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Title Page

Differential effects of normoxic and hyperoxic reperfusion on global myocardial ischemia-reperfusion injury

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Conflict of Interest Statement

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Abbreviations

CPB = cardiopulmonary bypass (CPB)

HR = Hypoxia re-oxygenation (HR)

IR = Ischemia Reperfusion (IR)

LVDP = Left ventricular developed pressure (LVDP)

OS = oxidant stress (OS)

ROS = reactive oxygen species

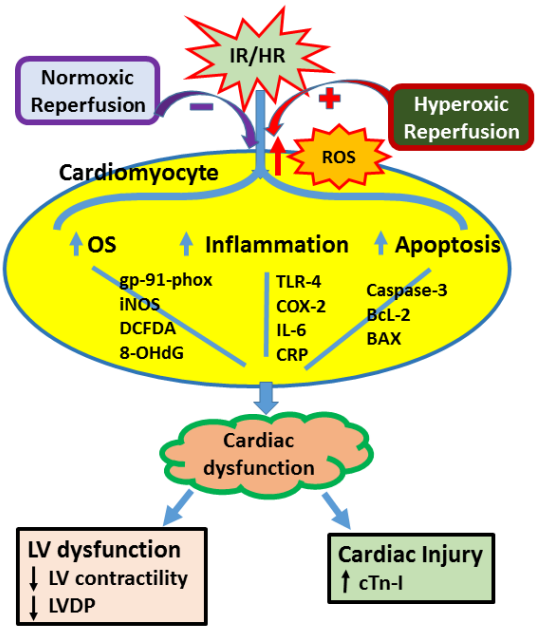
Abstract:

The objectives were to investigate if after hypoxia or ischemia, normoxic reperfusion is associated with less oxidant stress (OS), inflammation, and myocardial injury than hyperoxic reperfusion. In this study, cardiomyocytes (H9c2 cells) were cultured in hypoxia, followed by re-oxygenation in normoxia or hyperoxia. Cardiomyocyte OS, inflammation and apoptosis were measured. In parallel experiments, rabbits were cannulated for cardiopulmonary bypass (CPB). Following cardioplegic arrest and aortic cross-clamp removal, hearts were reperfused under normoxic or hyperoxic conditions. Left ventricular developed pressure (LVDP) and contractility (LV +dP/dt) were recorded, and blood samples and heart tissues were collected for measurement of OS, inflammation and cardiac injury. Results showed that H9c2 cells exposed to hyperoxic reoxygenation showed significant increases in OS, inflammation, and apoptosis compared to normoxic reoxygenation.. Following CPB and 2-hour hyperoxic reperfusion, LV +dP/dt and LVDP were significantly decreased compared with pre-CPB values (to 36±21%, p=0.002; and

53±20%, p=0.02, respectively), associated with significant increases in all plasma and tissue biomarkers for OS, inflammation, and myocardial injury. In contrast, LV +dP/dt was relatively well-preserved under normoxic reperfusion conditions (to 70±14% after 2-hour reperfusion), , and was associated with an attenuated myocardial OS, inflammatory, apoptotic, and injury response compared to the hyperoxia group (*eg* cTn-I: 5.9±1.5 vs. 20.2±7.6 ng/mL, respectively, p<0.0001) Overall, in both *in vitro* and *in vivo* experiments, normoxic reperfusion/re-oxygenation was associated with less robust OS, inflammation, apoptosis, and myocardial injury compared with hyperoxic reperfusion/re-oxygenation. These results suggest that hyperoxia should be avoided to minimize myocardial OS, inflammation and ventricular dysfunction after CPB.

Abstract word count: 250

Key words: ischemia, normoxic/hyperoxic reperfusion, cardiopulmonary bypass, myocardial injury.



Central picture
Cardiomyocyte under ischemia followed by normoxic or hyperoxic reperfusion

Central Message
Normoxic reperfusion is associated with preserved LV contractility and less oxidant stress, inflammation, apoptosis, and myocardial injury compared with hyperoxic reperfusion.

Perspective
CPB with cardioplegic arrest results in myocardial ischemia reperfusion injury associated with an increase in oxidant stress and inflammation that is attenuated by normoxic reperfusion compared with hyperoxic reperfusion in the *in vitro* and *in vivo* experiments. Thus, hyperoxia should be avoided to minimize myocardial ischemia reperfusion injury and ventricular dysfunction after open heart surgery.

Introduction:

Myocardial ischemia-reperfusion (IR) injury associated with cardiopulmonary bypass (CPB) and cardioplegic arrest contributes to ventricular dysfunction and adverse outcomes after cardiac surgery. The pathogenesis of IR injury is complex, but reactive oxygen species (ROS) generated during IR are hypothesized to play a pivotal role leading to lipid peroxidation, protein denaturation, and DNA fragmentation, all of which may result in irreversible myocyte injury and cell death. The cell damage induced by ROS can also initiate a local inflammatory response, which leads to further oxidative stress-mediated tissue injury (1-3).

Previous clinical and experimental studies suggest that after cardioplegic arrest, normoxic versus hyperoxic CPB may mitigate oxidant stress (OS) and myocardial re-oxygenation injury (4-7). Despite these findings, few institutions have adopted normoxic CPB as their standard perfusion practice for patients undergoing cardiac surgery. This apparent paradox may be due to: 1) the lack of a clear mechanistic link between myocardial OS, postoperative ventricular dysfunction, and important clinical outcomes after heart surgery; and, 2) the wide variation in practice among institutions regarding myocardial protective strategies (e.g. intraoperative steroids, cold versus warm cardioplegia, etc) and perfusion techniques, such as modified ultrafiltration, that may also influence postoperative ventricular function and outcomes.

The main objective of this study was to show that myocardial OS and ventricular dysfunction were mechanistically linked after CPB with cardioplegic arrest. A second objective was to compare the effects of different re-oxygenation/reperfusion conditions on myocardial OS and ventricular dysfunction using both *in vitro* and *in vivo* experimental

models. We hypothesized that after CPB with cardioplegic arrest, normoxic reperfusion would be associated with less OS and myocardial injury than hyperoxic reperfusion. These experimental results will provide the necessary preclinical data for a future randomized clinical trial evaluating the impact of normoxic versus hyperoxic CPB (current standard of care in most institutions) on outcomes after cardiac surgery.

