to autoregulate (i.e., maintain blood flow in the face of changing perfusion pressure) has been investigated.\(^5\) The general consensus is that there is a linear correlation between choroidal blood flow and perfusion pressure (MAP – IOP) when IOP is sequentially elevated.\(^1\)\(^-\)\(^4\)\(^,\)\(^10\)\(^-\)\(^14\) However, there is experimental evidence suggesting that autoregulation may be present in the iris and ciliary body that is masked by the relatively higher choroidal blood flow when total uveal blood flow is measured.\(^6\) Others have presented evidence for autoregulatory mechanisms in the cat LPCA and in the rabbit posterior choroid, especially when blood pressure rather than IOP is the controlling variable.\(^8\)\(^,\)\(^15\) With direct measurement of LPCA blood flow, we found no evidence for autoregulatory control mechanisms; there was a linear drop in blood flow with every stepwise increase of IOP. Although it is possible that autoregulation would be seen if the blood pressure was used as the controlling variable as observed by others (see earlier). However, other investigators found that the choroid does not autoregulate if the arterial blood pressure is reduced by hemorrhage.\(^14\)\(^,\)\(^16\)

In conclusion, we have shown that blood flow can be continuously measured in the cat LPCA by means of ultrasonic flowmetry. Blood flow in this conduit artery is approximately 0.6 mm/min and, under controlled conditions, is stable for periods of several hours. Vascular beds supplied by the LPCA are not reactive to alterations of arterial oxygen tensions and respond only modestly to large increases of arterial carbon dioxide levels. The linear pressure-response correlations that occur when IOP is elevated suggest an absence of autoregulatory mechanisms in vascular beds supplied by the long posterior ciliary arteries.

**Acknowledgments**

The author thanks Linda Hess for technical support.

**References**


**Metabolic Acidosis–Induced Retinopathy in the Neonatal Rat**

Jonathan M. Holmes,\(^1\) Shuichen Zhang,\(^1\) David A. Leske,\(^1\) and William L. Lanier\(^2\)

**Purpose.** Carbon dioxide (CO\(_2\))-induced retinopathy (CDIR) in the neonatal rat, analogous to human retinopathy of prematurity (ROP), was previously described by our group. In this model, it is possible that CO\(_2\)–associated acidosis provides a biochemical mechanism for CDIR. Therefore, the effect of pure metabolic acidosis on the developing retinal vasculature of the neonatal rat was investigated.

**Methods.** A preliminary study of arterial blood pH was performed to confirm acidosis in our model. In neonatal rats with preplaced left carotid artery catheters, acute blood gas samples were taken 1 to 24 hours after gavage with either NH\(_4\)Cl 1 millimole/100 g body weight or saline. In the subsequent formal retinopathy study, 150 newborn Sprague-Dawley rats were raised in litters of 25 and randomly assigned to be gavaged twice daily with either NH\(_4\)Cl 1 millimole/100 g body weight (\(n = 75\)) or saline (\(n = 75\)) from day 2 to day 7. After 5 days of recovery, rats were killed, and retinal vasculature was assessed using fluorescein perfusion and ADPase staining techniques.

**Results.** In the preliminary pH study, the minimum pH after NH\(_4\)Cl gavage was 7.10 ± 0.10 at 3 hours (versus 7.37 ± 0.03 in controls, mean ± SD, P < 0.01). In the formal retinopathy study, preretinal neovascularization occurred in 36% of acidic rats versus 5% of controls (P < 0.001). Acidotic rats showed growth retardation (final weight 16.5 ± 3.0 g versus 20.2 ± 2.6 g, P < 0.001). The ratio of vascularized to total retinal area was smaller in acidic rats (94% ± 4% versus 96% ± 2%, P < 0.001).
CONCLUSIONS. Metabolic acidosis alone induces neovascularization similar to ROP in the neonatal rat. This suggests a possible biochemical mechanism by which high levels of CO₂ induce neovascularization and supports the suggestion that acidosis may be an independent risk factor for ROP. (Invest Ophthalmol Vis Sci. 1999;40:804-809)

Clinical and laboratory studies suggest that a high level of CO₂ is a risk factor for retinopathy of prematurity (ROP).1,2 We have recently reported a "carbon dioxide-induced retinopathy" (CDIR) in the neonatal rat that has many of the features of ROP.1 The mechanism by which a high level of CO₂ induces neovascularization is currently unknown. Neonatal human and animals with elevated PaCO₂ have acidosis.3 Acidosis alone is known to affect the endothelial cells and pericytes of the developing vasculature,4 and acidosis has been suggested as a possible independent risk factor for the development of ROP.1 We therefore hypothesized that acidosis may provide a biochemical mechanism for CDIR.

