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Original Article

Apoptotic Change and NOS Activity in the Experimental Animal Diffuse Axonal Injury Model

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Although nitric oxide (NO) plays an important role in the pathophysiological process of cerebral ischemia or severe traumatic brain injury, its contribution to the pathogenesis of moderate diffuse axonal injury (mDAI) remains to be clarified. The alterations in nitric oxide synthase (NOS) activity and the histopathological response after mDAI was investigated. Forty anesthetized Sprague-Dawley adult rats were injured with a Marmarou's weight-drop device through a Plexiglas guide tube. These rats were divided into 8 groups (control, 1 hr, 2 hr, 3 hr, 6 hr, 12 hr, 24 hr, 48 hr after trauma). The temporal pattern of apoptosis in the adult rat brain after mDAI was characterized using TUNEL histochemistry. In addition, the cDNA for NOS activity was amplified using RT-PCR. The PCR products were electrophoresed on a 2% agarose gel. eNOS activity was not detected, but nNOS activity was expressed after 3 hr and continuously 48 hr after impact, which was approximately double that of the control group at 12 and 24 hr. Subsequently, there was a decrease in activity after 48 hr. The iNOS activity increased dramatically after 12 hr and was constant for a further 12 hr followed by a dramatic decrease below the level of the control group. Significant apoptotic changes occurred 12 and 24 hr. after insult. nNOS and iNOS activity were affected after moderate diffuse axonal injury in a time-dependent manner and there was a close relation between the apoptotic changes and NOS activity. Although the nNOS activity was expressed early, its activity was not stronger than iNOS, which was expressed later.

Key Words: DAI, nitric oxide synthase, apoptosis, TUNEL stain

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INTRODUCTION

Nitric oxide (NO) is a messenger molecule involved in diverse processes in many tissues. For example, NO may be responsible for the antitumor and antibacterial activities of macrophages. In blood vessel, it accounts for the endothelium-derived relaxing factor activity. In the brain, NO mediates the ability of the excitatory neurotransmitter, glutamate, to stimulate the cyclic GMP levels via the N-methyl-D-aspartate (NMDA) receptors. NO is synthesized from the semi-essential amino acid, L-arginine, by a group of isozymes known as NO synthases (NOSs).[1-4] The expanding family of NOS isoforms generally falls into two categories: (i) a constitutive form regulated by Ca^{2+} and calmodulin (cNOS); neuronal NOS (nNOS; type I) and endithelial NOS (eNOS; type III), and (ii) a cytokine-inducible form that is not known to be regulated post-transcriptionally (iNOS; type II).[1,4-8]

Neuronal NOS is primarily expressed in a small population of neurons throughout the central nervous system and the perivascular nerves. The NO produced by nNOS is thought to mediate the synaptic plasticity and neuronal signaling and may play an important role in neuronal injury under pathological conditions.[1,8,9] It has been established that by increasing the Ca^{2+} influx NMDA receptor activation is implicated in the cerebral damage that follows traumatic brain injury (TBI). Furthermore, NO itself may cause neuronal injury by reacting with superoxide (O^{2-}) to form peroxynitrite($ONOO^-$), a highly toxic free radical anion.[10-12]

NO produced by eNOS is believed to maintain a resting cerebral blood flow (CBF) and inhibits

platelet aggregation and leukocyte adhesion.[4,6] This suggests that early eNOS activation might reduce the severity of neuronal damage. However, both eNOS and nNOS produce NO for short periods after NOS activation by calcium/calmodulin.[6,9,13] In the nervous system, astocytes, microglia, vascular smooth muscles, endothelial cells, and macrophages can be induced to express iNOS by variety of cytokines. The iNOS expression in astrocytes and microglia requires protein synthesis, and iNOS produces large quantities of NO for sustained periods.[14-16]

Recent reports suggest that an active process similar to apoptosis may contribute to neuron death following a traumatic insult. In many cases, intranucleosomal DNA fragmentation has been described, supporting the hypothesis that DNA fragmentation may be an important component of the apoptotic cell death process in neurons.[17,18] TUNEL staining and subsequent quantitative analysis provide a tool to localize the DNA fragmentation within the context of the brain architecture.[17,19]

A time course study of NOS activity and apoptotic change after experimental head trauma in rats was undertaken.

