Attenuation of Acute Renal Injury After the Post-resuscitation Administration of Doxycycline in Surviving Newborn Piglets With Severe Hypoxia-Reoxygenation

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Background: Asphyxiated neonates often have myocardial dysfunction and renal insufficiency. Previously we demonstrated that doxycycline improved cardio-renal function through matrix metalloproteinase (MMP)-2 inhibition in an acute swine model of neonatal hypoxia-reoxygenation. The prolonged cardio-renal protective effects of doxycycline in neonates still remained unknown. We therefore hypothesized that the protective effects of doxycycline persisted in surviving subjects.

Methods: Newborn piglets were instrumented and subjected to 1 h of hypoxia followed by reoxygenation with 21–25% oxygen and observed for 4 days. Intravenous doxycycline (30 mg/kg) or normal saline (1 mL, saline-control group) was given at 5 min of reoxygenation (n = 8/group) in a randomized, blinded fashion. Sham-operated piglets (n = 5) received no hypoxia-reoxygenation. At 96 h after reoxygenation, the left ventricular function was assessed by Millar® catheter. Renal injury was investigated by measuring plasma creatinine, urinary N-acetyl-D-glucosaminidase activity, renal tissue lactate and MMP-2 activity.

Results: Both hypoxia-reoxygenation groups had similar hypoxic stress with severe lactate acidosis, and hemodynamic recovery. Doxycycline-treated piglets had higher urine output with lower urine N-acetyl-D-glucosaminidase, plasma creatinine, and renal MMP-2 activity (vs. saline-controls; all p < 0.05). These markers were all negatively correlated with urine output.

Conclusions: In newborn piglets surviving hypoxia-reoxygenation, we observed a weak but significant and persistent attenuation of renal injury and improved recovery with the post-resuscitation administration of doxycycline.

Keywords: renal failure, asphyxia, MMP, doxycycline, newborn
INTRODUCTION

Despite recent advances in obstetrical care and newborn resuscitation, asphyxia remains a major cause of neonatal mortality and morbidity. Annually, of the estimated 4 million neonatal deaths, approximately 23% are a result of asphyxia (1–3). Asphyxiated neonates commonly have multi-organ dysfunction and/or failure (4). Because of preferential perfusion of the vital organs such as the heart and brain during hypoxia, kidney is among the first organ injured by an hypoxic-ischemic insult due to regional vasoconstriction (5–7). Indeed, acute kidney injury (AKI) has been reported to be present in 30–70% of asphyxiated neonates (4–6). AKI has been shown to be associated with neonatal asphyxia, the outcome and its subsequent neurodevelopment in early childhood (5). Perlman and Tack previously suggested that oliguria in the perinatal period was a sensitive indicator of infants at risk for long-term neurologic deficits (8).

In addition to the hypoxic and ischemic injury to kidneys, the production of oxygen free radical species and the resultant oxidative stress contribute to further renal dysfunction during reoxygenation. It has been shown that increased oxidative stress can lead to the activation of matrix metalloproteinases (MMP), a family of zinc dependent endopeptidases with a variety of intracellular and extracellular proteolytic substrates (9). They are involved in the remodeling of the extracellular matrix in tissue during various physiological and pathologic conditions. Previous findings suggest that MMPs, commonly MMP-2 and MMP-9, play a crucial role in the pathogenesis of acute ischemia-reperfusion (I-R) injury of the kidney (10, 11). Further, it has been shown that the degree of acute tubular injury, necrosis, apoptosis and renal dysfunction was markedly less in the MMP-2 deficient transgenic mice compared to that seen in the wild type mice (12).

Accordingly, inhibition of MMPs has been suggested to be used as a clinically useful target for minimizing AKI after hypoxia-reoxygenation (H-R). Doxycycline (DOX) is an antibiotic that is approved for clinical use in neonates and other patient populations (13). DOX has selective inhibitory effects on MMP-2 and MMP-9, which is independent of its antimicrobial properties (14, 15). The renal protective effects of DOX have been shown in adult rat models of I-R renal injury (16, 17). Its protective effect in neonates against AKI induced by H-R or I-R is largely unknown. Previously, we also demonstrated that post-resuscitation administration of DOX attenuated AKI associated with the inhibition of MMP-2 activity in newborn piglets with severe H-R (18). Despite providing functional and biochemical data for the beneficial effects of DOX in neonatal H-R, the short time course (4 h after reoxygenation) precludes us from understanding if the renal protection of DOX will persist beyond the acute stage.