The neonatal rat has been used by us1,2,5 and others6,7 as a model for ROP. The retinal vasculature of the neonatal rat is incompletely developed at birth and is analogous to the premature human neonate.

Ammonium chloride gavage has been used by others8 to induce metabolic acidosis in rodents, and we adapted this technique to the neonatal rat. The present study was designed to investigate the effect of pure metabolic acidosis on the developing retinal vasculature and to determine whether or not metabolic acidosis alone is sufficient to induce neovascularization similar to ROP in the neonatal rat.

METHODS

The following studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Mayo Foundation.

Preliminary Arterial pH Study

In a blood pH and blood gas study, we gavaged neonatal rats with NH₄Cl and obtained at predetermined time points arterial blood gas samples for pH analysis. Sixty-six neonatal rats, 9 to 10 days of age, weighing 16.0 to 24.5 g, underwent placement of a left carotid artery catheter under general anesthesia as previously described by us.1,2 Body temperature was maintained throughout the study with a heating pad set at 39°C (K-MOD 100 heat therapy pump; Baxter, Deerfield, IL). After complete recovery from anesthetic (1-2 hours), the rats' stomachs were gavaged with either NH₄Cl or saline. See also Table 1. Data at time point zero (*) were obtained from nongavaged room air rats in a previous study.9

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Proprietary interest category: N.

Retinopathy Study

In the formal retinopathy study, 150 newborn Sprague-Dawley rats were raised in six expanded litters of 25 each. Newborn pups from mothers delivering on the same day were mixed and randomly assigned to each litter to control for initial weight. One mother was assigned to each litter of 25 for the entire study. Newborns were randomly assigned within litters to receive either NH₄Cl (1 millimole/100 g body weight) or saline in an approximate volume of 100 μl. In pilot studies, greater concentrations were associated with a high mortality rate (data not shown). Gavage was performed using polyethylene tubing (PE-20; Becton-Dickinson, Sparks, MD) attached to a 0.5-ml tuberculin syringe fitted with a 28-gauge needle (Monoject; Sherwood Medical, St. Louis, MO). The distal smooth end was lubricated with K-Y Jelly (Johnson & Johnson, Arlington, TX) before insertion down the esophagus into the stomach. Rats were then placed in a custom-designed plastic tube1 to limit their movements, so that they could not dislodge the arterial catheter. A 50-μl sample of arterial blood was drawn from the catheter of each rat at either 1, 3, 5, 7, 12, or 24 hours after gavage (Fig. 1). Arterial blood samples were analyzed for pH using a blood gas analyzer (System 1306; Instrumentation Laboratory, Lexington, MA).

In the formal retinopathy study, 150 newborn Sprague-Dawley rats were raised in six expanded litters of 25 each. Newborn pups from mothers delivering on the same day were mixed and randomly assigned to each litter to control for initial weight. One mother was assigned to each litter of 25 for the entire study. Newborns were randomly assigned within litters to receive either NH₄Cl (1 millimole/100 g body weight) or saline in an approximate volume of 100 μl. Mothers received standard laboratory diet and water ad libitum. Light was cycled on a 12-hour on/12-hour off schedule, and room temperature was maintained at approximately 70°F. After gavage (days 2-7), rats were allowed to recover for 5 days, analogous to our previous CO₂-induced retinopathy study.1

On day 13 of life, all rats were deeply anesthetized, and their left retinae were processed for fluorescent microscopy and ADPase staining.1,2,5,9,10 ADPase stained retinae were graded for neovascularization (yes or no) in a masked manner by three independent observers using a method that has become standard in our laboratory.1,2 As in our previous studies,1,2 neovascularization was defined as abnormal vascular
ANOVA, and a two-tailed Mest (pH, retinal vascular area) with laboratory (i.e., pH = 7.37). 2 In contrast to control rats, those with control rats; Table 1).

The pH values for saline controls in this study (Table 1) are the same as the room air values previously reported from our laboratory (i.e., pH = 7.37). 2 In contrast to control rats, those rats gavaged with NH4C1 had lower arterial pHs, at all time points (Fig. 1). The minimum pH after NH4C1 gavage was 7.10 ± 0.10 at 3 hours (versus 7.37 ± 0.03 in controls, P < 0.01, Fig. 1, Table 1).

