

# Effect of Moderate Hypocapnic Ventilation on Nuclear DNA Fragmentation and Energy Metabolism in the Cerebral Cortex of Newborn Piglets

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## ABSTRACT

Previous studies have shown that severe hypocapnic ventilation [arterial carbon dioxide partial pressure ( $P_{aCO_2}$ ) 7–10 mm Hg] in newborn animals results in decreased cerebral blood flow and decreased tissue oxidative metabolism. The present study tests the hypothesis that moderate hypocapnic ventilation ( $P_{aCO_2}$  20 mm Hg) will result in decreased cerebral oxidative metabolism and nuclear DNA fragmentation in the cerebral cortex of normoxic newborn piglets. Studies were performed in 10 anesthetized newborn piglets. The animals were ventilated for 1 h to achieve a  $P_{aCO_2}$  of 20 mm Hg in the hypocapnic (H) group ( $n = 5$ ) and a  $P_{aCO_2}$  of 40 mm Hg in the normocapnic, control (C) group ( $n = 5$ ). Tissue oxidative metabolism, reflecting tissue oxygenation, was documented biochemically by measuring tissue ATP and phosphocreatine (PCr) levels. Cerebral cortical nuclei were purified, nuclear DNA was isolated, and DNA content was determined. DNA samples were separated, stained, and compared with a standard DNA ladder. Tissue PCr levels were significantly lower in the H group than the C group ( $2.32 \pm 0.66$  versus  $3.73 \pm 0.32$   $\mu\text{mol/g}$  brain,  $p < 0.05$ ), but ATP levels were preserved. Unlike C samples, H samples displayed a smear pattern of small molecular weight fragments between 100 and

12,000 bp. The density of DNA fragments was eight times higher in the H group than the C group, and DNA fragmentation varied inversely with levels of PCr ( $r = 0.93$ ). These data demonstrate that moderate hypocapnia of 1 h duration results in decreased oxidative metabolism that is associated with DNA fragmentation in the cerebral cortex of newborn piglets. We speculate that hypocapnia-induced hypoxia results in increased intranuclear  $Ca^{2+}$  flux, which causes protease and endonuclease activation, DNA fragmentation, and periventricular leukomalacia in newborn infants. (*Pediatr Res* 50: 586–589, 2001)

## Abbreviations

C, control  
CBF, cerebral blood flow  
CP, cerebral palsy  
H, hypocapnic  
NMDA, N-methyl-D-aspartate  
 $P_{aCO_2}$ , partial pressure of carbon dioxide in arterial blood  
 $P_{aO_2}$ , partial pressure of oxygen in arterial blood  
PCr, phosphocreatine  
PVL, periventricular leukomalacia

Preterm and ill term infants are at risk for brain injury, subsequent neurodevelopmental delay, and CP, partially because of alterations in CBF (1). In neonates, white matter ischemia often results in ultrasonographic evidence of PVL, which has been associated with the development of CP in human infants (2).

Brain ischemia has been associated with hypocapnia owing to the effect of pH and  $CO_2$  on cerebral vascular tone (3). In

multiple studies both the degree and duration of hypocapnia have been associated with an increased incidence of PVL and CP in preterm infants (4–6). In one study of 251 infants <34 wk gestation, 50% of ventilated preterm infants with a  $P_{aCO_2}$  <17 mm Hg at least once during the first 3 d of life, and 30% of those with  $P_{aCO_2}$  of 7–20 mm Hg, developed CP or PVL or both (6). In a study of 26 ventilated preterm infants <1500 g, 23% of infants with  $P_{aCO_2}$  values <20 mm Hg during the first 24 h of life developed cystic PVL compared with 6% of infants who had normal  $P_{aCO_2}$  values during the same period (7). In another study of 103 preterm infants, the duration of hypocapnia (defined as a  $P_{aCO_2} \leq 30$  mm Hg) during the first 72 h of life was an independent predictor of PVL and was associated with

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the development of CP by 2 years of age (4). A recent study of 26 intubated 27- to 32-wk-old infants found that the time-averaged  $P_{aCO_2}$  was lower and time-averaged pH higher in infants with PVL than those with normal development on the third day of life (8). However, other studies have not found a relationship between hypocapnia and brain injury (9). In addition, in clinical studies many confounding variables contributing to brain injury may exist. To determine whether moderate hypocapnia does induce cerebral injury, newborn piglets were used to test the hypothesis that arterial blood  $P_{aCO_2}$  values of 20 mm Hg for 1 h result in alterations of cerebral energy metabolism and nuclear DNA fragmentation in the cerebral cortex.

