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## Cardio-renal recovery of hypoxic newborn pigs after 18%, 21% and 100% reoxygenation

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**Abstract Objectives:** We examined the effects of 18%, 21% or 100% oxygen on the recovery of the heart and

kidneys in a short-term survival model of neonatal hypoxia–reoxygenation (HR). **Design:** Controlled, block-randomized animal study. **Setting:** University animal research laboratory. **Subject:** Large white piglets (1–3 days, 1.7–2.5 kg). **Interventions:** Piglets received normocapnic hypoxia (15% oxygen) (2 h) and were reoxygenated with 18%, 21% or 100% oxygen (1 h) ( $n = 7$  per group) then 21% oxygen (2 h). Sham-operated pigs ( $n = 7$ ) had no HR. **Measurements and results:** Seventeen of 21 HR piglets recovered from moderate hypoxemia (mean  $\text{PaO}_2$  27–33 mmHg and pH 7.20–7.22, associated with tachycardia and hypotension). Systemic arterial pressure, heart rate, left renal arterial flow, oxygen transport, plasma troponin-I and creatinine levels were monitored and recovered with no differences among HR groups over 4 days after resuscitation. The 100% group had increased myocardial oxidative stress (oxidized glutathione levels) and the most cardiac HR-induced injury. There were no differences in renal oxidative stress and HR-induced injury among groups. Early oxygenation at 1 h after resus-

citation correlated with the plasma troponin-I level at 6 h ( $r = -0.51$  and 0.64 for  $\text{SaO}_2$  and systemic oxygen extraction ratio,  $p < 0.05$ , respectively) and renal HR-induced injury at 4 days ( $r = 0.61$  for renal oxygen delivery,  $p < 0.05$ ). **Conclusions:** In hypoxic piglets, 18%, 21% and 100% reoxygenation caused similar systemic and renal hemodynamic and functional recovery. The indicators of oxidative stress and HR injury in myocardial and renal tissues suggest that the reoxygenation with 100% oxygen appears sub-optimal and the use of 18% oxygen offers no further benefit, when compared with 21% oxygen.

**Keywords** Hypoxia–reoxygenation · Resuscitation · Hemodynamics · Histology

**Abbreviations** *cTnI*: Cardiac-specific troponin-I · *FiO<sub>2</sub>*: Fractional inspired oxygen concentration · *GSH*: Total glutathione · *GSSG*: Oxidized glutathione · *HR*: Hypoxia–reoxygenation · *MAP*: Mean arterial pressure · *OFR*: Oxygen free radicals · *RA*: Renal artery

### Introduction

Resuscitation of asphyxiated neonates traditionally involves the use of 100% oxygen. This practice may

expose neonates to the potentially detrimental effects of oxygen-derived free radicals (OFRs) generated during reoxygenation/reperfusion. Despite their similar effectiveness [1–4] controversies remain over the consequences

of using 21% instead of 100% oxygen in neonatal resuscitation [5, 6]. Indeed, the current guidelines on the appropriate concentration of oxygen to be used during neonatal resuscitation differ among countries [7, 8]. Markers of oxidative stress were found to be increased in the plasma or tissues after the resuscitation with 100% oxygen [9–13]. Vento et al. recently reported higher levels of markers of myocardial and reno-tubular injury in neonates resuscitated with 100% oxygen than with 21% oxygen [14]. However, more information is needed to investigate the relationship between early oxygenation during resuscitation and markers of OFR production, functional recovery, and hypoxia-reoxygenation (HR)-induced injury of the organ.

Away from the two extreme oxygen concentrations for normoxic and hyperoxic resuscitation, Haase et al. demonstrated that using an intermediate concentration of 50% oxygen also had higher oxidative stress with no additional improvement in the hemodynamic recovery of asphyxiated newborn piglets [11]. Given the increased oxidative stress even in neonates resuscitated with 21% oxygen compared with healthy controls [3, 4], it is uncertain whether hypoxic resuscitation with < 21% oxygen could result in less oxidative stress and the related injury. Interestingly, 17–19% oxygen has been used in infants with univentricular heart disease to achieve hypoxemia with no obvious harmful side effects [15]. In severely hypoxic newborn piglets, Feet et al. showed similar recovery in 18% and 21% reoxygenation groups over an observation of 3 h [16].

Using a survival model of neonatal HR, we primarily examined the hemodynamic effects of 18%, 21% and 100% oxygen used in the reoxygenation of hypoxic newborn piglets on the recovery of the cardiovascular and renal systems. We also investigated the functional recovery, the biochemical markers of tissue injury and OFR-related oxidative stress, and histological features of reoxygenation injury.

