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A Thesis Submitted to the

Yale University School of Medicine

in Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

by

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THE EFFECTS OF CHRONIC HYPOXIA ON THE DEVELOPING RAT BRAIN.

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The developing brain in premature infants is vulnerable to hypoxic injury. Clinical studies have correlated chronic postnatal hypoxia in very low birth weight infants with the development of neurological handicap later in life. Animal studies investigating this phenomenon have found that hypoxia induces angiogenesis in the developing brain but have not established a cause and effect mechanism for hypoxic injury. Since new vessels may have different permeability properties than mature vessels, we hypothesized that chronic hypoxia causes changes in properties of the blood brain barrier.

Rat pups were raised, beginning on postnatal day three (PND 3), in a chamber with an F_iO_2 of 9.5±1% for a period of 10 or 30 days (H10, H30). A subgroup of H30 rat pups was returned to normoxia for an additional 30 days prior to sacrifice (H30/N30). The brains of these animals were evaluated using immunohistochemical markers and electron microscopy. A separate subset of the H30 animals was injected with horseradish peroxidase (HRP), which circulated for five minutes prior to sacrifice. The brains of the HRP perfused animals were examined for leakage of HRP across the blood brain barrier.

We found a pronounced increase in vascular permeability in both H10 and H30 animals compared to controls. Additionally, a dramatic increase in the size of the lateral ventricles and associated decreases in cross sectional area of the corpus callosum and cortical volume were noted in H30/N30 animals. These findings suggest that chronic hypoxia increases blood brain barrier permeability as well as ventricular volume in the developing rat brain.

ACKNOWLEDGEMENTS

This project would not have been possible without the continuous help and support of several people.

William B. Stewart, PhD, for guidance and support for the duration of this project.

Laura R. Ment, MD, for supporting my research and fostering my interest in pediatrics.

Michael Schwartz, PhD, for compiling additional ventricular volume data.

Edward Novotny, MD, for generously housing our hypoxia chamber in his lab.

The Department of Surgery for generously supporting my time in lab.

ABBREVIATIONS USED

BBB	blood brain barrier
BPD	bronchopulmonary dysplasia
DAB	diaminobenzidine
F_iO_2	fraction of inspired oxygen
GFAP	glial fibrillary acidic protein
H10	10 days in hypoxia
H30	30 days in hypoxia
H30/N30	30 days in hypoxia followed by 30 days in normoxia
HRP	horseradish peroxidase
IVH	intraventricular hemorrhage
N10	10 day control animals
N30	30 day control animals
N60	60 day control animals
PND	postnatal day
SD	standard deviation
TMB	tetramethyl benzizdine
VEGF	vascular endothelial growth factor
VM	ventriculomegaly

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INTRODUCTION

Hypoxia and the premature infant

Prematurity carries with it many risks to the newborn infant. Recent data show that preterm infants weighing less than 1250 grams at birth constitute just over 1% of live births [1]. While improvements in perinatal care have led to a survival rate among these infants of greater than 85%, the incidence of neurodevelopmental handicap remains between 15% and 36%, the same level it was a decade ago [2,3]. Although many premature infants experience episodes of hypoxia, little is known about the mechanisms that may be involved in the pathogenesis of subsequent injury.

Chronic hypoxia in prematurity

The effects of chronic hypoxia that many premature infants experience in their early newborn life may be secondary to immaturity of their lungs and development of bronchopulmonary dysplasia (BPD). In fact, BPD is defined by the presence of characteristic radiographic findings in association with a requirement for supplemental oxygen at 28 days of life [4,5]. In a study of 27 premature infants, Luchi et al found no correlation between duration of mechanical ventilation and cognitive outcome at three years of age [6]. However, several other investigators demonstrate findings correlating increased duration of ventilation with development of neurological handicap [7,8]. Although much data exist regarding the clinical outcomes following illness, less in known about the pathogenesis of injury in the setting of chronic hypoxia.

A rat model for chronic sublethal hypoxia

The newborn rat brain has been a good model for examination of the brain of infants born at the end of the second trimester; newborn rats are at a similar stage of

development as 25-week-old infants. While corticogenesis has completed, there is still active branching of dendrites and growth of axons. Synapses are being formed at a rapid pace, and programmed cell death of neurons is abundant [9]. In chronically hypoxic animals it is known that the number of neurons is increased while that at the same time the volume of the cortex decreased [10]. It is also known that rats raised in chronic hypoxia have a marked increase in cortical blood vessels [11]. The dendritic branching in the brains of these animals is also decreased [12]. The studies reported here are at the same stages of development of the rat brain.