MATERIALS AND METHODS

Injury device

The trauma device introduced by Marmarou et al. (Fig. 1)[20,21] was used in this study. The trauma device consisted of a column of brass weights falling freely onto a metallic helmet fixed by dental acrylic to the skull vertex of a rat. In order to obtain high acceleration upon impact, the head was supported lightly allowing rapid displacement after impact. Brass weights, each 50 gm, were threaded so that they could be connected to produce a falling weight ranging from 50 to 500 gm. The weight fell through a 2-m and 1-m vertical sections of a transparent Plexglas tube held in place with a ring stand. The helmet consisted of stainless-steel disc. Previously, a 400 gm-2m impact was considered to be the upper limit for producing severe head trauma. Therefore, the energy delivery was decreased to 50% by reducing the height to 1 m[21] in order to induce moderate head injury in nonventilated animals.

Induction of head trauma

A total of 40 adult Sprague-Dawley rats, each weighing 300 to 350 gm, were anesthetized with an intraperitoneal injection of Nembutal (pentobarbital, 35 mg/kg). The animals were allowed to breathe spontaneously without tracheal intubation. A midline scalp incision was performed followed by periosteal elevation to expose the central area of the skull vault between the coronal and lambdoid sutures. The 1 cm diameter helmet was firmly attached by dental acrylic to the exposed area of the skull vault after drying the area. The animal was placed in a prone position on a foam bed with the disc centered directly under the lower end of the Plexiglas tube of the trauma

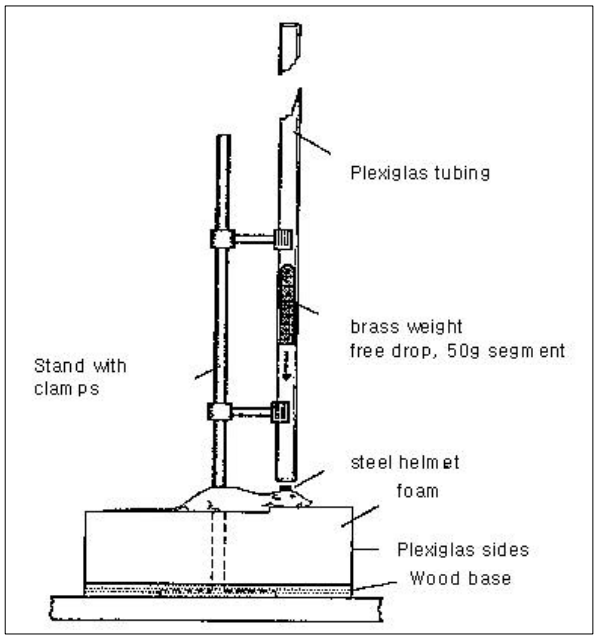

Fig. 1. Diagram of the head-injury device. The upper weight is attached to a string and the segmented brass weights elevated to the desired height. A brass weight was made of 50 gram segments, 18 mm in diameter. The bottom opening of the Plexiglas cylinder is positioned in close proximity to the head of the rat and centered for the mass to strike the helmet directly. The helmet consists of a stainless- steel disc, 10 mm in diameter and 3 mm thick, cemented to the calvaria with a thin layer of dental acrylic. The foam (of known spring constant) is cut to fit in the Plexiglas frame without being compressed. After release of the weight and contact, the Plexiglas frame is removed rapidly to prevent a second impact.

device. Two belts were fastened around the trunk of the rat to prevent it from falling off the foam bed after inducing trauma. The weight was allowed to drop freely from a predetermined height through the Plexiglas tube onto the disc. Simply sliding the foam bed containing the animal away from the tube immediately following the initial impact prevented rebound impact. The scalp was sutured and the rat was then allowed to recover from the anesthesia. Rats that died on impact and those with skull fractures were excluded.

