Oxidative–Nitrosative Stress in a Rabbit Pup Model of Germinal Matrix Hemorrhage
Role of NAD(P)H Oxidase

Muhammad T. Zia, MD; Anna Csiszar, MD, PhD; Nazar Labinskyy, MD; Furong Hu, BA; Govindaiah Vinukonda, PhD; Edmund F. LaGamma, MD; Zoltan Ungvari, MD, PhD; Praveen Ballabh, MD

Background and Purpose—Germinal matrix hemorrhage–intraventricular hemorrhage is the most common neurological problem of premature infants. Despite this, mechanisms of brain injury from intraventricular hemorrhage are elusive. We hypothesized that germinal matrix hemorrhage–intraventricular hemorrhage, by induction of NAD(P)H oxidases, might cause oxidative/nitrosative stress contributing to brain injuries and that NAD(P)H oxidase inhibition could offer neuroprotection.

Methods—To test this hypothesis, we exploited our rabbit pup model of glycerol-induced germinal matrix hemorrhage–intraventricular hemorrhage. We delivered rabbit pups prematurely (E29) by cesarean section and administered intra-peritoneal glycerol at 2 hours postnatal age. Free-radical adducts, including nitrotyrosine, 4-hydroxy-nonenal, and 8-hydroxy-deoxyguanosine as well as O2\(^{\cdot-}\) and H\(_2\)O\(_2\) levels were measured in the forebrain. To determine the source of free-radical generation, we used inhibitors for NAD(P)H oxidase (apocynin), xanthine oxidase (allopurinol), cyclo-oxygenase-2 (indomethacin), or nitric oxide synthases (L-NAME). Intraventricular hemorrhage pups were treated with apocynin and cell death was compared between apocynin-treated and vehicle-treated pups.

Results—Nitrotyrosine, 4-hydroxy-nonenal, and 8-hydroxy-deoxyguanosine levels were higher in pups with intraventricular hemorrhage than controls. Likewise, O2\(^{\cdot-}\) and H\(_2\)O\(_2\) levels were significantly greater in both the periventricular area and cerebral cortex of pups with intraventricular hemorrhage than controls. In pups with intraventricular hemorrhage, reactive oxygen species production was more in the periventricular area than in the cortex. Apocynin, but not allopurinol, indomethacin, or nitric oxide synthases, inhibited reactive oxygen species generation. Importantly, apocynin reduced cell death in pups with intraventricular hemorrhage.

Conclusion—Activation of NAD(P)H oxidase was the predominant mechanism of free-radical generation in pups with intraventricular hemorrhage. NAD(P)H oxidase inhibition by apocynin might suppress reactive oxygen species production and confer neuroprotection in premature infants with intraventricular hemorrhage. (Stroke. 2009;40:00-00.)

Key Words: apocynin ■ germinal matrix hemorrhage–intraventricular hemorrhage ■ NADPH oxidase ■ nitrotyrosine ■ oxidative stress ■ reactive oxygen species ■ reactive nitrogen species ■ superoxide

Germinal matrix hemorrhage–intraventricular hemorrhage is the most common neurological disorder of premature newborns. Germinal matrix hemorrhage–intraventricular hemorrhage predisposes preterm infants to cerebral palsy, cognitive deficits, and posthemorrhagic hydrocephalus.1 Germinal matrix hemorrhage–intraventricular hemorrhage is not a substantially preventable disorder; and unfortunately, its treatment is primarily supportive and not therapeutic. Therefore, it is crucial to determine the basis of brain injury from intraventricular hemorrhage (IVH) to identify a mechanism-based strategy to protect the brain in affected premature infants with IVH. The mechanism of brain injury after intracerebral hemorrhage is complex and involves inflammatory reaction, mass effect of hemorrhage, and ischemia around the hemorrhagic areas.2,3 These responses are associated with the generation of free radicals, cytokines, and a number of other immunomodulators. We ask whether the development of IVH in premature newborns is attended by an enhanced production of reactive oxygen (ROS) and nitrogen species, and if so, what is the predominant source of free radicals.

