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Effect of Post-Hypoxic Reoxygenation on DNA Fragmentation in Cortical Neuronal Nuclei of Newborn Piglets

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Programmed cell death, or apoptosis, is an active process that occurs naturally in the developing brain. Apoptosis has been implicated in several disorders of the central nervous system [1] as well as after a cerebral hypoxic–ischemic (HI) insult [2,3]. HI-induced apoptosis as a common cause of injury in the developing brain is currently a subject of intense investigation. The effect of the focal HI model has been evaluated over many years. HI injury in young and adult rat brains has been shown to induce necrosis and apoptosis, which respectively cause rapid and delayed death of neurons in the ischemic and penumbra regions [4].

Programmed cell death has been well described in a number of organs of the body during various developmental, physiological, as well as pathological states [5,6]. It is characterized morphologically by cellular shrinkage, membrane blebbing, and, in most cases, by the fragmentation of nuclear DNA into multiple segments of approximately 200 bp in length. Among a variety of proteins that are produced in response to cellular injury mediate DNA fragmentation, such as Bax, Fas, Bcl-x_S, and Bak, as well as the anti-apoptotic ones, such as the Bcl-2 and Bcl-x_L [7,8]. Apoptosis is an important mechanism of cell death necessary for fundamental biological process required for normal development, tissue maintenance, and pathogenesis following damage or disease [9]. Cell death following damage/disease is of particular interest because of its direct relation to clinical conditions such as hypoxia leading to neuronal degeneration/death and an effective approach to prevent or limit neuronal damage remains elusive. It is widely believed that development of strategies to prevent or limit neuronal loss following ischemic insult will come from studying apoptotic events using animal model systems.

The nature of the injury to the cell populations at risk is highlighted by the possibility that apoptosis may be triggered in neurons in models of neonatal hypoxia. Apoptotic mechanisms have been identified in the development of the rodent CNS [10]. The identification of apoptotic processes among specific cell populations at risk of ischemic injury might offer potential targets for limiting these types of early CNS injury.

Controversy exists as to the precise mode of cell death after cerebral hypoxia. However, evidence has been mounting that neuronal death, including ischemia-induced death, occurs via apoptosis as well as necrosis. Apoptosis may represent a different mode of cell death in cerebral ischemia, which may have implications for stroke therapies. Necrosis may predominate in more intense forms of ischemic damage, whereas apoptosis may occur in milder forms of ischemic damage [11]. In addition, unlike the acute damage of necrosis, apoptotic injury may take time to develop [12]. Although no universal definition of apoptosis currently exists, characteristics of apoptosis have been described that are distinctly different from necrosis. Recent evidence demonstrates that apoptosis involves the activation of caspases, a unique family of structurally related, highly conserved, aspartate-specific, cysteine proteases that are necessary to carry out the signal for apoptotic cell death [13]. Other markers for apoptosis include biochemical evidence of oligonucleosomal DNA fragmentation into approximately 180-bp multiples resulting from endonuclease activation that can be demonstrated with a typical “laddering” appearance on agarose gel electrophoresis. Despite the existence of several potential markers of apoptosis, an unequivocal definition of apoptosis remains elusive. The combination of evidence of caspase activation, demonstration of apoptotic bodies by EM and ethidium bromide-staining, TUNEL reactivity, and the presence of oligonucleosomal DNA fragmentation as detected by electrophoresis may be sufficient to define apoptosis [14].

DNA injury is subject to alternative interpretations like; DNA nick labeling techniques may suggest processes of DNA scission, but also of DNA repair. Evidence of DNA segmentation by gel electrophoretic methods has been found extremely useful in demonstrating nuclear DNA scission; however, the specific nature of the fragments may be very relevant. MacManus et al [15] have clearly demonstrated that the ends of fragmented DNA appearing in injured brain tissue of rats undergoing focal cerebral ischemia differ from those seen in isolated cells or in cardiac myocytes, which may undergo apoptosis. The morphological criteria for apoptotic injury in the CNS cannot be so readily borrowed from the injuries seen in cells or organs in which apoptosis is common (e.g., thymocytes). The present study tests the hypothesis that DNA fragmentation following hypoxia persists during reoxygenation in cortical neuronal nuclei of newborn piglets.

