Hyperoxia causes oxygen free radical-mediated membrane injury and alters myocardial function and hemodynamics in the newborn

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During the medical and/or surgical management of these patients, exposure to high levels of oxygen (hyperoxia) for varying durations can occur (1, 2, 35, 41). For example, during ECMO or CPB, systemic oxygen levels can reach an arterial PO2 (Pao2) of up to 500 mmHg, which can last several days during extracorporeal membrane oxygenation or for 2–5 h during a cardiac operation (1, 35). The original rationale for the use of hyperoxia was to ensure adequate oxygen delivery, assuming that higher levels of oxygen could only benefit the patient receiving critical care (6, 41). The potentially detrimental effects of hyperoxia itself were rarely considered.

In adults, the hemodynamic effects of hyperoxia have been extensively explored in animal models and patients with heart failure, coronary artery disease, and myocardial infarction (17, 25, 30–32). Most of these adult studies have reported significant decreases in heart rate (HR) after oxygen treatment (11, 14, 26, 31). However, the effects of oxygen on blood pressure are contradictory, with some studies reporting increases and others reporting no change or even a decrease (11, 17, 25, 26, 31). When focusing on the effects in the heart, research using adult dogs showed a reduction in cardiac output and compromised ventricular work rates (28).

In the newborn, however, there are only a few studies that have examined the hemodynamic effects of hyperoxia, and those were confounded by the presence of congenital heart disease in children or prior exposure to low oxygen (hypoxia) (7, 19). In these studies, hyperoxia was found to increase hemodynamics (7, 19). Work in the newborn pig heart by Ihnken and colleagues (19, 33) only addressed the myocardial effects of hyperoxia after a period of acute hypoxia or ischemia and identified the development of significant myocardial dysfunction. Interestingly, clinical evidence in children with acyanotic congenital heart disease also shows that hyperoxia decreases cardiac index and stroke index (7). Whether clinically relevant levels of hyperoxia alone affect hemodynamics and impairs myocardial function in newborns in the absence of confounding variables such as low oxygen or preexisting disease remains unknown.

Hyperoxia has been associated with the release of oxygen free radicals (22). If these radicals are not fully neutralized by key naturally occurring antioxidant enzymes [superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT)], they can cause membrane injury and may represent a potential mechanism by which hyperoxia may partly contribute to an impairment of myocardial function. Whether the newborn is particularly susceptible to this is unknown.
Therefore, the purpose of this study was to examine whether hyperoxia alone in the absence of any confounding variables can compromise cardiac function and hemodynamics in the newborn pig and to explore whether the underlying mechanism involves the release of oxygen free radicals that overwhelm myocardial antioxidant enzymes (SOD, GPx, and CAT) leading to subsequent membrane injury marked by increases in malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) levels. The findings from this work may significantly contribute to understanding the effects of hyperoxia in the newborn and its mechanisms.

**MATERIALS AND METHODS**

Neonatal Yorkshire pigs were chosen as the animal model in which to study the effects of hyperoxia because the cardiac and pulmonary systems of newborn humans and pigs have many structural and functional similarities (12, 15). Yorkshire pigs (3 days old, 1.5–2.5 kg) were anesthetized with an intraperitoneal injection of pentobarbital sodium (65 mg/kg, MTC Pharmaceuticals; Cambridge, Ontario, Canada), intubated, and mechanically ventilated to normal blood gases \([P_{A_0_2} = 88 \pm 6 \text{ mmHg}, \text{ arterial } P_{O_2} (P_{A_0_2}) = 38 \pm 5 \text{ mmHg}]\) with medical air. Normothermia (37.9 ± 0.2°C) was maintained in all piglets. A catheter was inserted into the right carotid artery and advanced to the aortic arch to monitor arterial blood pressure. After a sternotomy was performed and the heart was exposed, a second catheter was placed in the left ventricle (LV) to monitor left intraventricular pressure. Both catheters were connected to pressure transducers (COBE; Lakewood, CO) and a physiological recorder (BIO PAC Systems; Goleta, CA). The right carotid artery catheter was also used for sampling of blood gases.