Extraction of brain cerebral rna and reverse transcription-polymerase chain reaction (RT-PCR)

The rats were sacrificed by decapitation at 1 hr, 2 hr, 3 hr, 6 hr, 12 hr, 24 hr, 48 hr after TBI or a sham injury (the control group). Their brains were rapidly removed and immediately frozen by immersion in liquid nitrogen. The brain samples were stored at -80℃ in order to isolate the brain RNAs at a late stage. The frozen brain tissues stored at -80℃ were ground to a fine powder with a mortar and pestle under liquid nitrogen and then homogenized in the presence of a highly denaturing guanidinium isothiocyanate buffer by repeated suction through a 20-gauge needle using a syringe. Ethanol was added to provide an appropriate binding condition for the brain RNA to an RNA binding column where the brain RNAs would bind favorably to the silica-gel based membrane. After the contaminants were washed away, the RNA was then eluted from the column with distilled water containing diethylpyrocarbonate to inhibit the RNase. The purity and concentration of isolated brain RNAs were determined spectrophotometrically. The brain RNAs (1.5 μg) were subsequently reverse transcribed for 30 min at 42℃ in the presence of the viral reverse transcriptase of the avian meyloblastosis (AMV Rtase) and oligo-dT adapt primer (Takara, Shiga, Japan). Each isotype s of the nitric oxide synthase (eNOS, iNOS, nNOS) cDNA fragment was amplified using adequate sense and antisense primers (Fig. 2). To normalize the PCR products in a quantitative manner, β-actin cDNA fragments were used as the control and were amplified with sense and antisense primers (Fig. 3). The PCR amplification was carried out in the reaction mixture containing 4 μl of the single strand cDNAs. Denaturation, annealing, and elongation were performed for 30 cycles at 94℃, 55℃ and 72℃, and for the durations of 40, 40, and 90 seconds, respectively. The PCR products were electrophoresed on a 2% agarose gel (Nusieve 3:1; FMC, Rockland, ME, Fig. 4) containing 1 μg/ml ethidium bromide and were subsequently observed under a UV-transilluminator. An enhanced chemiluminescence method (Pierce, Rockford, IL, U.S.A) was used to visualize the protein bands, and quantification was performed using the image analysis software, Bio-1D (ver.97; Vilber Lourmat, France).[22]

DNA sequencing

To confirm the DNA sequences of the RT-PCR products shown in Fig. 4, direct sequencing of DNA was carried out by the dideoxy chain-termination method using Sequenase version 2.0 T7 DNA polymerase(U.S. biochemicals, Cleveland, OH, U.S.A) and [α-35 S]-labeled dATP (Amersham, Buckinghamshire, UK).

Histological staining (TUNEL staining)

TUNEL staining of the brain sections was performed according to the method reported by Gavrieli et al.[23] Briefly, the brains were removed from the skull and perfusion fixed with 100 ml of 4% paraformaldehyde in phosphate buffered saline (PBS). They were then postfixed for 24 hr at 4℃, and stored in 70% EtOH until processing. The processed brains were embedded in paraffin and cut into 6 μm thick sections. The 6 μm coronal sections at 3.5 - 3.8 mm posterior to the bregma were placed on lysine treated slides and deparaffinized by heating at 70℃ for 10 min. the slides were rehydrated by successive incubation in xylene, 96% EtOH, 90% EtOH, 80% EtOH, and finally water. The nuclei in the tissue sections were stripped of protein by treating with protein K (20 μg/ml) for 15 mim, followed by incubation with a 2% H_2O_2 solution to prevent endogenous peroxidase activation. The slides were rinsed with tap water, immersed in phosphate buffered saline (PBS) for 5 mim and incubated with terminal