## Methods

The study conformed to the regulations of the Canadian Council of Animal Care (Revised 1993) and was approved by the institutional committee. Twenty-eight Large White piglets (1–3 days old, 1.7–2.5 kg) were obtained on the day of experimentation and observed for 30–60 min for accommodation.

## Animal preparation

Spontaneously breathing piglets were gently given halothane (2–3%) for inhalational anesthesia, followed by oral intubation with an endotracheal tube (3.0 mm; Portex Inc., Wilmington, MA, USA). Single-lumen silicone cath-

eters (SIUVC5.0, NeoCare®; Klein-Baker Medical Co., San Antonio, TX, USA) were inserted to the level of the thoracic aorta and the right atrium via the left common carotid artery and external jugular vein, respectively. Intravenous cefazolin (100 mg in 1 ml) was given after venous access was established. Maintenance fluids consisted of 0.9% NaCl at 2 ml/kg/h and 10% dextrose at 10 ml/kg/h.

Boluses of fentanyl (20 µg) and pancuronium (0.6 mg) were given, followed by intravenous infusions of fentanyl (5–15 µg/kg/h) and midazolam (0.1–0.2 mg/kg/h) with the discontinuation of inhalational anesthesia. Thereafter, mechanical ventilation was commenced (Sechrist infant ventilator model IV-100; Sechrist Industries Inc. Anaheim, CA, USA) with pressures of 16/4 cmH<sub>2</sub>O at a rate of 12–20 breaths/min and fractional inspired oxygen concentration (FiO<sub>2</sub>) to 0.21–0.24 to maintain transcutaneous oxygen saturation between 89–95%, measured by a pulse oximeter (Nellcor N-200; Nellcor Inc., Hayward, CA, USA).

Following a dose of acepromazine (0.25 mg/kg) and additional boluses of fentanyl as needed, a left flank incision was performed. The left renal artery (RA) was isolated and encircled with a 2-mm transit time ultrasound flow probe (2SB; Transonic Systems Inc., Ithaca, NY, USA) to measure blood flow continuously. The flow probe cable and neck catheters were subcutaneously tunneled and the wounds were stitched.

The surgical procedure usually finished within 1 h. The rectal temperature was maintained at 38.5–39.5°C by an electrical heating blanket and an overhead warmer. All piglets were stabilized for at least 30 min.

## Experimental protocol

Moderate normocapnic alveolar hypoxia, which correlated to PaO<sub>2</sub> of 25–35 mmHg and SaO<sub>2</sub> of 40–50%, was induced by reducing the FiO<sub>2</sub> to 0.15, with the addition of nitrogen to the ventilator gases. After 2 h of hypoxia, the FiO<sub>2</sub> was abruptly increased to 0.18, 0.21 or 1.0 according to the block-randomization of piglets (18%, 21% and 100% HR groups, respectively; *n* = 7 per group). This was maintained for 1 h and then those animals in the 18% or 100% HR groups had their FiO<sub>2</sub> adjusted to 0.21. After a further 2 h, piglets were extubated and mechanical ventilation discontinued. During the first 2 h of extubation, supplemental oxygen (up to 30%) was given to maintain SaO<sub>2</sub> at 88–94%. Sham-operated control piglets (*n* = 7) were ventilated with FiO<sub>2</sub> of 0.21 for 5 h with no HR.

Post-operative pain and discomfort were monitored and treated accordingly with adjusting fentanyl and midazolam infusions, giving acetaminophen (15 mg/kg) and/or chloral hydrate (20 mg/kg) through an orogastric tube. The animals were cared for in warm, clean cages with background music and minimal lighting at ambient temperature of 30°C. The piglets were allowed to recover

for 4 days, fed via the orogastric tube with piglet milk replacer (Brown's Feeds, Clive, AB, Canada), with no further intervention done during this period.

### Data measurement and sample collection

Heart rate (via a three-lead ECG), mean arterial pressure (MAP), transcutaneous oxygen saturation and RA flow were continuously monitored. Hemodynamic recordings (averaged over a 2-min period) and simultaneous arterial and central venous blood samples were taken at normoxic baseline, at the end of hypoxia (2 h), and then at 1 h, 3 h and 6 h of reoxygenation and on recovery days 1, 2 and 4 in a blinded fashion. Blood gas measurements (ABL500 blood gas analyzer, Radiometer, Copenhagen) and co-oximetry (OSM3 Hemo-oximeter, Radiometer) were performed immediately after sampling. The total volume of blood sampling was limited to 8 ml (~5% of circulating volume), which was replaced by an equal volume of Lactated Ringer's Solution, to avoid significant hypovolemia or anemia.