Materials and Methods:

(For further details of the methods used, please see the Supplementary Material section)

Cell culture and hypoxia re-oxygenation protocol

Based on our earlier time-course experiments, we used embryonic rat heart derived H9c2 cells as an *in vitro* model to assess the direct effects of HR on cardiomyocyte oxidant stress, inflammation, and apoptosis. After 12-hour hypoxia (< 1% O_2), cells were returned to 95% room air /5% CO_2 or 95% O_2/5% CO_2 for 2-hour reoxygenation (8-10).

Immunoblotting

For detection of biomarkers for inflammation (TLR-4) and apoptosis (caspase-3, BcL-2, and BAX), H9c2 cell lysates were analyzed by immunoblotting assays (10-13).

Real-time quantitative polymerase chain reaction (RT-PCR)

RT-PCR was used to quantify mRNA expression of markers for oxidant stress (gp91-phox and iNOS) and inflammation (COX-2).

Intracellular ROS generation

The cell permeable probe 2',7'-dichlorofluorescein diacetate (DCFDA) was used to directly monitor intracellular ROS production, and detected using a fluorescence microscope (11-13).

Mitochondrial transmembrane potential (Δψ)

Mitochondrial transmembrane potential ($\Delta\psi$) was assessed using the polychromatic 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidoazolyl-carbocyanino iodide stain (JC-1), and detected using a fluorescence microscope (11-13).

Animal model

Male New Zealand white rabbits (2.9-3.3 kg) were assigned to two groups (N=6-7 per group): (1) normoxic reperfusion (with 21% O_2 CPB and inspired FiO_2 0.21), or (2) hyperoxic reperfusion (with 100% O_2 CPB and inspired FiO_2 1.0). The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Michigan. All animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institute of Health.

Surgical preparation

Anesthesia was induced with intramuscular ketamine and xylazine, and maintained by isoflurane inhalation (14). A high-fidelity 4-Fr Millar pressure catheter (Millar, Inc., Houston, TX) was placed into the LV apex for measuring left ventricular developed pressure (LVDP) and LV contractility (LV +dP/dt).

Experimental protocol

For veno-arterial CPB, blood was drained from the right jugular vein and infused into the right carotid artery. Following 30-minute hemodynamic stability on CPB, an ascending aortic cross-clamp was placed and cold (4°C) crystalloid cardioplegic solution (Plegisol, Abbott Lab, North Chicago, IL) infused into the aortic root. After 60-minute cross-clamp time, the clamp was removed, and the rabbit was reperfused and mechanically ventilated under normoxic or hyperoxic conditions for 60 minutes on partial CPB (50 mL/kg/min) followed by complete separation from CPB for an additional 60 minutes (14, 15). Mean arterial

pressure, heart rate, LVDP, LV +dP/dt, ECG and rectal temperature were monitored continuously using a computer equipped with a data acquisition system (PowerLab and LabChart, ADInstruments, Colorado Springs, CO).

Biomarkers of OS, inflammation and cardiac injury

Blood samples were collected from the femoral artery at multiple time points (pre-CPB, during CPB, and 0.5, 3, 10, 30, 60, 90, 120 min after aortic cross-clamp removal) for measurement of plasma ROS. DNA oxidation [8-hydroxy-2'-deoxyguanosine (8-OHdG)], inflammation [interleukin-6 (IL-6) and C-reactive protein (CRP)], apoptosis (caspase-3), and cardiomyocyte injury [troponin-I (cTn-I)] (5, 15-18).

Heart tissue specimen preparation

Rabbit heart tissues were collected immediately following 120-minute reperfusion. Tissue samples were fixed in 10% buffered formalin for Haemotoxylin and Eosin (H&E) staining, and the remaining heart tissue was snap frozen in liquid nitrogen and stored at -80ºC for measurement of OS and inflammation. The mRNA expression of gp91-phox, iNOS, and COX-2 were measured by RT-PCR.

Data analysis:

Data are presented as mean±S.E. Differences in messenger RNA levels and protein expression between control and each experimental group and/or between experimental groups were examined using Student's t-test for unpaired comparisons, with a significant p-value < 0.05. For normoxic and hyperoxic reperfusion, change in LVDP and LV +dP/dt, plasma OS, inflammatory markers, and myocardial apoptosis and injury at each time point

from pre-CPB was evaluated using one-way Analysis of Variance (ANOVA). In addition, group comparisons between reperfusion methods were also made in change in each measurement listed above at each time point from pre-CPB, using two-way ANOVA. A p-value < 0.004 was considered statistically significant for the results from ANOVA with Bonferroni correction for multiple comparison. All of the statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA).

Results:

HR-induced OS and inflammation in embryonic rat cardiomyocytes

Based on results from our previously published time course experiments (10), H9c2 cells exposed to 12-hour hypoxia and 2-hour room-air re-oxygenation showed a maximal OS response compared to control cells not exposed to HR. Therefore, all subsequent *in vitro* experiments were performed using 12-hour hypoxia followed by 2-hour re-oxygenation (12H/2R) in 95% room-air/5% CO_2 (normoxia) or 95% O_2/5% CO_2 (hyperoxia).

The mRNA levels of gp91-phox and iNOS were significantly increased in 12H/2R-treated H9c2 cells compared with control cardiomyocytes. Furthermore, mRNA expression of gp91-phox and iNOS was increased by 1.5- and 2-fold respectively with hyperoxia compared with normoxia (Figures 1A & 1B). To directly observe intracellular ROS generation, the cell permeable probe DCFDA was used. DCFDA was oxidized to yield high intensity green fluorescence (DCF) in the presence of ROS. The blue fluorescent dye Hoechst 33342 displayed nuclear morphology. We demonstrated an increase in DCF fluorescence in 12H/2R-treated H9c2 cells (compared with control cells) that was greater under hyperoxic compared with normoxic conditions (Figure 1C).

In addition to OS, inflammation plays an important role in the pathogenesis of myocardial IR injury after CPB. Figure 2 illustrates the substantial increase in COX-2 and TLR-4 expression with 12H/2R compared to control H9c2 cells. Furthermore, both COX-2 and TLR-4 expression were increased nearly 2-fold under hyperoxic compared with normoxic conditions.

These results demonstrate that 12H/2R increases both OS and inflammation in cardiomyocytes, and hyperoxic re-oxygenation conditions are associated with greater OS and inflammation than normoxic re-oxygenation.