## METHODS

Studies were performed in two groups of anesthetized, ventilated 1- to 3-d-old piglets, five normocapnic (C) and five H. The experimental protocol was approved by the Institutional Animal Care and Use Committee of MCP Hahnemann University. Anesthesia was induced with 4% halothane and maintained with 0.8% halothane. Lidocaine 1% was injected locally for performance of a tracheostomy and insertion of aortic and inferior vena caval catheters. Intravenous fentanyl (10  $\mu$ g/kg initially and every hour) and tubocurarine (0.3 mg/kg) were given, and the animals were placed on a volume ventilator using 75% nitrous oxide and 25% oxygen. Arterial blood pH,  $P_{aO_2}$ ,  $P_{aCO_2}$ , glucose, lactate, heart rate, and blood pressure were recorded every 15 min in all animals. Temperature was maintained with a warming blanket.

After 1 h of baseline ventilation, the piglets were either ventilated as normocapnic, control animals (C) keeping pH > 7.30,  $P_{aO_2}$  80–100 mm Hg, and  $P_{aCO_2}$  40 mm Hg, or as H with  $P_{aCO_2}$  20 mm Hg and  $P_{aO_2}$  80–100 mm Hg by adjusting the ventilator rate, usually from 25 to 50 breaths per minute. End-tidal  $CO_2$  values were monitored continuously in all animals. After 1 h of study ventilation the piglets were given an additional dose of fentanyl (10  $\mu$ g/kg i.v.), and the brains were removed, placed in liquid nitrogen within 4 s, and stored at  $-80^\circ C$  for biochemical analysis.

Brain concentrations of ATP and PCr were determined by a coupled enzyme reaction (10). Cerebral cortical neuronal nuclei were isolated according to a modification of the method by Giuffrida *et al.* (11). Cortical tissue was homogenized in 15 volumes of a medium containing 0.32 M sucrose, 10 mM Tris-HCl (pH 6.8), and 1 mM  $MgCl_2$ , filtered through nylon cloth (mesh 100), and centrifuged at  $850 \times g$  for 10 min. The pellet was resuspended and mixed with a medium containing 2.4 M sucrose, 10 mM Tris-HCl (pH 6.8), and 1 mM  $MgCl_2$  to achieve a final concentration of 2.1 M sucrose. The nuclei were purified by centrifugation at  $53,000 \times g$  for 60 min. Purity was assessed by phase-contrast microscopy. This method of neuronal nuclei isolation yields a 90–95% pure preparation (12).

DNA was isolated according to the method described by Higuchi and Linn (13). Cortical nuclei were centrifuged and resuspended in 50 mM Tris-HCl (pH 8.0), 100 mM EDTA, and 0.5% SDS, then incubated with 10 mg/mL proteinase K at  $55^\circ C$ . The digest was extracted with phenol (equilibrated with Tris pH 8.0), the phases were separated by centrifugation, and