The neurological state of animals was assessed daily using an established scoring scale with minor modifications [17].

At 96 h of recovery, piglets were euthanised humanely with pentobarbital (100 mg/kg). The placement of all catheters and flow probe was confirmed. Tissue samples from the left ventricle and right kidney were immediately snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until subsequent analysis. Additional samples were placed in 10% formalin for histological analysis.

### Hemodynamic calculations

Left RA flow was corrected for individual piglet weight and expressed as ml/kg/min. Renal oxygen delivery was calculated ( $\text{RA flow} \times \text{CaO}_2$ ) and systemic oxygen extraction ratio was estimated  $[(\text{SaO}_2 - \text{ScvO}_2) \div \text{SaO}_2 \times 100\%]$ . To study the cardiac function, we used rate-pressure product (product of heart rate and MAP).

### Assay of tissue glutathione content and lipid hydroperoxides

Total glutathione (GSH), oxidized glutathione (GSSG) and lipid hydroperoxide levels (expressed in  $\mu\text{mol/mg}$  protein) in the myocardial and renal tissues were measured using ELISA kits (#703002 and #705002, respectively; Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The protein content was determined by bicinchoninic acid assay (Sigma, St. Louis, MO, USA). The tissue GSSG:GSH ratio was calculated.

Plasma lactate, troponin-I and creatinine levels determination

Plasma was collected and kept at  $-80^{\circ}\text{C}$  until assay. Plasma lactate was determined using enzyme-linked metabolite assay with measurement of NADH levels by spectrophotometry at 340 nm. Plasma levels of porcine cardiac-specific troponin-I (cTnI) and creatinine were determined using ELISA (#2010-4; Life Diagnostics Inc., West Chester, PA, USA) and i-STAT (#714183; Abbott Point of Care Inc., East Windsor, NJ, USA), respectively. Increased plasma cTnI level was defined as  $> 0.03$  ng/ml [18].

### Histology

Heart and kidney tissues were examined by two pathologists (Y.B. and L.J.), who were unaware of group allocation. Histological grading of HR injury was assessed using the scoring systems previously reported [19, 20].

### Statistical analysis

Results are expressed as mean  $\pm$  SD. Data were analyzed by two-way ANOVA or ANOVA on ranks if tests for both normality and equal variance failed. Fisher's Least Significant Difference or Dunn's test was used for post-hoc analysis. Relationship between categorical, parametric and non-parametric variables was studied by  $\chi^2$ , Pearson Moment and Spearman tests, respectively. The sample size ( $n = 7$  per group) was estimated based on our previous experience with acutely instrumented piglets [11, 21]. The threshold for significance was  $\alpha < 0.05$ . A statistics package (Sigma Stat 2.0; Jandel Scientific, San Rafael, CA, USA) was used.

## Results

Of 28 piglets (age  $2 \pm 1$  day, weight  $2.0 \pm 0.2$  kg), 24 survived until the end of the experiment. Four died because of cardio-pulmonary arrest (1 in 18% HR group on day 1) or intestinal perforation (1 in the 21% HR group on day 3; 2 in the 100% HR group at 4 h and 8 h after reoxygenation). The neurological scores of HR piglets were not sig-

**Table 1** The neurological score of hypoxic-reoxygenated (HR) and sham-operated piglets on days 1–4 of recovery

	Day 1	Day 2	Day 3	Day 4
Sham-operated	19 $\pm$ 2	19 $\pm$ 2	20 $\pm$ 1	20 $\pm$ 0
18% HR	13 $\pm$ 4	16 $\pm$ 4	18 $\pm$ 2	18 $\pm$ 3
21% HR	15 $\pm$ 2	18 $\pm$ 3	18 $\pm$ 3	19 $\pm$ 2
100% HR	15 $\pm$ 3	19 $\pm$ 2	20 $\pm$ 1	20 $\pm$ 0

No significant difference among groups (ANOVA)

nificantly different from those of sham-operated controls at 24 h after HR ( $p = 0.08$ ) (Table 1). All the surviving animals fed well and gained weight with no difference among groups ( $0.48 \pm 0.22$  vs.  $0.43 \pm 0.21$  kg for HR and sham-operated groups, respectively;  $p > 0.05$ ).

