrating consistent findings were used in subsequent analyses.

All quantitative PCR measurements were normalized to the amount of GAPDH cDNA, which served as the endogenous control. Data were expressed as cycle threshold (Ct) values and used to determine ΔCt values (Ct = Ct of target gene minus Ct of endogenous control). Using the ΔΔCt values, relative mRNA expression levels of randomly selected genes were calculated as $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001).
RESULTS
The study population consisted of 30 infants who were randomly assigned in a double-blind manner to receive either standard cardioplegia (SC) or insulin-enhanced cardioplegia (IC). A total of 15 infants with simple TOF were allocated to each group, with a total sample size (n) of 30. Their ages ranged from 1 mo. to 12 mos. and there were no significant differences in age between the SC and IC groups (Table 1). Their weights ranged from 4.00 kg to 10.00 kg with an observed trend toward a higher weight in the SC group compared to the IC group, 7.14 kg vs. 6.11 kg, respectively (Table 1). Patients in the IC group had a significantly smaller body surface area (BSA) compared to patients in the SC group, .35 m² vs. .4 m², respectively (Table 1). There were no significant gender differences between the two groups as well as within (Table 1). There were no differences between the SC and IC group with respect to coronary sinus serum lactate and arterial serum lactate levels (Table 1). There were no previous cardiac operations associated with this study population.
**Table 1**: Demographic and clinical characteristics, at the time of surgery, of 30 infants with tetralogy of Fallot (TOF) randomly assigned to receive either Standard Cardioplegia (SC) or Insulin Supplemented Cardioplegia (IC) during cardiac surgery. Age, weight, pre-CPB coronary and arterial lactate are expressed as mean ± SD. Note: n = 15 unless otherwise noted in brackets.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>SC</th>
<th>IC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>15</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Age (mos.)</td>
<td>6.4 ± 2.8</td>
<td>6 ± 3.3</td>
<td>NS</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>7.14 ± 1.3</td>
<td>6.11 ± 1.5</td>
<td>.06*</td>
</tr>
<tr>
<td>BSA</td>
<td>0.4 ± .06</td>
<td>0.35 ± .05</td>
<td>.02</td>
</tr>
<tr>
<td>Male (n)</td>
<td>6</td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>Female (n)</td>
<td>9</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>Male:Female (n:n)</td>
<td>6:9</td>
<td>10:5</td>
<td>NS</td>
</tr>
<tr>
<td>Pre-CPB Coronary SS Lactate (mmol/L)</td>
<td>2.0 ± 0.61</td>
<td>2.2 ± 0.85</td>
<td>NS</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(n = 13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-CPB Arterial S Lactate (mmol/L)</td>
<td>1.6 ± 0.95</td>
<td>2.5 ± 1.6</td>
<td>NS</td>
</tr>
<tr>
<td>(n = 15)</td>
<td>(n = 14)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NS = not significant (> .05), BSA = body surface area (m²), n = sample size, CPB = cardiopulmonary bypass, SS = sinus serum, S = serum.
All patients underwent elective cardiopulmonary bypass (CPB) for repair of TOF with a 100% survival rate. There were no significant differences in CPB duration (mins) between the SC and IC group, 118.1 ± 59.7 vs. 98.2 ± 31.5, respectively (Figure 8). Cross-clamp time (mins.) or ischemia was similar between the SC and IC group; 59.07 ± 14.2 vs. 55.7 ± 10.5, respectively (Figure 9). Reperfusion duration was calculated as the aortic cross-clamp time off minus CBP off time. Patients in the IC group demonstrated a trend (.06) toward a 1.8 fold decrease in time spent in reperfusion compared to patients in the SC group, 35.2 ± 23.2 vs. 61.9 ± 61.1, respectively (Figure 10).

Figure 8: CPB duration (minutes) in infants with TOF randomly assigned to receive either SC or IC during cardiac surgery.
Figure 9: Duration of cross-clamp time/ischemia (minutes) in infants with TOF randomly assigned to receive either SC or IC during cardiac surgery.
Figure 10: Duration of reperfusion (minutes) in infants with TOF randomly assigned to receive either SC or IC during cardiac surgery.
There were also no significant differences between the SC and IC group with respect to HGL and LGL (Table 2). There were no significant differences between the SC and IC group with respect to HLL and LLL (Table 2).

**Table 2**: Perioperative highest glucose levels (HGL), lowest glucose levels (LGL), highest lactate levels (HLL) and lowest lactate levels (LLL) expressed as mmol/L in infants with TOF randomly assigned to receive either SC or IC during cardiac surgery.

<table>
<thead>
<tr>
<th>Level (mmol/L)</th>
<th>SC</th>
<th>IC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGL</td>
<td>3.8 ± 1.5</td>
<td>4.8 ± 1.5</td>
<td>0.55</td>
</tr>
<tr>
<td>LGL</td>
<td>1.02 ± 0.25</td>
<td>1.22 ± 0.66</td>
<td>0.29</td>
</tr>
<tr>
<td>HLL</td>
<td>13.0 ± 4.6</td>
<td>12.9 ± 4.1</td>
<td>0.47</td>
</tr>
<tr>
<td>LLL</td>
<td>4.29 ± 2.0</td>
<td>3.87 ± 1.3</td>
<td>0.18</td>
</tr>
</tbody>
</table>
Compared to the SC group, 1 minute post aortic cross-clamping coronary serum sinus lactate levels were significantly greater, by 63%, in the IC group; levels were $2.87 \pm .98$ vs. $4.69 \pm 1.6$ ($p = .003$), respectively (Figure 11). At 5 minutes post aortic cross-clamping there were no significant differences ($p = .07$) in CSS lactate levels between the SC and IC group, $2.85 \pm 1.18$ vs. $3.67 \pm 1.02$, respectively (Figure 11).

**Figure 11:** Post-aortic cross-clamp coronary serum sinus lactate (mmol/L) levels at 1 minute and 5 minutes. SC = standard cardioplegia, IC = Insulin supplemented cardioplegia. * vs. SC group at 1 minute.
Compared to the SC group, arterial serum lactate levels (mmol/L) at 1 minute post aortic cross-clamp were significantly greater, by 57%, in the IC group (Figure 12), lactate levels were 2.37 ± .93 vs. 3.72 ± 1.4 (p = .005), respectively. Similarly, lactate levels at 5 minutes were significantly greater by 33% in the IC group compared to the SC group; 3.71 ± 0.92 vs. 2.78 ± 0.98 (p = .02), respectively (Figure 12).

**Figure 12:** Post-aortic cross-clamp arterial serum lactate (mmol/L) levels at 1 and 5 minutes. SC = standard cardioplegia, IC = Insulin supplemented.

* vs. SC group at 1 minute. ‡ vs. SC group at 5 minutes.

There were no significant differences (p > 0.20) between the SC group and the IC group with respect to the following pressures (mmHg); RV PA, Aortic Systemic, Pulmonary artery systemic, RV chamber and gradient across RVOT (Table 3).
**PRIMARY PARAMETERS**

Compared to infants in the SC group, infants in the IC group demonstrated a significant 2-fold decrease in length of ICU stay (days); 4.2 ± 3.9 vs. 2.3 ± 1.1 (p = .04, 1-tail), respectively (Figure 13). There were no significant differences between the SC group and the IC group with respect to length of hospital (HSC) stay (days); 10.2 ± 6.3 vs. 9.6 ± 6.7 (p = 0.84), respectively (Figure 14).

**Figure 13:** Length of ICU stay (days) for infants with TOF randomly assigned to receive either SC or IC during cardiac surgery. Values are mean ± SD.
Infants in the IC group demonstrated a trend ($p = .06$) toward a 2.5 fold decrease in mechanical ventilation duration compared to infants in the SC group; $20.5 \pm 12.2$ vs. $55.0 \pm 67$, respectively (Figure 15).
Figure 15: Mechanical ventilation duration (hours) for infants with TOF randomly assigned to receive SC or IC during cardiac surgery. Values are mean ± SD.

There were no significant differences with respect to inotrope duration, frequency and number between patients in the SC group and those in the IC group (Table 4). When score per drug was calculated according to Moise et al. (1995) as, the sum of the rate (μg/kg/min) x number of hours delivered during the first 48 hours, there were no significant differences between patients in the SC group compared to the IC group (Table 4). However, when inotropic score was calculated using the method of Besogul et al. (1989) patients in the IC group demonstrated a significantly lower score (ANOVA = .01) compared to patients in the SC group (Figure 16).
Table 3: Inotrope support as a measure of duration, frequency (# patients receiving that particular inotrope), score and number of intotropes per patient in each group in infants with TOF randomly assigned to receive either SC or IC during cardiac surgery.

<table>
<thead>
<tr>
<th>Inotrope Measure</th>
<th>Inotrope</th>
<th>SC</th>
<th>IC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration (hrs.)</td>
<td>Dopamine</td>
<td>13.3 ± 19.1</td>
<td>9.13 ± 12.8</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>Nipride</td>
<td>5.87 ± 12.9</td>
<td>9.3 ± 17.5</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>Milrinone</td>
<td>24.5 ± 21.2</td>
<td>19.2 ± 22.1</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Epinephrine</td>
<td>6.4 ± 16.9</td>
<td>0 ± 0</td>
<td>0.15</td>
</tr>
<tr>
<td>Frequency</td>
<td>Dopamine</td>
<td>8/15</td>
<td>9/15</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>Nipride</td>
<td>4/15</td>
<td>2/15</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>Milrinone</td>
<td>10/15</td>
<td>7/15</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>Epinephrine</td>
<td>2/15</td>
<td>0/15</td>
<td>0.48</td>
</tr>
<tr>
<td>Score*</td>
<td>Dopamine</td>
<td>27.5 ± 45.0</td>
<td>11.34 ± 16.8</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Nipride</td>
<td>2.8 ± 6.9</td>
<td>2.0 ± 5.3</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>Milrinone</td>
<td>4.0 ± 4.3</td>
<td>3.0 ± 3.9</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Epinephrine</td>
<td>.78 ± 3.0</td>
<td>0 ± 0</td>
<td>0.32</td>
</tr>
<tr>
<td># Inotropes</td>
<td>0 – 4</td>
<td>1.6 ± 1.2</td>
<td>1.2 ± 0.78</td>
<td>0.28</td>
</tr>
</tbody>
</table>

*Sum of the rate (µg/kg/min) x number of hours delivered during the first 48 hours.
Figure 16: Inotropic score, as per Besogul et al., (1999) in infants with TOF randomly assigned to receive either SC or IC during cardiac surgery. The main effect of Group was found to be significant. Values are mean ± SD.
SECONDARY PARAMETERS

ICU HGL (mmol/L) did not differ (p = 0.54) between the SC and IC group (Table 5). However, ICU LGL (mmol/L) was significantly lower by 18% in the IC group compared to the SC group (Table 5). There were no significant differences in ICU HLL and ICU LLL between the SC and IC group (Table 5).

Table 4: ICU highest glucose levels (HGL), lowest glucose levels (LGL), highest lactate levels (HLL) and lowest lactate levels (LLL) expressed as mmol/L in infants with TOF randomly assigned to receive either SC or IC during cardiac surgery.

<table>
<thead>
<tr>
<th>Level (mmol/L)</th>
<th>SC</th>
<th>IC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGL</td>
<td>10.1 ± 2.9</td>
<td>9.5 ± 1.8</td>
<td>0.54</td>
</tr>
<tr>
<td>LGL</td>
<td>5.57 ± 0.78</td>
<td>4.55 ± 1.4</td>
<td>0.02</td>
</tr>
<tr>
<td>HLL</td>
<td>2.6 ± 1.5</td>
<td>2.2 ± .46</td>
<td>0.32</td>
</tr>
<tr>
<td>LLL</td>
<td>1.08 ± 0.38</td>
<td>1.11 ± 0.36</td>
<td>0.83</td>
</tr>
</tbody>
</table>

There were no significant differences between the SC and IC group with respect to mixed venous oxygen saturation (%) (MVO2), 58.89 ± 2.0 vs. 63.04 ± 2.2 (p = .19), respectively.