In adult rat models, oxidative stress plays a key role in the pathogenesis of cerebral injury after intracerebral hemor-
ROS damages neurons and oligodendrocytes, decreases cerebral blood flow, and opens up the blood–brain barrier to large molecules like neurotoxins and inflammatory cells, collectively leading to accumulation of oxidized aggregated proteins. The brain of premature infants is highly vulnerable to oxidative damage because it is inadequately equipped with antioxidant defense systems. Indeed, studies on autopsy specimens of premature infants with white matter (periventricular leukomalacia) and cortical injury have shown evidence of oxidative stress in various regions of the forebrain. However, free radical generation has neither been evaluated in premature infants with IVH nor in animal models of IVH. Therefore, the present study explores oxidative and nitrosative stress in IVH that might contribute to brain injuries in these premature newborns.

The potential cellular sources of ROS include NAD(P)H oxidase, cyclo-oxygenase, xanthine oxidase, or nitric oxide synthase and mitochondria. Presently, it is unknown how oxidative stress results from NAD(P)H oxidase largely contributes to the brain injury. In addition, NAD(P)H oxidase inhibition is neuroprotective in rodent models of hypoxia–ischemia and surgically induced brain injury. On this basis, we hypothesized that IVH, by induction of NAD(P)H oxidase, might result in formation of ROS in the brain of premature rabbit pups and that NADPH oxidase inhibition might confer neuroprotection.

**Materials and Methods**

**Animal Studies**

The animal protocol was approved by the Institutional Animal Care and Use Committee of New York Medical College. We used a rabbit model of glycerol-induced IVH in the study. The details of the model have been previously established and published. Timed pregnant New Zealand rabbits were obtained from Charles River Laboratories Inc (Wilmington, Mass). The pups were delivered prematurely by cesarean section at a gestational age of 29 days (full-term, 31 to 32 days). Pups were immediately dried and kept warm in an incubator, which was maintained at a temperature of 35°C and 60% humidity. After stabilization, the pups were weighed. Pups were fed formula and then 2 mL every 12 hours (100 mL/kg per day) using a 3.5-Fr feeding tube. At 2 hours postnatal age, the pups alternatively received an intraperitoneal injection of 50% glycerol (6.5 g/kg) solution or saline treatment. Glycerol induces intracranial hypertension leading to an increase in the transmural pressure across the vessel walls and, thus, causing vessels to rupture. Head ultrasound was performed 4 hours later to assess for the presence and severity of IVH. The pups were euthanized at 6 and 24 hours of age. Although pups were evaluated for IVH both on head ultrasound and after euthanasia, the final determination of the presence and severity of IVH was done on gross examination of the sectioned brain. Based on the presence of IVH, the pups were subdivided as either glycerol-treated IVH or glycerol-treated non-IVH.

**Head Ultrasound and Grading of IVH**

Head ultrasound was performed on all pups at approximately 6 hours postnatal age to determine the presence and severity of IVH using an Acuson Sequoia C256, Siemens ultrasound machine. As reported before, we classified IVH into: (1) mild, no gross hemorrhage and hemorrhage detected on microscopy of hematotoxyl and eosin-stained brain sections; (2) moderate, gross hemorrhage into lateral ventricles without significant ventricular enlargement (2 separate lateral ventricles discerned); and (3) severe, IVH with significant ventricular enlargement (fusion of ventricles into a common chamber) and/or intraparenchymal hemorrhage. In the IVH group, only brains with moderate and severe IVH were included in the study.

**Immunohistochemistry**

Immunolabeling of coronal brain sections was performed as described to detect markers of oxidative–nitrosative stress in situ. 4-Hydroxynonenal (trans-4-hydroxy-2-nonenal or 4-HNE) is the primary α, β-unsaturated hydroxalkenal, which is produced by lipid peroxidation in cells. 8-hydroxydeoxyguanosine (8-OHdG), an oxidized nucleoside of DNA, is a sensitive marker of DNA damage caused by increased cellular production of ROS. ROS such as peroxynitrite can nitrate specific amino acids such as tyrosine, altering the protein function. 3-Nitrotyrosine is widely used as a sensitive marker of this reaction indicating in vivo nitrosative stress.