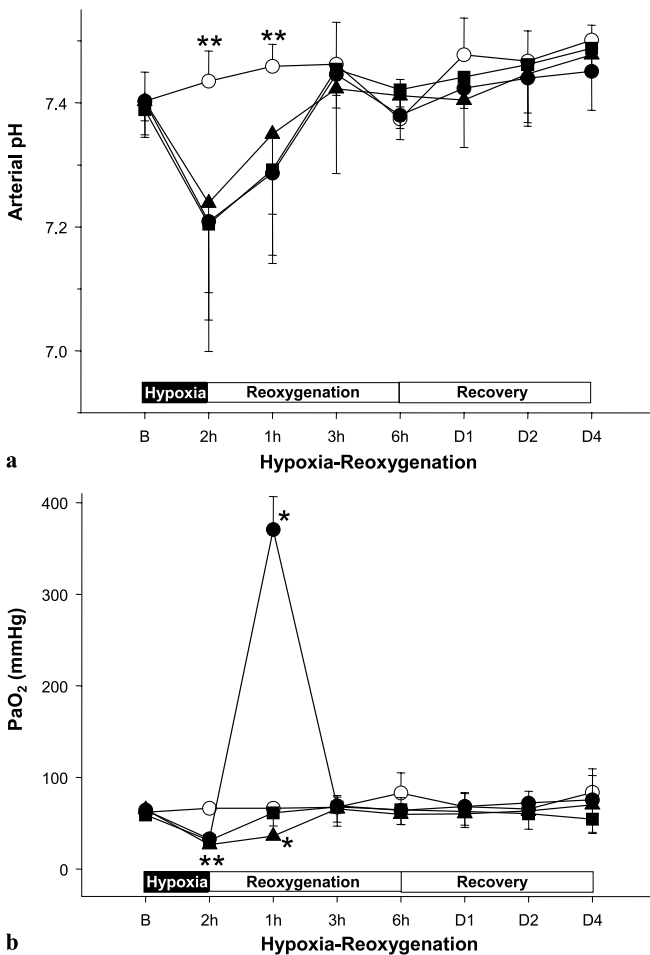
The hemodynamic and physiologic measurements at baseline were not different among groups. The  $\text{PaCO}_2$  throughout the study period did not differ among groups (data not shown).

During hypoxia (mean  $\text{SaO}_2$  of 44–45%; mean  $\text{PaO}_2$  27–32 mmHg), arterial pH decreased and base deficit increased with no difference among HR groups (Fig. 1a; mean base deficit of  $-8$  to  $-12$  vs.  $2 \pm 3$  mmol/l in sham-operated controls;  $p < 0.05$ ). Upon reoxygenation, 18% and 100% HR groups had  $\text{PaO}_2$  and  $\text{SaO}_2$  different from those of sham-operated controls (Fig. 1b;  $\text{SaO}_2$  of  $71 \pm 8\%$  and  $100 \pm 0\%$  vs.  $94 \pm 2\%$ , respectively;

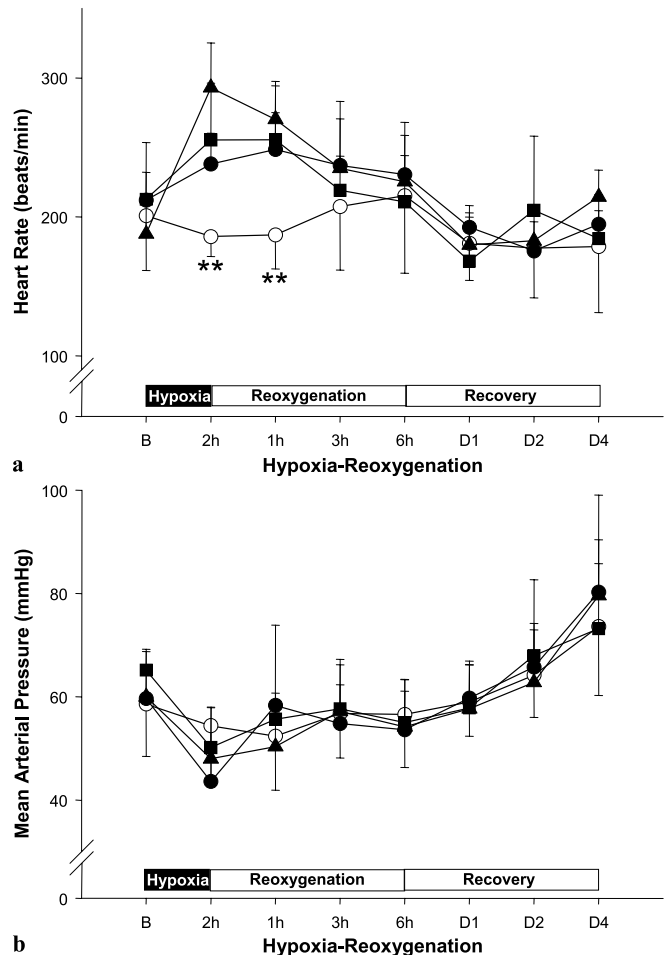
$p < 0.05$ ). After the first hour of reoxygenation, the blood gases of all HR groups normalized and were not different among groups (Fig. 1). During the early post-extubation period, there were no differences in oxygen supplementation and  $\text{PaO}_2$  among groups (Fig. 1b).