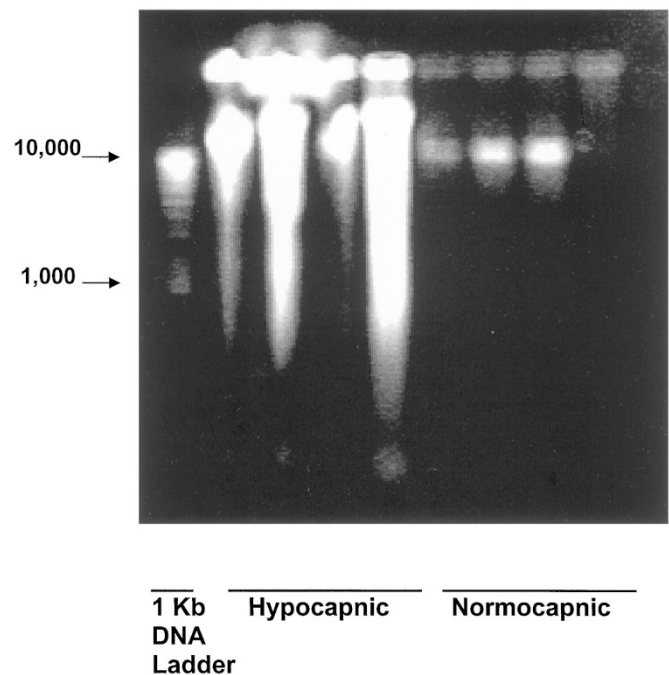
the aqueous phase was isolated. The extraction was repeated with phenol–chloroform (1:1), the digest was shaken and centrifuged, and the aqueous phase was removed. DNA was precipitated with 3 M sodium acetate (pH 6.0) and 100% ethanol at room temperature, then pelleted and washed with 70% ethanol. DNA was air-dried overnight and suspended with 10 mM Tris (pH 8.0)–1 mM EDTA. DNA content was measured spectrophotometrically by absorbance at 260 nm, and purity was confirmed by a ratio of >1.7 at 260/280 nm.

Nuclear DNA (0.2–0.5  $\mu$ g) was dissolved in gel loading buffer [0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% (vol/vol) glycerol], and separated on a 1% agarose gel in Tris-boric acid-EDTA (TBE) buffer (89 mM Tris boric acid, 2 mM EDTA, pH 8.0). After electrophoresis the gel was stained with ethidium bromide (0.5  $\mu$ g/mL in TBE) and analyzed by Gel Doc-1000 system (Bio Rad, Hercules, CA). A ready load 1-kb DNA ladder was used as a molecular weight standard. The densities of the DNA fragments were assessed using Molecular Analyst (BioRad, Hercules, CA) and expressed as the  $OD \times mm^2$ .

Statistical analysis between the two groups was performed by *t* tests. A *p* value <0.05 and an *r* value >0.05 were considered statistically significant. Analysis of Figure 2 was performed using regression analysis and the best-curve fitting program of SigmaPlot/SigmaStat (Jandel Scientific, San Rafael, CA).

## RESULTS

Baseline physiologic and blood measurements were similar in both the baseline H and C groups as shown in Table 1. After hypocapnia there was a significant increase in pH and decrease in  $P_{aCO_2}$  in the H group compared with the C group (*p* < 0.001)



**Figure 1.** Gel electrophoresis of cortical DNA fragmentation in hypocapnic and normocapnic newborn piglets using a standard 1-kb DNA ladder.

**Table 1.** Physiologic data of normocapnic and hypocapnic newborn piglets

Groups	pH	Paco <sub>2</sub> (mm Hg)	PaO <sub>2</sub> (mm Hg)	Mean			
				BP (mm Hg)	Heart rate (bpm)	Lactate (nmol/dL)	Glucose (mM)
Normocapnic	7.45 ± 0.03	40 ± 2	92 ± 7	70 ± 7	190 ± 40	1.6 ± 0.5	6.5 ± 0.7
Hypocapnic baseline	7.45 ± 0.04	41 ± 3	103 ± 15	71 ± 12	171 ± 27	1.9 ± 0.5	6.4 ± 1.2
Hypocapnic experimental	7.72 ± 0.04*	20 ± 1*	92 ± 9	69 ± 10	253 ± 40*	2.3 ± 0.6*	6.3 ± 0.8

Mean ± SD.

Abbreviation used: BP, blood pressure.

\*  $p < 0.05$  vs normocapnic and hypocapnic baseline.

and H baseline period ( $p < 0.01$ ). There was also a significant increase in serum lactate and heart rate in the H group compared with the C group ( $p < 0.01$ ) and the H baseline period ( $p < 0.01$ ). However, there was no significant difference in mean blood pressure or glucose during hypocapnia.

Tissue PCr levels were 38% lower in the H group than the C group ( $2.32 \pm 0.66$  versus  $3.73 \pm 0.32$   $\mu\text{mol/g}$  brain,  $p < 0.05$ ), but ATP levels were preserved ( $4.6 \pm 0.5$  in the H group versus  $4.6 \pm 0.3$   $\mu\text{mol/g}$  brain in the C group).