Chest tube volume (ml) and duration (hrs.) did not differ between the two groups (Table 6).

Table 5: ICU chest tube volume (ml) and duration (hours) in infants with TOF randomly assigned to receive either SC or IC during cardiac surgery. Values are mean ± SD. N = 15/group.

<table>
<thead>
<tr>
<th>CHEST TUBE</th>
<th>SC</th>
<th>IC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>232.5 ± 263</td>
<td>158.3 ± 128.7</td>
<td>0.34</td>
</tr>
<tr>
<td>Duration (hrs.)</td>
<td>63.9 ± 70.8</td>
<td>45.3 ± 25.3</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Total urine volume was significantly greater by 71% in the IC group compared to the SC group (p = .03) (Figure 17).
Every 4 hours for 24 hours in the ICU

Groups did not differ in the ICU every 4 hours for 24 hours with respect to arterial lactate, pH, bicarbonate, base excess and PO\textsubscript{2} (P > .11) (Appendix E). Arterial glucose levels were significantly greater in the SC group compared to the IC group at time 0 only (p = .02) (Figure 18); the levels did not differ between the two groups beyond time 0 (Appendix E).
**Figure 18**: ICU serum glucose levels at time 0 in infants with TOF randomly assigned to receive SC or IC during cardiac surgery. Values are mean ± SD.

Over the 24 hour period in the ICU, arterial PCO₂ (mmHg) was significantly greater in the SC group compared to the IC group; 40.79 ± .67 vs. 38.34 ± 0.68, respectively (p = .02) (Figure 19).
Figure 19: ICU arterial PCO₂ levels every 4 hours for 24 hours in infants with TOF randomly assigned to receive either SC or IC during cardiac surgery. Values are mean ± SD over time.
Performing a classical statistical method where the output is a probability (p value) is a highly effective alternative to methods such as ‘fold change’ (Pavlidis, 2003). A sensitive balance exists between clinical significance and statistical significance (Hopkins, 2000) and thus, genes with small fold changes, less than 2, as found in the nervous system and the heart (Podgoreanu et al., 2005 and Gaborit et al., 2005) are both highly significant and biologically relevant (Pavlidis, 2003). In determining statistically relevant genes, the above approach was employed using two softwares.

In AA, using a volcano plot set to a p value < .05 the differential expression (DE) limit was manipulated until genes related to insulin or insulin’s beneficial actions were found. Quantitative RT-PCR was used to verify microarray gene expression patterns on randomly selected genes from two separate gene lists generated (Table 6). The DE cut-off was found to be 0.6 at a p ≤ .05.

All patients were included in the ANOVA analysis using AffylmGUI. Due to the limited amount of RNA remaining from the microarray experiments RT-PCR was performed on the most significantly altered genes, hemoglobin beta and alpha-1, and expression patterns were verified (Table 6). A gene list was generated using the minimum DE of 0.6 and p ≤ .05 as in ArrayAssist, for Surgery (IC) and genes were selected based on the top 100 by un-adjusted p value. A second list was generated based on top 100 by M (DE). In the latter, ILKAP was statistically differentially expressed in PP_AA at a DE > 0.6 and p = .015, however in AffylmGUI the DE was 0.35 with a p = .019. Thus, based on the ILKAP gene, a gene related to the insulin-signaling pathway, genes with a DE > .35 were added to the list of differentially expressed genes. When Benjamini-Hochberg/FDR was employed as a multiple test correction, no significant genes were found in any of the contrasts. However, that is not to say there are no
significant genes. When searching for common genes between ArrayAssist and AffyImGUI (+/− FDR), more genes were found to be in common with AffyImGUI + FDR. Although with FDR there were no statistically relevant genes, p = .99, this does not exclude the fact that there are biologically relevant genes. The fact that the highly expressed Hb family of genes continued to appear at the top of the list (+/− FDR), in addition to insulin related genes such as DUSP1 and ILKAP, confirms biological relevance despite lack of statistical significance.

Table 6: RT-PCR results for randomly and insulin based selected genes.

<table>
<thead>
<tr>
<th>Gene List</th>
<th>Gene List</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(DE/M, p value)</td>
<td>(%) Change or FC ± SD</td>
</tr>
<tr>
<td>ArrayAssist –</td>
<td>TNF (0.8, .03)</td>
<td>+ 15%</td>
</tr>
<tr>
<td>Surgery Control</td>
<td>CytoP450 (- 1.3, .003)</td>
<td>- 35%</td>
</tr>
<tr>
<td>(Ischemia (n = 4) vs. Reperfusion (n = 2))</td>
<td>Inhibin (- .91, .029)</td>
<td>- 72%</td>
</tr>
<tr>
<td></td>
<td>Calcitonin-like(- 1.03, .006)</td>
<td>- 30%</td>
</tr>
<tr>
<td>ArrayAssist –</td>
<td>IGF-1 (0.6, .045)</td>
<td>1.6 ± 0.9</td>
</tr>
<tr>
<td>Reperfusion</td>
<td>IGFBP3 (.91, .016)</td>
<td>1.5 ± 0.27</td>
</tr>
<tr>
<td>(SC (n = 8) vs. IC)</td>
<td>IL10RB (.91, 1 x 10^-7)</td>
<td>1.2 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>PI3Kinase-1 (-.65, .025)</td>
<td>-.9 ± .38</td>
</tr>
<tr>
<td>AffyImGUI –</td>
<td>Hemoglobin-β</td>
<td>5.2 ± 1.17</td>
</tr>
<tr>
<td>Surgery_SC (n = 3 vs. n = 5)</td>
<td>(M &gt; 1.7, p &lt; .0005)</td>
<td></td>
</tr>
<tr>
<td>Reperfusion_SC vs. IC (n = 5 vs. n = 4)</td>
<td>(M &gt; 1.0, p = .05)‡</td>
<td>0.9 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin-α1</td>
<td>3.5 ± 1.2</td>
</tr>
<tr>
<td>Surgery_SC (n = 3 vs. n = 6)</td>
<td>(M &gt; 1.7, p &lt; .002)</td>
<td></td>
</tr>
<tr>
<td>Reperfusion_SC vs. IC (n = 6 vs. n = 5)</td>
<td>(M &gt; .7, p = .04)*</td>
<td>1.2 ± 1.5</td>
</tr>
<tr>
<td>Ischemia_SC vs. IC (n = 3 vs. n = 2)</td>
<td>(M &gt; .9, p &lt; .05)</td>
<td>3.2 ± .98</td>
</tr>
<tr>
<td>Surgery_IC (n = 2 vs. n = 5)</td>
<td>(M &gt; 1.5, p &lt; .003)</td>
<td>1.3 ± 1.5</td>
</tr>
</tbody>
</table>

DE/M = differential expression, * this gene came up 4x, but only one was significant; ‡ this gene came up 2x, but only one as significant.
I – All Patients
Patient Characteristics – Pre, Peri and Postoperative

A total of 18 patients comprised the microarray study. Twelve patients were arrayed in the control (SC) group and 6 patients were arrayed in the treated (IC) group. A total of 27 samples were arrayed between the two groups (Figure 20). A Two-Way ANOVA was performed to detect any significant differences among the following null hypotheses corresponding to (1) The main effect of cardioplegia type, (2) the main effect of time of surgery and (3) the interaction between cardioplegia type and time of surgery.

**“Standard Cardioplegia-SC”**

| ISCHEMIA (n = 8) | REPERFUSION (n = 8) |

**“Insulin-Cardioplegia-IC”**

| ISCHEMIA (n = 5) | REPERFUSION (n = 6) |

*Figure 20:* A total of 12 patients were arrayed in the SC group and 6 in the IC group. (n = #) denotes the number of biopsies. In total 16 and 11 biopsies were arrayed from the SC and IC group, respectively.

**Demographics**

There were no statistical differences (p > 0.41) for each main effect and interaction with respect to the following: age (months), weight (kg), gender and associated cardiac lesions.

**Pre-operative**

Coronary sinus serum lactate (p > .79) and arterial serum lactate (p > .76) did not differ significantly for each main effect and interaction.
Peri-operative

Total cardiopulmonary bypass time (CPB TT), total ischemia time (aortic cross-clamp time) and duration of reperfusion were not significantly different for each main effect and interaction. An un-paired t-test determined no significant differences (p = .61) between the SC and IC group with respect to average ischemic biopsy time (mins.), 49.9 ± 14.3 vs. 45.8 ± 12.9, respectively.

Post-operative

There was a statistical difference (p = .05) with respect to ICU stay between patients in the SC group and those in the IC group, 4.3 ± 0.85 days vs. 1.6 ± 1.0 days, respectively. However there were no statistical differences for the main effect of surgery time and any interactions with respect to ICU stay (p >.97). Length of hospital stay (HSC) and chest tube duration (hrs) did not differ significantly for each main effect and interaction. There was a trend (p = .09) toward a decrease in intubation duration (hrs) in the IC group relative to the SC group, 21.1 ± 17.5 vs. 60.1 ± 14.5, respectively. The main effect of surgery and interaction did not differ with respect to intubation duration (p > .96).

Gene Expression

Eighteen genes were differentially expressed in patients with tetralogy of Fallot during surgery with insulin-enhanced cardioplegia (IC) (Table 7). Seven were upregulated, of which 43% were from the hemoglobin subunit family. The remaining 57% were related to anti-apoptosis and calcium regulation. Eleven genes, with various functions, were down-regulated.
### Table 7: Myocardial gene expression during surgery (Surgery_IC) in infants with tetralogy of Fallot randomly assigned to receive insulin-enhanced cardioplegia (IC).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Symbol</th>
<th>Affymetrix ID</th>
<th>P Value</th>
<th>Fold Change</th>
<th>Pathway or Biological Process</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Down</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrial ribosomal protein</td>
<td>MRPS12</td>
<td>204331</td>
<td>.003</td>
<td>- 1.6</td>
<td>Gene expression (mitochondrion)</td>
</tr>
<tr>
<td>Stress-induced-Phosphoprotein</td>
<td>STIP1</td>
<td>213330</td>
<td>.004</td>
<td>- 1.39</td>
<td>Mediates association between hsp70 and hsp90, Phosphoprotein</td>
</tr>
<tr>
<td>Basigin</td>
<td>BSG</td>
<td>208677</td>
<td>.005</td>
<td>- 1.5</td>
<td>Stimulate fibroblasts to produce matrix metalloproteinases.</td>
</tr>
<tr>
<td>Interferon regulatory factor</td>
<td>IRF6</td>
<td>202597</td>
<td>.009</td>
<td>- 1.9</td>
<td>Unknown, The F0 of ATP synthase/H+ pumping.</td>
</tr>
<tr>
<td>ATP synthase, H+ transporting</td>
<td>ATP5H</td>
<td>210149</td>
<td>.01</td>
<td>- 1.1</td>
<td>The F0 of ATP synthase/H+ pumping.</td>
</tr>
<tr>
<td>Nuclear protein 3</td>
<td>NOL3</td>
<td>221566</td>
<td>.011</td>
<td>- 1.59</td>
<td>Unknown, Pore-forming (alpha) subunit of voltage-gated channel</td>
</tr>
<tr>
<td>Histone 1</td>
<td>HIST1H1E</td>
<td>215071</td>
<td>.011</td>
<td>- 1.5</td>
<td>Unknown, Pore-forming (alpha) subunit of voltage-gated channel</td>
</tr>
<tr>
<td>Potassium voltage-gated channel</td>
<td>KCNH2</td>
<td>205262</td>
<td>.011</td>
<td>- 1.55</td>
<td>Unknown, Pore-forming (alpha) subunit of voltage-gated channel</td>
</tr>
<tr>
<td>PDZ &amp; LIM domain</td>
<td>PDLIM7</td>
<td>203370</td>
<td>.017</td>
<td>- 1.11</td>
<td>Unknown, Pore-forming (alpha) subunit of voltage-gated channel</td>
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<tr>
<td>Potassium channel modulatory factor</td>
<td>KCMF1</td>
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<td>.018</td>
<td>- 1.18</td>
<td>Ion channel activity/unknown</td>
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<tr>
<td>Dual specificity Phosphatase 23</td>
<td>DUSP23</td>
<td>221419</td>
<td>.023</td>
<td>- 1.98</td>
<td>Protein dephosphorylation</td>
</tr>
<tr>
<td>Gelsolin</td>
<td>GSN</td>
<td>214040</td>
<td>.05</td>
<td>- 2.73</td>
<td>Actin modulating/severing protein</td>
</tr>
</tbody>
</table>