During the first hour of reoxygenation, the systemic oxygen extraction ratio was reduced in the 100% HR group ( $13 \pm 8\%$  vs.  $28 \pm 8\%$  of baseline;  $p < 0.05$ ), and were not different among groups afterward when the  $\text{FiO}_2$  was changed to 0.21. Plasma lactate at 2 h of hypoxia increased (mean plasma lactate 10.9–11.6 vs.  $2.4 \pm 0.9$  mmol/l in sham-operated controls;  $p < 0.05$ ) and normalized by 3 h of reoxygenation, with no difference among groups (mean plasma lactate 1.9–3.4 mmol/l).

On day 4 of recovery, the experimental groups had mean arterial pH of 7.45–7.50, mean  $\text{PaO}_2$  of 60–84 mmHg (Fig. 1), mean systemic oxygen extraction



**Fig. 1** Temporal changes of (a) arterial pH and (b)  $\text{PaO}_2$  in hypoxic piglets reoxygenated with 18% (filled triangles), 21% (filled squares) and 100% (filled circles) oxygen. Sham-operated control piglets (open circles) had no hypoxia and reoxygenation.  $n = 7$  per group. B, Normoxic baseline (time 0 min). \*\* $p < 0.05$  all hypoxic-reoxygenated groups vs. sham-operated controls; \* $p < 0.05$  vs. sham-operated controls



**Fig. 2** Temporal changes of (a) heart rate and (b) mean arterial pressure in hypoxic piglets reoxygenated with 18% (filled triangles), 21% (filled squares) and 100% (filled circles) oxygen. Sham-operated control piglets (open circles) had no hypoxia and reoxygenation.  $n = 7$  per group. B, Normoxic baseline (time 0 min). \*\* $p < 0.05$  all hypoxic-reoxygenated groups vs. sham-operated controls

ratio of 31–36% and mean plasma lactate concentrations of 1.8–2.1 mmol/l, with no differences among groups.

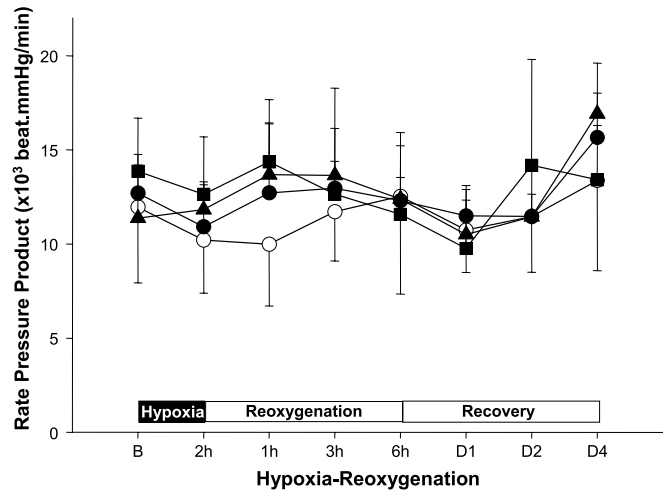
**Systemic hemodynamic and the left ventricular myocardial parameters**

During hypoxia, there was tachycardia (vs. sham-operated controls;  $p < 0.05$ ) and mild hypotension (MAP = 44–50 mmHg;  $p < 0.05$  vs. baseline,  $p > 0.05$  vs. sham-operated controls) (Fig. 1). Heart rate remained significantly increased in all HR groups at 1 h of reoxygenation and subsequently returned to levels not different from that of sham-operated controls by 3 h of reoxygenation (Fig. 2a). After reoxygenation, MAP improved without differences among groups (Fig. 2b). The rate–pressure product did not change significantly at 2 h of hypoxia, and the apparent increase at 1 h of reoxygenation was not significant ( $p = 0.1$ ; Fig. 3). The rate–pressure product did not differ among groups during the remaining experimental period.