HR-induced apoptosis in embryonic rat cardiomyocytes

Caspase-3 (a key mediator of apoptosis), BcL-2 (an anti-apoptotic factor), and BAX (a pro-apoptotic factor) were measured in H9c2 cells exposed to 12H/2R. Compared with untreated control cells, 12H/2R significantly increased caspase-3 protein expression and decreased the BcL-2/BAX ratio (e.g. more pro-apoptotic environment; Figure 3A & 3B). Furthermore, caspase-3 expression was increased 2-fold in response to hyperoxic compared to normoxic re-oxygenation conditions (Figure 3A). The BcL-2/BAX ratio was attenuated significantly in response to hyperoxia compared with normoxia.

Additional experiments addressed the hypothesis that the mitochondrial electron transport chain plays a key role in HR-induced apoptosis in H9c2 cells. We measured mitochondrial membrane potential ($\Delta\psi$) using JC-1, a cationic dye that enters the mitochondria and changes its fluorescent properties based on aggregation of the probe. Figure 3C(a) demonstrates that untreated, control cardiomyocytes with high $\Delta\psi$ show red J-aggregate accumulated in the mitochondria with no diffuse J-monomer (green fluorescence). In contrast, after 12H/2R,

apoptotic cardiomyocytes show more J-monomer with less J-aggregate under both hyperoxic and normoxic re-oxygenation conditions (Figures 3C(b) & 3C(c)).

These data suggest that HR is associated with cardiomyocyte apoptosis *in vitro*, and that hyperoxic re-oxygenation increases apoptosis compared with normoxic re-oxygenation.

Rabbit CPB model – left ventricular function

Following 60-minute of ischemia and hyperoxic reperfusion, LV +dP/dt and LVDP were significantly reduced at all time points compared to pre-CPB baseline measurements (Figure 4A and 4B). For example, under hyperoxic reperfusion conditions, LV +dP/dt and LVDP were 36±21% (p=0.002) and 53±20% (p=0.02) of pre-CPB values, respectively, at 2-hour reperfusion (R-120). In contrast, after 2-hour reperfusion under normoxic reperfusion conditions, LV function was relatively preserved (LV +dP/dt: 70±14%, p=0.42; LVDP: 80±13%, p>1.0, compared to pre-CPB values). Furthermore, LV +dP/dt was significantly reduced in the hyperoxic reperfusion group compared to the normoxic reperfusion group (p=0.004) (Figure 4A). LVDP also tended to be reduced in the hyperoxic reperfusion group compared with the normoxic reperfusion group, but this difference was not statistically significant (p=0.10) (Figure 4B). Neither blood pressures nor heart rates were significantly different between the normoxic and hyperoxic reperfusion groups throughout the experimental protocol (data not shown).

These data suggest that after CPB with cardioplegic arrest *in vivo*, LV function is better preserved with normoxic reperfusion compared with hyperoxic reperfusion.

Rabbit CPB model - plasma OS and inflammatory markers

To assess IR-induced OS and nucleic acid oxidative damage, we measured plasma ROS formation and 8-OHdG production, respectively. Under hyperoxic reperfusion conditions,

plasma ROS was increased greater than 2-fold within 0.5-minute aortic cross-clamp removal, and remained elevated throughout the entire reperfusion period compared to pre-CPB levels (Figure 5A). In contrast, in the normoxic reperfusion cohort, there was no significant change in plasma ROS production during reperfusion compared to pre-CPB levels. In addition, Figure 5A shows that hyperoxic reperfusion is associated with higher ROS formation than normoxic reperfusion. For example, at 10-minute reperfusion, ROS formation was 447±54 ng/mL in the hyperoxic group compared with 289±35 ng/mL in the normoxic group ($p=0.02$).

Consistent with the increase in ROS during reperfusion, 8-OHdG was increased following cross-clamp removal compared with pre-CPB levels, with a further increase beginning with complete separation from CPB (at 60-minute reperfusion). Furthermore, the hyperoxic reperfusion cohort showed significantly higher levels of plasma 8-OHdG than the normoxic reperfusion group. For example, at 120-minute reperfusion, 8-OHdG was 51.2±20.6 ng/mL in the hyperoxic group compared to 27.4±7.0 ng/mL in the normoxic group ($p=0.002$) (Figure 5B).

Pro-inflammatory cytokines play a key role in the inflammatory cascade associated with CPB and contribute to myocardial dysfunction and hemodynamic instability after CPB. Our study investigated the time course of two well-characterized pro-inflammatory cytokines in plasma from rabbits exposed to CPB with hyperoxic and normoxic reperfusion. Our data showed that plasma IL-6 was significantly increased in all rabbits during reperfusion compared with pre-CPB levels ($p=0.0003$ for hyperoxia and $p=0.001$ for normoxia, Figure 5C). Furthermore, IL-6 was increased further in rabbits exposed to hyperoxic reperfusion

compared to normoxic reperfusion (436±177 ng/mL vs.152±58 ng/mL, respectively, at 120-minute reperfusion; p=0.0004).

Compared to pre-CPB levels, we observed an increase in plasma CRP during reperfusion in the hyperoxic group, but not in the rabbits exposed to normoxic reperfusion (Figure 5D). After 120-minute reperfusion, plasma CRP was 514±98 ng/mL in the hyperoxic group vs. 200±26 ng/mL in the normoxic group (p=0.002).

These data indicate that CPB-induced IR is associated with an increase in OS, nucleic acid damage, and inflammation that is greatest under hyperoxic reperfusion conditions, and attenuated with normoxic reperfusion.

Rabbit CPB model - plasma apoptosis and myocardial injury

To assess the impact of IR on myocardial apoptosis and injury in our rabbit CPB model, we measured the change in plasma concentrations of caspase-3 and cTn-I. We observed a rapid increase in caspase-3 levels within 0.5 minutes following aortic cross-clamp removal that was sustained throughout reperfusion in the rabbits exposed to hyperoxia (Figure 6A). In contrast, rabbits exposed to normoxic reperfusion showed a modest, non-significant increase in plasma caspase-3 from pre-CPB levels.

Similar to the caspase-3 data, cTn-I levels progressively increased (particularly after complete separation from CPB at 60-minute reperfusion) in both hyperoxic and normoxic groups ($p<0.0001$ for both groups; Figure 6B). However, plasma cTn-I levels were significantly higher in the hyperoxic group compared with the normoxic group (20.2±7.6 vs.5.9±1.5 ng/mL, $p<0.0001$).