*cont’n on next page...*
Table 7 cont’n: Myocardial gene expression during surgery (Surgery_IC) in infants with tetralogy of Fallot randomly assigned to receive insulin-enhanced cardioplegia (IC).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Symbol</th>
<th>Affymetrix Probe Set ID</th>
<th>P Value</th>
<th>Fold Change</th>
<th>Pathway or Biological Process</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Up</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>HBA2</td>
<td>217414</td>
<td>.001</td>
<td>+ 3.2</td>
<td>Oxygen Transport</td>
</tr>
<tr>
<td></td>
<td></td>
<td>211745</td>
<td>.001</td>
<td>+ 3.2</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>HBA1</td>
<td>211699</td>
<td>.001</td>
<td>+ 3.48</td>
<td>Oxygen Transport</td>
</tr>
<tr>
<td></td>
<td></td>
<td>214414</td>
<td>.002</td>
<td>+ 2.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HBA1</td>
<td>204018</td>
<td>.003</td>
<td>+ 2.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HBA1</td>
<td>209458</td>
<td>.004</td>
<td>+ 2.96</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>HBB</td>
<td>209116</td>
<td>.001</td>
<td>+ 3.66</td>
<td>Oxygen Transport</td>
</tr>
<tr>
<td></td>
<td></td>
<td>211696</td>
<td>.001</td>
<td>+ 3.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>217232</td>
<td>.002</td>
<td>+ 3.7</td>
<td></td>
</tr>
<tr>
<td>Suppressor of cytokine signaling</td>
<td>SOCS3</td>
<td>206360</td>
<td>.003</td>
<td>+ 1.15</td>
<td>Anti-apoptosis</td>
</tr>
<tr>
<td>Fas apoptotic Inhibitory</td>
<td>FAIM</td>
<td>220643</td>
<td>.008</td>
<td>+ 1.19</td>
<td>Anti-apoptosis</td>
</tr>
<tr>
<td>Calcium channel Inhibitory</td>
<td>CACNB1</td>
<td>206996</td>
<td>.019</td>
<td>+ 1.07</td>
<td>Calcium regulation</td>
</tr>
<tr>
<td>Integrin-linked kinase</td>
<td>ILKAP</td>
<td>221548</td>
<td>.019</td>
<td>+ 1.27</td>
<td>PP2C family, regulated ILK, integrin mediated pathway</td>
</tr>
</tbody>
</table>
II – Paired Patients
In the SC group 4 patients were paired, i.e. both ischemic and reperfusion biopsies were arrayed, and 5 patients were paired in the IC group. A total of 18 samples were arrayed between the two groups.

**Demographics**

There were no statistical differences between patients in the SC group and the IC group with respect to: age (months), weight (kg), gender and associated cardiac lesions (Table 8).

**Pre-operative**

Pre-coronary sinus serum lactate (p = .66) and pre-arterial serum lactate (p = .34) did not differ significantly between the SC and IC group (Table 8).

**Peri-operative**

There were no statistical differences between patients in the SC group and those in the IC group with respect to CPB duration (p = .55), aortic cross-clamp total time (AXC TT) (p = .51), reperfusion duration (p = .67) (Table 8) and average ischemic biopsy time (p = .57), 51 ± 12.9 vs. 45.8 ± 12.9, respectively.

**Post-operative**

While patients in the IC group demonstrated a trend (p = .09) toward a decreased ICU stay, 1.6 ± .6 vs. 4.3 ± 4 days, respectively, HSC stay did not differ significantly (Table 8). Both intubation and chest tube duration (hrs.) did not differ significantly between the two groups (Table 8). Although both inotrope support and score did not differ between the two groups, dopamine score was 10x greater in the SC compared to the IC group.
**Table 8:** Demographics and clinical characteristics (Pre, Peri and Post-operative parameters) of infants in the microarray paired patient analysis. Values are expressed as mean ± SD. N denotes sample size.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SC</th>
<th>IC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (mos.)</td>
<td>6 ± 1.4</td>
<td>6 ± 5</td>
<td>.93</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>6.7 ± 1.1</td>
<td>6.3 ± 2.4</td>
<td>.75</td>
</tr>
<tr>
<td>Gender</td>
<td>3♂:1♀</td>
<td>4♂:1♀</td>
<td>.86</td>
</tr>
<tr>
<td>Associated Cardiac Lesions</td>
<td></td>
<td></td>
<td>.40</td>
</tr>
<tr>
<td><strong>Pre-operative</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary Sinus Serum Lactate</td>
<td>1.6 ± .5</td>
<td>1.8 ± .6</td>
<td>.66</td>
</tr>
<tr>
<td>Arterial Serum Lactate</td>
<td>1.3 ± .8</td>
<td>1.7 ± .4</td>
<td>.34</td>
</tr>
<tr>
<td><strong>Peri-operative</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of CPB (mins.)</td>
<td>122.3 ± 77</td>
<td>99.4 ± 27.0</td>
<td>.55</td>
</tr>
<tr>
<td>Aortic cross-clamp Time (mins.)</td>
<td>60.8 ± 14.4</td>
<td>54.4 ± 13.1</td>
<td>.51</td>
</tr>
<tr>
<td>Reperfusion Time (mins.)</td>
<td>58.3 ± 66.2</td>
<td>43.6 ± 30.1</td>
<td>.67</td>
</tr>
<tr>
<td><strong>Post-operative</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICU Stay</td>
<td>4.3 ± 4.0</td>
<td>1.6 ± .6</td>
<td>.09*</td>
</tr>
<tr>
<td>HSC Stay</td>
<td>10 ± 5.9</td>
<td>10.6 ± 6.1</td>
<td>.89</td>
</tr>
<tr>
<td>Chest Tube Duration (hrs.)</td>
<td>74.8 ± 61.9</td>
<td>38.2 ± 16.2</td>
<td>.24</td>
</tr>
<tr>
<td>Intubation Duration (hrs.)</td>
<td>66.8 ± 75.3</td>
<td>21.8 ± 13.6</td>
<td>.23</td>
</tr>
<tr>
<td>Inotropic Score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>27.4 ± 42.5</td>
<td>2.6 ± 2.2</td>
<td>.23</td>
</tr>
<tr>
<td>Nipride</td>
<td>2.5 ± 2.9</td>
<td>0.0 ± 0.0</td>
<td>.29</td>
</tr>
<tr>
<td>Milrinone</td>
<td>6.5 ± 5.6</td>
<td>6.2 ± 4.5</td>
<td>.94</td>
</tr>
<tr>
<td>Duration (hrs.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>22.7 ± 11.4</td>
<td>2.2 ± .98</td>
<td>.22</td>
</tr>
<tr>
<td>Nipride</td>
<td>3.0 ± 6</td>
<td>0.0 ± 0.0</td>
<td>.29</td>
</tr>
<tr>
<td>Milrinone</td>
<td>31 ± 22.7</td>
<td>32.8 ± 20.9</td>
<td>.91</td>
</tr>
</tbody>
</table>

CPB = cardiopulmonary bypass, ICU = intensive care unit, HSC = hospital stay, Lactate levels expressed as mmol/L, * = trend.

**Gene Expression**

At a DE of 0.6 (1.5 fold change) and p < .05, 20 genes were found to be differentially expressed in infants with tetralogy of Fallot during surgery treated with insulin-enhanced cardioplegia (Table 9). The hemoglobin family constituted over 50% of the genes differentially up-regulated, while 26% were related to decreasing inflammation and apoptosis, and the remaining 21% were related to other functions.
Down-regulated genes included those related to apoptosis, calcium signaling and other functions (Table 9).
Table 9: Myocardial gene expression profile during surgery (Surgery_IC) in infants with tetralogy of Fallot randomly assigned to receive insulin-enhanced cardioplegia. (Gene expression profiles derived from Paired Patients)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Symbol</th>
<th>Affymetrix Probe Set ID</th>
<th>P Value</th>
<th>Fold Change</th>
<th>Pathway or Biological Process</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Down</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transmembrane 7 superfamily 2</td>
<td>TM7SF2</td>
<td>210130</td>
<td>.018</td>
<td>- 1.71</td>
<td>Redox reaction, cholesterol biosynthesis</td>
</tr>
<tr>
<td>Polymerase RNAII</td>
<td>POLR2E</td>
<td>213887</td>
<td>.015</td>
<td>- 1.53</td>
<td>RNA transcription</td>
</tr>
<tr>
<td>Gelsolin</td>
<td>GSN</td>
<td>214040</td>
<td>.02</td>
<td>- 2.60</td>
<td>Actin and calcium binding</td>
</tr>
<tr>
<td>NDRG family member 2</td>
<td>NDRG2</td>
<td>214279</td>
<td>.03</td>
<td>- 1.56</td>
<td>cell differentiation/unknown</td>
</tr>
<tr>
<td>Prostaglandin E synthase 2</td>
<td>PTGES2</td>
<td>218083</td>
<td>.018</td>
<td>- 1.64</td>
<td>Prostaglandin biosynthesis</td>
</tr>
<tr>
<td>ATPase Ca2+ Transporting..</td>
<td>ATPA2A</td>
<td>212362</td>
<td>.036</td>
<td>- 1.53</td>
<td>Calcium signaling pathway</td>
</tr>
<tr>
<td>Death-associated protein Kinase 3</td>
<td>DAPK3</td>
<td>203890</td>
<td>.045</td>
<td>- 1.77</td>
<td>Apoptosis induction</td>
</tr>
<tr>
<td><strong>Up</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dual specificity phosphatase 1</td>
<td>DUSP1</td>
<td>201041</td>
<td>.037</td>
<td>+1.54</td>
<td>Regulator of cellular response to stress</td>
</tr>
<tr>
<td>Zinc finger protein</td>
<td>ZFP36</td>
<td>201531</td>
<td>.04</td>
<td>+2.1</td>
<td>Decrease inflammation</td>
</tr>
<tr>
<td>Early growth response</td>
<td>EGR1</td>
<td>201693</td>
<td>.02</td>
<td>+2.57</td>
<td>Transcription Regulation &amp; Survival</td>
</tr>
<tr>
<td>FBJ murine osteosarcoma</td>
<td>FOSB</td>
<td>202768</td>
<td>.021</td>
<td>+4.0</td>
<td>Transcription factor, may act to limit transcription of jun and fos</td>
</tr>
</tbody>
</table>