At 3 hours postgavage, the PaO2 values were higher in the acidic animals than in the saline gavaged animals, although the absolute difference was small and the PaO2 values would still be considered to be near the normal range (94 ± 10 mm Hg). 1,2 PaCO2 values were lower in the acidic animals, reflecting partial respiratory compensation for metabolic acidosis (Table 1). However, the compensatory PaCO2 changes were small (i.e., a reduction of no more than 6 mm Hg compared with control rats; Table 1).

In reviewing these data, it is important to note that these arterial pH and blood gas values were obtained from older neonatal animals (days 9–10 of life) in a separate study from the retinopathy study below.

Retinopathy Study

As in our previous studies using expanded litters, not all the rat pups survived. Forty-two (56%) of the acidotic rats and 59 (79%) of the controls completed the study. Data from all surviving rats were used in the analysis. All 101 ADPase-stained retinae in the acidosis study were technically readable and could be scored. All but 2 retinae were adequately perfused by fluorescein (1 from the acidotic group and 1 from controls), and data from the remaining 99 retinae were used for analysis of retinal areas.

Preretinal neovascularization occurred in 36% of acidic rats compared with 5% of controls (P < 0.001; Fig. 2, Table 2). The range of severity of neovascularization in the acidotic group was 0 clock hours to 5 clock hours: 7 retinae had 1 clock hour; 5 had 2 clock hours; 1 each had 3 clock hours, 4 clock hours, and 5 clock hours of neovascularization. Preretinal neovascularization was confirmed on thin section (Fig. 2C). No such abnormalities were seen in normal retinae or areas of retinae that scored negative for neovascularization (data not shown). Severity of neovascularization, when present, was lower in the control group (P < 0.001). Of the 3 control retinae scored positive for neovascularization, each had only 1 clock hour of neovascularization.

Acidotic rats demonstrated growth retardation at day 7 (intermediate weight = 10.1 ± 1.9 g versus 12.5 ± 1.8 g in controls, P < 0.001) and at day 13 (final weight = 16.5 ± 3.0 g versus 20.2 ± 2.6 g, P < 0.001). Ratios of vascularized to total retinal areas were smaller in acidic rats than in controls (94% ± 4% versus 96% ± 2%, P < 0.001), although the absolute differences between groups were small compared with other studies in our laboratory. 2

DISCUSSION

In this controlled study of metabolic acidosis in neonatal rats, 36% of acidic rats developed preretal neovascularization analogous to human ROP. This condition might be termed "metabolic acidosis-induced retinopathy" (MAIR). It is important to note that MAIR is a hyperoxia-independent form of preretinal neovascularization in immature retinae. As such, MAIR may help explain why some critically ill premature infants who are at risk for acidosis, but have never experienced elevated partial pressures of oxygen (e.g., children with congenital heart disease), still experience ROP. 1 Additionally, our
finding of MAIR suggests that systemic acidosis per se may be an important triggering factor in CDIR and perhaps other forms of retinopathy.

In interpreting our data, several methodological issues should be considered. The arterial blood gas and pH data were obtained in older animals (9-10 days of age) than those used in the retinopathy study (2-7 days of age during NH₄Cl exposure). Our decision to use older rats in the blood gas study was based solely on technical constraints that prevented us from obtaining reliable arterial blood gas values in younger neonatal rats. Specifically, the small size of the carotid artery made vessel cannulation difficult in younger rats. Once the catheters were placed, younger rats were more likely to experience catheter failure from blood clotting. We considered other previously reported techniques such as cutdown onto the descending aorta in the anesthetized rat. However, we elected not to use this technique out of concern for the near-instantaneous confounding effects on blood gases and pH that result from background anesthetic, surgical stimulation, disruption of accessory muscles of respiration, blood loss, heparin administration, and exposure of blood to ambient air. Instead, we believed that it was appropriate to perform our blood gas study in slightly older rats who were awake, unanesthetized, and spontaneously breathing. The physiological values we obtained in control rats (Table 1) and from room air control animals in previous studies, particularly the pH data, support our approach. We further believe it is appropriate to assume that both ages of rats we studied developed a metabolic acidosis in response to gavage with NH₄Cl.

Our high mortality rate is similar to our previous studies using expanded litters. This mortality may be a prerequisite in our model for severe neovascularization and may parallel the human situation in which the sickest infants have the most severe ROP.