These results suggest that CPB with cardioplegic arrest is associated with IR-induced myocardial injury and apoptosis that is significantly attenuated by normoxic reperfusion compared with hyperoxic reperfusion.

Rabbit CPB model - myocardial OS, inflammation, and histology

To directly assess myocardial OS and inflammation in our rabbit CPB model, we measured gp91-phox, iNOS and COX-2 mRNA expression in heart tissue homogenates after 60-minutes ischemia and 120-minutes reperfusion. These data showed that myocardial gp91-phox, iNOS, and COX-2 expression were significantly increased in hearts from rabbits exposed to CPB and cardioplegic arrest compared with control (no CPB) hearts. Furthermore, there was a significant reduction in gp91-phox, iNOS, and COX-2 expression in rabbits reperfused with normoxic conditions compared with hyperoxic conditions. As shown in Figure 7, mRNA expression of gp91-phox, iNOS and COX-2 were attenuated in rabbit hearts that were reperfused with normoxia compared to rabbit hearts reperfused with hyperoxia (gp91-phox: 2.63±0.57 vs. 5.18±0.89 fold of control, p=0.04; iNOS: 0.93±0.12 vs. 7.92±2.21 fold of control, p=0.03; COX-2: 6.88±1.88 vs. 21.40±3.89 fold of control, p=0.01).

Histological examination showed minor hemorrhage, myofiber hypereosinophilia, and focal vacuolation in cardiac tissue collected from rabbits independent of reperfusion conditions. However, in the rabbit hearts reperfused with hyperoxia, there were multifocal inflammatory infiltrates composed predominantly of heterophils within the myocardial vasculature as well as multifocally within the cardiac interstitium (Supplementary Materials). These multifocal heterophilic infiltrates suggests a more robust tissue response to reperfusion injury in the hyperoxic rabbits.

These results suggest that CPB with cardioplegic arrest results in direct myocardial IR injury associated with an increase in OS and inflammation that is attenuated by normoxic reperfusion compared with hyperoxic reperfusion.

Discussion

Cardiac surgery with CPB and cardiplegic arrest is associated with OS and a systemic inflammatory response that may contribute to significant postoperative organ dysfunction (19-23). In particular, CPB with cardioplegic arrest necessitates a period of global myocardial IR that triggers local ROS production *in vivo*, and subsequent activation of pro-inflammatory and apoptotic signaling pathways (24, 25). Other clinical studies confirm that cardiac surgery with CPB leads to systemic OS, as evidenced by an increase in multiple OS markers and a decrease in antioxidant reserves, associated with increased postoperative morbidity and prolonged hospital stay (26-29).

Our experimental results demonstrate that in isolated cardiomyocytes in culture, HR (a simulated model of IR) is associated with an increase in cellular OS, inflammation, injury, and programmed cell death. Hyperoxic re-oxygenation further exacerbates cardiomyocyte injury *in vitro*. In our *in vivo* rabbit CPB model, global myocardial IR (due to aortic cross-clamping and cardioplegic arrest) significantly impaired LV systolic function associated with significant increases in plasma and myocardial tissue OS, inflammation, and apoptosis. Compared to hearts reperfused with hyperoxic conditions, hearts reperfused with room air (normoxia) showed relatively preserved LV contractility, and attenuated increases in plasma and myocardial tissue OS, inflammation and cardiac injury.

We confirm that OS generated by HR or IR plays an important role in initiating the series of pathological events causing cardiomyocyte injury, apoptosis, and ventricular dysfunction. Our results also confirm our hypothesis that normoxic reperfusion mitigates OS, inflammation, cardiomyocyte injury, and LV dysfunction compared with hyperoxic reperfusion. The results of this study provide important preclinical data to support a clinical trial of normoxic CPB after cardiac surgery, and to support development of other therapeutic strategies to target oxidant stress and inflammation to prevent or reduce IR injury after open-heart surgery.

There are multiple reports in the literature of oxygen-dependent reperfusion injury in both patients and animal models. In previous studies, Buckberg (30, 31), Beyersdorf (32) and Caputo (33) reported that controlled re-oxygenation during CPB is associated with reduced myocardial damage compared with hyperoxic CPB during cardiac surgery. Also, Morita and colleagues (34) addressed the importance of controlling PaO_2 at the onset of CPB to avoid multi-organ injury after open-heart surgery in children with cyanotic congenital heart disease. Our experimental results are consistent with these findings, and further suggest that oxygen-dependent reperfusion injury is mediated by OS, inflammation, and apoptosis in the heart, and may be mitigated by normoxic reperfusion. In contrast to these previous publications, Smith et al (35) reported that controlled cardiac re-oxygenation did not improve myocardial function following global myocardial ischemia in a swine model. The reasons for this apparent discrepancy remain unclear. However, one potentially important distinction in our experimental protocol compared to these earlier studies, is that we controlled re-oxygenation conditions both through the CPB circuit and through the mechanical ventilator throughout the reperfusion period to maximize the potentially beneficial effects of normoxia on IR injury.

Although we developed an *in vivo* rabbit model to validate our *in vitro* observations regarding the contribution of oxidant stress, inflammation, and apoptosis to ventricular dysfunction after CPB, there were several limitations to our study. First, in the clinical setting, there are a variety of intraoperative strategies employed, in part, to preserve myocardial function after open-heart surgery. These strategies include intraoperative steroids, systemic cooling, cardioplegia additives, aprotinin, and modified ultrafiltration. We made no attempt to address the impact of these various therapies on myocardial function, but we plan to extend our experimental observations to investigate the impact of some of these strategies in the future. Second, we weaned the rabbits to partial CPB (50 mL/kg) for one hour following aortic cross-clamp removal before weaning the rabbits completely from CPB. Although this weaning strategy differs from usual clinical practice, we needed to employ a more gradual wean in the absence of any fluid resuscitation or vasoactive-inotropic medications that could have influenced heart rate, preload, afterload or contractility. Furthermore, we initially extended our experiments beyond 120 minutes of reperfusion (as well as with variable ischemic times), but we found that many of the rabbit hearts (particularly in the hyperoxic group) did not survive in our model in the absence of pharmacological support. Therefore, we chose to compare LV functional data for the two groups of rabbits during early reperfusion when all hearts survived with reasonable contractility. Third, the CPB oxygenator employed in our experimental model was specifically designed for use in rabbits by investigators from Aachen University (Aachen, Germany). Although it is much smaller than average pediatric oxygenators, our circuit still required a prime volume of 25-30 mL leading to a 40-50% reduction in hemoglobin concentrations. Although we recognize that use of blood prime would have resulted in less