Studies were performed in 36 anesthetized, ventilated 1- to 3 day old newborn piglets divided into 6 groups: normoxic (Nx), hypoxic (Hx) and hypoxic/reoxygenated for 6, 12, 24 hrs and 7 days. The Hx groups were exposed to 7% oxygen for 1 hr followed by reoxygenation to room air for 6, 12, 24 hrs and 7 days. The piglets were allowed to breathe and eat spontaneously following exposure to hypoxia. All animal procedures were conducted in accordance with NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Drexel University College of Medicine.

Brain concentrations of ATP and PCr were determined by a coupled enzyme reaction according to the method of Lamprecht et al. [16]. Frozen cortical brain tissue was powdered under liquid nitrogen, extracted in 6% (weight by volume) perchloric acid. The extract was thawed on ice and centrifuged at 2000×g for 15 min at 4 °C. The supernatant was neutralized to pH 7.6 by using 2.23 M K₂CO₃/0.5 M triethanolamine/50 mM EDTA buffer and then centrifuged at 2000×g for 15 min at 4 °C. Supernatant (300 µl) was added to 1 ml of buffer (50 mM triethanolamine, 5 mM MgCl₂, 2 mM EDTA, 2 mM glucose, pH 7.6) and 20 µl NADP. Glucose 6-phosphate dehydrogenase (10 µl) was added and the samples were incubated and read after 8 min. Hexokinase (10 µl) was then added, absorbance readings were taken until a steady state was reached and the ATP concentration was calculated from the increase in the absorbance at 340 nm. Next, ADP (20 µl) and creatine kinase (20 µl) were added to the solution. The samples were read every 5 min for 60 min and phosphocreatine concentrations were calculated from the increase in absorbance at 340 nm.

Cerebral cortical neuronal nuclei were isolated according to a modification of the method by Giuffrida *et al* [17]. 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 MgCl₂ in a Dounce type glass homogenizer (200 µm clearance) in 22 strokes. The homogenate was filtered through nylon mesh bolting cloth (mesh 110) and centrifuged at 850×g for 10 min. The resulting pellet was resuspended in homogenizing buffer. The suspension was mixed with a medium containing 2.4 M sucrose, 10 mM Tris-HCl (pH 6.8) and 1 mM MgCl₂ to achieve a final concentration of 2.1 M sucrose, which increases the yield of large neuronal nuclei. The nuclei were then purified by centrifugation at 53000×g for 60 min. All procedures were carried out at 0–4 °C. The above method of neuronal nuclei isolation yields 85–90% pure preparation. The purity of each nuclear preparation was assessed by phase contrast microscopy (Olympus).

DNA was isolated according to the method described by Higuchi and Linn [18]. 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°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 µg) was dissolved in gel loading buffer [0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% (vol/vol) glycerol], and was loaded in each lane and run at 70 V on a 1.25% agarose gel stained with ethidium bromide (0.5 µg/mL). After electrophoresis the gel was stained with ethidium bromide (0.5 µg/mL in TBE) and analyzed by Gel Doc-1000 system (Bio Rad, Hercules, CA). A 1-kb ready load DNA standard (GIBCO-BRL, Gaithersburg, MD) was run in a separate lane with each gel. The densities of the DNA fragments were assessed using Molecular Analyst (BioRad, Hercules, CA) and expressed as the OD / mm². Statistical analysis of the data was analyzed using one-way analysis of variance (ANOVA) test. A *p* value <0.05 was considered statistically significant.

Cerebral tissue hypoxia was confirmed by measuring ATP and phosphocreatine levels. ATP/PCr (µmol/g brain) were 4.11±0.15/3.67±0.30 for Nx, 1.31±0.68/0.74±0.30 for Hx, 3.81±0.11/3.24±0.14 for 6 hr reoxygenation, 4.21±0.12/3.27±0.09 for 12 hr reoxygenation, 4.63±0.09/3.75±0.27 for 24 hr reoxygenation and 4.31±0.12/3.70±0.21 for 7 days reoxygenation. There was a significant difference in the ATP and PCr values between Nx and Hx groups (*p*<0.05 and *p*<0.01 respectively) and between Hx and hypoxic reoxygenated groups (*p*<0.05). The data demonstrate that cerebral tissue hypoxia was achieved in the hypoxic and hypoxic reoxygenated groups, Figure 1. It appears that the cerebral oxidative metabolism has reached a steady state following 6 hours of reoxygenation, reflecting that between 6 and 24 hours no significant dropout of cortical neurons occurs.