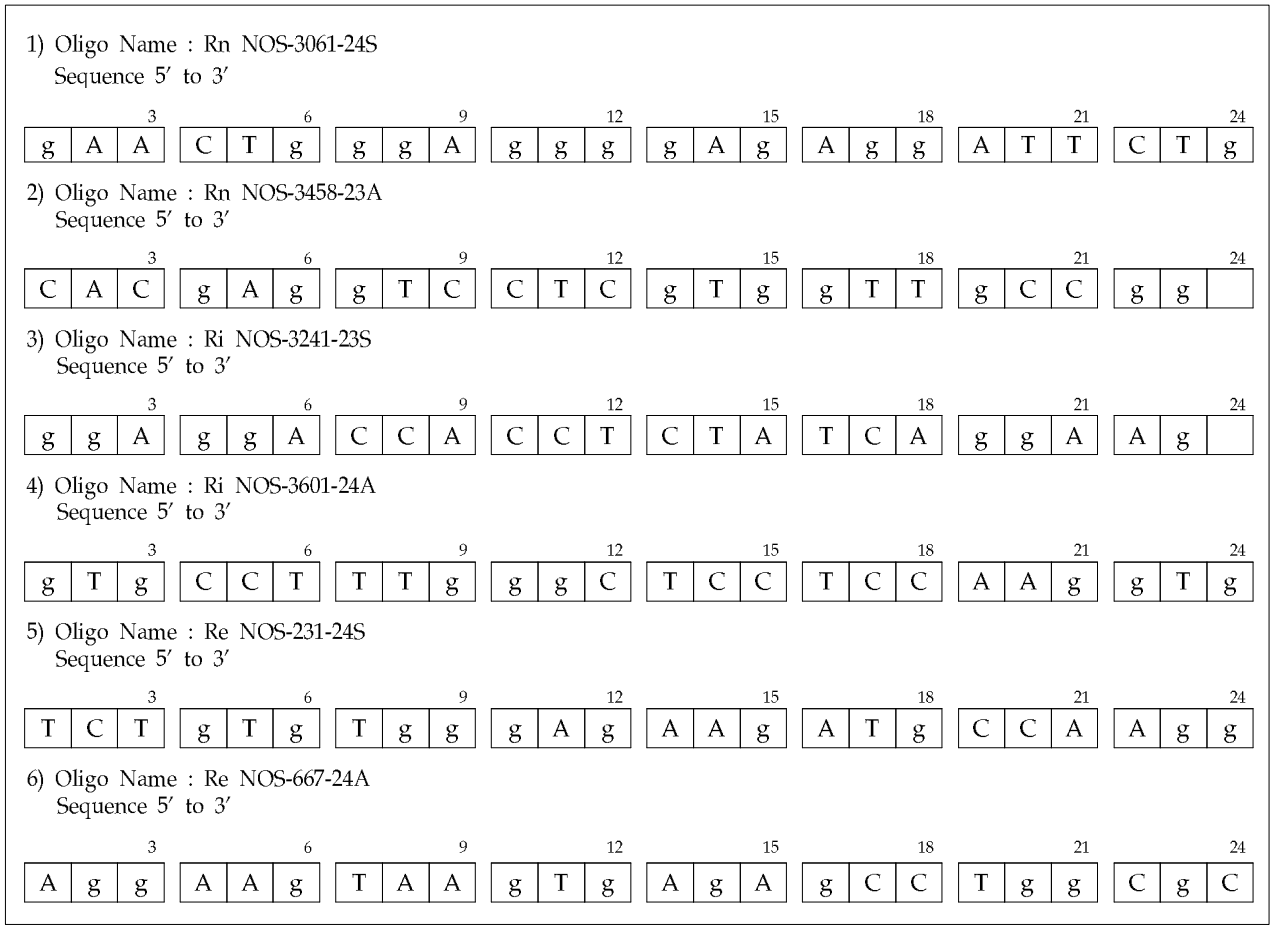

Fig. 2. Showing the Sense Primer Sequence and Antisense Primer Sequence Used for the Amplification of the Rat cDNA for eNOS, nNOS, and iNOS.

a) Code No: 2000110310P01 : Rβ-actin S Grade: PCR
5′-AgA AgA gCT Atg AgC TgC CTg Acg
Base Length: 24 Molecular wt: 7426.9

b) Code No: 2000110310P02 : Rβ-actin A Grade: PCR
5′-CTT CTg CAT CCT gTC Agc gAT gC
Base Length: 23 Molecular wt: 6966.6

Fig. 3. Showing the Sense (a) and Antisense (b) Primer Sequence Used for the Amplication of the β-Actin cDNA as the Control.

deoxynucleotidyl transferase (TdT) and biotin-21-dUTP at 37℃ for 60 min in a humid chamber. After washing with PBS for 5 min, the tissue was mounted in a crystal medium.