Plasma cTnI levels at 6 h of reoxygenation were elevated in the HR groups ( $0.45 \pm 0.2$  ng/ml vs. undetectable levels of sham-operated controls;  $p < 0.05$ ), with no differences among HR groups. By day 4 of recovery, the plasma cTnI levels decreased to 0–0.65 ng/ml. Increased plasma cTnI levels were found in 7 HR piglets (2, 2 and 3 in the 18%, 21% and 100% HR groups, respectively).

On day 4 of recovery, the 100% HR group had significantly higher myocardial GSSG levels than those of other groups, whereas the GSH levels were not different among groups (Table 2). The myocardial GSSG:GSH ratio tended to be higher in the 100% HR group ( $p = 0.06$ ). The myocardial lipid hydroperoxide levels were not different among groups (Table 2).

The left ventricular myocardium of the 100% HR group had the most reoxygenation-induced injury, evidenced by marked architectural disruption and overt myocardial necrosis. The myocardial injury in the 100% HR group was worse ( $p < 0.05$ ,  $\chi^2$  test) than that observed in the sham-operated and 18% HR, but not the 21% HR,



**Fig. 3** Temporal changes of rate pressure product, a parameter to measure cardiac function in hypoxic piglets reoxygenated with 18% (filled triangles), 21% (filled squares) and 100% (filled circles) oxygen. Sham-operated control piglets (open circles) had no hypoxia and reoxygenation.  $n = 7$  per group. B, Normoxic baseline (time 0 min)

groups. Overt myocardial necrosis was observed in 75% of the 100% HR group and in 20% of the 21% HR group, but not seen in the sham-operated and 18% HR groups. No significant differences were observed among the sham-operated, 18% and 21% HR groups.

**Left renal arterial flow and the kidney parameters**

The RA blood flow decreased during hypoxia and recovered upon reoxygenation with no difference among groups (Fig. 4a). The reduced renal oxygen delivery normalized with the use of 100% reoxygenation after hypoxia, whereas the oxygen delivery remained significantly lower than that of sham-operated controls during the first hour of reoxygenation in the 18% and 21% HR groups (Fig. 4b). No significant differences were found among groups during the next 5 h of reoxygenation. On day 1 of

**Table 2** Tissue glutathione content and lipid hydroperoxides (LOOH) levels in hypoxic-reoxygenated (HR) and sham-operated piglets on day 4 of recovery

	GSH ( $\mu\text{mol}/\text{mg}$ protein)	GSSG ( $\mu\text{mol}/\text{mg}$ protein)	GSSG:GSH ratio	LOOH ( $\mu\text{mol}/\text{mg}$ protein)
<b>Left ventricle</b>				
Sham-operated	547 $\pm$ 74	51 $\pm$ 14	0.11 $\pm$ 0.03	46 $\pm$ 17
18% HR	522 $\pm$ 67	62 $\pm$ 7	0.14 $\pm$ 0.02	64 $\pm$ 36
21% HR	537 $\pm$ 32	77 $\pm$ 9	0.18 $\pm$ 0.03	63 $\pm$ 27
100% HR	652 $\pm$ 157	142 $\pm$ 40*	0.29 $\pm$ 0.09†	118 $\pm$ 75
<b>Right kidney</b>				
Sham-operated	32 $\pm$ 4	3.4 $\pm$ 2.0	0.13 $\pm$ 0.07	33 $\pm$ 13
18% HR	43 $\pm$ 5	6.4 $\pm$ 1.2	0.25 $\pm$ 0.09	58 $\pm$ 17
21% HR	32 $\pm$ 4	2.2 $\pm$ 0.6	0.08 $\pm$ 0.03	43 $\pm$ 19
100% HR	31 $\pm$ 3	1.9 $\pm$ 0.4	0.09 $\pm$ 0.05	84 $\pm$ 29

GSH, Total glutathione; GSSG, oxidized glutathione  
\* $p < 0.05$ ; † $p = 0.06$  vs. sham-operated group

recovery, the reduced renal oxygen delivery of HR piglets was not significantly different ( $p=0.059$ ), and it then recovered with no difference among groups (Fig. 4b).

The plasma creatinine levels increased during the experimental period (day 4:  $69 \pm 18$  vs.  $49 \pm 14 \mu\text{mol/l}$  at baseline,  $p < 0.05$ ), with no differences among groups.