Using a surviving model of neonatal H-R, we aimed to examine whether the renal protective effects of intravenous DOX persisted for days after the newborn piglets recovered from H-R, which is important in the translation of findings to clinical trials. We also examined the mechanism of action of the renal protective actions of DOX in neonatal H-R. We hypothesized that post-resuscitation administration of DOX in newborn piglets with severe H-R would improve renal function with alleviated renal injury and inhibition of MMP-2 activity.

METHODS

Twenty-one newborn mixed breed piglets (1–4 days of age, weighing 1.7–2.4 kg) were obtained on the day of experimentation from the University Swine Research Technology Center. All experiments were conducted in strict accordance with the guidelines and approval of the Animal Care and Use Committee (Health Sciences), University of Alberta. The ARRIVE guidelines were also followed (19).

Animal Preparation

Piglets were anesthetized with inhaled isoflurane (1–5%) throughout the surgical procedure. During the experiment anesthesia was maintained with intravenous propofol (5–10 mg/kg/h) and morphine (0.1 mg/kg/h). Additional doses of propofol (1–2 mg/kg) and morphine (0.05–0.1 mg/kg) were also given as needed. The animals were orally intubated with an endotracheal tube (#3.5 mm, Mallinckrodt™, Covidien IIC, Mansfield, MA). Piglets were mechanically ventilated at a rate of 16–20 breaths/min at pressures of 20/5 cmH2O. Oxygen saturation was kept within 90–100%, hydration was maintained with an intravenous infusion of 10% dextrose solution at 10 mL/kg/h. The piglet’s body temperature was maintained at 38.5–39.5°C using an overhead warmer and a water heating pad.

Surgical Procedures and Hemodynamic Parameters Monitoring

Via a neck incision, a 5F double-lumen umbilical catheter (Argyle®, Klein-Baker Medical, San Antonio, TX) was subcutaneously tunneled and inserted into the right external jugular vein for the administration of medications and fluids. Another single-lumen umbilical catheter (5F) was subcutaneously tunneled and inserted into the right common carotid artery for systemic blood pressure monitoring and blood sampling. At the end of the surgical procedure, the neck incision was closed with sutures. On the 4th day of the experiment, piglet was anesthetized and a Millar® catheter (MPVS Ultra®, ADInstruments, Houston, TX) was inserted into the left ventricle via the left common carotid artery for continuous measurement of left ventricular contractile function.

Piglets recovered from surgical instrumentation until baseline hemodynamic parameters were stable (defined as ±<10% changes). Ventilator rate was adjusted to maintain normocapnia (paCO2 35–45 mmHg). Systemic mean arterial pressure and heart rate were continuously measured and recorded throughout the experiment with a Hewlett Packard 78833B monitor (Hewlett Packard Co., Palo Alto, CA).

Experimental Protocol (Figure 1)

Piglets were block-randomized into two treatment groups (n = 8/group) and subjected to an 1-h period of normocapnic hypoxemia (FiO2 of 0.11–0.15) followed by a 4-h period of normoxic reoxygenation (FiO2 of 0.21–0.25). Five minutes into reoxygenation piglets received an intravenous bolus of either
1 mL normal saline (saline-control group) or 30 mg/kg DOX (DOX group), an optimal dose based on our previous study (18), blindly. Five sham-operated piglets underwent surgery with no hypoxia and reoxygenation at a FiO2 of 0.21–0.25. After fully recovered from H-R with satisfactory respiratory status, the piglets were extubated to spontaneous breathing in room air and recovered for 4 days. After hemodynamic measurements with Millar® catheter on day 4th, the animal was euthanized with an intravenous overdose of phenobarbital (100 mg/kg) and tissues were snap frozen in liquid nitrogen and stored in −80°C until subsequent analysis.