Values from the three slides measured in each animal were pooled to determine the total number of cells detected by the TUNEL stain and, in the adjacent sections, for H & E. An average total cell count was calculated from the three slides at each time point. The data was analyzed as the fraction of the total number of cells detected by H & E that stained positively for TUNEL (AI).

$$\text{Apoptotic Index (AI)} = \frac{\text{The number of Apoptotic Cells}}{\text{The total number of cells}} \times 100$$

Statistical analysis

The data is presented as a mean ± S.D. obtained from triplicate samples for each five individual rats and analyzed using SPSS/PC + 9.0. A p value < 0.05 was considered statistically significant.

RESULTS

The assay for detection of NOS activity in the area of brain injury

The equivalent amounts of β-actin from all samples were amplified, standardizing the conditions save for primer pair. The samples without a RNA template or in the absence of RT produced no band.

The time course of the calcium-dependent (cNOS) and calcium-independent (iNOS) activity in the cortical injury is shown in Fig. 4. However, eNOS was not expressed in all samples including the control group. Nevertheless, nNOS activity did increase 3 hr after the induction of brain injury and nNOS activity was not observed to change between 3 and 24 hr after injury (more than 2 times in comparison with control group, p < 0.05), although a peak time of nNOS activity occurred 12 hr after injury. It then showed a decreasing tendency 48 hr after injury. iNOS increased dramatically between 12 hr and 24 hr (more than 5 times in comparison with control group), but dramatically reduced 48 hr after the induction of brain injury(Fig. 5, p < 0.001). nNOS activity was expressed earlier than iNOS activity, but iNOs activity was expressed at between 12 and 24 hr more significantly than nNOS activity

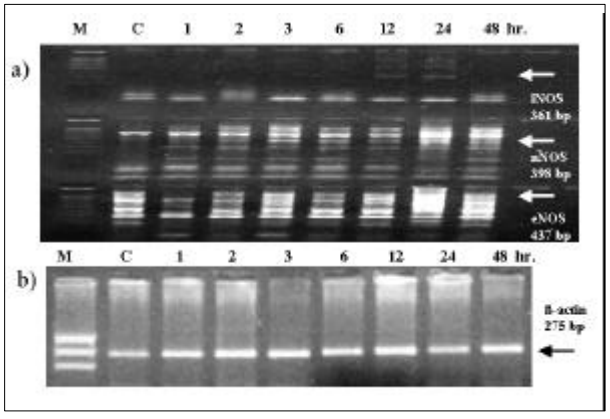

Fig. 4. Electrophorosis of cDNA for eNOS, nNOS, iNOS (a), and β-actin was shown. eNOS was not expressed within 48 hr. nNOS was expressed 3hr after injury, and was sustaining by 48 hr., but iNOS was expressed markedly between 12-24 hr, decreasing dramatically after 48 hr. *bp; base pair.

TUNEL stain

The apoptotic changes in the brain can be observed in as little as 3hr after injury. However, the percentage of apoptotic cells exhibiting positive TUNEL staining increases over time, reaching approximately 25% of the cells at 12-24 hr after injury (Table 1, p < 0.001). Although the number of apoptotic cells did not appear to change between 12 hr and 24 hr after injury (a peak of apoptosis was observed by 24 hr), they appeared to decline 48 hr after injury (p < 0.05, Fig. 6).

