

Platelet Dysfunction in Asphyxiated Newborn Piglets Resuscitated with 21% and 100% Oxygen

PO-YIN CHEUNG, JONATHAN P. STEVENS, ERIKA HAASE, LINDA STANG, DAVID L. BIGAM, WEI ETCHES, AND MAREK W. RADOMSKI

Departments of Pediatrics [P.-Y. C., J.P.S.], Surgery [E.H., D.L.B.], and Laboratory Medicine and Pathology [L.S., W.E.], University of Alberta, Edmonton, Alberta, Canada, T6G 2S2; Institute of Molecular Medicine [M.W.R.], Houston, TX 77030

ABSTRACT: Hemostatic disturbances are common in asphyxiated newborns after resuscitation. We compared platelet function in hypoxic newborn piglets reoxygenated with 21% or 100% oxygen. Piglets (1–3 d, 1.5–2.1 kg) were anesthetized and acutely instrumented for hemodynamic monitoring. After stabilization, normocapnic hypoxia was induced with an inspired oxygen concentration of 10–15% for 2 h. Piglets were then resuscitated for 1 h with 21% or 100% oxygen, followed by 3 h with 21% oxygen. Platelet counts and collagen (2, 5, and 10 $\mu\text{g/mL}$)-stimulated whole blood aggregation were studied before hypoxia and at 4 h of post-hypoxia/reoxygenation. Platelet function was studied using transmission electron microscopy and by measuring plasma thromboxane B_2 (TxB_2) and matrix metalloproteinase (MMP)-2 and -9 levels. Control piglets were sham-operated without hypoxia/reoxygenation. The hypoxemic (PaO_2 33 mm Hg) piglets developed hypotension with metabolic acidosis (pH 7.02–7.05). Upon reoxygenation, piglets recovered and blood gases gradually normalized. At 4 h reoxygenation, platelet aggregation *ex vivo* was impaired as evidenced by a rightward-downward shifting of the concentration-response curves. Electron microscopy showed features of platelet activation. Plasma MMP-9 but not MMP-2 activity significantly increased. Resuscitation with 100% but not 21% oxygen increased plasma TxB_2 levels. Platelet counts decreased after hypoxia/reoxygenation but were not different between groups during the experiment. Resuscitation of hypoxic newborn piglets caused platelet activation with significant deterioration of platelet aggregation *ex vivo* and increased plasma MMP-9 levels. High oxygen concentrations may aggravate the activation of prostaglandin-thromboxane mechanistic pathway. (*Pediatr Res* 59: 636–640, 2006)

Asphyxia in newborns may cause multiorgan injury including hemostatic disturbances with a bleeding diathesis and thromboembolism (1). During the resuscitation of asphyxiated newborns, platelets are subjected to hypoxia and reoxygenation injury. Of interest, free radicals may be associated with platelet activation during anoxia and reoxygenation (2,3). During hypoxia, Akahori *et al.* (4) demonstrated the reduction of *in vitro* platelet aggregation, whereas Castle *et al.* (5) found shortened *ex vivo* platelet survival. The platelet pathology,

however, has not been well investigated in intact newborn animals with asphyxia.

MMP-2 (constitutive isoform, 72 kD) and MMP-9 (inducible isoform, 92 kD) are zinc-dependent endopeptidases that play an important role in the regulation of platelet aggregation (6–9). MMPs are activated by proteolysis and oxidants including the peroxynitrite anion (6,10). Increased plasma MMP-2 and -9 activity with platelet dysfunction was observed in an animal model of extracorporeal circulation with a high oxygen environment (11). Lemke *et al.* (12) recently reported a surge in plasma MMP-9 activity in the first day of postnatal life, which is consistent with a change from a low to a high oxygen environment. Interestingly, in neonatal models of asphyxia, resuscitation with 100% oxygen is associated with higher oxidative stress and MMP-2 activities in the heart and brain than those with 21% oxygen (13,14). However, there is little information on the activity of MMPs in plasma and its relationship to platelet aggregatory function in the reoxygenation of asphyxiated newborns.