Additionally, it has been shown by Ment et al that chronic hypoxia causes several characteristic changes in the developing rat: the brains are significantly smaller when compared to controls, as are the overall body weights; the hearts and spleens, however, are significantly larger in the hypoxic animals in response to the low oxygen concentrations and the increased workload demanded of those organs [12]. Similarly, LaManna et al demonstrated a 48% increase in hematocrit following three weeks of hypobaric (0.5 atm) hypoxia [13].

Blood brain barrier development in the newborn rat

Morphologically, endothelial cells of the brain are continuous, non-fenestrated capillaries, and as a result form an impermeable barrier between the vascular and parenchymal spaces in the brain: the blood brain barrier (BBB). This barrier has three main functions: (1) protection of the brain from substances that are abundant in the vascular space, such as the neurotransmitters glutamate and glycine, which would be toxic to neurons at the levels present in blood, (2) selective transport via specialized transport of substances such a glucose, and (3) metabolism of blood- or brain-borne

substances [14]. In addition, the BBB endothelial tight junctions exhibit a very high electrical resistance unique to these cells [15].

Newborn rats do not develop a mature blood brain barrier before 15 days of life [15]. Endothelial cells continue to proliferate and mature after this period, but existing differentiated endothelial cells form vessels that prohibit leakage of the vascular contents into the brain parenchyma. Hypoxia has been shown to have many effects on the blood brain barrier. Muramatsu et al demonstrated an increased vulnerability to hypoxic injury in P7 rats, and correlated this finding with the permeability of the BBB that exists at that age [16]. Zhang et al report morphological changes in the endothelial cells of the rat blood brain barrier following neonatal hypoxia that do not resolve following a period of recovery [17]. There have also been in vitro studies of BBB function, utilizing an endothelial cell/astrocyte co-culture model, which have demonstrated similar increases in permeability in response to hypoxia [18]. A monoclonal antibody specific to a blood brain barrier protein (SMI-71) has been described [19], but it has not yet been used to study the BBB in hypoxia.

Role of VEGF in angiogenesis in the developing brain

The developing brain is vulnerable to hypoxic injury, as neurons and blood vessels are developing and remodeling at a high rate. The supporting cells of the nervous system, glia, are actively creating the framework for deposition of endothelial cells which will together become the blood brain barrier. Vascular endothelial growth factor (VEGF) has been shown to stimulate growth and differentiation of endothelial cells in developing brain [20], as well as in glial tumors [21]. VEGF is an essential growth factor that is active in early angiogenesis, and is necessary for the development of a viable vascular system; knockout mice lacking a single copy of its gene die *in utero* [22]. Weindel et al

showed that VEGF operates as a paracrine growth factor that is active in both normal and tumor angiogenesis [23]. Further data suggest that VEGF is up regulated in response hypoxia [21], and that this increase in VEGF mediates an increase in angiogenesis [11]. These results all suggest that VEGF is up regulated in hypoxia, and that this up regulation leads to an increase in angiogenesis. In addition to this increase in angiogenesis there is also an increase in vascular permeability secondary to the permeability inducing properties of the VEGF protein [24]. VEGF is elevated in malignant brain tumors, and these tumors are usually associated with surrounding edema secondary to the increase in permeability VEGF causes [23]. This permeability has been reported by Connelly to be 1000-fold greater than that of histamine [25]. It is not known, however, by what mechanism VEGF induces an increase in blood brain barrier permeability.

STATEMENT OF PURPOSE

The purpose of these experiments was to further characterize the nature of hypoxic injury to the developing brain, specifically the mechanisms leading to an increase in blood brain barrier permeability. Furthermore, we sought to describe the occurrence of ventricular enlargement in response to chronic sublethal hypoxia.

HYPOTHESIS

I hypothesized that chronic sublethal hypoxia leads to changes in the permeability properties of the blood brain barrier in the developing brain.

METHODS

All of the following studies were performed at the Yale University School of Medicine. The protocols and procedures were reviewed and approved by the Yale University Animal Care and Use Committee. All experiments and data analysis described were performed by the first author unless otherwise indicated.