cont’n on next page...
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Symbol</th>
<th>Affymetrix Probe Set ID</th>
<th>P Value</th>
<th>Fold Change</th>
<th>Pathway or Biological Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin alpha 2</td>
<td>HBA2</td>
<td>204018</td>
<td>.027</td>
<td>+ 3.03</td>
<td>Oxygen Transport</td>
</tr>
<tr>
<td></td>
<td>HBA2</td>
<td>209458</td>
<td>.016</td>
<td>+ 3.12</td>
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</tr>
<tr>
<td></td>
<td>HBA2</td>
<td>211699</td>
<td>.024</td>
<td>+ 3.48</td>
<td></td>
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<tr>
<td></td>
<td>HBA2</td>
<td>211745</td>
<td>.017</td>
<td>+ 3.25</td>
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<tr>
<td></td>
<td>HBA2</td>
<td>214414</td>
<td>.02</td>
<td>+ 3.03</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin gamma G</td>
<td>HBG2</td>
<td>204419</td>
<td>.037</td>
<td>+ 3.94</td>
<td>Oxygen Transport</td>
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<tr>
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<td>213515</td>
<td>.014</td>
<td>+ 4.53</td>
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<tr>
<td>Hemoglobin beta</td>
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<td>.019</td>
<td>+ 3.73</td>
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<td></td>
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<tr>
<td>Hemoglobin gamma A D component of complement</td>
<td>HBG1</td>
<td>204848</td>
<td>.02</td>
<td>+ 4.17</td>
<td>Oxygen Transport, G protein activity.</td>
</tr>
<tr>
<td></td>
<td>DF</td>
<td>205382</td>
<td>.02</td>
<td>+ 1.69</td>
<td>Innate immune response, humoral suppression of infectious agents Inhibits VSMC proliferation</td>
</tr>
<tr>
<td>Sulfatase 1 Eukaryotic translation initiation factor 4A</td>
<td>SULF1</td>
<td>212354</td>
<td>.016</td>
<td>+ 2.04</td>
<td>Protein biosynthesis</td>
</tr>
<tr>
<td>Leucine zipper transcription factor Integrin-linked Kinase PP2C</td>
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<td>214805</td>
<td>.02</td>
<td>+ 1.66</td>
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</tr>
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<td>LZTFL1</td>
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<td>+ 1.54</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>ILKAP</td>
<td>221548</td>
<td>.015</td>
<td>+ 1.54</td>
<td>PP2C Family, regulates ILK, Integrin mediated signaling pathway</td>
</tr>
</tbody>
</table>
Thirty infants with tetralogy of Fallot scheduled to undergo elective cardiac surgery were randomly assigned to receive either Standard Cardioplegia (SC) or Insulin-enhanced Cardioplegia (IC). A dosage of 10IU/L of insulin was chosen based on work done by Rao et al., (1999). Using TOF cardiomyocyte cultures exposed to various insulin concentrations, the enzymatic activity of the insulin specific enzyme, pyruvate dehydrogenase (PDH) was found to be maximal at 10IU/L. Shortly after this dosage was confirmed in a pediatric heart transplant setting by Dellgren et al., (2001) in which recipients whose donor hearts were perfused with 10IU/L of insulin demonstrated a significant decrease in mortality and significant increase in patient graft survival. To limit the degree of heterogeneity in a CHD patient population, subjects underwent the same anesthetic, surgical and perfusion techniques and who were phenotypically similar as possible, with the exception of gender and ethnicity, were chosen. To achieve balanced study arms, the effect of variation in patient population and potential bias in selection was accounted for by randomization.

At the time of surgery, both demographic and clinical characteristics of the patients in the two groups were similar. There were no significant differences between the two groups with respect to the proportions of females or males, age and associated cardiac lesions. There was a trend (p = .056) toward a lower body weight (kg) in the IC group and thus to avoid potential bias on clinical outcome, this factor was adjusted for in the statistical analyses. Although BSA was statistically different between the two groups, it is not biologically significant as all infants’ values fell within normal range (Li et al., 2000 and Najm et al., 2000).

Blood lactate concentration is proportional to tissue oxygen debt (Charpie et al., 2000) and levels are used as an indirect and non-invasive measure of tissue oxygen
metabolism (Shime et al., 2001), thus a marker of circulatory status (Toda et al., 2005). Higher than normal levels in the heart reflect a state of stress and is a strong predictor of a poor postoperative course after cardiac surgery (Colagrande et al., 2005, Todi et al., 2005 and Boldt, 2002). Neither group demonstrated inadequate oxygenation as evidenced by similar lactate levels from both the coronary sinus and arterial blood taken immediately before CPB start. All lactate levels fell within normal physiological range, 0.8 - 2.0 mmol/L (Ekouevi et al., 2006) and well below levels predictive of a complicated postoperative course, > 6 mmol/L (Hamamoto et al., 2006, Cheung et al., 2005 and Munoz et al., 2000).

**PERIOPERATIVE**

Cardiac surgery consists of a period of ischemia (cross-clamp time) followed by a period of reperfusion. Although reperfusion is required for tissue survival, the period itself elicits pathologic consequences resulting in reperfusion injury. A primary manifestation of the latter is contractile dysfunction (Hoffman et al., 2004 and Chen & Chow, 2005). Weaning off CPB entails the return of the heart and lungs to the circulation. The reperfusion period, which precedes complete separation from CPB, is critical and the duration often dictates myocardial recovery. Generally, an increase in weaning off CPB time indicates poor/impaired myocardial function (Souza & Elias, 2000) and when necessary inotropes may be administered during reperfusion to facilitate weaning. Since insulin is considered to exert inotropic effects, this would shorten weaning off CBP time. In the present study, total time spent in the ischemic period was not significantly different between the two groups. However, compared to patients in the SC group, patients in the IC group exhibited a trend toward a reduced
reperfusion period, $59.1 \pm 14.2$ mins. vs. $35.2 \pm 23.2$ mins., ($p = .06$). Insulin is known to enhance or promote oxidative metabolism during reperfusion by activating the enzyme PDH. Rao et al. (1996) discovered a positive lactate extraction value in patients demonstrating a significant improvement in cardiac output after receiving GIK during surgery, compared to controls. In this study, at 5 minutes post-aortic cross-clamp, the lactate extraction value for patients in the IC group was $+1.1\%$ compared to $-2.5\%$ in the SC group. The positive value indicates a switch from anaerobic glycolysis to oxidative metabolism and is an indication of improved cardiac functioning, which would hence lead to a decrease in weaning time and thus reperfusion duration. Furthermore, in both clinical and animal studies, insulin-enhanced cardioplegia has been shown to decrease inflammation and oxygen free radical production during reperfusion, as well as improve coronary blood flow and thus cardiac function (Perez et al., 2006 and Ma et al., 2006). According to the above, we may infer an improvement in cardiac function in patients randomly assigned to receive IC resulting in reduced time spent during reperfusion and thus early weaning off CPB, compared to SC patients.

**INTENSIVE CARE UNIT (ICU)**

Perioperative myocardial injury is a major determinant of postoperative cardiac dysfunction after surgery for congenital heart defects (Hasegawa et al., 2005). These hearts usually require pharmacological or mechanical support during the postoperative period. The latter clinical parameters, in addition to length of ICU stay, have been shown to correlate with postoperative complications as well as the degree of myocardial damage and dysfunction (Hirsch et al., 1998 and Durandy & Hulin, 2007).
The most common postoperative complication following repair of TOF is right ventricular diastolic dysfunction. Clinical indices of RV dysfunction include an increase in chest tube drainage times, prolonged duration of inotropic support and ventilation time as well as an increase in ICU stay and a higher dose of diuretics (Sachdev et al., 2006). Thus myocardial preservation during open-heart surgery is of vital importance in this group of pediatric patients. In our study, compared to patients randomly assigned to receive standard cardioplegia during cardiac surgery, patients randomly assigned to receive insulin-enhanced cardioplegia demonstrated a significant 2-fold reduction in the length of ICU stay (p = .04), a trend toward a 2.5 fold decrease in mechanical ventilation duration (p = .06), a significant decrease in inotropic support over a 48 hour period (p = .01) and a significant increase, +71%, in urine output (p = .03). Together, the above findings suggest an improvement in cardiac function in the IC group compared to the SC group.

**PRIMARY PARAMETERS**

*Mechanical Ventilation...*

Mechanical ventilation plays a vital role in the successful treatment of infants undergoing cardiac surgery. The patient’s respiratory function is supported during the operative period, recovery from anesthesia and hemodynamic stabilization during the ICU stay (Shime, 2004, Kocis, et al., 2001 and Hirsh et al., 1998). Duration of mechanical ventilation has been shown to correlate with length of ICU stay in children undergoing heart surgery (Baisch et al., 2005), mortality (Kuracheck et al., 2003 and Trachsel et al., 2005) and contractile dysfunction (Portman et al., 2004). Early weaning from mechanical ventilatory support after heart surgery represents improved postoperative outcome
(Durandy & Hulin, 2007) and thus often serves as a primary clinical outcome parameter (Portman et al., 2004 and Williams et al., 2006).

...reflects perioperative myocardial injury.

In pediatric patients undergoing congenital heart surgery, cardiac and lung dysfunction are common causes of prolonged mechanical ventilation (Harrison et al., 2002, Harkel et al., 2005, Portman et al., 2004 and Durandy & Hulin, 2007). After open-heart surgery in patients with TOF, lung injury is considered one of the most important complications. Wei et al. (2004) randomly assigned sixty-four children with TOF to either a control or protective pulmonary perfusion solution. Patients in the control group (n = 30) demonstrated enhanced lung dysfunction as well as extended intubation duration and ICU stay, compared to TOF patients in the protective solution group (Wei et al., 2004).

The release of the pro-inflammatory cytokines IL-6 and IL-8 during heart surgery are markers of perioperative myocardial injury (Anselmi et al., 2004 and Hauser et al., 1998) and serum levels have been correlated with postoperative compromised cardiopulmonary function in pediatric patients (Madhok et al., 2006 and Hauser et al., 1998). In children undergoing heart surgery, IL-6 levels correlated with a prolonged and worse postoperative course, described as, an increase in duration of mechanical ventilation, length of ICU stay and mortality (Hauser et al., 1998). Furthermore, Wei et al. (2004) demonstrated a significant increase in IL-6 and IL-8 levels as well as tumor necrosis factor-α levels, a marker of myocardial injury, in TOF patients with compromised postoperative pulmonary function compared to TOF children not displaying compromised pulmonary function.
Our study demonstrated a trend (.06) toward a 2.5 fold decrease in mechanical ventilation duration in patients randomly assigned to receive IC compared to control, SC. Based on the above, our data suggests improved myocardial and pulmonary function in the IC group compared to the SC group, possibly due to reduced perioperative myocardial injury.

**Insulin’s Role**

In CABG patients receiving GIK immediately before heart surgery, Lazar et al. (2000) demonstrated a significant decrease in ventilatory support time and subsequently a significant improvement in recovery time.

**Inotropic Support**

Inotropes are administered to avoid clinical signs of cardiac failure, thus by maintaining a certain level of mean arterial pressure (mmHg) and urine output (ml/hr) (Shime, 2004). Together, inotropic dosage, duration and number comprise inotropic support (Besogul et al., 1999). Clinically, inotropic support within the first 24 hours after surgery is used to assess cardiovascular performance as well as the degree of myocardial damage incurred during surgery (Portman et al., 2004 and Gessler et al., 2005). Using serum HFABP levels as a rapid indicator of myocardial damage, Hasegawa et al. (2005) found inotropic support correlated significantly with serum peak HFABP levels in pediatric patients (n = 98) undergoing heart surgery for repair of VSD. Hauser et al. (1998) found serum IL-6 levels, which correlate with the degree of perioperative injury, to correlate with inotropic support in children undergoing heart surgery.

Girard et al. (1992) did observe a significant increase in cardiac function despite no significance in inotrope requirement. In a double-blind study assessing GIK supply
before surgery in adult patients, patients receiving GIK one hour before surgery demonstrated a 2-fold lower requirement for inotropic drugs (NS), while cardiac index and right ventricular workload index were significantly greater in the GIK group compared to the control (Girard et al., 1992). However, when inotrope support was calculated in 4-hr intervals across the 48-hr ICU period, patients in the IC group demonstrated a significant decrease in inotropic support compared to patients in the SC group. Although cardiac index and RV workload were not measured in our study, based on clinical evidence from numerous studies employing GIK, our data is suggestive of an improved cardiac performance in TOF patients receiving insulin-enhanced cardioplegia during surgery.