The following primary antibodies were used: mouse monoclonal anti-4-hydroxy-2-nonenal (Shizuka; 1:20), goat polyclonal anti-8-hydroxydeoxyguanosine (Chemicon; 1:200), and mouse monoclonal anti-3-nitrotyrosine (Invitrogen). Peroxynitrite (Chemicon) was used as a positive control and degraded peroxynitrite (Chemicon) was used as a negative control. The secondary antibodies (Jackson Immunoresearch) included Cy-3 conjugate of goat antimouse and Cy3 conjugate of mouse anti-goat. After drying and several washes in PBS, the tissue sections were incubated with the primary antibody diluted in PBS at room temperature for 1 hour. After washing in PBS, the sections were incubated with the secondary antibody diluted in 1% normal goat serum in PBS at room temperature for 40 minutes. Finally, after washes in PBS, sections were mounted with Slow Fade Light Antifade reagent (Molecular Probes) and were visualized under fluorescent microscope (Axioskop 2 Plus; Carl Zeiss Inc).

To evaluate neuronal degeneration in apocynin-treated and vehicle-treated IVH pups, we performed Fluoro-Jade B (Chemicon) staining on fixed brain sections according to the manufacturer’s instruction. To detect apoptosis, fluorescent in situ detection of DNA fragmentation (TUNEL) was performed on fixed brain sections as described previously. The sections were air-dried on slides, hydrated in 0.01 mol/L PBS, and permeabilized for 5 minutes in 1:1 ethanol/acetic acid (−20°C). An ApoTag Fluorescein in situ DNA fragmentation detection kit (Chemicon) was used to visualize TUNEL-labeled nuclei. Tissue sections were counterstained with propidium iodide to visualize all the nuclei. We next quantified Fluoro-Jade B and TUNEL-positive nuclei in apocynin- and vehicle-treated pups. From each brain, a set of 3 to 5 coronal sections were taken as every 2-mm sections using a cryostat. From every section, approximately 5 images were acquired from both PVZ and cortex using 40× objective. Thus, we performed counting in 60 to 100 (5 images ×3 to 5 sections ×2 regions ×2 coronal levels) images per brain.

Rabbit Tissue Collection and Processing

Forebrains were sectioned into 2-mm slices on brain matrix starting from the cranial end. Sections of the second and third slice from the cranial end were used for immunohistochemistry. The second slice had structures including caudate nucleus, medial septal nucleus, gray matter, internal capsule, and others, whereas the third slice had caudate nucleus, hippocampus, thalamus, globus pallidus, internal capsule, and others. For immunohistochemistry, the brain slices were fixed in 4% paraformaldehyde in phosphate-buffered saline (0.01 mol/L PBS; pH 7.4) for 24 hours and were cryoprotected by immersing into 20% sucrose in PBS buffer for 18 hours followed by 30% sucrose for the next 24 hours. We froze tissues after embedding them into Optimum Cutting Temperature Compound (Sakura). Frozen coronal blocks were cut into 10-μm sections using a cryostat. For Western blot analysis, we used the second 2-mm slice from the cranial end to make homogenates in the sample buffer. For superoxide and hydrogen peroxide levels, the second 2-mm slice of the forebrain was taken and an approximately 1-mm area around the ventricle (periventricular zone) as well as the cortical plate was dissected with a surgical blade. The periventricular zone (PVZ) included germinal matrix, caudate nucleus, corona radiata, and corpus callosum. The dissected tissue pieces were further chopped into 0.5- to 1-mm cubes.
Western Blot Analysis
Protein extraction and Western blotting were performed under reducing conditions as described before. Briefly, frozen brain tissue was homogenized in sample buffer and boiled for 5 minutes. The protein concentration was determined. Total protein was separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis. The separated proteins were transferred to polyvinylidene difluoride membrane by electrotransfer. The membranes were then incubated with primary antibodies, washed, incubated with secondary antibody, and detected with a chemiluminescence ECL system (Amersham).