Unlike normocapnic piglets, hypocapnic piglets displayed a smear pattern of small molecular weight fragments between 100 and 12,000 bp (Fig. 1). The density of DNA fragments was eight times greater in the H group than the C group ( $1821 \pm 1229$  versus  $229 \pm 102$  OD  $\times$  mm<sup>2</sup>,  $p < 0.05$ ). DNA fragmentation varied inversely with levels of PCr ( $r = 0.93$ ; Fig. 2).

## DISCUSSION

These data demonstrate that moderate hypocapnia of 1 h duration results in a 38% decrease in brain tissue PCr levels and fragmentation of nuclear DNA in the cerebral cortex of newborn piglets. The decrease in tissue PCr levels indicates that there was a decrease in tissue oxygenation in the cerebral cortex during hypocapnia. In our previous studies we have shown that newborn piglets with lower Paco<sub>2</sub> levels (9–11 mm Hg) for 1 h had a reduction in tissue PCr values by 80% (14). Thus, the degree of reduction of tissue high-energy phosphates may correlate with the severity of hypocapnia. During ischemia-induced hypoxia, the storage form of high-energy phosphates, PCr, is used before ATP to preserve cellular function

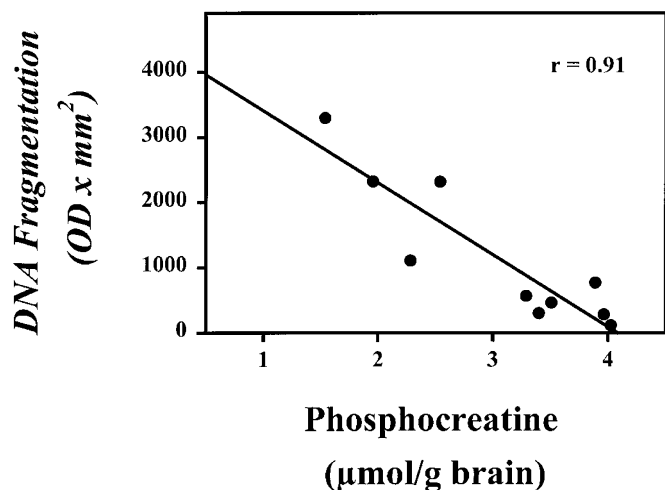
(15). As a result, it is not until tissue PCr levels have been almost fully used that ATP levels decrease. In our present study the ischemia-induced hypoxia may not have been severe or long enough to decrease tissue ATP levels.

Hypocapnia may decrease cerebral tissue high-energy phosphates by reducing CBF (3), thereby decreasing oxygen and nutrient delivery to the brain. In our piglet model hyperventilation to a Paco<sub>2</sub> of 16 mm Hg decreases CBF by 40% (16). Cerebral oxygenation may be further limited during hypocapnia as alkalosis shifts the oxyhemoglobin dissociation curve to the left and decreases tissue release of oxygen (3). Brain lactate levels are increased during hypocapnia and are proportional to Paco<sub>2</sub> levels (17), again suggesting that hypocapnia induces cerebral hypoxia. However, brain lactate production is also tissue pH-dependent, and as pH increases, lactate production increases independent of oxygen availability, thus brain and serum lactate levels may reflect tissue oxygenation or tissue pH (18). In the present study the piglets had an increase in serum lactate levels and heart rate during hypocapnia. This may be related to hypocapnia-induced peripheral vasoconstriction. It is possible that these changes may be caused by decreased cardiac output from alterations in the ventilator strategy used. But inasmuch as arterial blood pressures were constant, the increase in ventilator rate used to induce hypocapnia was not thought to decrease cardiac output.

In our study there was a correlation between DNA fragment density and tissue PCr levels (Fig. 2) indicating that as tissue oxygenation decreased there was increased fragmentation of nuclear DNA. The linear correlation of PCr and DNA fragmentation does not appear to reflect a direct effect of tissue PCr levels on DNA fragmentation but rather reflects the trend that as tissue hypoxia worsens there is increased fragmentation of DNA. The increase in DNA fragmentation may be caused by increased intranuclear Ca<sup>2+</sup> concentrations, which correlate with tissue PCr values during hypoxia (19).