DISCUSSION

NOS enzymes were originally classified as being

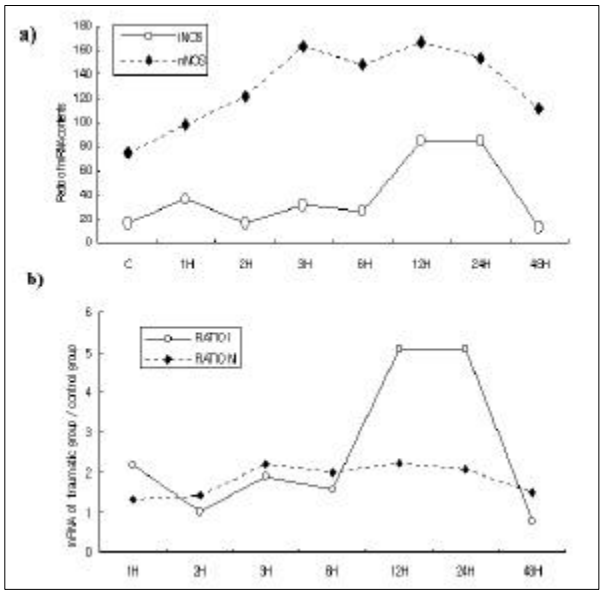

Fig. 5. The ratio of nNOS-mRNA / β-actin mRNA and iNOS-mRNA/ β-actin mRNA expression (a). nNOS-mRNA was expressed about 150% between 3-24 hr. in comparison with β-actin mRNA, but iNOS-mRNA was expressed about 85% between 12-24 hr. In comparison with the control group (b), nNOS-mRNA was expressed more than 2 times, but iNOS-mRNA was expressed more than 5 times.

Table 1. Apoptotic Index (AI) as a Function of Time

Time	Control	1hr	2hr	3hr	6hr	12hr	24hr	48hr
AI (%)	0	0	0	11.8	18.9	24.8	27.5	13.5

* AI was about 25% at 12-24 hr. after Injury, which was a peak time.