**Postoperative Care**
Postoperatively, the piglet was housed in individual kennel to which the animal was secured by a tether-swivel system (Lomir Biomedical Inc., Notre-Dame-de-l’Île-Perrot, QC). The
ambient temperature was maintained at 28 ± 1°C and lighting was established with a 12-h light/dark cycle. Piglets’ behaviors, including lethargy, lack of interest in surroundings, abnormal cry, vomiting, and temperature were monitored every 4 h per day (from 0800 to 2400 h). Post-operative pain and discomfort were minimized by intravenous buprenorphine (0.01 mg/kg) Q12h and oral acetaminophen (15 mg/kg) Q8h, Cefazolin (10 mg/kg) Q8h were given intravenously to prevent sepsis. Hibitane (Wyeth Animal Health, Guelph, ON) was also applied to the incision area to prevent potential wound infection. At 1200 h every day, the piglets were weighed, and nutrition solution infusion rates and urine samples were also collected and stored in −80°C until subsequent analysis.

Intravenous solution was given through a controlled pressure-sensitive infusion pump (IVAC Signature Gold Infusion Pump; ALARIS Medical Systems, San Diego, CA). Immediately after putting the piglet in the kennel (day 0), all piglets were given 10% dextrose solution at 10 mL/kg/h. Parenteral nutrition was initiated on day 1 and gradually increased to 10 mL/kg/h on day 2. The parenteral nutrition was estimated from daily nutritional requirements for sow-fed piglets and has previously demonstrated normal growth and body composition (20). The targeted energy intake was 270 kcal/kg/d, with amino acids providing 27%, carbohydrate 37%, and lipid 36% of energy.

### Preparation of Kidney Tissue and Determination of Kidney Injury Markers

At the termination of experiment, right kidneys were immediately removed en bloc and snap-frozen in liquid nitrogen for biochemical analyses. The whole kidney was homogenized. Renal tissue lactate levels were determined by a nicotinamide adenine dinucleotide enzyme coupled colorimetric assay as previously described (18). Gelatinolytic activities of MMP-2 and MMP-9 were quantified using gelatin zymography as previously described (21). Tissue protein content was quantified using a commercially available QuantiChrom assay kit (Sigma-Aldrich Canada Ltd., Oakville, ON).

Both plasma and urine creatinine (Cr) levels were measured using a commercially available QuantChrom assay kit (DICT-500; Bioassay Systems, Hayward, CA). N-Acetyl-D-glucosaminidase (NAG) activity was measured in the urine sample by a commercially available colorimetric assay kit (no. 875406; Roche, Indianapolis, IN) and normalized to urine Cr level.

### Statistical Analysis

Results are presented as the mean ± SEM. Based on our previous experience and a predicted 50% improvement in renal function, we required 8 animals in each H-R group.
RESULTS

Hemodynamic and biochemical variables were compared using one-way and two-way repeated measures analysis of variance as appropriate, followed by Tukey post-hoc testing (SigmaPlot v13; Systat Software Inc., San Jose, CA). Correlations were determined using the Pearson Product Moment test. Significance was defined as $p < 0.05$. 

Hemodynamic Parameters

As shown in Table 1, $\text{PaO}_2$, pH and plasma bicarbonate levels were significantly lower and plasma lactate significantly higher in piglets subjected to H-R compared with sham-operated piglets at the end of hypoxia. However, there was no difference between the two H-R groups regarding the degree of hypoxic stress as indicated by similar $\text{PaO}_2$, metabolic acidosis and hyperlactatemia. Heart rates of both H-R groups also increased markedly at the end of hypoxia, resulting in higher rate-pressure product as compared to the sham-operated group (Table 2).

Three piglets (one from saline-control and two from DOX) were euthanized earlier during the 4-day observation period because of progressive hypoxic respiratory failure. The body weight gain over 4 days was similar among three experimental groups ($0.29 \pm 0.02$, $0.31 \pm 0.02$, and $0.32 \pm 0.02$ kg for sham-operated, saline-control and DOX, respectively). As shown...
in Tables 1, 2, arterial blood gases, systemic blood pressure and heart rate were monitored and recovered with no differences among all three groups over 4 days after reoxygenation. Although cardiac output, systolic and diastolic functions (dp/dt\textsubscript{max}, dp/dt\textsubscript{min}, and Tau) measured by Millar\textsuperscript{®} assessment on the final day were slightly lower in the saline-control group, the differences were not statistically significant (Table 3).