**Insulin...improves cardiac function, reducing inotrope requirement.**

Low cardiac output, inotropic requirement and increased biochemical markers of myocyte damage, are all adverse prognostic indices associated with increased morbidity and mortality after heart surgery in both pediatric and adult patients (Ranasinghe et al., 2006). Insulin has been shown to exert direct inotropic effects on the reperfused heart and improve postoperative contractile performance (Doenst et al., 1999 and Lazar et al., 2000). Since inotropes are administered to improve cardiac contractile function, it is not surprising that numerous clinical studies have demonstrated a significant reduction in inotrope requirement with the administration of insulin during cardiac surgery. In a recent meta-analysis of all randomized GIK studies in cardiac surgery, GIK administration was found to considerably improve postoperative recovery of contractile function (Bothe et al., 2004).
In addition to insulin’s inotropic effects on the heart, improvement in cardiac function may also be attributed to insulin’s ability to attenuate perioperative myocardial damage as evidenced by a significant reduction in the levels of biochemical markers of myocardial injury such as cTnI and IL-6 (Quinn et al., 2006). In two randomized double-blind placebo-controlled trials investigating the effects of GIK on cardiovascular performance on CABG patients (n = 440), patients receiving GIK during CABG demonstrated a significant decrease in dopamine utilization as well as a significant increase in cardiac index, compared to CABG patients randomly assigned to receive 5% dextrose (control group). The latter was found to be associated with a significant decrease in the release of serum cardiac troponin I (cTnI) in the GIK group compared to control (Ranasinghe et al., 2006). In 280 patients undergoing CABG, Quinn et al. (2006) also demonstrated, a significant reduction in dopamine dosage concomitant with a significant increase in cardiac index, in patients (n = 138) randomly assigned to receive GIK during surgery compared to patients randomly assigned to the dextrose/placebo group (n = 142). In addition to the latter, incidence of low cardiac output (LCO) episodes, myocardial injury (defined as perioperative myocardial infarction, PMI) and serum cTnI levels, 6 hours post reperfusion, were significantly lower in the GIK group compared to patients in the control group (Quinn et al., 2006). In cardiac surgery for mitral valve replacement, Besogul et al. (1999), who administered the same amount of insulin as our study, also demonstrated a significant reduction in inotropic requirement (score) as well as a significant increase in cardiac index in patients (n = 15) receiving GIK 12 hours before surgery. In CABG patients receiving GIK immediately before surgery Lazar et al. (2000) reported similar findings to Besogul et al. (1999), as well as a significant decrease in both ventilatory and inotrope support.
Thus, the above evidence suggests an improved cardiac function in patients receiving IC, due a decrease in perioperative myocardial damage, compared to the SC group.

Length of ICU Stay

Length of ICU stay is a cardinal clinical marker routinely used in studies to assess patient performance after cardiac surgery. Numerous studies, in both pediatric and adult cardiac surgery patients, have correlated the length of ICU stay with perioperative myocardial injury (Hauser et al., 1998), mortality and postoperative complications such as, cardiac dysfunction, arrhythmias, renal failure and multiple organ dysfunction (Marshall et al., 1995, Careaga et al., 2001, Brown et al., 2003 and Stambouly et al., 1996). Thus, ICU duration is considered a cardinal clinical marker in assessing patient performance. Patients randomly assigned to receive insulin-enhanced cardioplegia demonstrated a significant 2-fold reduction in length of ICU stay. This may be due to the fact that patients also demonstrated an improved postoperative outcome, ie: decrease in the duration of mechanical ventilation, reduced inotropic support during the ICU period and increase in urine output volume.

The beneficial effects of insulin-enhanced cardioplegia at reducing perioperative myocardial injury, subsequently manifested as an improvement in mechanical ventilation, reduced need for inotropic support etc. was discussed earlier. Thus it is not surprising numerous clinical studies document a significant reduction in length of ICU stay with the administration of insulin-enhanced cardioplegia during cardiac surgery.
SECONDARY PARAMETERS

ICU Glucose

Upon ICU arrival, serum glucose levels were significantly lower in the IC group compared to the SC group. Insulin is known to facilitate the uptake of glucose from the circulation (Pessin & Saltiel, 2000) and this significant decrease in glucose levels verifies the presence and action of insulin. These results are consistent with others who have employed insulin-enhanced cardioplegia during cardiac surgery with observed beneficial effects (Koskenkari et al., 2005 and Gandhi et al., 2007).

ICU Total Urine Volume

Fluid accumulation in the interstitial space (edema) may continue once the pediatric patient is removed from bypass, for a period of 24 to 36 hours. Fluid retention has been shown to correlate with serum IL-6 levels, a sensitive indicator of the degree of inflammation incurred during surgery, in children after open-heart surgery (Hauser et al., 1998). Continued accumulation is commonly observed in infants following repair of tetralogy of Fallot and is related to low cardiac output syndrome and a consequent decrease in renal blood flow (Ungerleider, 2005). A decrease in urine output is the primary symptom of a decrease in renal blood flow. Acute renal dysfunction is a prevalent complication following cardiac surgery and has been recognized as an independent predictor of postoperative mortality (Stafford-Smith, 2005 and Morris et al., 2004), thus necessitating aggressive postoperative diuresis (Shime, 2004). An increase in diuresis is associated with an improved postoperative outcome (Costello et al., 2006). Thus urine output (ml), over a 48-hour period in the ICU, is viewed as a
marker of the insult of heart surgery (Stafford-Smith, 2005) and therefore a common study parameter to address patient outcome (Alkan et al., 2006 and Portman et al., 2004).

Compared to patients in the SC group, total urine volume (ml) over 2 days was significantly greater by 71% in patients receiving insulin-enhanced cardioplegia. The need for inotrope requirement is related to maintaining a mean arterial pressure, cardiac function as well as urine output (Besogul et al., 1999). In a study investigating pulsatile vs. non-pulsatile perfusion in pediatric patients undergoing heart surgery for repair of CHD, those demonstrating an improved postoperative outcome exhibited a significant decrease in inotropic support, shorter length of ICU stay and significantly higher urine output (Alkan et al., 2006). In examining the effect of milrinone on Low Cardiac Output Syndrome (LCOS) in pediatric patients (n = 238) after heart surgery for repair of various CHDs, patients with LCOS had a worse postoperative course; significantly lower urine output and a prolonged period of mechanical ventilation and hospital stay (Hoffman et al., 2003). Our data parallel the latter study in that patients in the IC group also demonstrated a significant decrease in the length of ICU stay, inotropic score at 4-hr intervals across a 48-hr period, a trend toward a reduced duration in mechanical ventilation with a significant increase in urine output. We may thus infer an improvement in cardiac function and decrease in myocardial injury in patients in the IC group.
**Summary**

The universal goal in cardiac surgery is to provide effective myocardial protection during the procedure. Our study demonstrated an improved postoperative outcome in infants with tetralogy of Fallot randomly assigned to receive insulin-enhanced cardioplegia during cardiac surgery, compared to those receiving standard cardioplegia. Patients in the IC group demonstrated a trend toward a 2.5 fold decrease in the duration of mechanical ventilation and a significant decrease in inotropic score during a 48-hour ICU period, suggestive of an improvement in cardiac function. Furthermore, concomitant to the reduced inotropic support, patients in the IC group demonstrated a 71% increase in urine output volume (ml). Finally, given the significant improvement in the former clinical parameters, patients in the IC group also demonstrated a significant 2-fold shorter ICU stay. Thus, our data suggests patients in the IC group incurred significantly less perioperative myocardial injury during surgery and as a result possibly an improvement in cardiac function.
A) Gene Expression & Cardiac Surgery

Cardiac surgery has been documented to induce a myocardial transcriptional stress response in both humans and animals (Ruel et al., 2003, Konstantinov et al., 2004, Voisine et al., 2004 and Podgoreanu et al., 2005). In sixteen CABG patients, Ruel et al. (2003) found approximately 1000 genes significantly expressed after surgery (CPB) with cardioplegia. Furthermore, independent of cardiac surgery, cardioplegia is documented as providing additional mechanisms of cardioprotection at the transcriptional level during cardiac surgery (Schomisch et al., 2005).

Insulin has been shown to exert myocardial protection at the transcriptional level during ischemia and reperfusion (Ranasinghe et al., 2006). Our study sought to investigate the myocardial protective gene expression profiles induced with insulin-enhanced cardioplegia during surgery in infants with tetralogy of Fallot. There were no significant differences between the two study groups with respect to ischemic biopsy time, thus permitting the direct comparison of IC during ischemia. Alterations in gene expression during cardiac surgery, as well as markers of perioperative injury, are documented to occur as early as five minutes upon reperfusion (Jonassen et al., 2000, Toledo-Pereyra et al., 2004 and Naito et al., 2000). At this time, insulin’s effects at the level of gene expression have also been documented (Beauloye et al., 2001). Given the latter, we chose to investigate gene expression profiles at five minutes during reperfusion.

The present study has generated several novel observations about broad patterns of transcription in an infant’s myocardium with TOF undergoing cardiac surgery receiving insulin-enhanced cardioplegia. However, for the purpose of this study, only genes related to perioperative myocardial injury will be discussed.
B). Insulin-enhanced Cardioplegia Induces A Cardioprotective Gene Expression

Ischemia-reperfusion during cardiac surgery is reported to trigger myocardial inflammation and apoptosis, two processes well documented to contribute to perioperative myocardial injury (Podgoreanu et al., 2005, Anselmi et al., 2004 and Wan et al., 2002). In our study, infants with tetralogy of Fallot randomly assigned to receive insulin-enhanced cardioplegia (IC) exhibited a gene expression profile related to attenuating perioperative myocardial injury and improving cardiac function.

All Patients

Insulin-enhanced cardioplegia significantly induced the expression of SOCS3 and FAIM genes reported to be involved in anti-inflammation and anti-apoptosis, respectively. FAIM encodes an inducible effector Fas apoptosis inhibitory molecule and is thus resistant to fas-mediated apoptosis. This gene, broadly expressed in various tissues, represents a unique class of antiapoptotic proteins associated with inhibition of Fas-induced poly-ADP ribose polymerase (PARP) cleavage (Schneider et al., 1999). PARP has been implicated in the pathogenesis of heart failure (Pillai et al., 2006) and tissue injury during myocardial ischemia and reperfusion (Erdélyi et al., 2005). SOCS3, suppressor of cytokine signaling, is highly expressed in the heart. This gene is induced by cytokines as well as insulin. SOCS3 is involved in the negative regulation of cytokines, inhibiting proinflammatory signaling, specifically IL-6 (Lehmann et al., 2003 and Wang et al., 2007). Induced by mechanical stress in cardiac muscle cells, SOCS3 also acts as a negative feedback switch for hypertrophy (Yasukawa et al., 2001). Greater SOCS3 expression has been associated with improved myocardial function in mice (Wang et al., 2007). Interestingly, this gene has been shown to be downregulated during
ischemia in rat hearts (Schomisch et al., 2005), and our study demonstrated a significant upregulation in gene expression in the IC group, substantiating insulin’s role/effect.