hemodilution, there are multiple reports about the potentially beneficial effects of hemodilution during bypass, and other studies that suggest that blood prime may actually increase the likelihood of post-CPB myocardial injury and lung edema (36). Therefore, use of blood vs. crystalloid prime and a "safe" degree of hemodilution is at a minimum controversial in the cardiac surgery literature. Regardless, for our studies, use of crystalloid circuit prime and the resulting hemodilution were identical for both the normoxic and hyperoxic groups of rabbits, and therefore does not explain any differences between the two groups with regards to myocardial edema, LV compliance, and systolic function. With regards to cardioplegia, this is another area of controversy and variability among institutions performing heart surgery. There are limited data that demonstrate a superior benefit for blood vs. crystalloid cardioplegia solution. Based on our usual clinical practice at the University of Michigan, cold crystalloid cardioplegia was used in this study. Finally, in our cell-based model, we did not directly measure cellular oxygen tension during reoxygenation under either normoxic or hyperoxic conditions. Therefore, we do not know how well these cell culture conditions mimic the cellular conditions in the tissues.

In conclusion, the results of this study contribute to an improved understanding of the impact of re-oxygenation conditions on myocardial injury and ventricular dysfunction after cardiac surgery with CPB and cardioplegic arrest. Mechanistically, myocardial IR injury is associated with an increase in OS, inflammation, and apoptosis. Furthermore, normoxic reperfusion (or re-oxygenation) is associated with preserved LV contractility and less OS, inflammation, apoptosis, and myocardial injury compared with hyperoxic reperfusion (or re-oxygenation). At a minimum, this study strongly suggests that hyperoxia should be avoided to minimize myocardial IR injury and ventricular dysfunction after open-heart surgery.

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Figure Legends

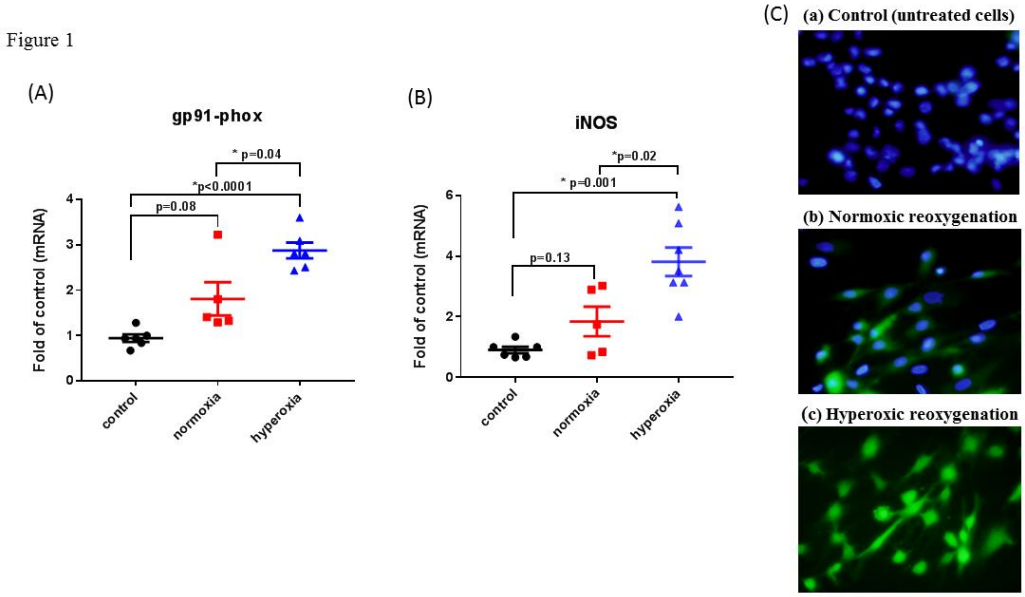

Figure 1. Oxidant stress (OS) in H9c2 cells in response to 12 hours of hypoxia and 2 hours of re-oxygenation.

Messenger RNA levels of (A) gp-91-phox, and (B) iNOS were analyzed by RT-PCR. The results are expressed as mean ± S.E. of 5-6 different cell culture experiments. (C) Intracellular ROS were visualized using fluorescence microscopy. (a) Untreated, control cells, (b) Cells exposed to normoxic reoxygenation, (c) Cells exposed to hyperoxic reoxygenation. Blue = Hoechst 33342, green = DCF. Normoxia = Cells exposed to hypoxia and 21% O_2 re-oxygenation, Hyperoxia = Cells exposed to hypoxia and 95% O_2 re-oxygenation.

Figure 2

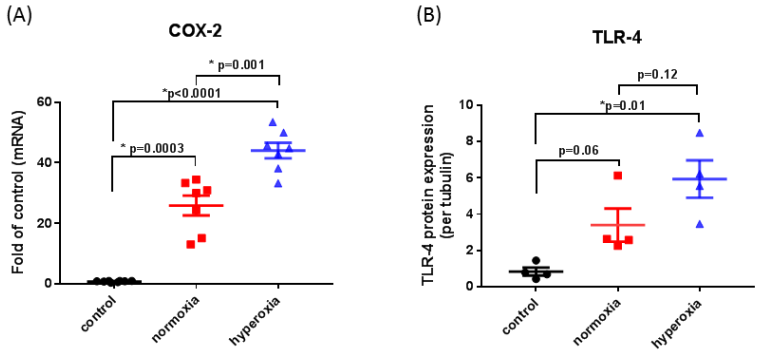

Figure 2. Inflammation in H9c2 cells in response to 12 hours of hypoxia and 2 hours of re-oxygenation.

Messenger RNA levels of (A) COX-2, and protein levels of (B) TLR-4 were analyzed by RT-PCR or immunoblotting assay, respectively. The results are expressed as mean ± S.E. of 4-6 different cell culture experiments. Normoxia = Cells exposed to hypoxia and 21% O_2 re-oxygenation, Hyperoxia = Cells exposed to hypoxia and 95% O_2 re-oxygenation.