**Urine Output**
The urine output for both sham-operated and DOX groups gradually increased with time, whereas the output from saline-control group remained unchanged throughout the 4-day observation period (Figure 2, Table 4). Consequently, the total urine output of saline-control group was significantly lower than that of both sham-operated and DOX groups. There was no evidence of volume overload in the animals of saline-control group.

**Biochemical Parameters**
Table 4 summarized changes in all renal biomarkers among all experimental groups. Plasma Cr levels of both sham-operated and DOX groups increased during the experimental period, and then declined back to the baseline level during the recovery period (Figure 3A). In contrast, the plasma Cr level of saline-control group remained high during recovery and was significantly higher than both sham-operated and DOX group on day 2. The plasma Cr on day 2 was negatively correlated with total urine output ($r = -0.62, p = 0.008$) (Figure 4A).

As shown in Figure 5, total MMP-2 activity of the kidney was higher in saline-control piglets. The gelatinolytic activities of MMP-2 at 75, 72, and 64 kDa were studied with significant increased activity of MMP-2 at 64 kDa of saline-control piglets, compared with sham-operated and DOX groups, but not at 75 and 72 kDa. Total MMP-2 activity had negative correlation with total urine output ($r = -0.57, p = 0.016$) (Figure 4B) and a positive correlation trend with plasma Cr level ($r = 0.44, p = 0.07$) (Figure 6A). No difference was found in MMP-9 activity among different experimental groups (data not shown).

Urine NAG/Cr ratio, an index of renal tubular function associated with H-R, of the sham-operated group remained about the same throughout the 4 days observation period (Figure 3B). The urinary NAG activity of both saline-control and DOX groups were higher than that of sham-operated group on day 1. However, the urinary NAG activity of DOX group declined with time and was significantly lower than that of saline-control group on day 3 (Figure 3B). Urinary NAG activity on day 3 was found to significantly correlate with total urine output ($r = -0.78, p < 0.001$) (Figure 4C) and total MMP-2 activity ($r = -0.51, p = 0.04$) (Figure 6B).

Four days after H-R, renal tissue lactate levels were elevated in H-R saline-control piglets (0.24 ± 0.04 µmol/mg protein) when compared with that of sham-operated and DOX piglets (0.17 ± 0.01 and 0.19 ± 0.02 µmol/mg protein, respectively), but the difference was not significant (Table 4). The renal lactate level did not correlate with total urine output ($r = -0.41, p = 0.11$).

**DISCUSSION**
The current study confirmed that single bolus DOX injection immediately after hypoxic insult preserved renal function for a prolonged period in surviving newborn piglets. The findings

### Table 3

|                 | Sham-operated | Saline-control | DOX   | p-value |
|-----------------|---------------|----------------|-------|---------|
| Heart rate (bpm) | 219 ± 2       | 192 ± 13       | 215 ± 10 | 0.19    |
| Cardiac output (mL/kg/min) | 211 ± 17 | 187 ± 21 | 226 ± 36 | 0.55    |
| Developed pressure (mmHg) | 76 ± 1 | 96 ± 10 | 89 ± 6 | 0.19 |
| dp/dt\textsubscript{max} (mmHg) | 3,341 ± 446 | 3,481 ± 377 | 3,790 ± 343 | 0.73 |
| dp/dt\textsubscript{min} (mmHg) | −4,158 ± 233 | −5717 ± 728 | −4,313 ± 629 | 0.17 |
| Tau (ms) | 17 ± 2 | 15 ± 1 | 14 ± 1 | 0.42 |