Arterial blood samples were obtained, and appropriate adjustments were made to ensure normal physiological values for \(P_{A_0_2}\) and \(P_{A_CO_2}\), as well as acid-base status [\(pH\) and bicarbonate \(\text{HCO}_3^-\)] using an ABL30 Acid-Base Analyzer (Radiometer; Copenhagen, Denmark).

**Experimental Protocol**

Animals were then randomly allocated to 5 h of normoxia control \((P_{A_0_2} = 83 \pm 5 \text{ mmHg}, n = 4)\) or hyperoxia \((P_{A_0_2} = 422 \pm 33 \text{ mmHg}, n = 6)\). The 5-h normoxia ventilatory experiments were performed to monitor the stability of the model. To assess hemodynamic performance, systolic blood pressure (SBP), diastolic blood pressure (DBP), developed pressure, and mean arterial blood pressure (MAP) as well as HR were recorded hourly. Myocardial performance was derived hourly by differentiating the LV intraventricular pressure trace to yield indexes of positive \(+dP/dt_{max}\) and negative maximum first derivative of LV pressure (-dP/dt_{max}), which were used to assess myocardial contractility and relaxation, respectively. To account for the load-dependent nature of these measurements, all derived values of \(+dP/dt_{max}\) were also normalized for preload \([\text{LV end-diastolic pressure (LVEDP)}]\), afterload \([\text{LV systolic pressure (LVSP)}]\), and HR. At the end of the 5-h ventilatory protocol, LV myocardial biopsies were taken to measure antioxidant enzyme activities (SOD, GPx, and CAT) as well as for MDA and 4-HNE measurements, which reflect the extent of lipid peroxidation (29) secondary to oxygen free radical-mediated membrane injury.

All experimental procedures and protocols used in this investigation were reviewed and approved by the University of Toronto Animal Care and Use Committee and are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1985) and Canadian Council on Animal Care guidelines.

**Assessment of Antioxidant Enzyme Activity**

SOD. LV myocardial biopsies were homogenized using cold (0.25 M sucrose) buffer and spun for 10 min at 8,500 g at 4°C. The supernatant was then analyzed based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzoquinone in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. The method determines both Cu-Zn-SOD and Mn-SOD activity (Bioxytech, Oxish Health Products; Portland, OR). SOD activity was expressed per milligram of protein.

GPx. LV myocardial biopsies were homogenized using cold buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 1 mM 2-mercaptoethanol] and spun for 15 min at 8,500 g at 4°C. In this assay, analysis of the supernatant for GPx activity was based on oxidized glutathione produced upon reduction of an organic peroxide by GPx, which is recycled to its reduced state by the enzyme glutathione reductase. The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm, which provides the spectrophotometric means of monitoring GPx activity (Calbiochem; San Diego, CA). GPx activity was expressed as milligram of protein.

**CAT.** In the catalase assay, LV tissue samples were first homogenized using cold buffer (100 mM KCl, 50 mM K_2HPO_4, 2 mM EDTA, 50 mM Tris-HCl, and 5 mM MgSO_4) and spun for 10 min at 1,500 g at 4°C. Supernatants containing CAT were incubated in the presence of a known concentration of \(H_2O_2\). After incubation for exactly 1 min, the reaction was quenched with sodium azide. The amount of \(H_2O_2\) remaining in the reaction mixture was then determined by the oxidative coupling reaction of 4-aminoazobenzenone and 3,5-dichloro-2-hydroxybenzenesulfonic acid in the presence of \(H_2O_2\) and catalyzed by horseradish peroxidase. The resulting quinoneimine dye was measured at 520 nm as an indicator of CAT activity (Calbiochem). CAT activity was expressed as milligram of protein.

**MDA and 4-HNE Measurements**

Sample preparation. Heart tissue (~100 mg) ground into a powder in liquid nitrogen was suspended in 0.5 ml deionized water followed by the addition of EDTA at a final concentration of 400 μM and butylated hydroxytoluene (BHT) and desferal at final concentrations of 20 μM. Samples were then analyzed by a modification of the previously described method (29) and analyzed by gas chromatography-mass spectrometry (29).

**Statistical Analysis**

Paired t-tests were used to determine significant differences in functional and hemodynamic parameters between baseline and the end of 5 h of hyperoxic ventilation. Independent Student’s t-tests were used to determine significant differences in MDA, 4-HNE, and antioxidant enzyme levels between normoxic and hyperoxic piglets. All data are expressed as means ± SE, and statistical significance was defined as \(P < 0.05\).