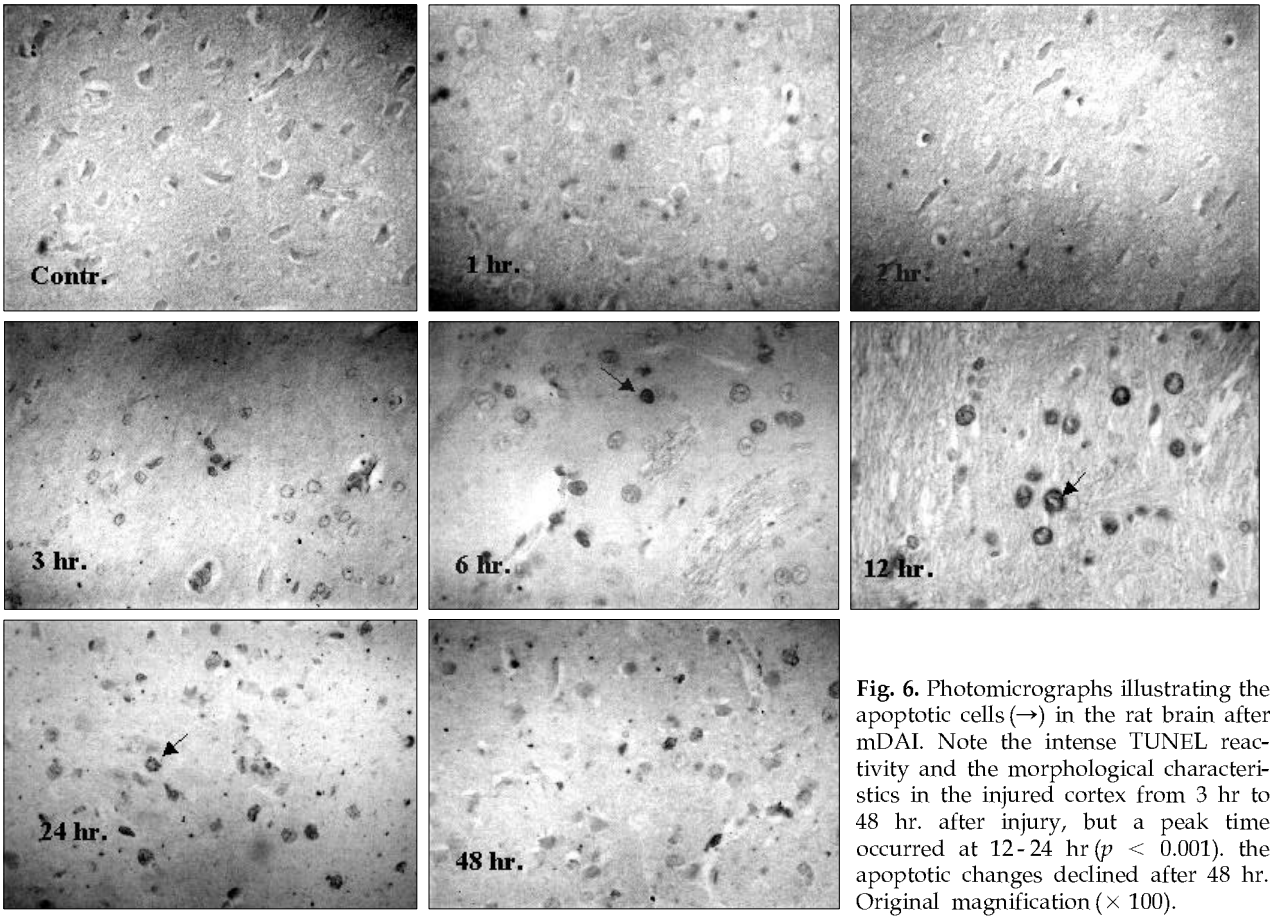

Fig. 6. Photomicrographs illustrating the apoptotic cells (→) in the rat brain after mDAI. Note the intense TUNEL reactivity and the morphological characteristics in the injured cortex from 3 hr to 48 hr. after injury, but a peak time occurred at 12-24 hr ($p < 0.001$). the apoptotic changes declined after 48 hr. Original magnification (× 100).

constitutive or inducible. The neuronal (nNOS, type 1) and endothelial (eNOS, type 3) forms of NOS have been classified as constitutive, in that the activation of these enzymes to produce NO does not require new enzyme protein synthesis. However, both of these NOS isoforms are inducible, in that, primarily under conditions of traumatic or pathological insults, new enzyme protein synthesis does occur. Both eNOS and nNOS produce NO for short periods (several minutes, < 30 min) after activation of NOS by calcium/calmodulin.[2,7,18,24] An increase in the intracellular calcium concentration from 100 to 500 nM changes the rate of NO synthesis from < 5% to > 95% of the maximum.[25] This Ca^{2+} sensitivity is typical for a calmodulin-activated enzyme. Macrophages normally do not contain detectable NOS proteins, but synthesize NOS proteins in response to stimulation by a variety of cytokines. This isoform is now termed immunological or inducible NOS (iNOS, type 2). Once iNOS is induced in macrophages, it produces large quantities of NO for sustained period.[18] Under normal condition, nNOS can be detected in neurons and the perivascular nerves, and eNOS in the cerebrovascular endithelium and in some neurons. In the nervous system, astrocytes, microglia, vascular smooth muscle and endothelial cells can express iNOS upon induction and iNOS expression can be induced by a variety of cytokines.[3,14,25,26] The iNOS

expression in astrocyes and microglia requires protein synthesis, and iNOS produces large quantities of NO for sustained periods (several hours to days). NO may cause cytotoxicity by disrupting mitochondrial respiration due to inhibition of aconitase and the mitochondrial electron transport complex I and II.[24,27] In addition to disrupting the cellular metabolism, NO inhibits DNA synthesis by depressing ribonucleotide reductase activity and damaging the DNA structures by several possible pathways, including DNA nitration, deamination and oxidation.[28] Recently, it has been proposed that DNA damage plays a central role in NO toxicity. NO produced by the astrocytes and microglia may contribute to neurological damage in traumatic or pathological insults.[29]