Hypoxia model

A model of chronic hypoxia was developed in which Sprague-Dawley rats were placed in a Plexiglas chamber on day three of life (Stewart and Ment). Newborn rat pups were placed into the hypoxia chamber with their mothers on the third day of life. The oxygen content of this chamber was maintained at 9.5 +/- 1% using bottled nitrogen gas in combination with a pump that circulated room air through the chamber. The humidity of the chamber was also controlled by means of a circulating filter and desiccants. The oxygen concentration was monitored constantly by a laboratory computer connected to two separate oxygen sensors (Cameron Instrument Co.). Rats were exposed to hypoxia for a period of either 10 or 30 days (H10 or H30); a subgroup of the H30 rats were raised an additional 30 days in normoxic conditions following the initial 30 day hypoxic period (H30/N30). Animals were exposed to room air two times per week for a period of less than ten minutes for cleaning and changing of food and cages.

Immunohistochemical studies

In each of the three groups the experimental and control animals were anesthetized with pentobarbital and perfused using 4% paraformaldehyde in phosphate buffer. The brains were removed and dehydrated in 0.1M phosphate buffer with 20% sucrose. Serial coronal sections of 60 μm were cut using a freezing microtome and

stored in order in a cryoprotectant solution (30% sucrose, 30% ethylene glycol, and 2% polyvinylpyrrolidone in 0.1M phosphate buffer) at -20°C. Serial sectioning allowed characterization of both anatomical features and three-dimensional characteristics of these brains. Tissue was stained with cresyl violet and with immunohistochemical markers specific for vascular endothelial cells, glia, and blood brain barrier proteins, as described below. Every ninth section was mounted for each study, with a minimum of 24 sections per animal.

Immunohistochemical studies were performed using antibodies to vascular endothelial growth factor (VEGF), glial fibrillary acidic protein (GFAP), and smi-71 (Sternberger Monoclonals, Inc. #71) - a marker of blood brain barrier maturity. In each case, tissue sections were removed from cryoprotectant, rinsed in phosphate buffer, blocked, and incubated in primary antibody for a period of 48 hours. Secondary antibody labeling was performed with either standard HRP conjugated antibody reacted with diaminobenzidine (DAB) [VEGF, SMI-71], or tetra-methylrhodamine (TRITC), or flourescene (FITC) conjugated secondary antibody [GFAP and VEGF co-localization studies] viewed under the appropriate immunoflourescence filter. A minimum of seven animals (hypoxic and control) were studied with each antibody.

HRP permeability studies

A subgroup of H30 and H30/N30 rats was given intra-cardiac injections of 40 mg horseradish peroxidase (Sigma, type IV) dissolved in phosphate buffer that was allowed to circulate for five minutes prior to sacrifice and perfusion with 4% paraformaldehyde in phosphate buffer. These brains (N=7 control, N=6 experimental) were subsequently sectioned in the coronal plane at 60 μm using a freezing microtome and stored in cryoprotectant solution at –20°C. HRP perfused sections were incubated with

tetramethylbenzidine and reacted in the standard fashion to visualize extravasation of HRP across the blood brain barrier. Of approximately 200 tissue sections per animal, at least 24 (every ninth section) were studied per animal. The entire cortex in each section was sampled for leakage of HRP.

Ventriculomegaly

It was observed during staining of the brain sections that the cross sectional area of the lateral ventricles appeared larger in the hypoxic animals. Subsequently, every ninth section was analyzed using a projection grid for cross sectional area, and these results were tabulated in order to estimate the volume of the ventricles in the hypoxic versus the control animals. A more detailed stereological analysis of sagittal sections was subsequently performed using celluloidin embedded serial 50 μm sections (sagittal sectioning performed in the laboratory of Michael Schwartz). Following analysis of ventricular volume, the cross sectional area of the corpus callosum and the volume of subcortical white matter were also measured using standard stereology methods (analysis performed by William B. Stewart).

Statistics

All statistics were performed using Statview software. All statistical findings are presented as mean ± SD unless otherwise indicated. Statistical significance was assumed for $p < 0.05$.

RESULTS

Immunohistochemistry

Coronal sections from hypoxic and control brain from each of the three age groups were stained with antibodies directed against both GFAP and VEGF. Figure 1 shows co-localization of both markers within glia from a representative tissue section. Staining was most prominent in glial cells surrounding the large blood vessels.