Figure 3

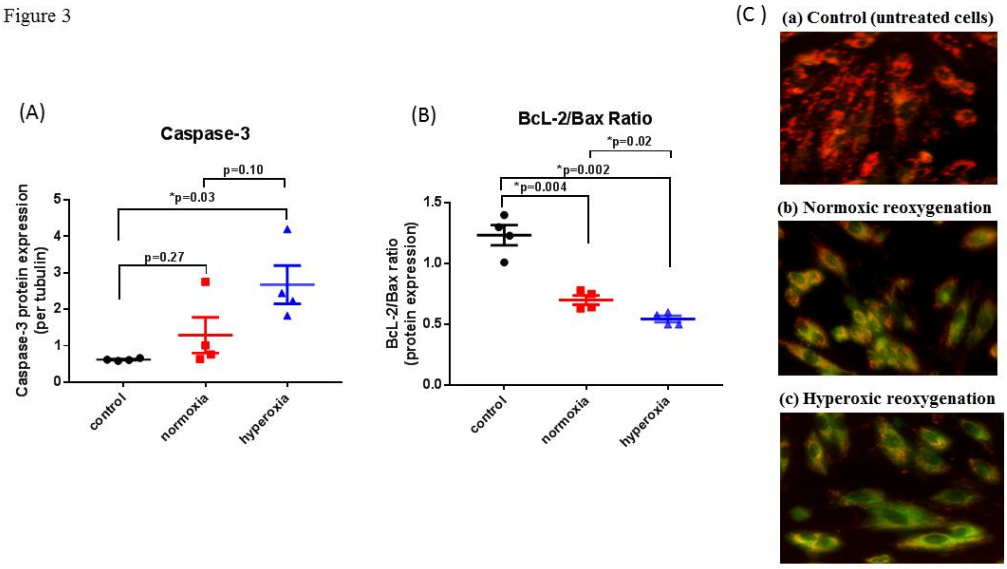

Figure 3. Apoptosis in H9c2 cells in response to 12 hours of hypoxia and 2 hours of re-oxygenation.

Protein expression of (A) caspase-3, and (B) BcL-2 and BAX (for BcL-2/BAX ratio) were analyzed by immunoblotting assay. The results are expressed as mean ± S.E. of 4 different cell culture experiments. (C) Representative JC-1 staining in H9c2 cells. (a) Untreated, control cells. (b) Cells exposed to normoxic reoxygenation. (c) Cells exposed to hyperoxic reoxygenation. Red = healthy cells with high $\Delta\psi$, green= apoptotic cells with low $\Delta\psi$. Normoxia = Cells exposed to hypoxia with 21% O_2 re-oxygenation, Hyperoxia = Cells exposed to hypoxia and 95% O_2 re-oxygenation.

Figure 4

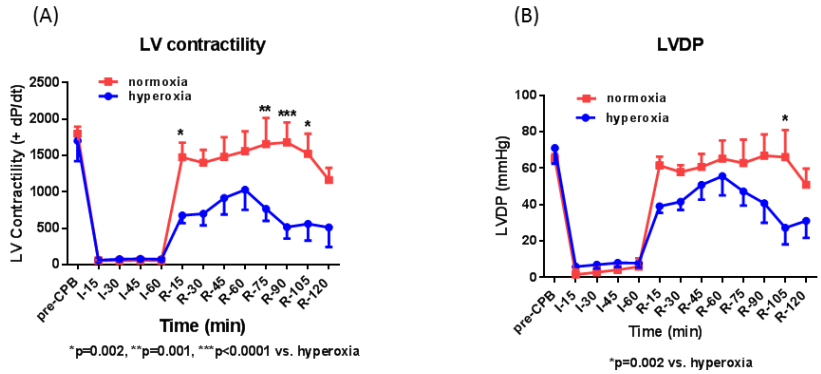

Figure 4. Left ventricular function after CPB and cardioplegic arrest.

Following 60 minutes of global myocardial ischemia and 2 hours of reperfusion with normoxic (21% O_2) or hyperoxic (100% O_2) conditions, (A) LV contractility (LV +dP/dt); and (B) LV developed pressure (LVDP) (mmHg) were measured. I = Ischemic time. R = Reperfusion time. The results are expressed as mean ± S.E of 6-7 rabbits per group.

Figure 5

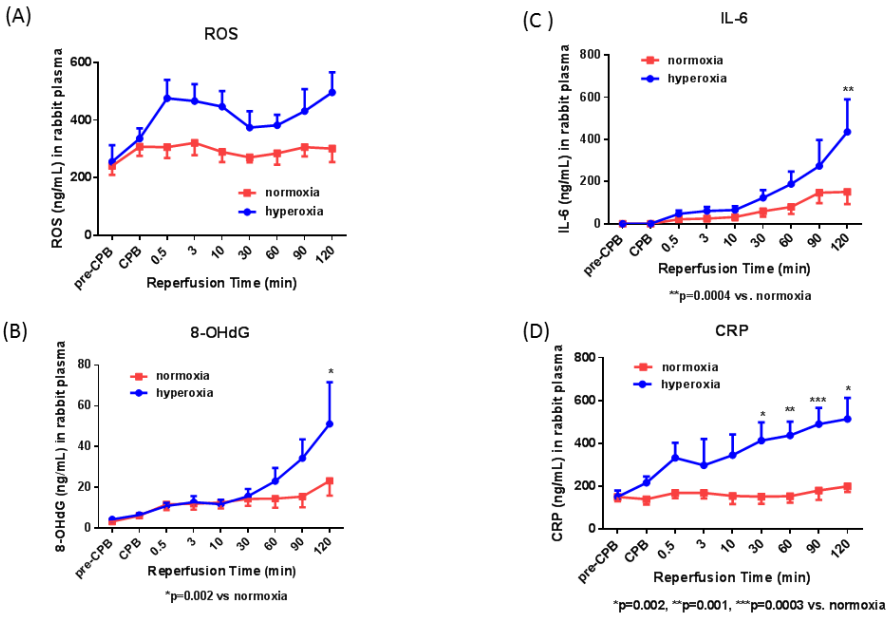

Figure 5. Time-course of plasma biomarkers for oxidant stress and inflammation after CPB and cardioplegic arrest. (A) ROS; (B) 8-OHdG; (C) IL-6; and, (D) CRP under normoxic (21% O_2) or hyperoxic (100% O_2) reperfusion conditions. The results are expressed as mean ± S.E from 5-7 rabbits per group.