The expression of ILKAP, integrin-linked kinase associated protein phosphatase 2C, was significantly upregulated in the IC group. ILKAP is a physiological regulator of ILK-mediated signaling. At increased ILK expression levels, ILKAP complexes with ILK to selectively inhibit ILK mediated GSK3\(\beta\) signaling (Kumar et al., 2004 and Leungs-Hagesteijn et al., 2001). ILK phosphorylates GSK3\(\beta\) whose downstream effects have been implicated in pathological hypertrophy. Elevated levels of ILK protein in human hypertrophy caused by congenital or acquired outflow tract obstruction have been reported (Lu et al., 2006). ILK specific markers of overload hypertrophy were elevated in these patients as well, suggestive of ILK upregulation (Hannigan et al., 2007). ILK overexpression has been shown to elevate cyclin D1 protein levels (D’Amico et al., 2000). Cyclin D family of proteins play a crucial role in the development of cardiac hypertrophy, a pathology leading to heart failure (Busk & Hinrichsen, 2003), and inhibition of D cyclins impairs hypertrophic growth in neonatal cardiomyocytes (Busk et al., 2002). In prostate cancer cells, expression levels of cyclin D1 were inversely correlated with ILKAP protein levels (Kumar et al., 2004).

A significant increase in the expression levels of the hemoglobin subunits: A2, A1 and B were found with IC during surgery. A similar profile was observed by Konstantinov et al. (2004) during surgery with IC in infants with VSD and Schomisch et al. (2005) in rats. Schomisch et al. (2005) attribute this increase due to the cardioprotective effects of cardioplegia. Two alpha and two beta chains constitute hemoglobin A (HbA), which comprises 97% of the total Hb in adults. Hb’s main role is the transport of oxygen (Ganong, 2001). In 90 children undergoing cardiac surgery for
repair of various CHD, Duke et al. (1997) found maintenance of oxygen delivery in the ICU was due to a significant increase in blood hemoglobin levels during the first four hours. Oxygenation recovery during reperfusion has been shown to correlate with recovery of hemodynamic and contractile function in the heart (Ilangovan et al., 2004). Hb has been shown to improve oxygenation in various ischemic tissues as well as reduce ischemia-reperfusion injury in the canine myocardium by providing an ‘oxygen bridge’. However the mechanisms remain to be elucidated (Burkhof and Lefer, 2005). In studying the effects of modified ultrafiltration (MUF) in pediatric patients with CHD undergoing heart surgery, patients with a significant decrease in postoperative chest tube drainage also demonstrated a significant increase in hemoglobin levels (Gaynor², 2003). In adults, low Hb levels (anemia) is common in patients admitted to the cardiac ICU and has been shown to be a main cause of impaired cerebral oxygenation during cardiac surgery (Freudenberger & Carson, 2003 and Nollart et al., 1995) and thus associated with the development of neurological complications after coronary bypass (Shaw et al., 1989). Furthermore, anemia has been correlated to renal impairment after heart transplant surgery in adults (Gleissner et al., 2004) and was found to be associated with major adverse events, postoperative peak troponin levels, increase in ICU stay and survival after one year in patients undergoing percutaneous coronary intervention (Lee et al., 2004). Our study found an increase in urine output, suggestive of improved renal function compared to control, a decrease in the length of ICU stay as well as a decrease in troponin-T gene expression (discussed later under Ischemia section).

Expression of the beta-1 polypeptide of the L-type voltage-dependent calcium channel (CACNB1) was significantly upregulated during surgery with IC. L-type voltage-dependent calcium channels are heterotetrameric polypeptide complexes
comprising the $\alpha_1$ and accessory subunits $\alpha_2/\delta$ and $\beta$. The $\beta_1$ subunit functions in the assembly and expression of the $\alpha_1$ subunit, acting as a chaperone in trafficking $\alpha_1$ from the endoplasmic reticulum to the plasma membrane (Schaub et al., 2006 and Bodi et al., 2005). Calcium entry via the L-type channel is the major trigger for sarcoplasmic reticulum (SR) – calcium release, termed excitation-contraction coupling (EC). The $\beta_1$-subunit has been shown to plays a crucial role in the EC coupling process. In $\beta_1$ – knockout mice, impaired coupling and early lethality was observed. In the human heart, a disruption in EC coupling has been shown to lead to heart failure. In allografts from patients with diastolic heart failure, transcript and protein expression levels of the $\beta_1$-subunits were significantly decreased, while the expression levels of the other subunits were unchanged. Although the exact mechanism is unknown, deficiency in the assembly process of the $\alpha_1/\beta_1$ complex is believed to be responsible (Bodi et al., 2005). In addition to the above, decreased L-type calcium channel mRNA expression was found to be associated with atrial fibrillation in human atrial tissue from patients with valvular heart disease (Gaborit et al., 2005).

In addition to the above highly expressed genes, related to reducing perioperative myocardial injury and improving cardiac function, additional genes related to augmenting harmful effects on the heart were downregulated. Such genes include $KCNH2$, thought to be related to long QT syndrome (Watanabe et al., 2007), however the exact relevance to this study is unknown, as well as $DUSP23$ (increases activation of JNK activity, a mediator of apoptosis and inflammation (Takagaki et al., 2004 and Francescato et al., 2007). $PDLIM7$, a heart specific gene related to enhancing cardiac hypertrophic signaling (Nakagawa et al., 2000) and $basigin$, which stimulates
the production of matrix metalloproteinases known to be involved in the progression of heart failure (Yndestad et al., 2007).

Post-operative clinical parameters in this microarray study group complemented the above potential cardioprotective profile observed in IC patients. Compared to the SC group of patients, IC patients exhibited a 2-fold significant shorter ICU stay as well as a trend toward a decrease intubation duration by 4-fold.

**Paired Patients**

Probes sets yielded by two methods is a means of ensuring the avoidance of false-positives (Ruel et al., 2003). In an attempt to minimize the heterogeneity in the study, a gene expression profile was generated using paired patients. In this respect, an ischemic biopsy was analyzed and compared against a reperfusion biopsy for each patient. Paired patient analysis (paired-sample \( t \) test in this case) is more powerful than a two-sample \( t \) test in that it significantly reduces the chances of committing a Type I error (Zar, 1984).

As observed in surgery with IC (All Patients), transcript expression levels of the \( Hb \alpha \) and \( \beta \) subunits as well as \( ILKAP \) were significantly increased in the paired patients microarray analysis as well. Paired patient analysis also revealed an increase in expression levels of \( DUSP1 \), a regulator of cellular response to stress (Ruel et al., 2003). DUSP1 acts to inhibit MAP kinase signaling, processes implicated in myocardial reperfusion injury and ultimately heart failure (Khan et al., 2004 and Lal et al., 2007). Expression levels of \( ZFP36 \), involved in decreasing TNF inflammation (Ruel et al., 2003) and \( ERG-1 \), involved in anti-apoptosis (Simkhovich et al., 2003) and considered cardioprotective (Konstantinov et al., 2004), were also significantly upregulated. \( FOSB \)
may act to limit the expression of the proto-oncogenes, Fos and Jun, known to be immediately expressed after heart surgery and responsible for inducing apoptosis and thus myocardial damage (Aebert et al., 1997 and Webster et al., 1993).

Of interest, transcript levels of *gelsolin* and *ATPA2A* (an ATPase calcium transporter, slow-twitch muscle) were significantly downregulated. Gelsolin is a calcium-dependent severing protein implicated in collagen phagocytosis (Chan et al., 2007) and calcium channel activation via the L-type calcium channel (Lader et al., 1999).

Postoperatively, compared to patients in the SC group, patients in the IC group demonstrated a trend toward a shorter ICU stay, by approximately 2-fold, and a 10-fold decrease in dopamine duration, although this difference did not achieve statistical significance.

**C). Insulin-enhanced Cardioplegia & Gene Expression During Ischemia & Reperfusion**

*Ischemia*

*Downregulated Genes*

During ischemia with IC, the *expression of genes* either implicated in the development of or related to myocardial injury, were decreased relative to the control/SC group (Appendix 2). *Calsequestrin-1*, which is found in the heart, binds and stores calcium in the mitochondria and sarcoplasmic reticulum. This is of particular importance since mitochondrial calcium overload is associated with myocardial dysfunction (Halestrap, 2004 and Piper et al., 2003). CASQ1 has been shown to be involved in the pathogenesis of myocardial stunning (Frass et al., 1993) and the
overexpression of this gene in mice was found to be associated with a depression in cardiac function and cardiac hypertrophy (Sato et al., 2000). ADP-ribosyltransferase-1, involved in reactive oxygen species induced signaling (Habon et al., 2001) and PARP activation (heart failure and I/R injury), was also downregulated with IC. The exact biological function of clusterin is unknown, however it is believed to be activated by TGF-beta activation. High levels of clusterin were observed in rats during cerebral ischemia induced by cardiac arrest (Kida et al., 1995) and during the development of atherosclerosis, and thus is thought of as an indicator of vascular damage (Trougakos et al., 2002).

Cardiac troponin I (cTnI) and T (cTnT) are well-documented markers of myocardial injury due to ischemia-reperfusion after heart surgery and thus are routinely employed in the assessment of myocardial protective strategies (Gunnewiek & Hoeven, 2004, Modi et al., 2003, Taggart et al., 1997 and Perrault et al., 1999). Increased serum levels of cTnI and T have been shown to predict the early postoperative course after pediatric heart surgery (Berroëta et al., 2006, Modi et al., 2003, Hirsch et al., 1998, and Immer et al., 1999) and has been linked to renal dysfunction (Zoccali & Mallamaci, 2006 and Choy et al., 2003). Immer et al. (1999) found a significant correlation between the need for inotropic support, renal dysfunction and duration of intubation with high serum cTnI levels. A decrease in serum cTnI levels were noted in CABG patients receiving insulin at the start of CABG surgery, compared to the control group (Ranasinghe et al., 2006). In this study, TOF patients receiving insulin demonstrated a decrease in the length of ICU stay, a decrease in inotropic score at 4-hr intervals during
a 48-hr ICU period and an increase in urine output volume. The latter improved postoperative outcome and decrease in the transcription levels of cTnT imply that TOF patients receiving IC incurred significantly less perioperative myocardial injury, compared to the SC group of patients.

**Upregulated Genes**

*MADH7*, or Smad, was significantly upregulated during ischemia. This gene is activated in response to stress and inhibits TGF-beta1 signaling thereby preventing Smad-2 access and increasing Smad-7 expression. TGF-beta1 signaling has been shown to induce fibrosis in the heart after ischemia (Li *et al.*, 2007) a process linked to atrial fibrillation susceptibility and heart failure (Everett 4th & Olgin, 2007 and Wang *et al.*, 2007). Smad-7 mRNA expression levels increase with an inhibition of TGF-beta1 activity and have been shown to reduce fibrosis and hypertrophic scar formation (Schultze-Mossgau *et al.*, 2004). Thus this gene may play a vital role in patients with TOF undergoing surgery as the development of fibrosis and thus right heart failure following surgery is common in patients with TOF (Alexiou *et al.*, 2001 and Chaturvedi *et al.*, 2007).

**Reperfusion**

Insulin has been shown to attenuate apoptotic processes in the heart (Jonassen *et al.*, 2001, Jonassen *et al.*, 2000 and Sack & Yellon, 2003) as early as five minutes into reperfusion (Beauloye *et al.*, 2001). Reperfusion is believed to be the main period in which insulin exerts its cardioprotective effects (Jonassen* et al.*, 2000). IC attenuated the expression of genes involved in myocardial injury and induced the expression of potential cardioprotective genes (Appendix 3).
**Downregulated Genes**

Reperfusion with IC significantly attenuated the expression of the gene *four and a half LIM domains 1* (FHL1), a gene exclusive to the heart (McGrath *et al.*, 2006). This gene was also downregulated at reperfusion in findings by Konstantinov *et al.* (2004) in infants with VSD administered insulin-enhanced cardioplegia during surgery. FHL1 is believed to be proportional to the degree of myocardial injury after heart surgery. Although the biological function of FHL1 is not completely understood an increase in expression levels are believed to play a role in the development of cardiac hypertrophy (Gaussin *et al.*, 2003), atrial fibrillation (Chen *et al.*, 2007) and the last steps of apoptosis (Scholl *et al.*, 2000). Furthermore, FHL1 is believed to play a role in sacromere assembly, where overexpression of FHL1 was associated with impaired myosin thick filament assembly (McGrath *et al.*, 2006). Increased expression levels of this gene were found to be associated with increased IL-6 and cTnI levels in patients after undergoing valvular or coronary artery surgery (Wan *et al.*, 2002). It is interesting to note, two separate probe set IDs for this gene revealed a downregulation.