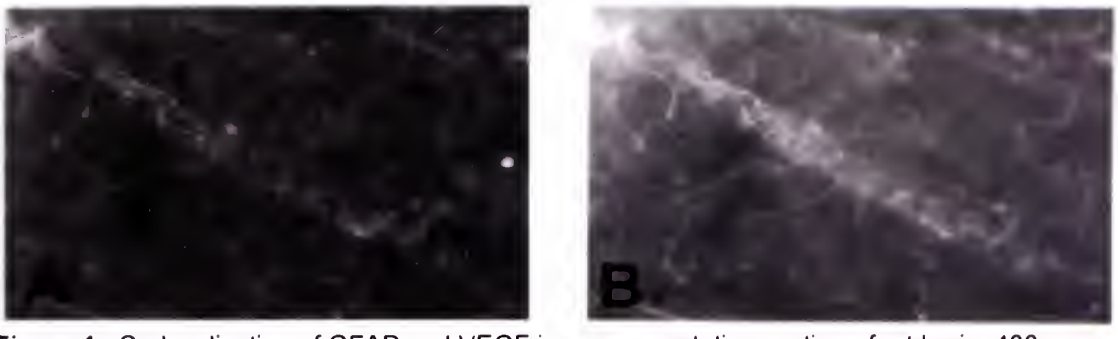

Figure 1. Co-localization of GFAP and VEGF in a representative section of rat brain. 400x original magnification. Panel A is FITC conjugated VEGF; panel B, TRITC conjugated GFAP.

Tissue sections from H30 and N30 animals stained with VEGF are shown in figure 2. Note an increase in neuron density in the hypoxic versus the control animal, with VEGF labeling neuron cell bodies [11].

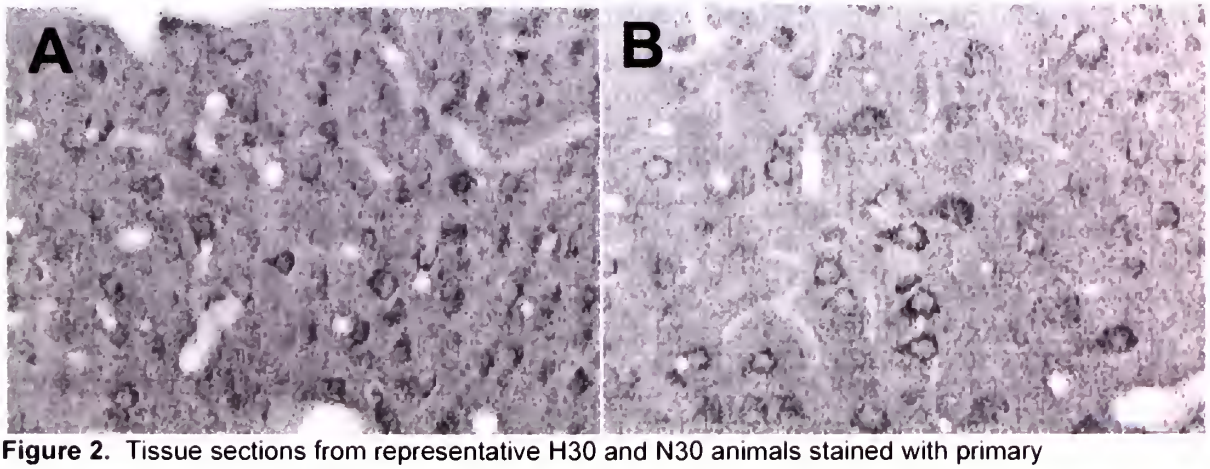

Figure 2. Tissue sections from representative H30 and N30 animals stained with primary monoclonal antibody directed towards VEGF (HRP conjugated secondary antibody). Panel A is an H30 animal; panel B, an N30 control. 40x mag.

Figure 3 shows tissue from H10, H30, and N10 N30 animals stained with SMI-71 (Sternberger Monoclonals Inc.) in an attempt to characterize deficiencies in proteins specific to mature blood brain barrier. No difference in the presence of staining was detected between the experimental and control groups. However, between 10 (panels A and B) and 30 (panels C and D) days there was a large increase in SMI-71 staining. This increase is consistent with previously cited data concerning the changes in the quantity of mature vessels in the cortex during this time period. By 30 days all of the cortical vessels appeared to be labeled throughout. Also note the increased vascular density between hypoxic and control animals, most prominent in the 30 day animals (panels C and D).