The densitometric data on DNA fragmentation (OD/mm²), show increased from 1776±267 in the Nx group to 3211±285 in the Hx group (*p*<0.05). In the reoxygenation groups, DNA fragments (OD/mm²) decreased to 2018±249 after 6 hrs (*p*<0.05 vs. Hx) but increased to 3408±206 after 12 hrs, 2782±406 after 24 hrs and 3256±302 in 7 days respectively (Figure 2). The data demonstrate that hypoxia of 1 hr duration results in an increased DNA fragmentation while there was a decrease in DNA fragmentation in the early phase (6 hrs) of reoxygenation but is comparable to acute hypoxia during the later phases (12, 24 hrs and 7 days) of reoxygenation in the cerebral cortex of newborn piglets.

A number of biochemical and cellular mechanisms have been implicated in neuronal cell death in ischemic and hypoxic-ischemic brain injury. However, unlike those undergoing necrosis, neurons undergoing apoptosis activate a genetic program that is believed to lead to cell death [19]. The goal of our study was to demonstrate the extent of DNA fragmentation in the cerebral cortical neurons in the piglet brain was greater with hypoxia (60 min) preceding reoxygenation (6hr, 12h, 24hr and up to 7days). In this study, DNA fragmentation was observed in neurons exposed to low levels of hypoxia and reoxygenation. Since the pattern of DNA fragments does not necessarily determine the pathway of neuronal cell death the data presented here indicates delayed cell death is either due to apoptosis or necrosis. In our previous studies we have shown that the time course for in situ fragmentation of neuronal DNA varied with the hypoxic severity: as the severity of hypoxia increased, the time elapsed for neurons to exhibit in situ DNA fragmentation decreased [20].

It has been debated as to what constitutes the trigger for delayed cell death in circumstances such as in vivo models of hypoxia-ischemia [21]. These results support the notion that the degree of oxidative injury likely depends in part on the duration of the hypoxic insult. Oxidative injury in the brain have been studied in both hypoxia/reoxygenation and ischemia/reperfusion models [22]. However, the influence of age on the expression of apoptotic proteins and the extent of DNA fragmentation after exposure to hypoxia/reoxygenation has not been extensively studied [23].

Programmed cell death has also been assessed by noting cleavage of genomic DNA and has been shown to occur in the brain following ischemia and oxygen deprivation [24,25]. Two distinct patterns of DNA degradation during hypoxic-ischemic brain cell death have been observed. First, there is random cleavage of the DNA, including nick formation in a single strand that appears as a 'smear' after agarose gel electrophoresis. The second pattern is characterized by double-stranded DNA degradation into oligonucleosomal fragments that appears as a 'ladder' after agarose gel electrophoresis. The ladder pattern of DNA fragmentation has been associated with programmed cell death, although morphologic characteristics of programmed cell death are not always associated with ladder-type DNA fragmentation and similarly ladder-type fragments have been observed in some necrotic cells. Mechanistically, the cleavage of DNA at its internucleosomal linker regions is produced by a specific endonuclease that is Ca^{++} dependent [26].

We have previously demonstrated that nuclear Ca^{++} signals can regulate events that lead to hypoxia-induced neuronal death. The increased intranuclear Ca^{++} may activate Ca^{++} -dependent endonucleases leading to nuclear DNA fragmentation. In addition, the increased intranuclear Ca^{++} can activate Ca^{++} /calmodulin-dependent protein kinase IV (CaM kinase IV) leading to increased phosphorylation of cyclic AMP response element binding (CREB) protein [27] that triggers transcription of apoptotic proteins such as Bax and Bad and initiates early events of caspase-mediated programmed neuronal death. The Bax expression during hypoxia increased as a function of the increase in cerebral tissue hypoxia as measured by cerebral tissue high-energy phosphates. The increased Bax to Bcl-2 ratio may initiate the cascade of activation of initiator caspase-9 through the activation of apoptotic protease activating factor-1 and subsequently activate the executioner caspase-3. Furthermore, the caspase-3-dependent cleavage of caspase-activated Dnase (CAD) and inhibitor of CAD (ICAD) may lead to CAD-mediated fragmentation of nuclear DNA. In addition, increased intranuclear Ca^{++} may activate calcium magnesium-dependent endonucleases, which may lead to DNA fragmentation at internucleosomal sites. Studies from our laboratory have shown that hypoxia results in increased fragmentation of neuronal nuclear DNA and the degree of DNA fragmentation increases with the degree of cerebral tissue hypoxia [20,28]. Since our previous studies have shown that the increased influx of calcium in nuclei and increased expression of proapoptotic