Asphyxiated infants are often resuscitated with 100% oxygen, and this is associated with increased oxidative stress up to 28 d after birth (15). In the midst of the debate between the use of 21% or 100% oxygen in the resuscitation of asphyxiated newborns, we designed this study to examine *ex vivo* platelet aggregation after hypoxia and subsequent reoxygenation with either 21% or 100% oxygen in a newborn model of asphyxia. We hypothesized that in newborn piglets with asphyxia, resuscitation with 100% oxygen would be associated with increased platelet activation and higher plasma MMP-2 and -9 activities than those with 21% oxygen.

METHODS

The study conformed to the regulations of the Canadian Council of Animal Care (Revised 1993) and was approved by the Health Sciences Animal Welfare Committee, University of Alberta.

Mixed-breed newborn piglets between 1–3 d old and weighing 1.5–2.1 kg were used. Halothane anesthesia was used initially and followed by i.v.

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Correspondence: Po-Yin Cheung, MBBS, Ph.D., Royal Alexandra Hospital, NICU, Room 5027, 10240 Kingsway Avenue, Edmonton, AB, Canada T5H 3V9; e-mail: poyin@ualberta.ca

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Abbreviations: MMP, matrix metalloproteinase; TxB_2 , thromboxane B_2

fentanyl 5–10 $\mu\text{g}/\text{kg}/\text{h}$, Midazolam 0.1–0.2 $\text{mg}/\text{kg}/\text{h}$, and pancuronium 0.05–0.1 $\text{mg}/\text{kg}/\text{h}$ after mechanical ventilation was started. A dose of i.v. acepromazine (0.25 mg/kg) was also given with additional i.v. boluses of fentanyl (10 $\mu\text{g}/\text{kg}$) and pancuronium (0.1 mg/kg) as needed. Argyle catheters (5F, Sherwood Medical Co., St. Louis, MO) were inserted *via* the right femoral artery and vein for blood sampling, mean arterial pressure measurement, and i.v. administration of medications and fluids, respectively. *Via* a tracheotomy, pressure-controlled assisted ventilation was started (Sechrist infant ventilator model IV-100, Sechrist Industries Inc., Anaheim, CA) at pressures of 19/4 cm H_2O , a rate of 18–20 breaths per minute, and fractional inspired oxygen concentration (FiO_2) of 0.21–0.24 to maintain oxygen saturation of 90–100%. Oxygen saturation was continuously monitored with a pulse oximeter (Nellcor, Hayward, CA), and heart rate and blood pressure were measured with a Hewlett Packard 78833B monitor (Hewlett Packard Co., Palo Alto, CA). Maintenance fluids during experimentation consisted of 5% dextrose at 7.5 mL/kg/h and 0.9% normal saline at 2.5 mL/kg/h. The piglet's temperature was maintained at 38.5–39.5°C using an overhead warmer and a heating pad. Stabilization was defined as a heart rate and blood pressure change <10%; arterial Po_2 75–100 mm Hg; Pco_2 35–55 mm Hg; pH 7.35–7.45.

After a 30-min stabilization period, hypoxia was induced for 2 h, with the FiO_2 at 0.10–0.15 aiming for arterial oxygen saturations of 30–40% and a Po_2 of 20–40 mm Hg. Piglets were resuscitated with either 100% oxygen ($n = 9$) or 21% oxygen ($n = 8$) for 1 h, then with 21% oxygen for 3 h. In the control animals ($n = 9$), the same operative and sampling procedures were followed, but the piglets were not subjected to hypoxia or reoxygenation during the 6-h experiment.

Arterial blood samples were collected in 3.15% Na citrate (9:1, vol:vol) at baseline, at the end of hypoxia and after 4 h of reoxygenation. Hemoglobin concentration and platelet counts were measured using a hematology analyzer (MicroDiff 16, Coulter, Hialeah, FL). Platelet aggregation was measured by collagen-stimulated whole blood impedance aggregation using a Chronolog Aggregometer (Chrono-log Corp., Havertown, PA). Collagen concentrations of 2, 5, or 10 $\mu\text{g}/\text{mL}$ (10 μL) were added to 495 μL of citrated whole blood diluted with normal saline (1:1, vol:vol). Due to the limited volume of blood sampling during the experiment and the usual recommended testing procedure of the Chronolog Aggregometer, we did not test the aggregatory response with >10 $\mu\text{g}/\text{mL}$ collagen. The concentration of collagen that produced 50% of the maximum platelet aggregatory response with 2–10 $\mu\text{g}/\text{mL}$ of collagen (EC_{50}) was estimated.