Figure 6

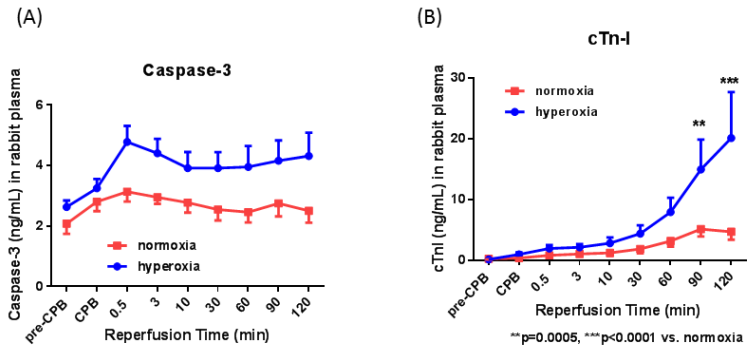

Figure 6. Time-course of plasma markers for apoptosis and cardiomyocyte injury after CPB and cardioplegic arrest. (A) caspase-3; and, (B) cardiac troponin-I under normoxic (21% O_2) or hyperoxic (100% O_2) reperfusion conditions. The results are expressed as mean ± S.E. from 6-7 rabbits per group.

Figure 7

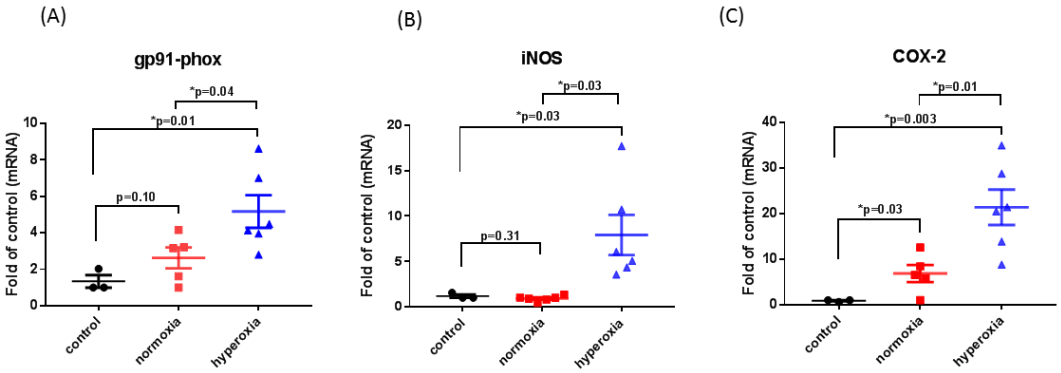

Figure 7. Tissue OS and inflammation in rabbit myocardium.

Messenger RNA levels of (A) gp-91-phox; (B) iNOS; and (C) COX-2 in rabbit heart homogenates were measured by RT-PCR. The results are expressed as mean ± S.E. from 5-6 rabbits per experimental group, and 3 rabbits of control group

Video legends

This video shows the rabbit CPB/IR study as performed at our laboratory.

(1) The experimental protocol included 60-minute ischemia/cardioplegic arrest and 120-minute reperfusion.

(2) The rabbit CPB model was veno-arterial CPB. The blood was drained from the jugular vein and infused into the carotid artery. A millar catheter was placed at LV for ventricular function

measurement. The blood pressure measurement and blood sample collection were from femoral artery.

(3) Eight parameters were recorded throughout the whole experiment.

After aortic cross-clamping and injection of cardioplegia, the LVDP and LV contractility were decreased immediately. Also, heart rate was reduced dramatically. However, after reperfusion, LVDP and LV contractility as well as heart rate recovered quickly.

References

1. Zakkar M, Guida G, Suleiman MS, Angelini GD. Cardiopulmonary bypass and oxidative stress. Oxidative Medicine and Cellular Longevity. 2015; ID 189863, 8 pages

2. Rodrigo R, Libuy M, Feliu F, Hasson D. Oxidative stress-related biomarkers in essential hypertension and ischemia-reperfusion myocardial damage. Disease Markers 2013; 35:773-90.

3. Nissinen J, Biancari F, Wistbacka J, PeltolaT, LoponenP, Tarkiainen P, et al. Safe time limits of aortic cross-clamping and cardiopulmonary bypass in adult cardiac surgery. Perfusion 2009; 24:297-305

4. Mokhtari A, Lewis M. Normoxix and hyperoxic cardiopulmonary bypass in congenital heart disease. BioMed Research International 2014; 678268, 11 pages

5. Caputo M, Mokhtari A, Miceli A, Ghorbel MT, Angelini GD, Parry AJ, et al. Controlled reoxygenation during cardiopulmonary bypass decreases markers of organ damage,

inflammation, and oxidative stress in single-ventricle patients undergoing pediatric heart surgery. J Thorac Cardiovasc Surg 2014; 148:792-801.

6. Kutala VK, Khan M, Angelos MG, Kuppusamy P. Role of oxygen in postischemic myocardial injury. Antioxid. Redox Signal 2007; 9:1193-1206.

7. Fugelseth D, Borke W, Lenes K, Matthews I, Saugstad O, Thaulow E. Restoration of cardiopulmonary function with 21% verse 100% oxygen after hypoxaemia in newborn pigs. Arch Dis Child Fetal Neonatal Ed 2005; 90: F229-34.

8. Hafez P, Chowdhury SR, Jose S, Law JX, Ruszymah BHI, Modh Ramzisham AR, et al. Development of an in vitro cardiac ischemic model using primary human cardiomyocytes. Cardiovasc Eng Technol 2018; June (10 pages)

9. Chen S, Yang B, Xu Y, Rong Y, Qiu Y. Protection of luteolin-7-O-glucoside against apoptosis induced by hypoxia/reoxygenation through the MAPK pathways in H9c2 cells. Mol Med Rep 2018; 17: 7156-62.

10. Peng YW, Buller CL, Charpie JR. Impact of N-acetylcysteine on neonatal cardiomyocyte ischemia-reperfusion injury. Pediatr Res 2011; 70:61-6.

11. Miao Y, Zhou J, Zhao M, Liu J, Sun L, Yu X, et al. Acetylcholine attenuates hypoxia/reoxygenation-induced mitochondrial and cytosolic ROS formation in H9c2 cells via M2 acetylcholine receptor. Cell Physiol Biochem 2013; 31:189-98.