### Table 4

|                      | Sham-operated | Saline-control | DOX   |
|----------------------|---------------|----------------|-------|
| **URINE OUTPUT** (mL/kg) |               |                |       |
| Day 1                | 27 ± 9        | 33 ± 7         | 36 ± 7 |
| Day 2                | 15 ± 3        | 17 ± 6         | 30 ± 5 |
| Day 3                | 52 ± 7\*      | 25 ± 5         | 51 ± 5\* |
| Day 4                | 61 ± 15\*     | 26 ± 5         | 63 ± 7\* |
| Total                | 154 ± 21\*    | 101 ± 11       | 181 ± 17\* |
| **PLASMA CREATININE** (mg/dL) |       |                |       |
| Day 1                | 1.87 ± 0.31   | 2.18 ± 0.59    | 1.78 ± 0.47 |
| Day 2                | 1.65 ± 0.19\* | 2.33 ± 0.34    | 1.45 ± 0.23\* |
| Day 3                | 1.71 ± 0.26   | 1.88 ± 0.38    | 1.65 ± 0.21 |
| Day 4                | 1.57 ± 0.41   | 1.94 ± 0.16    | 1.82 ± 0.24 |
| **NAG/Cr RATIO (U/mg)** |               |                |       |
| Day 1                | 1.96 ± 0.38\* | 3.13 ± 0.43    | 2.56 ± 0.34 |
| Day 2                | 1.74 ± 0.28\* | 2.88 ± 0.43    | 2.20 ± 0.26 |
| Day 3                | 1.93 ± 0.27\* | 3.00 ± 0.36    | 2.05 ± 0.10\* |
| Day 4                | 1.94 ± 0.36   | 2.69 ± 0.26    | 2.18 ± 0.34 |
| **TOTAL MMP-2 ACTIVITY (AU)** |       |                |       |
|                      | 0.97 ± 0.04\* | 1.32 ± 0.11    | 1.06 ± 0.05\* |
| **TISSUE LACTATE (µmol/mg PROTEIN)** |       |                |       |
|                      | 0.17 ± 0.01   | 0.24 ± 0.04    | 0.19 ± 0.02 |

*p < 0.05 vs. saline-control group (two-ways repeated measures or one-way ANOVA).
are original in an intact large animal surviving model of neonatal hypoxia and are important for the translation into clinical trials. By measuring the renal blood flow directly, we previously demonstrated a significant decrease in renal perfusion during hypoxia (18). It has also been shown that vital organs such as the heart, brain, and adrenal glands are preferentially perfused at the expense of other organ systems during hypoxia (5, 22). Reducing renal blood flow has been suggested to be the initial cause of kidney injury in hypoxic-ischemic insult. Further injury occurs during reoxygenation with the increased production of reactive oxygen and nitrogen species (23, 24). AKI in the neonatal period has been associated with oliguria/anuria and or elevated plasma Cr levels (25, 26). The daily urine output of the saline-control group remained low throughout the observation period, whereas the output of DOX group increased drastically after day 2 with a consequently higher total urine output (Figure 2). We further observed that plasma Cr level of saline-control piglets increased after H-R and remained high during the recovery period, whereas plasma Cr levels of DOX piglets gradually declined back to around the baseline level over 4 days (Figure 3A). NAG is a lysosomal enzyme present in cells of the proximal tubules. Urinary NAG activity has been suggested to be used as an indicator of AKI (26, 27), and correlated positively with the severity of asphyxia in term neonates (28). Our results demonstrated that the H-R induced AKI could be protected by DOX treatment in these newborn piglets surviving the H-R.

MMP has been shown to play a crucial role in the pathogenesis of acute ischemia/reperfusion injury of the kidney (10, 11). Our findings of increased renal MMP-2 activity and the negative correlation with urine output support this notion. By using MMP-2 deficient transgenic mouse model, the degree of acute tubular injury and renal dysfunction was markedly less in the MMP-2 deficient transgenic mice compared to that seen in the wild type mice after renal ischemia-reperfusion (12). Interestingly, we observed the increased MMP-2 activity was attenuated by DOX treatment in this hypoxia-reoxygenation
injury of the kidneys. Previously, it was suggested that activated MMPs targeted cell adhesion molecules in the renal tubular cells, leading to renal tubular injury and dysfunction (29–31). This seems to be the case as there was a positive correlation between MMP-2 and NAG in our study. However, we cannot exclude other potential pathophysiologic mechanisms that may mediate AKI in H-R injury. Of note, although the use of supplemental oxygen at FiO$_2$ 0.21–0.25 (which is often needed in post-surgical and anesthetic animals) might have influenced the oxidative stress and therefore MMP-2 activation, both H-R groups were treated similarly resulting in normoxic reoxygenation with FiO$_2$ of 0.21–0.25 and PaO$_2$ similar to baseline according to the protocol (Table 1).