Ethidium Fluorescence
Dihydroethidine, an oxidative fluorescent dye, was used to measure superoxide levels in situ as we previously reported. In brief, thin sections of the brain were incubated with hydroethidine (3 × 10⁻⁶ mol/L at 37°C for 30 minutes) and then the samples were washed 3 times. Unstained sections and samples preincubated with PEG-SOD were used for background correction and negative control, respectively. For quantitative measurements, the time course of the buildup of ethidium bromide fluorescence in the tissue samples was recorded for 30 minutes as described. The slope factor was calculated and normalized to Hoechst 33258 fluorescence representing DNA content/cell mass. The effect of diphenylene iodonium (10⁻⁶ mol/L, an inhibitor of flavin containing oxidases, including NAD(P)H oxidase), apocynin (3 × 10⁻⁴ mol/L, an inhibitor of NAD(P)H oxidase activation), indomethacin (10⁻⁵ mol/L, a specific inhibitor of the cyclooxygenase), allopurinol (10⁻⁴ mol/L, a specific inhibitor of the xanthine oxidase), and L-N⁵-nitroarginine methyl ester (L-NAME, 3 × 10⁻⁴ mol/L, a specific inhibitor of nitric oxide synthase) on tissue O₂⁻ production were also determined.

Lucigenin Chemiluminescence and NADPH Oxidase Activity Assay
Tissue O₂⁻ levels ex vivo was also assessed by measuring SOD-inhibitable lucigenin (5 μmol/L) chemiluminescence (as we reported before). To assess NADPH oxidase activity, samples from the periventricular region and cortex were homogenized and NADPH-driven lucigenin chemiluminescence was measured as reported.

Measurement of Oxidative Stress by DCF and H₂O₂ Production by Amplex Red Fluorescence Assays
The cell-permeant oxidative fluorescent indicator dye C-H,DCFDA (5 and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-acetyl ester, abbreviated DCF throughout the text; Invitrogen, Carlsbad, Calif) was used to assess oxidative stress in the periventricular region and cortex as we reported elsewhere. Unstained sections and samples preincubated with PEG-catalase were used for background correction and negative control respectively. In separate experiments, the time course of the buildup of DCF fluorescence in the tissue samples was recorded for 30 minutes. The slope factor was calculated and normalized to Hoechst 33258 fluorescence representing DNA content/cell mass. In separate experiments, H₂O₂ production was measured fluorometrically in the periventricular region and the cortex using the Amplex Red/horseradish peroxidase assay according to our published protocols. H₂O₂ generation rate was compared by measuring the time course of the buildup of resorufin fluorescence for 60 minutes in the presence and absence of apocynin, diphenylene iodonium, indomethacin, allopurinol, or L-NAME.

Quantitative Real-Time Polymerase Chain Reaction
Quantitative real-time polymerase chain reaction was used to assess changes in mRNA expression of the NOX2 and NOX4 subunits of NAD(P)H oxidase in the periventricular region and cortex of IVH pups compared with saline controls. These subunits have been shown to be induced by intracerebral hemorrhage and hypoxia–ischemia in the adult mouse model. Total RNA from the periventricular region of rabbit pups with and without IVH was isolated using the Mini RNA isolation kit (Zymo Research) and was reverse-transcribed using Superscript II kit (Invitrogen) as described previously. The real-time polymerase chain reaction technique was used to analyze mRNA expression using the Strategen MX3000 as reported before. Quantification was performed using the efficiency-adjusted ΔΔCT method. The housekeeping gene GAPDH was used for internal normalization. For the ΔΔCT calculation to be valid, we calculated the efficiency of the target amplification and the efficiency of the reference amplification (the housekeeping gene GAPDH) by constructing a standard curve using dilution series of brain mRNA samples (efficiency of primers, 1.95 to 2.0). We next normalized the target gene to the reference gene to control for cDNA loaded into the reaction. Fidelity of the polymerase chain reaction was determined by melting temperature analysis and visualization of product on a 2% agarose gel.

Apocynin Treatment
Prematurely delivered (E29) pups were sequentially treated with glycerol or saline at 2 hours postnatal age. We next alternately assigned the glycerol-treated pups to receive either intravenous apocynin or vehicle at 3 hours of age. Apocynin (2.5 mg/kg per dose) was administered intravenously in the jugular vein at 3 hours and 12 hours of age using an insulin syringe with a 30-gauge needle. Dose of apocynin was based on its previous use in rat experiments. At 24 hours of age, the pups were euthanized and evaluated for the presence and severity of IVH. The randomization and stratification procedure ensured that 2 groups of pups—apocynin- and vehicle-treated pups with IVH—were balanced with respect to severity (moderate or severe) of IVH. The 3 groups of pups, including apocynin-treated and vehicle-treated pups with IVH as well as saline-treated non-IVH pups, were compared for the evidence of cell death—apoptosis and neuronal degeneration.