Platelet poor plasma was prepared by centrifugation at 10,000 $\times g$ for 15 min and stored at -80°C for subsequent biochemical analysis. TxB_2 is a stable metabolite of thromboxane A_2 , which is a marker of platelet activation, and the plasma levels of this eicosanoid were assayed using a commercially available immunoassay kit (R&D Systems Inc., Minneapolis, MN). MMP-2 and -9 activity in plasma was studied by gelatin zymography, as described previously (11). Briefly, samples were mixed with sodium dodecylsulfate sample buffer and subjected to 8% polyacrylamide separating gels containing gelatin (2 mg/mL , Sigma Chemical Co.). Human recombinant MMP-2 and MMP-9 (Oncogene Research Products, San Diego, CA) were used as standards. Following electrophoresis, the gels were washed with 2.5% Triton X-100 and incubated overnight at 37°C in 50 mmol/L Tris-HCl buffer (0.15 mol/L NaCl, 5 mmol/L CaCl_2 , 50 mmol/L Tris HCl, and 0.05% NaN_3 , pH 7.6). Gelatinolytic activities were visualized as transparent bands against a blue background through staining with Coomassie blue. The zymograms were scanned, and the intensity of the digitalized bands was analyzed by SigmaGel measurement software (Jandel Corp., San Rafael, CA). With the plasma protein concentration assessed by bicichoninic acid assay (Sigma Chemical Co.) using BSA as a standard, MMP activity was expressed in arbitrary units per amount of protein and compared with its respective baseline activity.

Platelet morphology was examined by transmission electron microscopy at the Surgical Medical Research Institute, University of Alberta. Findings were assessed by two investigators (P.Y.C. and J.S.) with no knowledge of the group assignment. The presence of pseudopodia and granule centralization was identified qualitatively to confirm that the morphology was consistent with platelet activation.

Statistical analysis. Data are expressed in mean \pm SE of mean. Differences between groups were compared using analysis of variance (ANOVA) or the Kruskal-Wallis test, with Fisher's least significant difference or Dunn's test for *post hoc* analysis for parametric and nonparametric variables, respectively. The paired *t* test was used to compare platelet counts before and after hypoxia/reoxygenation. A statistical program (SigmaStat, v.2.0, Jandel Corp., San Rafael, CA) was used, and $p < 0.05$ was considered statistically significant.

RESULTS

Nineteen piglets were studied. There were no significant differences between groups in age (1.8 ± 0.1 , 1.7 ± 0.1 , and 1.8 ± 0.1 d for control, 21% and 100% reoxygenated groups, respectively) or baseline physiologic parameters (Table 1).

At 1 h of hypoxemia, piglets had tachycardia and hypotension compared with baseline values ($p < 0.05$ with a heart rate of 225 ± 19 and 204 ± 16 bpm and a mean blood pressure of 52 ± 6 and 47 ± 3 mm Hg, for 21% and 100% reoxygenated groups, respectively). After 2 h of alveolar hypoxia, the piglets were severely hypoxemic (mean PaO_2 33 mm Hg) with a metabolic acidosis (mean pH 7.02–7.05), and systemic hypotension (mean blood pressure 26–27 mm Hg) (Table 1). Upon resuscitation with 100% oxygen, the mean PaO_2 was 367 ± 15 mm Hg compared with 82 ± 8 and 74 ± 5 mm Hg for 21% oxygen reoxygenated group and controls, respectively (both $p < 0.05$). The blood pressure and acid-base balance gradually recovered during reoxygenation compared with baseline and did not differ from controls.