High glycolytic rates and low glucose oxidation rates can result in uncoupling of glycolysis from glucose oxidation. During reperfusion, this uncoupling contributes to the production of protons from glucose metabolism thus contributing to ischemic injury (Lopaschuk & Stanley, 1997). Phosphorylase glycogen (PYGM) is highly expressed in the heart and is responsible for glycogen breakdown and promoting glycolysis. PYGM transcript levels were significantly downregulated in reperfusion with IC. An inhibition in glycolysis may thus aid in attenuating uncoupling during reperfusion and thus potential myocardial damage.
In addition to the above, IC attenuated mRNA expression levels of \textit{PLA2G4C} (phospholipase A2) and \textit{TCF8} (transcription factor 8), genes related to inducing an inflammatory response, as well as of \textit{Calsequestrin 1}, as observed in ischemia with IC.

\textit{Upregulated Genes}

Transcript levels of \textit{prostaglandin E receptor} (PTGER4) were significantly increased with IC. PTGER4 increases ERG-1 expression and phosphorylation of GSK3 (glycogen synthase kinase 3), thereby enhancing cell survival and inhibiting apoptosis, respectively (Simkhovich \textit{et al.}, 2003, Kaga \textit{et al.}, 2006 and Fang \textit{et al.}, 2000). PTGER4 is also believed to play a protective role at preventing kidney failure via attenuating apoptosis (Vukicevic \textit{et al.}, 2006 and Nagamatsu \textit{et al.}, 2006).

In addition to the above, transcript levels of the following genes were increased: \textit{tenascin XB}, \textit{Collagen type VI} and \textit{Fibulin 2}, all of which play a role in collagen scaffolding, hence transmission of contraction (de Simone & de Divitiis, 2002), and coronary vasculogenesis and angiogenesis (Tsuda \textit{et al.}, 2001). \textit{Aprataxin}, a gene expressed in the brain and associated with neurodegeneration (Rass \textit{et al.}, 2004), was significantly upregulated with IC. \textit{Hb-\alpha2} expression levels were also increased and the relevance was discussed earlier in section B.
D). Summary

Transcript profiling of samples acquired from the right ventricular outflow tract from infants with TOF administered insulin-enhanced cardioplegia during cardiac surgery, generated a cardioprotective gene expression profile. The expression profile includes reducing or inhibiting apoptosis, inflammation, cardiac hypertrophy, atrial fibrillation, fibrosis and at increasing or preserving myocardial function. Together this gene expression profile suggests a reduction in the perioperative myocardial injury incurred in IC patients compared to SC patients. Postoperative clinical parameters further substantiate the latter implication by complimenting the transcript profile in that IC patients demonstrated an improved postoperative outcome.
CONCLUSIONS
In conclusion, perioperative myocardial injury is a major determinant of postoperative cardiac dysfunction after surgery for congenital heart defects. Postoperative complications associated with the degree of perioperative injury include, an increase in the duration of mechanical ventilation, an increased need for inotropic support and a decrease in urine output, all of which contribute to an increase in the length of ICU stay. Perioperative myocardial injury is the most common cause of low cardiac output syndrome, a major contributor to mortality and morbidity after pediatric cardiac surgery. Infants with tetralogy of Fallot, when compared to infants with VSD and or children with TOF, have been shown to develop greater perioperative myocardial injury. Thus, this sub-population of pediatrics with CHD represents a ‘high-risk’ group of patients. The composition of cardioplegia is known to play a vital role in the successful operative management in pediatric heart surgery, with inadequate protection accounting for over 50% of LCOS cases.

Our institution recently identified a significant decrease in mortality in pediatric transplant patients receiving donor hearts perfused with insulin-enhanced cardioplegia. Insulin administered during cardiac surgery has been shown to improved cardiac function and reduce ischemia-reperfusion injury associated with cardiac surgery, however the great majority of studies involve the adult population. Thus to date there are relatively few studies on pediatric myocardial protection. Furthermore, studies lack the link between evaluation of clinical outcome and underlying basic mechanisms. Thus this study addressed clinical outcome in addition to potential mechanisms of action, using transcript profiling, in the first randomized, double blind, placebo-controlled
study investigating the potential beneficial effects of insulin-enhanced cardioplegia during cardiac surgery in infants with tetralogy of Fallot.

Compared to patients randomly assigned to receive standard cardioplegia (SC), patients randomly assigned to receive insulin-enhanced cardioplegia (IC) demonstrated a trend \( p = .06 \) toward a 1.8 fold reduction in reperfusion duration, a significant 2-fold decrease in the length of ICU stay and a trend \( p = .06 \) toward a 2.5 fold decrease in the duration of intubation. Over a 48-hour period, inotropic score, as per Besogul (1999), was found to be significantly lower in the IC group compared to the SC group. Furthermore, total urine volume was significantly greater by 71% in patients in the IC group. Together the above clinical parameters indicate an improved postoperative outcome, which studies have correlated to a reduction in perioperative myocardial injury and improvement in cardiac function. However, due to the lack of hemodynamic data, our data infers an improved cardiac function.

Transcript profiling data revealed a potential cardioprotective profile complimentary to the clinical outcome observed. Surgery with IC upregulated the expression of genes involved in improving cardiac function, attenuating apoptosis and inflammation while at the same time downregulated the expression of genes related to the development of cardiac hypertrophy, arrhythmias and fibrosis. Studies have documented insulin’s beneficial actions to occur during reperfusion, and our study demonstrated a significant downregulation in the four and a half LIM domain 1 (FHL1) gene responsible not only in cardiac hypertrophy and atrial fibrillation, but most notably apoptosis. We also demonstrated a significant decrease in the expression levels of cTnT, a well known marker of myocardial injury due to ischemia-reperfusion predictive of a complicated postoperative course and consistently shown to be higher in
infants and/or TOF patients. The ILKAP gene appeared in both software gene profile results and was significantly upregulated during surgery with IC. This gene is related to ILK functions and suggests an upregulation of ILK activity or expression in TOF patients, as studies suggest. The above potential mechanisms of action of insulin administered with cardioplegia is of particular importance to this population of pediatrics with CHD, due to the high incidence of right ventricular dysfunction, development of fibrosis and arrythmias.

Thus, compared to standard cardioplegia, insulin-enhanced cardioplegia during cardiac surgery for repair of TOF in infants significantly improved postoperative outcome. The observed improvement in postoperative outcome may be due to the cardioprotective gene expression profile generated during surgery in infants with IC. Based on the results of this study, insulin-enhanced cardioplegia appears to reduce perioperative myocardial injury and is thus a promising myocardial protective strategy in infants with TOF. The results from this thesis analysis provide a better understanding toward the molecular mechanisms pertaining to the cardiac response to surgery in infants with TOF and their associated clinical implications. Further investigation based on these data may lead to the development of tailored cardioplegia approaches resulting in an improved post-operative outcome in these high-risk patients.
LIMITATIONS

Although this thesis has documented relevant data into the cardioprotective effects of insulin-enhanced cardioplegia during heart surgery in infants with TOF, as typical of any research, this study has several limitations.

1. The main limitation in this study is the small sample size. Increasing the number of patients may have change the results reporting a trend toward reporting significance. Nonetheless, beneficial effects with IC during surgery were found.

2. Female pediatric patients have been recently shown to fare worse after heart surgery for CHD compared to males. Although gender was not significantly different between the two groups, our study did include both males and females.

3. Numerous studies document that infants are at a greater risk of perioperative myocardial injury compared to children. Further to that, the younger the infant the worse they do. Another limitation in our study is the variation in age (months) between 1 and 12 months. Due to the low n# we were unable to create subpopulations within our group which may have allowed us to identify higher risk patients as well as those who would benefit most from IC during surgery.

4. Despite their immense potential, microarray techniques have inherent shortcomings related to lack of standardized methods for statistical analyses of data. It provides a measure of transcribed mRNA rather than a functional gene product.
Second, although we demonstrated changes in mRNA expression, the latter have been analyzed in a small cohort of patients. Another limitation is that we have demonstrated differences in gene expression at an mRNA level, but due to limitations in biopsy size and time points of sampling we were unable to investigate directly changes in protein expression (or function) and correlate the levels to the mRNA expression levels observed. Third, at the time the study was conducted 5mg were required for microarray analysis. This amount was too great to be obtained from the very small biopsies and thus greatly limited the n# in the study. To date, less than 20μg is required. This amount is feasible to attain from the biopsy size and thus would have permitted all thirty infants to be included in microarray analysis. Further to the latter, more RNA would remain for RT-PCR and one would be able to verify many more genes both randomly as well as genes of interest.

5. Another limitation is the lack cardiovascular function assessment; both pre and post surgery as well as follow-up over time. This data would have provided insight into the myocardial function state of the patient, and thus would allow us to address whether insulin-enhanced cardioplegia significantly attenuates the most common postoperative complication in pediatric patients after CHD surgery, cardiac dysfunction.

Despite the aforementioned limitations, the results from this thesis analysis provide a better understanding toward the molecular mechanisms and beneficial effects of insulin-enhanced cardioplegia during cardiac surgery in infants with TOF and their associated clinical implications.
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Title of Research Project:
Insulin and Glucose Enriched Cardioplegia in Infants Undergoing Cardiac Surgery.

Investigators:
Glen Van Arsdel, MD  
Viveck Rao, MD  
Jean Luc Bigras, MD  
John Coles, MD

Purpose of the Research:
The purpose of this research study is to see if adding insulin and glucose to your child’s heart during the surgery will help improve recovery of your child’s heart function after the surgery. Insulin is a hormone that is normally produced in the body. Glucose is a simple sugar that is normally used by the body to produce energy.

Description of the Study:
Your child was born with a problem called a heart defect that may be fixed with open-heart surgery. For part of this operation, your child will be on a heart and lung machine and your child’s heart will not get the normal blood flow. This means that the heart will not get a regular supply of oxygen. Normally the heart uses oxygen in the blood to make energy. When there is no oxygen, the heart has to find another way to make processes, but at the same time, it makes a harmful chemical called lactic acid. A lot of lactic acid can damage the heart and may make it harder for the heart to return to normal after the surgery.

During the operation, a liquid will be given to your child’s heart to make the heart stop beating so the defect can be fixed. This liquid is called the “cardioplegia solution.” By adding insulin and glucose to this cardioplegia solution, it may help the heart in 3 different ways. The insulin and glucose solution may: (1) help store more energy for the heart to use during the operation; (2) help reduce the amount of harmful lactic acid in the heart; and (3) help the heart muscle recover better from the surgery when blood flow is returned to the heart.
If you choose to let your child take part in this study, your child will be put randomly into 1 of 2 treatment groups ("randomly" means by chance). That means that the group your child is put in is chosen by a method that is like the flip of a coin. One group of children will be given the insulin and glucose in addition to the standard cardioplegia solution. The other group of children will receive the standard cardioplegia solution with no added insulin or glucose.

If your child takes part in this study, he or she will get the same standard treatment and care for babies having heart surgery and a 50% chance of getting insulin and glucose in the cardioplegia solution. We cannot promise that your child will get the added insulin and glucose. The cardioplegia solution with the added insulin and glucose or the standard cardioplegia solution will be given to your child during the operation through the heart and lung machine.

Your child will have these tests:

1. **Transesophageal Echocardiogram:**

   We will look at how well your child’s heart is working before going on the heart and lung machine and after coming off the heart and lung machine. This is done with a transesophageal echocardiogram and an arterial trace. This is a special test that allows the doctors to take a picture of your child’s heart. We will decrease your child’s blood pressure for a few seconds to obtain picture of the heart working at a lower pressure. The blood pressure will be allowed to return to normal after 5-7 seconds.