The renal tissue GSSG level of the 18% HR group tended to be higher than in the other experimental groups ( $p=0.06$ ), which did not differ among themselves (Table 2). The renal GSSG:GSH ratio, GSH and lipid hydroperoxide levels did not differ among groups (Table 2).

The histological features of HR-induced injury in the right kidney were not different among groups. Ragged, ill-defined proximal convoluted tubule lumina, but no necrosis, were noted in 17–60% of the experimental groups.

## Correlation between oxidative stress and tissue injury

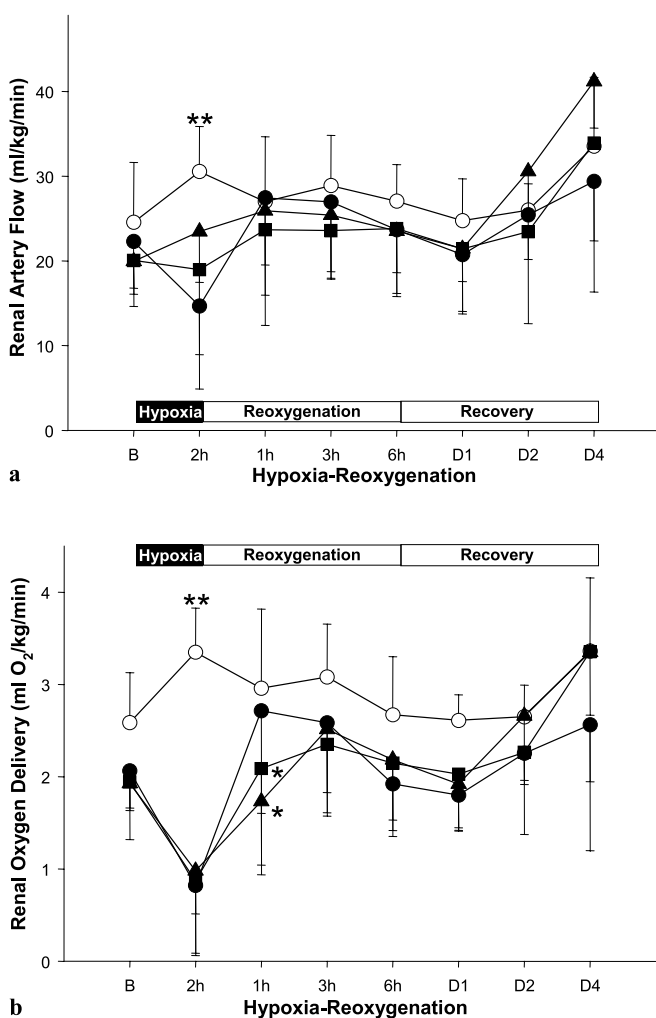
The cardiac and renal HR-induced injury were significantly correlated ( $r=0.76$ ,  $p < 0.001$ ). Plasma cTnI at 6 h of reoxygenation correlated significantly with the SaO<sub>2</sub> and systemic oxygen extraction ratio at 1 h of reoxygenation ( $r=-0.51$  and  $0.64$ ;  $p < 0.05$ , respectively). Plasma cTnI on day 4 of recovery did not correlate with the initial oxygenation, but increased levels were associated with increasing severity of HR-induced myocardial injury ( $p < 0.01$ ,  $\chi^2$  test). Regarding the renal injury, there was significant correlation between HR-induced renal injury and RA oxygen delivery at 1 h of reoxygenation ( $r=0.61$ ,  $p < 0.05$ ).

## Discussion

Asphyxia can result in multi-organ dysfunction and damage, which could be devastating to the cardiovascular, renal and central nervous systems [14, 22]. In piglets subjected to moderate hypoxemia, there was no significant difference in the hemodynamic and functional recovery with 18%, 21% and 100% reoxygenation, which had differential effects on the oxidative state and HR-induced injury of the heart and kidney.

### Cardiovascular recovery in response to 18%, 21% and 100% reoxygenation

Consistent with many studies comparing the cardiovascular recovery of 21% and 100% reoxygenation of newborn animals [9–13] we did not observe any differences in the heart rate, MAP and rate–pressure product among the groups. Vento et al. recently reported increased plasma troponin levels of asphyxiated neonates resuscitated with 100% oxygen, whereas those resuscitated with 21% oxygen had intermediate levels (0.060 and 0.046 vs. 0.014 ng/ml in controls, respectively) [14]. Although plasma troponin level indicates the degree of ischemic myocardial damage, the significance of these elevated levels in the presence of similar systemic hemodynamic state remains to be determined [23]. In this study, we further demonstrated significantly worse reoxygenation injury in the 100% HR group, as evidenced by the more abundant architectural disruption and necrosis in the myocardium, which was partially reflected in the plasma cTnI level, in addition to higher oxidative stress. Interestingly, early oxygenation after resuscitation inversely correlated with plasma cTnI levels at 6 h of reoxygenation with no apparent hemodynamic effect. Although we did not identify significant HR-induced injury at 4 h of reoxygenation [21], this cautions the use of hypoxic and normoxic resuscitation.