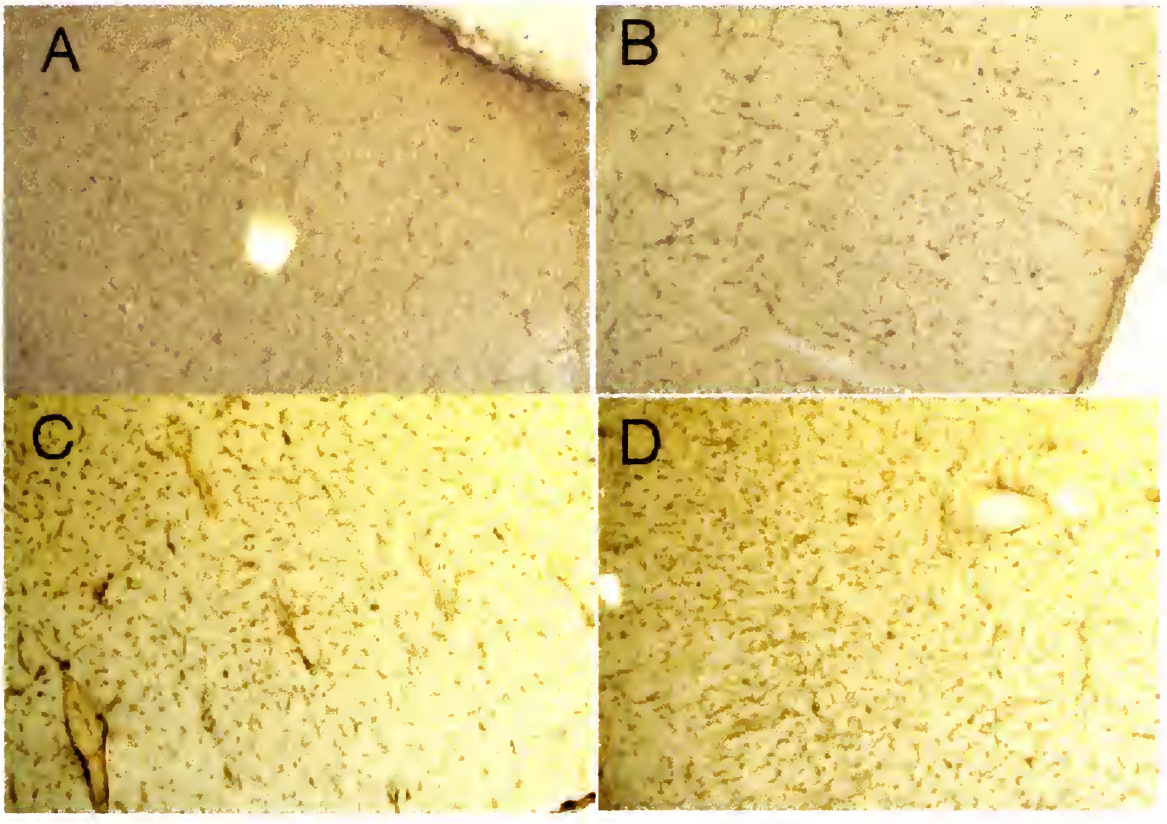

Figure 3. Tissue sections from representative H30 and N30 animals stained with monoclonal antibody directed towards SMI-71 (HRP conjugated secondary). Panel A is an H10 animal; panel B, an N10 control. Panel C is an H30 animal; panel D, an N30 control. 30x magnification.

Chronic hypoxia increases vascular permeability

Tissue sections from HRP perfused animals were examined for leakage of HRP across the BBB. Dark field examination of representative sections from 10 and 30 day hypoxic and control animals are shown in figure 4. At PND 10 there is leakage in the control animal (panel A); this is consistent with published data regarding BBB maturity at this age; note the intense leakage in the hypoxic animal at PND 10 (panel B). At PND 33 there is an absence of HRP leakage in the control animal (panel C) while the hypoxic animals shows extensive leakage (panel D).