proapoptotic protein mediated fragmentation of nuclear DNA.

In summary, in the present study we have shown that the brain is more vulnerable to hypoxia-reoxygenation induced injury and that a decrease in DNA fragmentation in the early phase (6 hrs) of reoxygenation occurs but is comparable to hypoxia during the later phases (12, 24 hrs and 7 days) of reoxygenation. We propose that the biphasic pattern of DNA fragmentation in reoxygenation occurs by an initial oxidative DNA injury followed by an enzymatic cleavage of DNA by endonucleases activation.

Reference

1. Barinaga M. Is apoptosis key in Alzheimer's disease? *Science* 1998;281:1303–1304. [PubMed: 9735049]
2. Pulera MR, Adams LM, Liu H, Santos DG, Nishimura RN, Yang F, Cole GM, Wasterlain CG. Apoptosis in a neonatal rat model of cerebral hypoxia–ischemia. *Stroke* 1998;29:2622–2630. [PubMed: 9836776]
3. Vannucci RC, Lyons DT, Vasta F. Regional cerebral blood flow during hypoxia–ischemia in immature rats. *Stroke* 1988;19:245–250. [PubMed: 3344541]
4. Hill IE, MacManus JP, Rasquinha I, Tuor UI. DNA fragmentation indicative of apoptosis following unilateral cerebral hypoxia–ischemia in the neonatal rat. *Brain Res* 1995;676:398–403. [PubMed: 7614012]
5. Columbano A. Cell death: current difficulties in discriminating apoptosis from necrosis in the context of pathological processes in vivo. *J. Cell. Biochem* 1995;58:181–190. [PubMed: 7673326]
6. King KL, Cidlowski JA. Cell cycle and apoptosis: common pathways to life and death. *J. Cell. Biochem* 1995;58:175–180. [PubMed: 7673325]
7. Chen J, Graham SH, Nakayama M, Zhu RL, Jin K, Stetler RA, Simon RP. Apoptosis repressor genes Bcl-2 and Bcl-x-long are expressed in rat brain following global ischemia. *J. Cereb. Blood Flow Metab* 1997;17:2–10. [PubMed: 8978381]
8. Kaneda K, Kashii S, Kaeko T, Akaike A, Honda Y, Minami M, Satoh M. Apoptotic DNA fragmentation and upregulation of Bax induced by transient ischemia of the rat retina. *Brain Res* 1998;814:11–20.
9. Kerr JF, Gobe GC, Winterford CM, Harmon BV. Anatomical methods in cell death. *Methods Cell Biol* 1995;46:1–27. [PubMed: 7609651]
10. Nitatori T, Sato N, Waguri S, Karasawa Y, Araki H, Shibana K, Kominami E, Uchiyama Y. Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. *J Neurosci* 1995;15:1001–1011. [PubMed: 7869078]
11. Bonfoco E, Krainc D, Ankarchona M, Nicotera P, Lipton SA. Apoptosis and necrosis: two distinct events induced respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci U S A* 1995;92:7162–7166. [PubMed: 7638161]
12. Linnik MK, Zobrist RH, Hatfield MD. Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. *Stroke* 1993;24:2002–2009. [PubMed: 8248983]
13. Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J. Human ICE/CED-3 protease nomenclature. *Cell* 1996;87:171. [PubMed: 8861900] Letter
14. Collins RJ, Harmon BV, Gobe GC, Kerr JF. Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. *Int J Radiat Biol* 1992;61:451–453. [PubMed: 1349327]
15. MacManus JP, Fliss H, Preston E, Rasquinha I, Tuor U. Cerebral ischemia produces ladder DNA fragments distinct from cardiac ischemia and archetypal apoptosis. *J Cereb Blood Flow Metab* May; 1999 19(5):502–10. [PubMed: 10326717]
16. Lamprecht, W. Creatine phosphate. In: Bergmeyer, HU., editor. *Methods of Enzymatic Analysis*. Academic Press; New York: 1974. p. 1777–1781.
17. Giuffrida AM, Cox D, Mathias AP. RNA polymerase activity in various classes of nuclei from different regions of rat brain during post-natal development. *J Neurochem* 1975;24:749–755. [PubMed: 1123629]