Statistical Analysis
Data are expressed as mean ± SEM. The parameters were compared between pups with and without IVH as well as a function of postnatal age. We used the t test (parametric variable) or Mann–Whitney U test (nonparametric variable) to perform pairwise comparisons. A probability value of < 0.05 was considered significant.

Results
Higher Expression of Free Radical Adducts in Pups With IVH Than Controls
To determine whether differences exist in the presence and location of oxidative stress markers between the brain of premature pups with glycerol-induced IVH and saline-treated controls (non-IVH), we evaluated free radical adducts, including 4-hydroxynonenal and 8-OHdG, by immunohistochemistry and Western blot analyses. The cellular content of 3-nitrotyrosine, a marker of nitrosative stress, was also assessed. Immunolabeling of coronal brain sections with 4-HNE-specific antibody revealed that 4-HNE was more abundant in neural cells of the PVZ of pups with IVH than in controls at both 6 (4 hours after induction of IVH) and 24 hours (22 hours after induction of IVH) postnatal age (Figure 1A). The HNE expression was weaker in the cortex compared with the PVZ in pups with IVH. Western blot analysis of brain homogenates of a full-thickness slice at the level of the midseptal nucleus confirmed that 4-HNE (25 to 60 kDa) levels were significantly higher in pups with IVH than saline-treated non-IVH controls at 6 hours (P < 0.05), but not at 24 hours postnatal age (Figure 1B).

We next evaluated the expression of 8-OHdG in pups with IVH compared with saline-treated non-IVH controls. We used human skin as the positive control, whereas no primary antibody on the section was the negative control. We noted...
that 8-OHdG immunoreactivities were greater in both the PVZ and cortex of pups with IVH compared with controls at 6 hours and 24 hours postnatal age (Supplemental Figure I, available online at http://stroke.ahajournals.org). In addition, the expression of 8-OHdG was higher in the PVZ than in the cortex of pups with IVH at both 6 hours and 24 hours postnatal age. We could not achieve satisfactory immunoblot with the commercially available 8-OHdG-specific antibodies and thus, 8-OHdG was not quantified by Western blot analysis.

Immunolabeling of coronal brain sections with nitrotyrosine-specific antibody revealed that this was intensely expressed in the neural cells around the ventricle (PVZ) in pups with IVH at both 6 and 24 hours postnatal age, but not in pups without IVH (Figure 2A). The immunoreactivities to nitrotyrosine in the cerebral cortex were weaker compared with the PVZ in the pups with IVH. We next quantified nitrotyrosine by Western blot analysis, which showed nitrotyrosine bands (20 to 75 kDa) were denser and stronger in pups with IVH than saline-treated non-IVH controls (Figure 2B). The optical density measurement of bands confirmed that nitrotyrosine levels were significantly higher in pups with IVH than non-IVH controls at 6 hours postnatal age *(P<0.05)*, but not at 24 hours.

Figure 1. 4-HNE levels are greater in pups with IVH than non-IVH controls. A, Representative immunofluorescence of cryosections from the brain of premature pups with IVH and saline-treated non-IVH controls labeled with 4-HNE-specific antibodies. The immunoreactivities were intense in the PVZ in pups with IVH at both 6 and 24 hours of age but weak in the cortex. The immunosignals were more abundant at 6 hours than 24 hours postnatal age in the PVZ of pups with IVH. Scale bar - 20 μm. V, ventricle; CP, choroid plexus. B, Representative Western blot analysis of 4-HNE in pups with IVH and saline-treated non-IVH controls at 6 and 24 hours. The molecular weight ranged from 25 to 60 kDa. The bar graph shows mean±SEM (n=6). The values were normalized to β-actin levels. 4-HNE levels were higher in pups with IVH than saline-treated non-IVH controls at 6 hours (arrowhead), but not at 24 hours of age. 4-HNE levels were 1.5- to 2-fold greater at 6 hours compared with 24 hours of age in pups with IVH, but the difference was not statistically significant. *(P<0.05)* for the comparison between IVH pups and non-IVH controls at 6 hours.