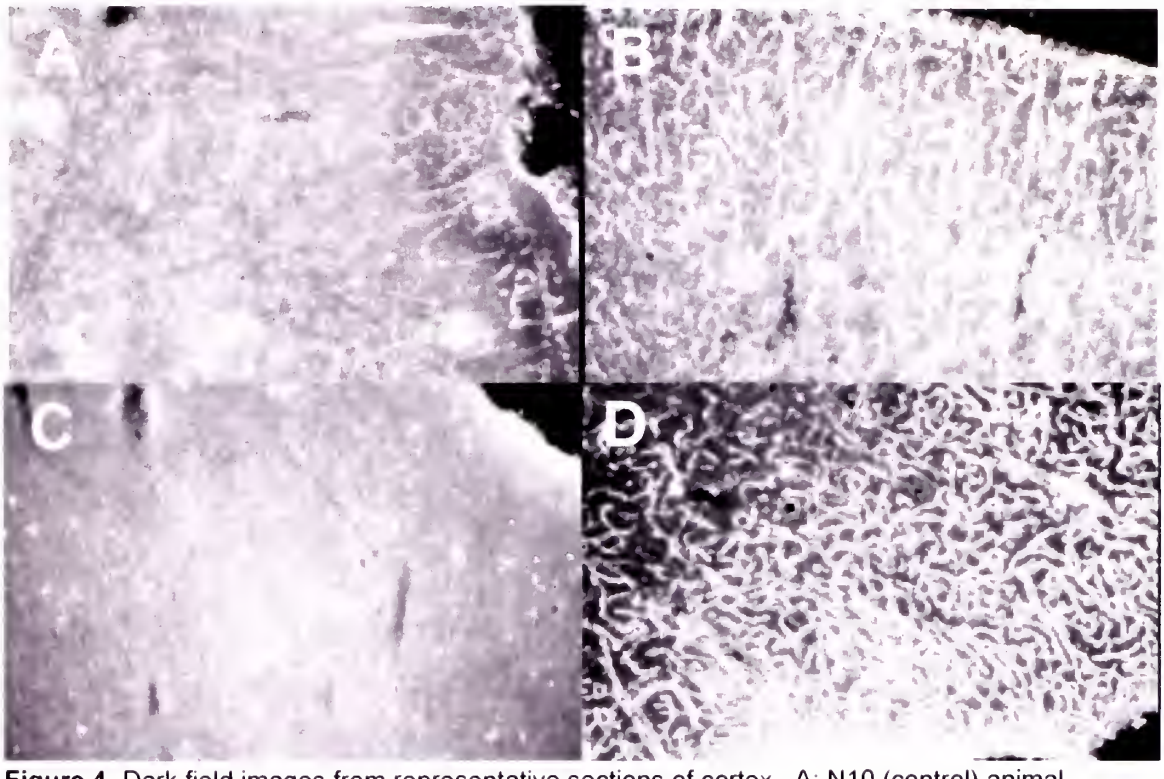

Figure 4. Dark field images from representative sections of cortex. A: N10 (control) animal shows some perivascular staining. B: H10 animal with markedly increased vasculature as well as increased perivascular staining. C: N30 (control) animal with no appreciable perivascular or parenchymal staining. D: H30 animal with increased vasculature and marked perivascular staining. 30x magnification.

The images in figure 4 also illustrate the increase in vascular density in the hypoxic animals. The vascular density is increased by PND 13 (H10, N10 animals) and it continues to be increased at PND 33.

Chronic hypoxia produces ventriculomegaly

Lateral ventricles were examined using matched coronal sections through the head of the caudate of H30/N30 and N60 animals; these were found to be larger in cross section when compared to controls (figure 5).

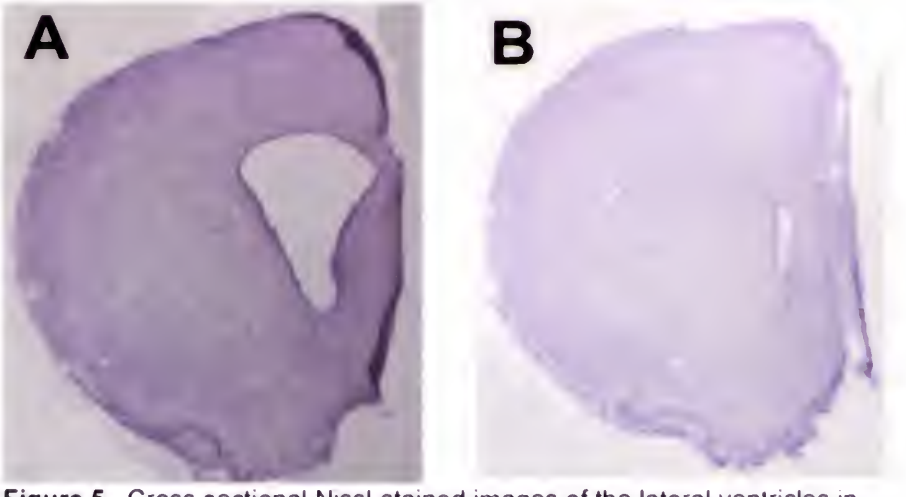

Figure 5. Cross sectional Nissl-stained images of the lateral ventricles in the coronal plane. Panel A is an H30/N30 animal; panel B, an N60 control. Note the marked increase in cross sectional ventricular area of the hypoxic P63 animal (H30/N30) compared to the control P63 animal. 10x magnification