2. **Transthoracic Echocardiogram:**

   We will do another test approximately 18 to 24 hours after the operation to look how well your child’s heart is working. This test is called a transthoracic echocardiogram (this test is also known as an “echo”.) An echo is a special test that takes a picture of your child’s heart. This test uses ultrasound to take a picture of the structure of your child’s heart. (Ultrasound uses sound waves to take pictures of the inside of the body.)

   This test is done by a technologist, a person who is specially trained to do echocardiograms. First, the technologist will put 3 electrodes (sticky pads) on your child’s chest and arms. These electrodes are connected by wires to an echo machine and are used to record your child’s heart beat during the test. Next, the technologist will put some jelly on your child’s chest. A probe with a rounded end will roll gently on this jelly so we can take pictures of the heart from different angles.

   It is unlikely that your child will experience any pain from this test. He or she may feel some pressure from the probe on the chest. This test is normally done for children after heart surgery.
2. **Myocardial Biopsy:**

We will take a small sample of the muscle tissue that was removed from your child’s heart for laboratory testing. The laboratory tests will measure the chemical activity in your child’s heart. We will take 3 samples of muscle tissue during the operation. It is unlikely that your child will experience any additional discomfort from this test because the heart muscle must be removed anyway in order to fix your child’s heart defect.

3. **Blood Testing for Lactate Levels:**

We will take a small amount of blood from a vein in your child’s heart to measure the lactate level in the heart at different times during the operation. We will take about 3 ml (less than 1 teaspoon) of blood during the operation for this test. This amount of blood will not require extra blood to be given to your child. This test will let us know how well your child’s heart is working.

After the operation, we will measure the lactate level in your child’s blood every 4 hours until the lactate level becomes normal or until 24 hours after the operation. This blood test is normally done for babies after heart surgery. Your child will not require extra transfusions because of this. We will monitor your child’s blood sugar on an hourly basis for 3 hours after surgery.

4. **Patient Information:**

We will also record what medicines your child takes, how much fluid he or she needs for the first 24 hours, how long he or she is in the intensive care unit, and how long he or she is in the hospital. We will get all this information from your child’s medical records.

Your child’s participation in this study will be completed at the time he or she leaves the hospital.

**Possible harms, injuries, discomorts or inconveniences:**

There are no known harms associated with participation in this research study. However, there may be some unforeseen harmful consequences that we do not know about. Because only a small number of adults have been treated with the insulin and glucose added cardioplegia solution, we do not know all the risks or side effects of this treatment.

One possible side effect that your child may experience is low sugar in his or her blood. If this happens, glucose will be given to your child through an intravenous line. An intravenous line, or IV, is a small tube put in a vein in an arm or leg to give medicines or other liquids. It is normal for a baby having heart surgery to have an IV.
Your child may also experience some mild chest discomfort from the transesophageal echocardiogram.

The heart muscle samples for this study will be collected from the heart muscle that was surgically removed in order to fix the defect. Collecting this sample of the muscle tissue will not likely pose a risk to your child.

The blood samples collected after the operation in this study are samples that a baby having open heart surgery would normally have done. We will be adding an extra blood test to be conducted.

There are no risks to your child from collecting information from his or her medical record.

**Potential Benefits:**
If our study finds that adding insulin and glucose to the cardioplegia solution during heart surgery is effective, your child’s heart function may recover better after surgery. There is no guarantee, or promise, that the added insulin and glucose will help your child.

Taking part in this study may not help your child directly. But what we learn from this study will help your doctor better understand the effects insulin and glucose on heart function. This information may help other babies and children who need open heart surgery in the future.

**Alternatives:**
If you choose to not let your child take part in this study, your child will get the standard therapy given to children undergoing open heart surgery. At present, there are no other treatment options available.

**Confidentiality:**
Confidentiality will be respected and no information that discloses the identity of your child will be published without consent unless required by law. For your information, the research consent form will be inserted in the patient health record.

**Participation:**
Participation in the research is voluntary. If you choose not to have your child participate, you and your family will continue to have access to quality care at the Hospital for Sick Children. If you choose on behalf of your child to participate in this study you can withdraw your child from the study at any time. Again, you and your family will continue to have access to quality care at The Hospital for Sick Children.

**Consent:**
I acknowledge that the research procedures described above have been explained to me and that any questions that I have asked have been answered to my satisfaction. I
have been informed of the alternatives to participation in this study, including the right not to participate and the right to withdraw without compromising the quality of medical care at The Hospital for Sick Children for my child and for other members of my family. As well, the potential harms and discomforts have been explained to me and I also understand the benefits (if any) of participating in the research study. I know that I may ask now, or in the future, any questions I have about the study or the research procedures. I have been assured that records relating to my child and my child's care will be kept confidential and that no information will be released or printed that would disclose personal identity without my permission unless required by law.

I hereby consent for my child ________________________________ to participate.

Name of Parent/Guardian_____________________________________

The persons who may be contacted about the research are:
Dr. Glen Van Arsdell, MD at (416) 813-6420
or Kourosh Dinyari, MB Clinical Research Coordinator at (416) 813-8369

Signature of Parent/Guardian______________________________

Date______________________________

Name of person who obtained consent/Witness________________

Signature of person who obtained consent/Witness________________

Date______________________________

00-09-06
Appendix 2
### Table 10: Ischemia_SC vs. IC

Myocardial gene expression profile at end ischemia in infants with tetralogy of Fallot randomly assigned to receive either standard cardioplegia (SC) or insulin supplemented-cardioplegia (IC) during cardiac surgery. (All Patients) (* = found in both AffylmGUI and Array Assist.)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Symbol</th>
<th>Affymetrix Probe Set ID</th>
<th>P Value</th>
<th>Fold Change (+/-)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Down</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Apolipoprotein A-1</td>
<td>APOA1</td>
<td>204450</td>
<td>.035</td>
<td>-1.79</td>
<td>Promotes cholesterol efflux</td>
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<tr>
<td>Osteocalcin</td>
<td>BGLAP</td>
<td>206956</td>
<td>.013</td>
<td>-1.60</td>
<td>Binds to calcium</td>
</tr>
<tr>
<td>ADP-ribosyltransferase 1</td>
<td>ART1</td>
<td>207919</td>
<td>.0003</td>
<td>-1.57</td>
<td>Apoptosis, heart failure</td>
</tr>
<tr>
<td>*Clusterin</td>
<td>CLU</td>
<td>208792</td>
<td>.015</td>
<td>-1.54</td>
<td>Apoptosis, vascular damage</td>
</tr>
<tr>
<td>*Myelin basic protein</td>
<td>MBP</td>
<td>210136</td>
<td>.026</td>
<td>-2.0</td>
<td>Indicator of brain damage</td>
</tr>
<tr>
<td>*Troponin T1</td>
<td>TNNT1</td>
<td>213201</td>
<td>.0006</td>
<td>-1.60</td>
<td>Cardiac damage</td>
</tr>
<tr>
<td>*PARK2 co-regulated</td>
<td>PAK2</td>
<td>214204</td>
<td>.02</td>
<td>-1.59</td>
<td>Unknown</td>
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<tr>
<td>*Calsequestrin 1</td>
<td>CASQ1</td>
<td>219645</td>
<td>.034</td>
<td>-1.87</td>
<td>Binds and stores calcium in mitochondria and sarcoplasmic reticulum</td>
</tr>
<tr>
<td>Hypothetical Protein</td>
<td>FLJ10204</td>
<td>FLJ10204</td>
<td>219060</td>
<td>-2.22</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Up</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Interferon regulatory</td>
<td>IRF6</td>
<td>202597</td>
<td>.037</td>
<td>+1.56</td>
<td>IRF family/unknown</td>
</tr>
<tr>
<td>Hemoglobin gamma</td>
<td>HBG2</td>
<td>204419</td>
<td>.035</td>
<td>+1.91</td>
<td>Oxygen transport</td>
</tr>
<tr>
<td>MAD</td>
<td>MADH7</td>
<td>204790</td>
<td>.0012</td>
<td>+1.54</td>
<td>Inhibit TGF beta signaling</td>
</tr>
<tr>
<td>Hypothetical protein</td>
<td>MGC10940</td>
<td>215436</td>
<td>.04</td>
<td>+1.71</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
Appendix 3
Table 11: Reperfusion_SC vs. IC

Myocardial gene expression profile at 5 minutes of reperfusion in infants with tetralogy of Fallot randomly assigned to receive either standard cardioplegia (SC) or insulin-supplemented cardioplegia (IC) during cardiac surgery. (All Patients) (* = found in both AffylmGUI and Array Assist.)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Symbol</th>
<th>Affymetrix Probe Set ID</th>
<th>P Value</th>
<th>Fold Change (+/-)</th>
<th>Function</th>
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<tr>
<td>Down</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>*Four and a half LIM domains 1</td>
<td>FHL1</td>
<td>201539</td>
<td>.039</td>
<td>- 1.8</td>
<td>Apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>210298_x</td>
<td>.03</td>
<td>- 1.56</td>
<td></td>
</tr>
<tr>
<td>*Phosphorylase Glycogen Solute carrier family 1</td>
<td>PYGM</td>
<td>205577</td>
<td>.007</td>
<td>- 1.57</td>
<td>Promote glycolysis</td>
</tr>
<tr>
<td>*Phospholipase A2 Transcription Factor 8</td>
<td>PLA2G4C</td>
<td>209785</td>
<td>.03</td>
<td>- 1.53</td>
<td>Inflammatory Response</td>
</tr>
<tr>
<td>*Calsequestrin 1 Troponin T</td>
<td>CASQ1</td>
<td>219645</td>
<td>.02</td>
<td>- 1.73</td>
<td>Binds and stores calcium</td>
</tr>
<tr>
<td></td>
<td>TNNT1</td>
<td>213201</td>
<td>.002</td>
<td>- 1.65</td>
<td>Cardiac damage</td>
</tr>
<tr>
<td>Up</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Prostaglandin E Receptor</td>
<td>PTGER4</td>
<td>204897</td>
<td>.029</td>
<td>+1.55</td>
<td>Inhibit GSK3 pathway</td>
</tr>
<tr>
<td>*Transcription Factor 21</td>
<td>TCF21</td>
<td>204931</td>
<td>.024</td>
<td>+1.55</td>
<td>RNA Pol II transcription</td>
</tr>
<tr>
<td>*Tenascin XB</td>
<td>TNXB</td>
<td>208609</td>
<td>.007</td>
<td>+1.57</td>
<td>Cell matrix adhesion</td>
</tr>
<tr>
<td>*Hemoglobin alpha</td>
<td>HBA2</td>
<td>214414</td>
<td>.028</td>
<td>+1.75</td>
<td>Oxygen transport</td>
</tr>
</tbody>
</table>

cont’n on next page...
Table 11 cont’n: Reperfusion_SC vs. IC Myocardial gene expression profile at 5 minutes of reperfusion in infants with tetralogy of Fallot randomly assigned to receive either standard cardioplegia (SC) or insulin-supplemented cardioplegia (IC) during cardiac surgery. (All Patients) (* = found in both AffylmGUI and Array Assist.)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Symbol</th>
<th>Affymetrix Probe Set ID</th>
<th>P Value</th>
<th>Fold Change (+/-)</th>
<th>Function</th>
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<tbody>
<tr>
<td>*Chromosome</td>
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<td>209183</td>
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<td>Collagen type VI</td>
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<td>213290</td>
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<td>Matrix organization</td>
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<td>.037</td>
<td>+2.46</td>
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</tr>
<tr>
<td>*S-phase 2</td>
<td>SULF1</td>
<td>212354</td>
<td>.05</td>
<td>+1.85</td>
<td>Inhibits VSMC proliferation</td>
</tr>
</tbody>
</table>