Platelet count and function. Platelet counts were 118–368 $\times 10^9/\text{L}$ during the experiment and were not different between groups at baseline and after hypoxia and reoxygenation ($p > 0.05$, ANOVA) (Table 2). Both hypoxia/reoxygenated groups had lower platelet counts than the respective baseline values, whereas the platelet counts did not change in the control piglets ($p < 0.05$, paired *t* test) (Table 2). Mean hemoglobin concentrations were 73–91 g/L and were not different between groups at baseline or after hypoxia and reoxygenation.

Collagen-induced platelet aggregation after 2 h of hypoxia was not altered from baseline and was not different from that of controls (data not shown). After 4 h of reoxygenation, there was an impaired aggregation with 5 $\mu\text{g}/\text{mL}$ collagen stimulation in the 100% reoxygenated group (12 ± 1 Ohms) ($p < 0.05$) but not in the 21% group (17 ± 3 Ohms), when compared with controls (22 ± 4 Ohms) (Fig. 1A). The aggre-

Table 1. Arterial oxygen partial pressure, pH, and mean blood pressure in hypoxic piglets resuscitated with 21% and 100% oxygen and control piglets with no hypoxia/reoxygenation

	Baseline	End of hypoxia	End of reoxygenation
PaO_2 (mm Hg)			
Control	80 ± 7	73 ± 5	73 ± 4
21%	95 ± 10	$33 \pm 2^*$	76 ± 5
100%	90 ± 6	$33 \pm 3^*$	74 ± 7
pH			
Control	7.42 ± 0.02	7.43 ± 0.02	7.40 ± 0.04
21%	7.47 ± 0.02	$7.02 \pm 0.04^*$	7.37 ± 0.04
100%	7.45 ± 0.02	$7.05 \pm 0.04^*$	7.38 ± 0.03
Heart rate (beats/min)			
Control	175 ± 8	186 ± 12	191 ± 22
21%	164 ± 13	170 ± 14	216 ± 19
100%	168 ± 14	145 ± 17	215 ± 21
Mean blood pressure (mm Hg)			
Control	74 ± 5	67 ± 4	53 ± 2
21%	71 ± 3	$26 \pm 3^*$	46 ± 4
100%	66 ± 4	$27 \pm 3^*$	49 ± 3

Values are mean \pm SEM.

* $p < 0.05$ vs controls (ANOVA).

Table 2. Platelet counts ($\times 10^9/L$) in hypoxic piglets resuscitated with 21% and 100% oxygen and control piglets with no hypoxia/reoxygenation

	Baseline	End of reoxygenation
Control	245 \pm 30	224 \pm 28
21%	211 \pm 23	185 \pm 21*
100%	280 \pm 19	235 \pm 20*

Values are mean \pm SEM.

* $p < 0.05$ vs baseline (paired t test).

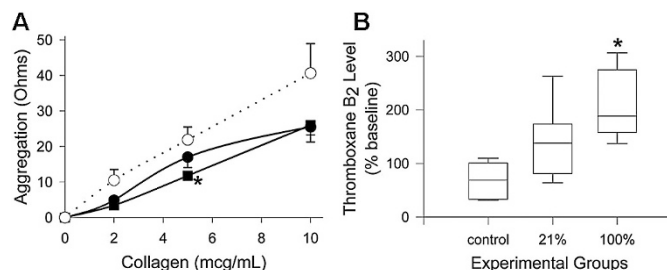


Figure 1. (A) Collagen-stimulated whole blood aggregatory responses in hypoxic piglets resuscitated with 21% (●) and 100% (■) oxygen and control (○) piglets with no hypoxia/reoxygenation at the end of experiment. * $p < 0.05$ vs corresponding response of controls at 5 $\mu\text{g/mL}$ of collagen (ANOVA). (B) Changes in plasma TxB_2 levels in hypoxic piglets resuscitated with 21% and 100% oxygen and control piglets with no hypoxia/reoxygenation at the end of experiment are shown as a plot of median, 25th and 75th percentiles in box and 5th and 95th percentiles with error bars. * $p < 0.05$ vs controls (Kruskal-Wallis test).

gatory responses induced by 2 and 10 $\mu\text{g/mL}$ collagen were not different between groups, but the concentration-response curves were shifted rightward and downward compared with controls ($p < 0.05$). There was a higher EC_{50} in the 100% reoxygenated group compared with both the 21% reoxygenated and control groups (5.5 ± 0.4 , 4.1 ± 0.5 , and 3.9 ± 0.5 $\mu\text{g/mL}$, respectively; $p < 0.05$).