**RESULTS**

**Cardiac Performance and Hemodynamics**

Control animals that underwent 5 h of normoxic ventilation did not show any significant changes in myocardial function or hemodynamics (Table 1), indicating the stability of the model. The values shown in Table 1 fall within the normal of physiological range of what is seen in healthy 3-day-old piglets (7, 19). The baseline (normoxic) values of the hyperoxic group fell within the range of those seen in the normoxic study group, thereby confirming the appropriate random allocation of the animals used in this study and their initial normal status.

Five hours of hyperoxia resulted in significant but variable reductions in myocardial contractility (+dP/dt_{max}), ranging from 3% to 48% (baseline; 2,023.89 ± 219.08 mmHg/s, 5 h of hyperoxia: 1,590.11 ± 177.65 mmHg/s; Fig. 1). Four of six animals also showed varying degrees of impairment in myo-
cardial relaxation (−dP/dt\text{max}), which did not reach statistical significance (baseline: −1,704.53 ± 330.79 mmHg/s, 5 h of hyperoxia: −1,616.05 ± 389.51 mmHg/s; Table 2). When the effect of hyperoxia on hemodynamics was examined, significant reductions were seen in SBP (Fig. 1) but not in DBP. All animals but one showed marked reductions in developed pressure by as much as 28%; however, due to the variability of this response, this did not reach statistical significance (Table 2). MAP was significantly reduced (Fig. 2), whereas HR was significantly increased (Fig. 2), after the 5-h hyperoxic exposure. Hyperoxia-mediated reductions in myocardial contractility remained significant even after +dP/dt\text{max} values were normalized for preload (LVEDP), afterload (LVSP), and HR (Table 3).

### Table 1. Left ventricular performance and hemodynamic measurements in normoxic piglets

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>After 5 h of Ventilation</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Contractility, mmHg/s</td>
<td>1,905.94±440.87</td>
<td>1,979.44±360.71</td>
<td>0.64</td>
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<tr>
<td>Relaxation, mmHg/s</td>
<td>−1,747.91±405.53</td>
<td>−1,650.47±170.90</td>
<td>0.80</td>
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<tr>
<td>SBP, mmHg</td>
<td>76.30±7.05</td>
<td>73.60±8.53</td>
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<tr>
<td>DBP, mmHg</td>
<td>38.20±5.83</td>
<td>34.54±3.43</td>
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</tr>
<tr>
<td>Developed pressure, mmHg</td>
<td>76.13±19.93</td>
<td>68.51±7.30</td>
<td>0.66</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>50.57±6.00</td>
<td>46.54±5.25</td>
<td>0.75</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>180.15±18.00</td>
<td>201.03±8.48</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Values are means ± SE. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial blood pressure; HR, heart rate.

### Table 2. Left ventricular relaxation and diastolic and developed blood pressure measurements in hyperoxic piglets

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>After 5 h of Ventilation</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relaxation, mmHg/s</td>
<td>−1,704.53±330.79</td>
<td>−1,616.05±389.51</td>
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<tr>
<td>DBP, mmHg</td>
<td>41.50±9.03</td>
<td>31.61±8.53</td>
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<tr>
<td>Developed pressure, mmHg</td>
<td>79.16±18.93</td>
<td>65.70±21.57</td>
<td>0.09</td>
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</table>

Values are means ± SE.

Fig. 1. Myocardial contractility (+dP/dt\text{max}; A) and systolic blood pressure (B) in newborns at baseline and at 5 h of hyperoxia.

Fig. 2. Mean arterial blood pressure (A) and heart rate [in beats/min (bpm); B] in newborns at baseline and at 5 h of hyperoxia.
Those newborns exposed to hyperoxia showed a modest but significant 11% decrease in SOD activity in the LV compared with those newborns exposed to normoxia (Fig. 3). In contrast, LV GPx activity was dramatically decreased by 61%, whereas no significant changes in CAT activity were seen in newborns after hyperoxic ventilation (Fig. 3). MDA and 4-HNE levels in the LV were significantly elevated by 52% and 38%, respectively, in piglets exposed to hyperoxia compared with the MDA and 4-HNE levels generated through normal cellular activity in normoxic animals (Fig. 4).