**Fig. 4** Temporal changes of (a) renal artery flow and (b) renal oxygen delivery in hypoxic piglets reoxygenated with 18% (filled triangles), 21% (filled squares) and 100% (filled circles) oxygen. Sham-operated control piglets (open circles) had no hypoxia and reoxygenation.  $n=7$  per group. B, Normoxic baseline (time 0 min). \*\* $p < 0.05$  all hypoxic-reoxygenated groups vs. sham-operated controls; \* $p < 0.05$  vs. sham-operated controls

### Renal recovery in response to 18%, 21% and 100% reoxygenation

The recovery of RA flow and oxygen delivery of HR piglets following reoxygenation was rapid, contrasting to the slow recovery over 4 h reported by Johnson et al. [21]. Differences in experimental design, including the severity of hypoxia, may explain the difference. Although the 100% reoxygenated piglets had faster normalization of renal oxygen delivery than the 21% and 18% reoxygenated piglets, we found a positive correlation between the HR-induced renal injury (day 4 recovery) and the RA oxygen delivery during early reoxygenation. Furthermore, there was a trend for higher renal tissue GSSG levels in the 18% HR group. However, we do not know the significance of these findings when all HR groups had similar recovery regarding renal perfusion, function and HR-induced injury.

### The choice of 18%, 21% and 100% reoxygenation in hypoxic newborn piglets

From the cardio-renal perspective, 100% reoxygenation after moderate hypoxemia should be avoided. The recovery of other organs needs to be considered. Some reports suggest that 100% reoxygenation may be a beneficial therapy; it has generally been thought to be non-toxic [24, 25] and to have faster oxygen debt replenishment [26, 27] despite the greater oxidative stress [3, 4, 9–13]. Interestingly, we did not find significant differences in the animals' neurological state during recovery, in contrast to the conflicting reports from Temesvari [17] and Presti [25].

Furthermore, 18% reoxygenation offers no further benefit regarding the myocardial and renal oxidative state and recovery. The inverse relationship between early SaO<sub>2</sub> and plasma cTnI level suggests perpetuating hypoxic-ischemic injury with hypoxic resuscitation. Indeed, we found worse hemodynamic recovery in asphyxiated newborn piglets resuscitated with 18% oxygen than with 21% oxygen [28]. Therefore, the appropriate oxygen concentration to be used in resuscitation would balance the OFR-related injury during the reoxygenation/reperfusion process against persistent hypoxia/ischemia damage in neonatal HR, both potentially resulting in more cellular damage/death.

### Limitations

Like all animal models, the swine model has limitations, although the newborn piglet has physiology similar to that of the 36- to 38-week gestation human fetus [29]. Adequate time for accommodating to the laboratory condition and chronic instrumentation would reduce the stress on these piglets but could increase their age at the time of experimentation. Great caution is required as the model examines reoxygenation of neonatal hypoxia, not perinatal asphyxia, as in a recent report [31], which is associated with severe combined metabolic and respiratory acidosis, multi-organ dysfunction and high mortality. Severe hypoxia and/or hypercapnia would have affected the survival in this model, which is undergoing further improvement. The duration of 1 h reoxygenation seems relatively long but this is not uncommon if the resuscitation occurs distant from a designated neonatal intensive care facility. Despite the lack of significant differences, oxygen supplementation during the early post-extubation period might have confounded the effect of reoxygenation. However, avoiding oxygen supplementation might increase the mortality of the model, deviate from the clinical management, and complicate the study with potential additional hypoxic injury. A dose-response study including other intermediate oxygen concentrations (e.g. 50%) will be interesting [11, 30, 32]. We provide several parameters that are commonly used in the clinical setting to measure the organ recovery from HR insult. Detailed assessment of the organ recovery will provide insights. Although the number of animals studied seems small, the sample size needs to be dramatically increased to detect the differences, given the similar recovery of the hemodynamic and functional parameters. Nonetheless, the negative findings deserve cautious interpretation.

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