The timing of our NH₄Cl gavage (days 2-7), and the conclusion of our study, were somewhat arbitrary, but were based on our experience with oxygen-induced retinopathy (OIR) and CDIR. A 7-day period of exposure, followed by a 5-day recovery period, has been used by us in our OIR and CDIR models. We have not addressed whether neovascularization occurs without a recovery period in either CDIR or MAIR. The choice of a twice-daily dosing schedule was based on the acidosis profile found in the preliminary blood pH study (Fig. 1).

As we have discussed before, in grading retinae, it is possible that we may have been liberal in our definition of neovascularization because we defined any abnormal clumps or sheets of cells, and abnormal vascular structures, as neovascularization. However, the inclusion of control retinae, masking of examiners, and use of a median score reduced the bias toward false positives. In the past we have made multiple thin sections of areas scored as positive for neovascularization and

**Figure 2.** (A) ADPase preparation of a retina demonstrating neovascularization at the junction of the avascular and vascular retina (arrow, see B). This retina is a representative (i.e., it is neither the least nor most severe) retina in the acidotic group. Scale bar, 1 mm. (B) Areas of neovascularization (arrow) labeled in (A). Scale bar, 0.2 mm. (C) Toluidine blue-stained thin section of the area labeled in (B), demonstrating preretinal neovascularization arising from within the retina (arrow). Scale bar, 0.02 mm.
confirmed the presence of preretinal cells. Performing retinal sections on all retinas, as a technique of identifying preretinal neovascularization, would require many hundreds of sections per eye to avoid missing areas of neovascularization and therefore was not performed in this study. We did not make serial sections of every retina to determine whether all the abnormal vascular structures were truly preretinal neovascularization. We did not observe leakage from the neovascularization, in part because the fluorescein marker we used is coupled to a high-molecular-weight dextran. This fluorescein-dextran complex would not be expected to leak from areas of neovascularization.

Regarding the incidence of neovascularization in the retinopathy study, it is noteworthy that 3 (5%) of the control retinas were scored positive for neovascularization. This rate of neovascularization in controls is higher than that in room air controls in our previous studies (0%). It is possible that twice-daily gavage with saline may have had an adverse effect on the rat pups, such as reduced feeding due to handling-related physiological stress response. We should also note that not all rats gavaged with NH₄Cl developed neovascularization. This is not surprising, because the incidence of neovascularization in our OIR model is only 50%. Although our study demonstrated that metabolic acidosis from NH₄Cl gavage resulted in retinal changes similar to those of CDIR, we have not yet evaluated whether the time course for retinopathy development is comparable between these two entities. Furthermore, we have not determined whether treatment of metabolic acidosis (e.g., using bicarbonate) will prevent neovascularization. These issues will require further study.

The mechanism of induction of neovascularization by acidosis alone is currently unknown, but appears to be independent of hyperoxia. Although we observed a small increase in PaO₂ (Table 1) in NH₄Cl-treated rats as a result of compensatory hyperventilation, the PaO₂ values of 108 mm Hg were very near the normal range for awake neonatal rats (94 ± 10 mm Hg). It is noteworthy that the small increases in PaO₂ observed in NH₄Cl-treated rats in the present study (Table 1) are in stark contrast to PaO₂ increases to 371 mm Hg encountered in our rat model of OIR.

Although the development of MAIR appears to be independent of increases in PaO₂, our research does not rule out the possible influence of acidosis as a contributing mechanism in OIR. Clinically, in preterm neonates, there is an association among oxygen use, hyperoxia, and ROP. It also has been observed that the sickest premature infants are the most likely to develop ROP, regardless of whether they experience hyperoxia or not. These same infants may experience episodes of acidosis as a result of systemic hypoperfusion, hypoxia (for which they may receive supplemental oxygen), sepsis, and other causes. The present study, and our previous research with CO₂ administration, suggests that episodes of acidosis, regardless of their origin, may trigger preretinal neovascularization either directly, or indirectly through other stimuli (e.g., hyperoxia or hypoxia).

Although it is possible to use animal models to resolve the confounding effects of acidosis as a trigger or facilitator in OIR, there are scant data on pH in those models. Several OIR models include periods of cyclic oxygenation such that animals experience both hyperoxia and hypoxia. The pH changes that occur during hypoxic cycles have not yet been studied. Further research will be needed to determine whether metabolic acidosis occurs during hypoxic cycles in such OIR models and whether the development of acidosis is critical to the development of neovascularization.