Preliminary volume estimations computed using the cross sectional area of serial sections (every 9th) were analyzed and it was found that H30/N30 animals had significantly greater ventricular volumes compared to N60 controls (H30/N30 ventricular volume = 5.44±1.49mm^3; N60 ventricular volume =3.00±1.80mm^3 $p<0.05$). Cortical volumes, subcortical white matter volumes, and corpus callosum areas were all examined using sagittal sections, and were found to be significantly decreased in all age groups studied (figure 6). Ventriculomegaly was not statistically significant until PND 63 (figure 6).

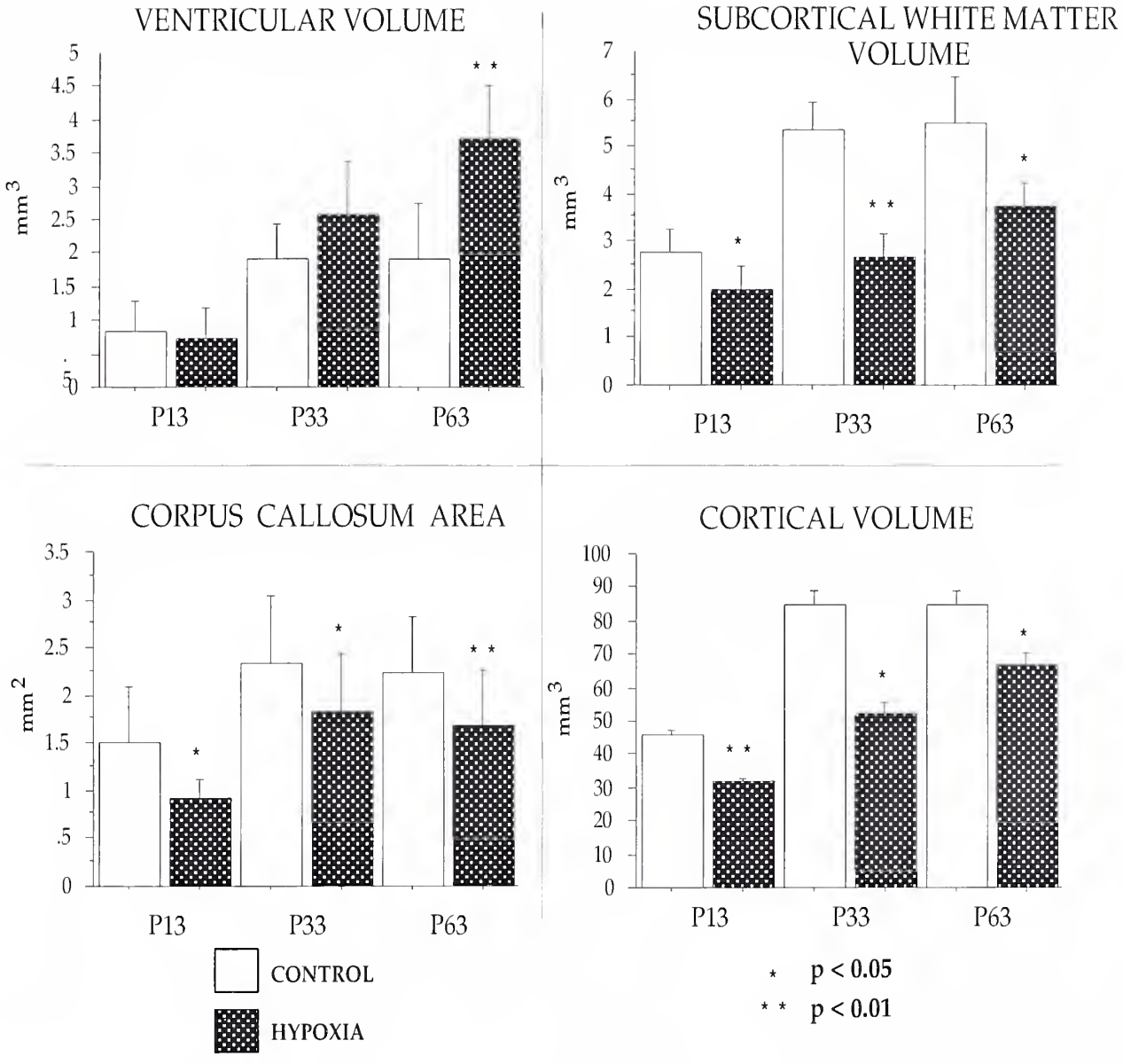

Figure 6. Measurements of lateral ventricular volume, cortical volume, corpus callosum area, and subcortical white matter volume. Hypoxic animals are represented by shaded colums. Control animals are represented by white colums. Statistical significance is noted by the asterix where appropriate.

DISCUSSION

Clinical relevance

Hypoxia has been implicated in many problems that premature infants face. The effects of acute hypoxia have been extensively studied in infants and animals [2,26-33]. Chronic hypoxia is less well studied; many of these tiny babies suffer from bronchopulmonary dysplasia, a condition defined by a long-term need for oxygen supplementation [4,5], and these babies have been shown to have an increase in the incidence of neurodevelopmental handicaps later in life [2,29]. Our studies attempt to mimic this chronic hypoxia in an attempt to characterize the pathogenesis of these neurodevelopmental handicaps.

Animal model for chronic hypoxia

In the early stages of postnatal development the rat brain has been shown to been very sensitive to hypoxic insult. In postnatal rats, extensive differentiation of dendrites and axons occurs during the first 20 days of development, a time that coincides with 25 weeks in a preterm human infant [34]. Our experimental model sought to replicate the chronic state of hypoxia experienced by premature infants with BPD.

VEGF and blood brain barrier permeability

Blood brain barrier permeability is increased in our H10 and H30 animals. Since VEGF is known to be up regulated in hypoxia [20], it would be expected that permeability of existing vessels would increase. Additionally, these animals experience a surge of VEGF-mediated angiogenesis in response to hypoxia, increasing the number of vessels in the brain [11]. The leakage of a large molecule such as HRP across the blood

brain barrier of hypoxic rats is suggestive that leakage of similar large molecules could also occur, some of which may cause damage to the developing brain (Figure 4).