12. Huang X, Zuo L, Lv Y, Chen C, Yang Y, Xin H, et al. Asiatic Acid attenuates myocardial ischemia/reperfusion injury via Akt/GSK-3 b/HIF-1a signaling in rat H9c2 cardiomyocytes. Molecules 2016; 21: 1248 (14 pages)

13. Guo W, Liu X, Li J, Shen Y, Zhou Z, Wang M, et al. Prdx1 alleviates cardiomyocyte apoptosis through ROS-activated MAPK pathway during myocardial ischemia/reperfusion injury. Int J Biol Macromol 2018; 112: 608-15.

14. Kim WG, Moon HJ**.** Rabbit model of cardiopulmonary bypass. Perfusion 1999; 14:101-05.

15. Sznycer-Taub N, Mackie S, Peng YW, Donohue J, Yu S, Charpie J. Myocardial oxidative stress in infants undergoing cardiac surgery. Pediatr Cardiol 2016; 37:746-50

16. Christen S, Finckh B, Lykkesfeldt J, Gessler P, Freses-Schaper M, Nielsen P, et al. Oxidative stress precedes peak systemic inflammatory response in pediatric patients undergoing cardiopulmonary bypass operation. Free Radic Biol Med 2005; 38:1323-32.

17. Amoureux S, Sicard P, Korandji C, Borey A, Benkhadra S, Sequeira-Le Grand A. et al. Increase in levels of BDNF is associated with inflammation and oxidative stress during cardiopulmonary bypass. Int J Biomed Sci 2008; 4: 204-11.

18. Solberg R, Enot D, Deigner H-P, Koal T, Scholl-Burgi S, Saugstad OD, et al. Metabolomic analyses of plasma reveals new insights into asphyxia and resuscitation in pigs. 2010, PLoS One 5: e9606

19. Brucken A, Kaab AB, Kottmann K, Rossaint R, Nolte KW, Weis J, et al. Reducing the duration of 100% oxygen ventilation in the early reperfusion period after cardiopulmonary resuscitation decreases striatal brain damage. Resuscitation 2010; 81:1698-703.

20. Garcia-de-la-Asuncion J, Pastor E, Perez-Griera J, Belda FJ, Moreno T, Garcia-del-Olmo E, et al. Oxidative stress injury after on-pump cardiac surgery: Effects of aortic cross clamp time and type of surgery. Redox Report 2013, 18:5, 193-9

21. Christen S, Finckh B, Lykkesfeldt J, Gessler P, Freses-Schaper M, Nielsen P, et al. Oxidative stress precedes peak systemic inflammatory response in pediatric patients undergoing cardiopulmonary bypass operation. Free Radic Biol Med 2005; 38:1323-32.

22. Baines CP. How and when do myocytes die during ischemia and reperfusion: the late phase. J of Cardiol Pharmaco Ther 2011; 16:239-43.

23. Dominguez-Rodriguez A, Abreu-Gonzalez P, Reiter R. Cardioprotection and pharmacological therapies in acute myocardial infarction: Challenges in the current era. World J Cardiol 2014: 26: 100-06.

24. Pilcher J, Weatherall M, Shirtcliffe P, Bellomo R, Young P, Beasley R. The effect of hyperoxia following cardiac arrest-A systematic review and meta-analysis of animal trials. Resuscitation 2012; 83: 417-22.

25. Dongworth R, Hall AR, Burke N, Hausenloy DJ. Targeting mitochondria for cardioprotection: examining the benefit for patients. Future Cardiol 2014; 10: 255-72.

26. Bainey KR, Armstrong PW. Clinical perspectives on reperfusion injury in acute myocardial infarction. Am Heart J 2014; 167:637-45.

27. Aiyagari R, Gelehrter S, Bove EL, Ohye RG, Devaney EJ, Hirsch JC, et al. Effects of N-acetylcysteine on renal dysfunction in neonates undergoing the arterial switch operation. J Thorac Cardiovasc Surg 2010, 139:956-961.

28. Morita K. Surgical reoxygenation injury of myocardium in cyanotic patients: clinical relevance and therapeutic strategies by normoxic management during cardiopulmonary bypass. Gen Thorac Cardiovasc Surg 2012; 60: 549-56.

29. Ghorbel MT, Mokhtarl A, Sheikh M, Angelini GD, Caputo M. Controlled reoxygenation cardiopulmonary bypass is associated with reduced transcriptomic changes in cyanotic tetralogy of Fallot patients undergoing surgery. Physiol Genomics 2012; 44: 1098-106.

30. Buckberg GD. Controlled reperfusion after ischemia may be the unifying recovery denominator. J Thorac Cardiovasc Surg. 2010; 140: 12-8

31. Ihnken K, Morita K, Buckberg GD, Winkelmann B, Beyersdorf F, Sherman MP. Reduced oxygen tension during cardiopulmonary bypass limits myocardial damage in acute hypoxic immature piglet hearts. Eur J Cardiothorac Surg. 1996; 10: 1127-34

32. Beyersdorf F. The use of controlled reperfusion strategies in cardiac surgery to minimize ischeaemia/reperfusion damage. Cardiovasc Res. 2009; 83: 262-8

33. Caputo M, Mokhtari A, Rogers CA, Panayiotou N, Chen Q, Ghorbel MT, et al. The effects of normoxic versus hyperoxic cardiopulmonary bypass on oxidative stress and inflammatory response in cyanotic pediatric patients undergoing open cardiac surgery: A randomized controlled trial. J Thorac Cardiovasc Surg 2009; 138: 206-214

34. Morita K. Surgical reoxygenation injury of the myocardium in cyanotic patients: clinical relevance and therapeutic strategies by normoxic management during cardiopulmonary bypass. Gen Thorac Cardiovasc Surg 2012; 60: 549-56

35. Smith JM, Roberts WH, Miller JD, Hasselfeld KA, Hendy MP. Controlled cardiac reoxygenation does not improve myocardial function following global myocardial ischemia. International Journal of Surgery 2006; 4: 153-9

36. You XM, Nasrallah F, Darling E, Robins M, Nieman G, Searles B. Rat cardiopulmonary bypass model: application of a miniature extracorporeal circuit composed of asanguinous prime. J Eetra Corpor Technol. 2005; 37: 60-5.