**Correlations**

Correlations performed to assess the impact of oxygen free radical-mediated injury on antioxidant enzyme activity showed significant inverse correlations between MDA levels and SOD activity as well as GPx activity (Fig. 5), whereas no correlation was found between MDA levels and CAT activity ($r = 0.49, P = 0.21$). When the relationship between $+dP/dt_{\text{max}}$ values and antioxidant enzyme activity was examined, a strong and significant inverse correlation was observed between $+dP/dt_{\text{max}}$ values and SOD activity ($r = 0.22, P = 0.54$) or $+dP/dt_{\text{max}}$ values and GPx activity ($r = 0.20, P = 0.61$).

**DISCUSSION**

This study for the first time demonstrates that exposure to high levels of oxygen alone in newborns leads to both a general impairment of myocardial function and hemodynamics as well as significant oxygen free radical-mediated membrane injury.

The changes in ventricular contractility seen in the present study may be due to the direct effect of hyperoxia on myocardial function in the newborn or alternatively may be mediated by secondary changes in preload, afterload, and/or HR. Previous studies have shown that hyperoxia has strong effects on vascular smooth muscle, and a number of oxygen-sensing mechanisms in smooth muscle cells have been proposed and identified (3, 4, 40). There is also evidence in the literature to suggest that hyperoxia acts as a systemic vasoconstrictor (11, 30, 31). Interestingly, when corrected for these parameters, contractility was still significantly reduced after hyperoxic exposure. It is important to note that there are several methods to measure ventricular function in a whole animal model, and each of these methods have been documented to have their advantages and disadvantages (8). The selection of a whole animal physiology model for this study was of particular relevance because it allowed for a systemic reaction to occur in response to the hyperoxic exposure. In the newborn, the derived indexes of $+dP/dt_{\text{max}}$ and $-dP/dt_{\text{max}}$ are well documented and accepted methods to assess myocardial contractil-
ity and relaxation, respectively (38, 39), and, interestingly, when corrected for load, the reduction in contractility seen in these newborns after 5 h of hyperoxia remained significant. This is consistent with work performed by Beekman and colleagues (7) investigating the effect of hyperoxia in children with acyanotic congenital heart disease in which even after HR was kept constant by atrial pacing, a significant decrease in cardiac index and stroke index was seen in children breathing 95% oxygen. These children as well as studies in adult dogs exposed to hyperoxia show that reductions in cardiac index and ventricular work rates occurred in the face of increased systemic vascular resistance and hemodynamics, possibly indicating an intracardiac effect (19, 28). The present study for the first time documents that in newborns, there is a distinct effect of hyperoxia on myocardial performance and hemodynamics in a whole animal preparation in the absence of cardiac disease.

Furthermore, our previous work has shown that plasma epinephrine levels were not significantly elevated in piglets exposed to hyperoxia (5), thereby suggesting that these animals did not compensate for cardiac dysfunction through a systemic adrenergic response. However, this does not exclude the possibility that hyperoxia-mediated reductions in MAP may evoke a cardiac-specific adrenergic response leading to an increase in HR through the local release of norepinephrine that would not manifest in a rise in plasma epinephrine levels.

The magnitude of response to hyperoxia was variable in the present study, with certain newborns showing a greater degree of functional and hemodynamic impairment compared with others. This variability was inherent to the hyperoxic exposure because those newborns that underwent normoxic ventilation were stable over the course of the 5-h study period. It is therefore apparent that certain newborns have a more exaggerated response to hyperoxia than others and hence are at greater

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Fig. 4. Left ventricular (LV) malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) levels in normoxic and hyperoxic piglets at the end of 5 h of ventilation. ‡P < 0.001; #P = 0.02.

Fig. 5. Significant and inverse correlation between cardiac MDA levels and SOD activity (r = −0.54, P = 0.03) as well as GPx activity (r = −0.79, P = 0.01) in piglets.