Plasma TxB_2 levels and platelet morphology on electron microscopy. After 2 h of hypoxia, plasma levels of TxB_2 were not different from baseline or between groups (data not shown). Subsequent 4-h reoxygenation caused increases in plasma TxB_2 levels compared with baseline. The fractional increase in the 100% reoxygenated group was almost 2-fold and significantly higher than that of controls (Fig. 1B). Electron microscopic examination showed similar features of platelet activation (centralization of granules and development of pseudopodia) were observed in both the 21% and 100% reoxygenated groups (Fig. 2).

Plasma MMP-2 and -9 activity. Gelatinolytic activity was detected at 72 and 92 kDa and was identified as proMMP-2 and -9, respectively. After 2 h of hypoxia, plasma MMP-2 and -9 activity was not different from that at baseline or of controls. Upon resuscitation, there was a dramatic increase in plasma MMP-9 activity but not MMP-2 activity at 30 min of reoxygenation with significant differences from controls (Fig. 3). The modestly higher plasma MMP-9 activity in piglets resuscitated with 100% oxygen was not different from that with 21% oxygen ($p > 0.05$). Thereafter, MMP-9 activity decreased and the plasma activity of MMPs were not different from baseline or between the groups at 4 h of reoxygenation (data not shown).

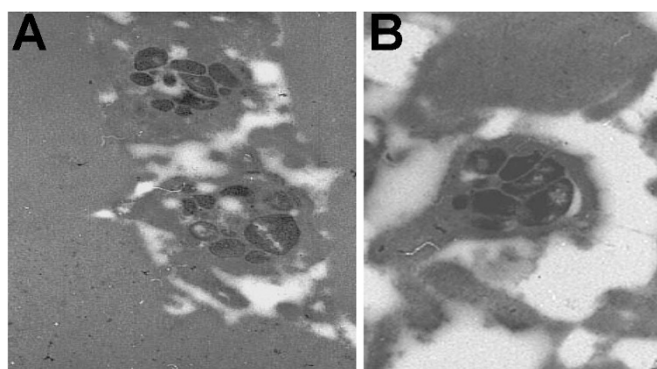


Figure 2. Features of platelet activation with granules centralization (A) and development of pseudopods (B) under transmission electron microscopy

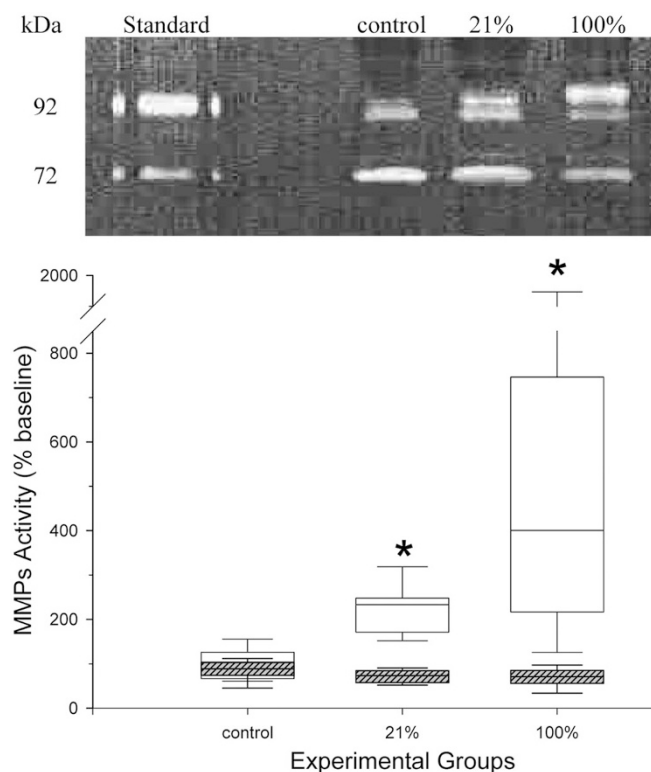


Figure 3. Changes in MMP-2 (hatched box) and MMP-9 (open box) activity in the plasma of hypoxic piglets resuscitated with 21% and 100% oxygen at 30 min of reoxygenation and controls at the corresponding time. A representative zymogram of the plasma MMP activity is shown in the upper panel and a plot of median, 25th, and 75th percentiles in box and 5th and 95th percentiles with error bars in the lower panel. * $p < 0.05$ vs controls (Kruskal-Wallis test).