Figure 2. Nitrotyrosine levels were higher in pups with IVH than without IVH. A, Cryo-sections were labeled with nitrotyrosine-specific antibody. Positive controls were made by adding peroxynitrite to the section and negative control by adding degraded peroxynitrite. Nitrotyrosine was strongly expressed in the PVZ of pups with IVH at both 6 and 24 hours of age, but weakly expressed or absent in saline-treated controls. Scale bar - 20 μm. V, ventricle. B, Representative Western blot analysis of nitrotyrosine in pups with IVH and saline-treated non-IVH controls at 6 hours and 24 hours. The molecular weight ranged from 20 to 75 kDa. The bar graph shows mean±SEM (n=6). The values were normalized to β-actin levels. Nitrotyrosine levels were significantly higher in pups with IVH than saline-treated non-IVH controls at 6 hours (arrowhead), but not at 24 hours of age. Nitrotyrosine levels were 1.5- to 2-fold greater at 6 hours compared with 24 hours of age in pups with IVH, but the difference was not statistically significant. *(P<0.05)* for the comparison between IVH pups and non-IVH controls at 6 hours.
Taken together, there was ample evidence of oxidative and nitrosative stress in pups with IVH in contrast to pups without IVH. In addition, generation of free radical was more around the ventricle than in the cortex of pups with IVH.

Enhanced Levels of Superoxide (O$_2$•−) and Hydrogen Peroxide in Pups With IVH

Because we observed a consistent elevation in the level of all the biomarkers of oxidative/nitrosative stress in the brains of pups with IVH at 6 hours of age (4 hours after induction of IVH), we evaluated O$_2$•− and H$_2$O$_2$ levels at this time point. Dihydroethidine fluorescence (Figure 3A) and Lucigenin chemiluminescence (Figure 3B) measurements demonstrated that O$_2$•− levels were significantly higher in both the PVZ and cortex of the pups with IVH than in the respective brain regions of glycerol- and saline-treated non-IVH controls. O$_2$•− levels were higher in the PVZ of pups with IVH than the cortex, and the difference was statistically significant for O$_2$•− levels measured by dehydroethidine fluorescence (P=0.03), but not for lucigenin chemiluminescence.

Using the DCF fluorescence (Figure 3C) and Amplex Red (Figure 4A) assays, we demonstrated that oxidative stress was significantly higher in the PVZ of pups with IVH than glycerol- and saline-treated non-IVH controls (P<0.03). Together, IVH results in enhanced generation of O$_2$•− and H$_2$O$_2$.

NADPH Inhibition Suppresses O$_2$•− and H$_2$O$_2$

Generation in IVH, but not Cyclo-Oxygenase, Xanthine Oxidase, or Nitric Oxide Synthase Suppression

Because IVH elicited significant oxidative stress, we sought to determine the main source of cerebral ROS production in the model. Using Amplex Red, DCF, and dihydroethidine fluorescence assays, we demonstrated that the NAD(P)H oxidase inhibitor, apocynin, significantly attenuated ROS production in the PVZ in the brains of pups with IVH compared with untreated IVH pups (P<0.01, 0.001, and 0.001 respectively; Figure 4). Similar results were obtained with diphenylene iodonium, which inhibits flavin containing oxidases, including the NAD(P)H oxidase (data not shown). In contrast, we found that allopurinol, indomethacin, and l-NNAME had no significant effect on cerebral ROS production in IVH. Hence, NAD(P)H oxidase is the primary source of free radical generation in our model of glycerol-induced IVH.

Evaluation of NAD(P)H Oxidase Activity and Gene Expression

To further characterize the role of NAD(P)H oxidases in cerebral ROS generation in IVH, we assessed tissue NADPH oxidase activity in vitro. We found that NADPH oxidase activity was significantly increased in both the PVZ and cortex of pups with IVH than glycerol- and saline-treated controls at 6 hours of age and that it remained substantially elevated at 24 hours postnatal age (Figure 5A). Because NOX-2 (gp91phox) and NOX-4 containing NAD(P)H oxidase are abundant in the adult brain with well-recognized roles in free radical production in both surgically induced brain injury and intracerebral hemorrhage in rodent models, we evaluated the effect of IVH induction on the expression of NOX-2 and NOX-4. There was no significant difference in their gene expression between IVH pups and non-IVH controls (Figure 5B).