Cerebral hypoxia results in increased intracellular Ca<sup>2+</sup>, which is proportional to the degree of hypoxia (20). Increased intracellular Ca<sup>2+</sup> in turn activates proteases, phospholipases, and nitric oxide synthase (21, 22). Activation of these enzymes results in the generation of oxygen free radicals, peroxidation of nuclear membrane lipids, and activation of endonucleases leading to fragmentation of nuclear DNA (22–24).

Hypocapnia-induced DNA fragmentation may also be the result of increased intranuclear Ca<sup>2+</sup> concentrations as a result of NMDA receptor activation (25, 26). In newborn piglets, severe hypocapnia (Paco<sub>2</sub> 9–11 mm Hg) results in increased



**Figure 2.** Linear correlation of DNA fragmentation and high-energy phosphates in the cerebral cortex of hypocapnic and normocapnic newborn piglets.

activation of the NMDA receptor by spermine and increased NMDA receptor sensitivity to activation by  $Mg^{2+}$  (14). Activation of NMDA receptors leads to increased intracellular  $Ca^{2+}$  flux (20). Intracellular  $Ca^{2+}$  is then transported into the nucleus by the high-affinity  $Ca^{2+}$ -ATPase enzyme and by inositol 1,4,5-triphosphate and inositol 1,3,4,5-tetrakisphosphate receptors (27, 28). Increased intranuclear calcium activates endonucleases, which cut DNA at intranuclear cleavage sites, resulting in DNA fragmentation (29). NMDA receptor activation also results in the generation of oxygen free radicals, which peroxidize nuclear membrane lipids and may result in increased intranuclear  $Ca^{2+}$  and subsequent endonuclease activation, DNA fragmentation, and cell death (21, 22, 24).

Neuronal cell death and DNA degradation have been described as occurring in two patterns—"early" or "necrotic" injury owing to increased  $Na^+$  flux, resulting in cell swelling and lysis, and "late" or "programmed" cell death because of increased intracellular  $Ca^{2+}$  flux leading to the activation of proteases, phospholipases, and endonucleases and the synthesis of apoptotic proteins (30, 31). Early cell death has been associated with random DNA cleavage caused by the breakage of single strands of DNA resulting in a smear-type pattern on gel electrophoresis (31). Late cell death has been characterized by a ladder-type pattern caused by DNA cleavage by endonucleases (31). Complete degradation of DNA by endonucleases results in a ladderlike pattern on gel electrophoresis with fragments 180–200 bp apart because of cleavage at internucleosomal sites (29, 30). During focal ischemic injury in the rat brain, both types of DNA fragmentation have been demonstrated (29).

In our study DNA fragmentation occurred after 1 h of hypocapnia in a smear-type pattern. The time frame (1 h) and smear pattern suggest that fragmentation is caused by the activation of proteases and necrotic cell injury rather than programmed cell death. However, the smear pattern of degradation observed in our study may be caused by either the action of proteases or the incomplete digestion of DNA by endonucleases. When intranuclear  $Ca^{2+}$  increases, nuclear proteases are activated, leading to the digestion of histone proteins. If protease activation precedes the activation of endonucleases, then degradation of histone proteins will allow random access of endonucleases to DNA and result in additional sites for the action of endonucleases and a nonspecific or smear pattern of DNA fragmentation. However, if endonucleases are activated first, before protease activation, they will cleave DNA at specific internucleosomal regions, producing a nucleosome-sized ladder pattern (29). In this study, as in others (30), DNA fragmentation is not a "hallmark" of programmed cell death, but indicates that even during a brief episode of moderate hypocapnia there is damage of nuclear DNA and cortical injury in the newborn brain.

### CONCLUSION

In summary, the data demonstrate that moderate hypocapnia of 1 h duration results in decreased oxidative metabolism and DNA degradation in the cerebral cortex of newborn piglets. We speculate that hypocapnia-induced hypoxia results in the modification of nuclear membranes, leading to increased intra-

nuclear  $Ca^{2+}$  flux, which results in protease and endonuclease activation, DNA fragmentation, and PVL in the newborn brain.

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