18. Higuchi Y, Linn S. Purification of all forms of HeLa cell mitochondrial DNA and assessment of damage to it caused by hydrogen peroxide treatment of mitochondria or cells. *J Biol Chem* 1995;270:7950–7956. [PubMed: 7713892]
19. Goldberg MP, Choi DW. Intracellular free calcium increases in cultured cortical neurons deprived of O₂ and glucose. *Stroke* 1990;21:III75–III77. [PubMed: 2237988]
20. Akhter W, Ashraf QM, Zanelli SA, Mishra OP, Delivoria-Papadopoulos M. Effect of graded hypoxia on cerebral cortical genomic DNA fragmentation in newborn piglets. *Biol Neonate* 2001;79(34):187–93. [PubMed: 11275649]
21. Mehmet H, Yue X, Squier MV, Lorek A, Cady E, Penrice J, Sarraf C, Wylezinska M, Kirkbride V, Cooper C, Brown GC, Wyatt JS, Reynolds EOR, Edwards AD. Increased apoptosis in the cingulate sulcus of newborn piglets following transient hypoxia–ischemia is related to degree of high energy phosphate depletion during the insult. *Neurosci. Lett* 1994;181:121–125. [PubMed: 7898750]
22. Sutherland GR, Dix GA, Auer RN. Effect of age in rodent models of focal and forebrain ischemia. *Stroke* 1996;27:1663–1668. [PubMed: 8784145]
23. Didier M, Bursztajn S, Adamec E, Passani L, Nixon RA, Coyle JT, Wei JY, Berman, S.A. DNA strand breaks induced by sustained glutamate excitotoxicity in neuronal cultures. *J. Neurosci* 1996;16:2238–2250. [PubMed: 8601804]
24. Ferrer I, Tortosa A, Macaya A, Sierra A, Moreno D, Munell F, Blanco R, Squier W. Evidence of nuclear DNA fragmentation following hypoxia–ischemia in the infant rat brain, and transient forebrain ischemia in the adult gerbil. *Brain Pathol* 1994;4:115–122. [PubMed: 8061857]
25. Gillardon F, Lenz C, Waschke KF, Krajewski S, Reed JC, Zimmermann M, Kuschinsky W. Altered expression of Bcl-2, Bcl-X, Bax, and c-Fos colocalizes with DNA fragmentation and ischemic cell damage following middle cerebral artery occlusion in rats. *Mol. Brain Res* 1996;40:254–260. [PubMed: 8872309]
26. Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. *Int. Rev. Cytol* 1980;68:251–306. [PubMed: 7014501]
27. Mishra OP, Ashraf QM, Delivoria-Papadopoulos M. Phosphorylation of cAMP response element binding (CREB) protein during hypoxia in cerebral cortex of newborn piglets and the effect of nitric oxide synthase inhibition. *Neuroscience* 2002;115(3):985–91. [PubMed: 12435435]
28. Ravishankar S, Ashraf QM, Fritz K, Mishra OP, Delivoria-Papadopoulos M. Expression of Bax and Bcl-2 proteins during hypoxia in cerebral cortical neuronal nuclei of newborn piglets: effect of administration of magnesium sulfate. *Brain Res* May 18;2001 901(12):23–9. [PubMed: 11368946]

Figure 1.
Levels of ATP (fig. 1a) and PCr (fig. 1b) in the cerebral cortex of normoxic, hypoxic and post-hypoxic reoxygenated piglets. The data are expressed as mean±S.D.

Figure 2.

The effect of post-hypoxic reoxygenation on DNA fragmentation in cortical neuronal nuclei of newborn piglets. The density (OD/mm²) of DNA fragments is presented on the y axis. The data are expressed as mean±S.D.