Apocynin Treatment Reduced Cell Death in Pups With IVH

To determine whether apocynin offers neuroprotection, we compared apoptosis (TUNEL staining) and neuronal degeneration among 3 groups of pups at 24 hours postnatal age: (1) apocynin-treated pups with IVH; (2) vehicle-treated pups with IVH; and (3) saline-treated pups without IVH (Figure 6). Apocynin- and vehicle-treated groups were balanced with respect to the severity of IVH. TUNEL staining showed lesser density of apoptotic cells in apocynin-treated pups with IVH compared with vehicle-treated pups with IVH in the PVZ (P=0.012) but not in the cortex (P=0.069 each). Likewise, density of Fluoro-Jade B(+) neurons were lesser in apocynin-treated pups with IVH than vehicle controls in the PVZ (P<0.02 each), but not in the cortex (P<0.09 each). In contrast to pups with IVH, TUNEL and Fluoro-Jade B(+) cells were scarce in saline-treated pups without IVH.
Discussion

Germinal matrix hemorrhage—intraventricular hemorrhage in premature infants is a major public health concern because of its high incidence and the attendant complications. The mechanism of brain injury in IVH has not been well elucidated, thus limiting development of therapeutic strategies to protect the brain in affected premature infants. We evaluated the generation of free radicals in our rabbit model of IVH as well as its mechanism of production. Four principal observations were made. First, induction of IVH in premature pups resulted in oxidative and nitrosative macromolecular damage as indicated by the higher level of 4-HNE, 8-OHdG, and nitrotyrosine in the brain of pups with IVH compared with controls. Second, $\text{O}_2^\bullet/$H$_2$O$_2$ levels in brain slices ex vivo were significantly greater in both the PVZ cortex as well as in glyceral- and saline-treated non-IVH controls. NADPH oxidase levels were significantly higher in both the PVZ and cortex of pups with IVH compared with controls. Second, $\text{O}_2^\bullet/$H$_2$O$_2$ levels in brain slices ex vivo were significantly greater in both the PVZ and cortex of pups with IVH compared with controls. Indomethacin, but not allopurinol, and L-NAME, inhibited ROS generation in the brain of preterm pups, suggesting that NAD(P)H oxidase is the predominant source of free radical generation in IVH. Fourth, apocynin treatment reduced apoptosis and neuronal degeneration in pups with IVH. Together, these studies highlight the oxidative and nitrosative stress in premature rabbit pups with IVH and suggest a mechanism-based strategy for the possibility of future therapeutic intervention to limit brain injury in premature infants with IVH.

The most important and novel observation made in the present study was that induction of IVH in premature pups resulted in significantly enhanced oxidative stress, suggesting key roles of ROS in acute brain injuries. Presently, the mechanisms by which ROS induces neural damage in premature newborns are not well understood. However, several studies in adult animal models of intracerebral hemorrhage have shown that ROS and reactive nitrogen species generation lead to damage in neurons and glia in the brain as well as altered blood–brain barrier permeability by several mechanisms. ROS induces release of excitatory amino acid and formation of lipid peroxidation products that damages neurons, neurofilaments, and glial cells. In addition, free radicals oxidize a number of neurotransmitters, including...
The second key observation made in the present study was that apocynin and diphenylene iodonium attenuated IVH-induced oxidative stress in the brain of preterm pups, suggesting that NAD(P)H oxidase is the predominant source of ROS generation in this model. This finding was further substantiated by the direct demonstration of elevated NADPH oxidase activity in the PVZ of the brains of pups with IVH (Figure 5). The primary initiating mechanisms for NAD(P)H oxidase activation in IVH of premature infants, albeit unknown, might include reperfusion injury of ischemic regions around the area of hemorrhage and destruction\(^2\) followed by an inflammatory response consisting of neutrophil and macrophage infiltration.\(^3\) In contrast, inhibition of cyclo-oxygenase, xanthine oxidase, and nitric oxide synthases had no significant effect on ROS production, suggesting that these sources do not play a key role in IVH-induced oxidative stress in preterm pups. More importantly and consistent with our ex vivo inhibition of oxidative stress with apocynin, we noted cytoprotection offered by apocynin treatment in pups with IVH compared with vehicle-treated controls. The finding of apocynin treatment suppressing generation of ROS and providing neuroprotection in premature pups with IVH is consistent with a number of in vivo studies in adult rodent model of stroke, in which neuroprotective effect of apocynin has been demonstrated.\(^28-31\) However, only 20% to 40% reduction in cell degeneration or death was observed in apocynin-treated IVH pups compared with vehicle-treated IVH pups. Hence, we speculate that postnatal apocynin treatment might offer some neuroprotection in premature infants after IVH.