Fig. 6. Significant and inverse correlation between +dP/dtmax values and CAT activity (r = −0.81, P = 0.02) in piglets.
risk for hemodynamic and functional impairment. This could also apply to children in the critical care setting, implying that every neonate may not mature at exactly the same rate and thus their susceptibility to stresses including exposure to high oxygen levels may vary.

This varying level of susceptibility in the newborn may in part be a result of their ability to tolerate oxidative injury. Although somewhat controversial, the antioxidant defensive systems are immature in the newborn, making them particularly susceptible to oxygen free radical-mediated injury (13, 34). One mechanism through which hyperoxia could reduce myocardial function is through membrane injury that is mediated by the release of oxygen free radicals, which could be a result of insufficient myocardial antioxidant defences in the newborn heart. The work in the present study, for the first time, clearly demonstrates that the newborn heart lacks the ability to upregulate its antioxidant enzyme activity to meet the challenge of an additional oxygen free radical stress that may be mediated by hyperoxia. Moreover, not only does this work document the lack of upregulation but further suggests that two of the three major antioxidant enzymes suffer a reduction in their activity after a hyperoxic exposure, thereby rendering the newborn heart at greater risk of oxygen free radical-mediated membrane injury. This is consistent with the significant increases in MDA levels, strongly suggesting a mechanism involving oxygen free radical-mediated injury that may partly account for the impairment in myocardial function and hemodynamics seen in newborns. This was further substantiated by reductions of both SOD and GPx activity levels being significantly correlated with increases in MDA levels in this study. GPx activity also showed the greatest reduction in response to hyperoxia, thereby confirming its role as the main antioxidant pathway in the heart. The reduction in GPx activity and the presence of oxygen free radical-mediated membrane injury do imply the development of some myocardial oxidative stress in the newborn in response to hyperoxia. Interestingly, however, GPx activity did not correlate with functional impairment. One limitation to this is that GPx activity alone does not take into account the GSH redox status (GSH-to-GSSG ratio) or GSH + GSSG pool size (21), which is required to fully elucidate the contribution of the GPx pathway to the hyperoxia-induced depression of myocardial function in newborns. In contrast to the GPx findings in response to hyperoxia, CAT activity did not correlate with MDA levels; however, it did significantly correlate with reductions in +dP/dmax values, leading to the possible speculation that CAT activity may serve as a marker of functional impairment in the newborn. However, it is also recognized that a direct negative inotropic effect of locally released oxygen free radicals (37) or other factors (42) on the myocardium could have also contributed to the functional reductions seen in this study.

Oxygen free radical-mediated injury and an impairment in myocardial function may also be linked through calcium cycling, which is critical in myocardial function and which can be susceptible to oxygen free radical-mediated injury. This is particularly important in the neonate due to its extensive reliance on sarcoclemma calcium influx for myocardial contraction because calcium sequestration in the neonatal heart has been reported to be immature (9). This greater reliance of the neonatal heart on extracellular calcium is also consistent with the greater number and sensitivity of L-type Ca2+ channels in the newborn heart sarcolemma (20). Work in transfected heart cells as well as in adult rats has shown that oxygen free radicals not only impair Ca2+ channel function but also reduce the number of Ca2+ channels in the cell membrane (10, 16, 23). Both of these effects contribute to a decrease in calcium influx into the cardiac cell (10, 16, 23).

If hyperoxia-induced oxygen free radical-mediated injury in the newborn changes membrane composition, this may not only lead to an impairment of L-type Ca2+ channel function but also a decrease in channel numbers in the neonatal heart. This impairment in channel function and reduction in the number of L-type Ca2+ channels may limit the ability to move calcium into the cell and potentially lead to a reduction in the number of actin-myosin cross-bridges that can be formed in the neonatal heart. This in turn would ultimately have a detrimental impact on myocardial function in the neonate. Oxygen free radicals have also been implicated in the inhibition of calcium binding to cardiac troponin in the myocardium (24). This, coupled with the strong evidence that oxygen free radicals have a detrimental effect on L-type Ca2+ channels, may explain the significant effect on myocardial contractility in this study.

In conclusion, this study presents novel findings identifying that in newborns, hyperoxia triggers oxygen free radical-mediated membrane injury together with an inability of the newborn heart to upregulate its antioxidant enzyme defenses while impairing myocardial function and hemodynamics.

GRANTS

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