Our findings are consistent with those of Risau [13], in that the BBB is not fully mature at PND 10, as indicated by some leakage in control animals, but that it has developed by PND 30. Since blood vessels are rapidly growing during this stage of development, one could hypothesize that the elevated levels of VEGF driving this growth may also play a role in the permeability of the BBB in normoxic animals at this age. As synaptogenesis continues it may be the case that proteins leaking across the blood brain barrier damage developing neurons; when the BBB continues to leak past the first 15 days of development [13], as is the case in chronic hypoxia, this damage may become more severe or perhaps irreparable. We were unable, however, to show a molecular defect in the blood brain barrier in any age group using SMI-71 (figure 3), a specific immunohistochemical marker for mature BBB [19].

Ventriculomegaly in response to chronic hypoxia

As damage occurs to the developing brain the size of the cortical neurons decreases [9]. We found an increase in the volume of the lateral ventricles in H30/N30 animals. These animals have enjoyed a period of recovery from chronic hypoxia, and their body growth has indeed exhibited catch-up growth [34]. The brains remain small, however, and the size of the cortical neurons is decreased. It is possible that the ventricles of these animals simply grow to fill the space created by an absence of cortical volume. This also would correlate with the findings in children with BPD and ventriculomegaly, in that the hydrocephalus is low pressure, is consistent with periventricular leukomalacia, and is merely a symptom of the cortical injuries caused by chronic hypoxia [34].

Conclusions

Several conclusions can be made based upon these results. Although no specific deficits in the blood brain barrier, with respect to immunohistochemical findings, were found, the increase in permeability in response to chronic hypoxia was clearly shown, and has been suggested by others. The concurrent increase in VEGF suggests an interplay between the two known functions of this protein (increasing vascular permeability and promoting angiogensis), and further studies could be focused on examining that interplay. The finding of ventriculomegaly is in parallel with what is seen in infants who survive hypoxia, and further study of this phenomenon could help elucidate the pathogenesis of ventriculomegaly in preterm infants.

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