Importantly, NAD(P)H oxidase activity was significantly elevated in the PVZ as well as in the cortex of premature pups with IVH compared with controls. However, gene expression of NOX-2 and NOX-4, homologs of NAD(P)H oxidase, was not significantly increased in the brain of pups with IVH compared with non-IVH controls. A number of other investigators working on a adult rodent model of hypoxia–ischemia and cerebral hemorrhage have also observed higher expression of NOX-2 (gp91phox) isoform of NAD(P)H oxidase compared with controls.\(^12,32,33\) NOX enzymes are already known to contribute to pathological processes. Indeed, genetic deficiency of the gp91phox subunit of NADPH oxidase reduces the neuropathological consequences of hemorrhage in a mouse model of collagen-induced ICH.\(^12\) NOX-2 is expressed in neurons, astrocytes, and microglia\(^33,34\); and NOX-4 is expressed in endothelium and vascular smooth cells in addition to neurons.\(^35,36\) NOX-4 has roles in ischemia–induced neangiogenesis and is upregulated in ischemia in peri-infarct region.\(^36\) Together, these findings support the notion that NOX-2 and NOX-4 subunits of NADPH oxidase serve an important function in free radical generation in premature rabbit pups with IVH and by extension, possibly, in human newborns.

Another key finding of the present study is demonstration of substantially elevated levels of 3-nitrotyrosine—a biomarker of increased peroxynitrite formation—in the PVZ of premature pups with IVH. \(\text{O}_2^-\) reacts with nitric oxide (likely derived from neuronal nitric oxide synthase) to form peroxynitrite, which is a major cytotoxic mediator of neuronal injury during stroke.\(^37\) This finding is also important because peroxynitrite in known to activate the poly ADP-ribose polymerase (PARP-1) that suppress neuronal viability in a PARP-dependent fashion.\(^38\) PARP(s) plays key roles in postischemic, traumatic, and other forms of brain injuries and are now considered interesting targets for therapies for neuroprotection.\(^39\) Thus, the present work sets stage for future studies on the use of peroxynitrite decomposition catalyst
and/or with PARP inhibitors to prevent the neurological sequelae in preterm infants with IVH.

In conclusion, the present study revealed enhanced oxidative–nitrosative stress in the brain regions around the ventricle of prematurely delivered rabbit pups with IVH. In addition, NADPH oxidase was identified as the major source of free radical generation. Accordingly, apocynin treatment in rabbit pups with IVH significantly reduced apoptosis and neuronal degeneration compared with vehicle-treated IVH pups. These data suggest that reducing oxidative–nitrosative stress by suppression of NAD(P)H oxidase, facilitating decomposition of peroxynitrite or disruption of pathways activated by oxidative stress may remarkably diminish the occurrence and severity of neurological sequelae in premature rabbit pups and, possibly, in humans.

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**References**

Figure 1. Greater expression of 8-OHdG in IVH than non-IVH pups. Cryosections from the forebrain of rabbit pups with IVH and saline-treated (non-IVH) controls were immunolabeled with 8-OHdG-specific antibody. 8-OHdG was expressed more in the PVZ than in the cortex of pups with IVH at both 6 and 24 hours of age. However, no such immunoreactivity was observed in non-IVH controls. Scale bar=20 μm. V, ventricle; CP, choroid plexus.
Oxidative–Nitrosative Stress in a Rabbit Pup Model of Germinal Matrix Hemorrhage.
Role of NAD(P)H Oxidase
Muhammad T. Zia, Anna Csiszar, Nazar Labinskyy, Furong Hu, Govindaiah Vinukonda, Edmund F. LaGamma, Zoltan Ungvari and Praveen Ballabh

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