DISCUSSION

Our study has demonstrated reduced *ex vivo* platelet aggregatory response to collagen stimulation after hypoxia and reoxygenation in an intact animal model of neonatal asphyxia. This impaired aggregatory response was associated with platelet activation, as shown by decreased platelet counts, increased plasma TxB_2 levels, and the changes in platelet morphology such as granular centralization and pseudopod formation. The treatment of hypoxic piglets with 100% oxygen resulted in impaired aggregation and was associated with increased plasma TxB_2 levels and MMP-9, but not MMP-2, levels upon reoxygenation compared with controls. The ef-

fects of 21% oxygen were intermediate and not statistically different from those of the other groups possibly due to small sample size. Because the differences are modest, it would require 30–40 animals in each group to achieve adequate power to test the hypothesis that in newborn piglets with asphyxia, resuscitation with 100% oxygen is associated with increased platelet activation and worse platelet aggregatory function than those resuscitated with 21% oxygen.

In this study, we examined the *ex vivo* platelet aggregatory function in newborn animals using whole blood impedance aggregometry. In contrast to *in vitro* studies using platelet-rich plasma, whole blood aggregometry can precisely determine platelet aggregatory function in a more physiologic milieu (16) because of interaction between blood cells under various pathophysiologic conditions (17). The technique provides comparable results with the optical method using platelet-rich plasma (18). Furthermore, the technique has the advantage of requiring a small volume of blood (0.5 mL per test), which is an important consideration in newborn subjects because of limited blood volume.

In the animals subjected to hypoxia/reoxygenation, the concentration-response curve of collagen-stimulated platelet aggregation was shifted rightward and downward. Whereas the EC_{50} of the 100%, but not 21%, reoxygenated group was increased, the negative finding on the maximum aggregatory response (at 10 $\mu\text{g/mL}$ collagen) requires cautious interpretation. Nonetheless, this may indicate the reduced number of receptors and responsiveness of the platelets to collagen stimulation (19). Although it has been shown that exposure to hydrogen peroxide *in vitro* (20) or brief hyperoxia *in vivo* (21), even in the absence of hypoxia, inhibits both adenosine diphosphate (ADP)- and collagen-induced platelet aggregation, we speculate that the platelet aggregatory dysfunction is secondary to the hypoxia and reoxygenation injury. It is known that several platelet functions are energy dependent including α -granule degranulation (22), aggregation, and arachidonic acid release. In anoxic washed platelets, decreased thrombin-induced aggregation correlates with both the duration of anoxia and decreased adenylate energy charge (4). Littleton-Kearney *et al.* (23) previously described thrombocytopenia with impaired collagen-stimulated platelet aggregation in isolated cerebral ischemia. In our model of prolonged systemic hypoxia, we have demonstrated both platelet activation and dysfunction. Because similar platelet dysfunction was detected in both the 21% and 100% groups, these findings support our proposal that hypoxia is a prerequisite for the observed platelet pathology during reoxygenation. The possibility that the deterioration in platelet function during reoxygenation is a delayed effect of hypoxia secondary to energy failure within the platelet seems unlikely. In a hypoxic environment with a P_{aO_2} of 33 mm Hg, there still may have been sufficient oxygen left to prevent the anoxic effects. It would be challenging to study a delayed hypoxic effect using the current model because the animals would not survive for a further 4 h at a similar level of systemic hypoxemia.