In this study we also examined the left ventricular systolic and diastolic functions by the placement of a Millar® catheter in these newborn animals. We previously demonstrated that the post-resuscitation administration of doxycycline attenuated cardiac injury (plasma troponin I, myocardial lactate and MMP-2 activity) and improved functional recovery in acutely instrumented piglets with H-R (32). We therefore speculated that there might be a persistence of improved left ventricular function after 4 days, which was associated with better cardiac output and regional (renal) perfusion, in the DOX piglets. However, we did not observe any significant improvement in the cardiac function on day 4. This may be related to a transient beneficial effect in the cardiac function which resolved during the recovery, confounding effects of general anesthesia, an isolated, renoprotective of doxycycline in renal function, in addition to a small sample size in a different (acute vs. survival) neonatal H-R model. Serial echocardiographic examinations including cardiac output and superior vena cava flow measurement, doppler studies of renal artery, and the use of near-infrared spectroscopy may help answer some of these questions regarding systemic blood flow and renal perfusion.

**LIMITATIONS**

Piglets have similar anatomy and physiology to humans (33). Further it has been shown that newborn piglets even experience asphyxia in a similar manner to human neonates (34, 35). Nevertheless, the translation of our findings needs extreme caution including the applicability of the model of hypoxia and asphyxia. The latter includes the absence of perinatal transition, hypercapnia and severe cardiovascular compromise in the current model despite of severe metabolic acidosis with hypoxia. Longer hypoxic challenge (120 vs. 60 min) and more invasive surgical procedures were carried out in previous acute study (18, 32) than those of current study. Further the application...
of therapeutic hypothermia, a standard practice for neonates with hypoxic-ischemic encephalopathy in developed countries, may confound our observations for it effects in oxidative stress-related injury.

Among all tetracyclines, DOX is approved for use in neonates and has a lesser side-effect profile (36, 37). The use of DOX is safe in children younger than 8 years, particularly for short term therapy. Nevertheless, DOX has not been widely used in neonates and has been restricted for the treatment of specific infections (13). Furthermore, it is uncertain if there were pharmacodynamic and pharmacokinetic differences in these 1–4 days-old piglets, which could be associated with the postnatal increase in renal function especially for surviving animal models. Retrospective analysis of urine output of 1–2 and 3–4 days-old piglets suggested similar trends of renal protection with the DOX treatment, compared with the saline-controls (p = 0.03 and p = 0.22, respectively). More pharmacokinetic and pharmacodynamic studies are needed for DOX in neonates. Although DOX may be the most clinically transferable MMP inhibitor for use in neonates at present, further studies on its safety in this patient population are required.

A prolonged survival experiment with investigations on the brain and intestinal microbiomes may be needed to study the safety of DOX treatment. Notwithstanding the challenges with chronic survival experiments, repetitive tissue sampling or invasive interventions will further understand the temporal changes in the mechanism of DOX including the inhibition of MMP-2 activation and other pathways such as improved mitochondrial function (38). The measurement of tissue inhibitors of MMP-2, use of in-situ zymography, detailed histological and electronic microscopic examinations will help study the mechanisms and location of renal injury.

CONCLUSIONS

Using a surviving swine model of neonatal H-R, we demonstrated that a single dose of DOX given immediately after hypoxic insult improved renal function and alleviated renal injury with inhibition of MMP-2 activation in the kidneys. The renoprotective effect was weak but significant; our results provide the foundation for future studies on the long-term effects of DOX and clinical investigations of DOX in neonatal AKI after H-R.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors upon request, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The study has been approved by the University of Alberta Animal Care and Use Committee and was conducted with full compliance to the Canadian guidelines on the use of animal for experiments.

AUTHOR CONTRIBUTIONS

GS and P-YC: conception and design; T-FL, ML, MP, GS, and P-YC: collection and assembly of data, analysis, and interpretation of the data; T-FL, GS, and P-YC: drafting of the article; T-FL, ML, MP, GS, and P-YC: critical revision of the article for important intellectual content and final approval.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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