Based on similarities between CDIR in our previous study and MAIR described in the present study, we hypothesize that these two entities share common pathomechanisms. These mechanisms have been reviewed by us previously. Damage to the developing endothelium may be a common mechanism in OIR, CDIR, MAIR, and clinical ROP. This is supported by our finding of reduced retinal vascularization in acidic rats of the present study. However, the absolute difference in retinal vascular areas between acidic and control rats was small at the conclusion (day 13) of the study (96% versus 94%, Table 2). We have not yet studied the effect of acidosis on retinal vascular area at earlier time points (e.g., before the recovery period, day 7), but, with such small absolute differences at the conclusion of the study (day 13); it is likely that factors other than retinal vascular area are important in the pathogenesis of neovascularization in this model.

Further studies might address the possible interactions of CO₂ and acidosis, growth retardation, growth hormone, vascular endothelial growth factor, and retinal neovascularization. Our results support the hypothesis that acidosis per se is a risk factor for the development of preretinal neovascularization in neonatal animals. Because human neonates at risk for ROP are prone to episodes of acidosis, we suggest that further studies should address the potential role of acid-base control in the pathogenesis of ROP.

### Table 2. Comparison of Data between the NH₄Cl- and Saline-Gavaged Rats

<table>
<thead>
<tr>
<th></th>
<th>NH₄Cl Group</th>
<th>Saline Group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of surviving pups*</td>
<td>42 (56)</td>
<td>59 (79)</td>
<td>0.005</td>
</tr>
<tr>
<td>Birth weight (g)†</td>
<td>6.3 ± 0.5</td>
<td>6.3 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Intermediate weight (day 7) (g)†</td>
<td>10.1 ± 1.9</td>
<td>12.5 ± 1.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Final weight (day 13) (g)†</td>
<td>16.5 ± 3.0</td>
<td>20.2 ± 2.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Number of pups with neovascularization*</td>
<td>15 (36)</td>
<td>5 (1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Range of severity (clock hours)</td>
<td>0-1</td>
<td>0-1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total retinal area (mm²)†</td>
<td>34.6 ± 2.1</td>
<td>35.4 ± 1.9</td>
<td>NS</td>
</tr>
<tr>
<td>Vascularized retinal area (mm²)†</td>
<td>32.5 ± 2.8</td>
<td>34.1 ± 2.1</td>
<td>0.002</td>
</tr>
<tr>
<td>Ratio of vascularized to total retinal area (%)†</td>
<td>94 ± 4</td>
<td>96 ± 2</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NS, not significant at the P < 0.05 level.
* Values in parentheses are percentages.
† Values are means ± SD.
Human Diabetic Neovascular Membranes Contain High Levels of Urokinase and Metalloproteinase Enzymes

Arup Das,1,2 Paul G. McGuire,3 Cheryl Eriqat,1 Richard R. Ober,4 Eugene Defuian, Jr,5 George A. Williams,6 Angela McLamore,1 Jyoti Biswas,7 and David W. Johnson8

**Purpose.** Retinal neovascularization is one of the leading causes of blindness. A crucial event in this process is the remodeling and penetration of the capillary basement membrane by migrating endothelial cells. This process requires proteolysis of basement membrane components by a variety of proteinases. The objective of the present study was to determine the expression of proteinases in human retinal tissues showing active neovascularization.

**Methods.** Epiretinal neovascular membranes surgically removed from patients with proliferative diabetic retinopathy were analyzed by zymography, and the types and amounts of proteinases present in the tissues were determined. Retinas from nondiabetic donor eyes served as control specimens.

**Results.** Both the high- (54 kDa) and low- (33 kDa) molecular-weight forms of urokinase were present at significantly higher levels in neovascular membranes than in normal retinas. The pro forms of the matrix metalloproteinases (MMP) MMP-2 and MMP-9 were significantly elevated in the neovascular membranes in comparison with levels in normal retinas. In addition, the active forms of these enzymes were present in the membranes, whereas there was no detectable level of the active forms in normal retinas.

**Conclusions.** Human diabetic neovascular membranes contain high levels of urokinase and MMP. The increased activity of proteinases in the final common pathway of retinal neovascularization indicates that inhibition of these enzymes may be a useful therapeutic target as an alternative approach in the management of proliferative retinopathies. (Invest Ophthalmol Vis Sci. 1999;40:809-813)

**References**