Most of the studies on neonatal resuscitation reported similar functional recovery of the asphyxiated subjects despite a greater oxidative stress in the 100% reoxygenated group than

that in 21% group (24). Interestingly, we demonstrated a similar functional impairment in platelet aggregation. In this study, the postresuscitation hyperoxia in the 100% reoxygenated group may contribute to the oxidant-induced changes in plasma TxB_2 levels and MMP-9 activities. The thromboxane pathway is one of the many mechanisms of platelet activation and is affected by free radicals generated during reoxygenation (2). Indeed, peroxy nitrite, a potent oxidant formed from nitric oxide and superoxide, causes platelet activation (25). Higher plasma levels of TxB_2 in the 100% reoxygenated piglets compared with controls suggests increased activation of prostaglandin-thromboxane mechanistic pathway, although the small sample size precluded us from evaluating this pathway in the 21% reoxygenation group. Study of a temporal marker of platelet activation such as CD62 may be helpful, but the commercially available human or rodent antibodies have uncertain cross-reactivity with porcine antigen.

Sawicki *et al.* (7) first demonstrated that MMP-2 is released and contributes to platelet aggregation. In contrast to MMP-2, MMP-9 inhibits platelet aggregation when released from platelets (8,9). We previously reported that the plasma MMP-9 activity was not different, whereas the plasma MMP-2 activity increased gradually over the first day of extracorporeal membrane oxygenation in critically ill newborns (26). Interestingly, the activity of both plasma MMP-2 and -9 was increased in adult rabbits that underwent extracorporeal membrane oxygenation (11). Oxidative stress can contribute to the activation of MMPs (10,12). Similar to oxidant-induced increases in MMP activity (13,14), we speculate that 100% reoxygenation (to a lesser extent, 21% reoxygenation) causes the activation and release of MMP-9, which contributes to platelet aggregatory dysfunction (8,9). The nonsignificant difference in MMP-9 activities between 100% and 21% reoxygenated groups may be related to the small number of animals used.

We used a hypoxia/reoxygenation protocol different from that of other reports (13,14). We believe that the duration and severity of hypoxemia will affect the response and thus may explain the difference in findings compared with the literature. Therefore, we controlled both the duration and severity, as stated, to a 2-h period of normocapnic hypoxemia with arterial oxygen saturation of 30–40% and P_{O_2} of 20–40 mm Hg. We believe that the 2-h hypoxemia is clinically appropriate as the time from the onset of perinatal asphyxia through detection, diagnosis to treatment, and emergent resuscitation is about 1.5–2 h. One-hour reoxygenation with 100% oxygen may be relatively long but is not uncommon if the resuscitation occurs distant from a designated neonatal intensive care facility. Furthermore, the use of 1- to 3-d-old piglets may account for a wide variation of physiologic baseline levels. In a previous report, we observed significant differences in the hemodynamic response to hypoxia between 3-d-old and 1- to 2-d-old piglets but not between 1-d-old and 2-d-old piglets (27). Hence, the translation of the current findings requires caution because of the controlled experimental setting in an animal model of neonatal but not perinatal hypoxia/reoxygenation.

It is likely that hypoxia/reoxygenation-induced platelet activation may exert systemic effects. Indeed, circulating acti-

vated platelets can potentiate the activation of other platelets, for example, ADP released from platelet dense granules increases activation caused by other agonists (28). Systemic activation of platelets may affect other sequelae of asphyxia including pulmonary hypertension and reperfusion damage of ischemic organs due to interaction with endothelial cells (29). Khandoga *et al.* (29) showed that this contributes to microvascular injury and cell damage in the liver. This may be coupled with cardiac dysfunction as cardiac reoxygenation injury is also known to be partly mediated by platelets exposed to superoxide (30). The lung and pancreas may also be affected (31,32). It is therefore likely that circulating platelets can play a role in multiorgan failure in intact hypoxic newborn animals or infants.

Therefore, in the choice of the appropriate oxygen concentration for neonatal resuscitation, one needs to consider the effect of hypoxia and reoxygenation on platelet function. Our findings on plasma TxB_2 levels and MMP-9 activity support the judicious use of oxygen when resuscitating hypoxic newborns. These findings should be considered in conjunction with other studies suggesting that 21% oxygen during resuscitation does not seem to cause any excess harm to infants (24). While the use of 100% oxygen in neonatal resuscitation requires caution, it is possible that 21% oxygen may be as beneficial as, and possibly less noxious than, 100% oxygen.

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