iNOS and nNOS activity has been reported to develop after moderate brain injury, but no eNOS activity was observed. It was proposed that the reasons for the lack eNOS expression were; 1) this study was started 1 hr after brain injury. However, according to previous reports, eNOS was usually expressed within a few minutes after insult, 2) because traumatic model used in this study was a moderate diffuse axonal injury model, vascular injury was minimal or absent. According to the results in this study, nNOS activity increased from 3 hr after the induction of brain injury, significantly increased after 24 hr and remained attenuated for up to 48 hr after brain injury. However, the iNOS level was much higher after 12 hr, but had dramatically reduced 48 hr after brain injury. This data was some different from previous reported data which showed that iNOS is continuously active and leads to long-lasting NO generation compared to calcium-dependent NO synthesis lasting a few minutes.[2,6,30] It was believed that because astrocyte, microglia, and macrophage normally contain very small amounts of the iNOS protein, they may not be induced enough to express iNOS by moderate brain insult. Therefore, iNOS activity may be dramatically increased from 12 hr to 24 hr after injury after which they rapidly return to levels observed in the control group. In this study nNOS activity was expressed earlier as reported by others,[1,2,6,9,13] but for sustained periods (2 days) as opposed to that observed for iNOS activity. The reason for this is unclear. However, because in this moderate injury model the diffuse axonal injury was mainly shown and the vascular injury was minimal or there were a few invading inflammatory cells in the injured cortex, it is suspected that iNOS expression may not be continuously active when compared to nNOS. In addition, it is believed that up-regulation of nNOS and its mRNA may occur, reaching a maximum in 12 hours and continuing for up to 24 hours after brain injury. Zhang et al.[29] reported that the up-regulation of nNOS and mRNA also occurs, reaching a maximum after 4 hr and continuing for up to 7 days after permanent MCA occlusion. Buttery et al.[3] reported that iNOS activity was demonstrating widespread induction of the enzyme at its optimum at around 6 hours after the insult and the expression of the enzyme then decreases with time and is markedly reduced by 24 hrs. This reduction in the number of cells that expressed iNOS by 24 hrs was accompanied by the development of granulomatous lesions in the liver of endotoxin-treated rats. If this model was the severe diffuse axonal injury model, which included vascular injury or a vascular infarction model, it is believed that the NOS activity would show different results from those in this study.

The neuronal death that accompanies an ischemic stroke or trauma has previously been attributed to passive necrotic or apoptotic processes. It has been suggested that traumatically induced cell death is primarily necrotic in nature and is characterized by a swelling of the nucleus and cytoplasmic organelles as well as an early loss of plasma-membrane integrity and cell lysis. In contrast to necrosis, apoptosis has been defined as a combination of morphological and biochemical criteria. Apoptosis is characterized by cell shrinkage, chromatin condensation, and the formation of apoptotic bodies. However, these latter two features are not prominent. Nevertheless, the biochemical definition of apoptosis may include any or all of the following three conditions: expression of cell death specific proteins, a dependency on macromolecular synthesis, and the endonucleolytic degradation of DNA into nucleosomal fragmentation.[17,31-33]

Apoptosis is associated with normal CNS development and specific pathological conditions in the CNS, including Alzheimer's, and Huntington's

disease, cerebral ischemia, and brain trauma. In many cases with programmed cell death in cultured neurons and neuronal cell lines, intranucleosomal DNA fragmentation has been described, which supports the idea that DNA fragmentation may be an important component and a step leading to the apoptotic cell death process in neurons.[17,31-33]

The question remains as to whether DNA fragmentation is causal in the ischemic death of neurons or whether it exists as a "tombstone" to mark those cells which will be deleted. Pharmacological studies have demonstrated that the endonuclease inhibitor, aurintricarboxylic acid, can reduce excitotic death *in vitro*[34] and ischemic damage *in vivo*.[35] This suggests that DNA fragmentation may be a causal factor in ischemic neuronal death.

Both the TUNEL staining and subsequent quantitative analysis provide a tool to localize DNA fragmentation within the context of the brain architecture. Linnik et al.[17] reported that a dissection approximated the core of the infarction, the transition zone, and the midline region in the ischemic model and revealed classical intranucleosomal DNA fragmentation. They suggested that this specific fragmentation contributed to the positive TUNEL staining. It also revealed that positive TUNEL staining can be observed as little as 1 hr after the infarction, and the percentage of cells exhibiting positive TUNEL staining increases over time, reaching approximately 23% of cells 24 hr after the infarction. This suggests that DNA fragmentation had spread significantly into the transitional zone. Conti et al.,[31] using TUNEL histochemistry, reported that significant neuronal apoptosis occurs acutely in the first 12 - 24 hr with a second, more pronounced response 1 week after the brain injury. According to our data, although apoptosis was shown to occur in as little as 3 hr after injury, significant apoptotic changes had occurred 12 - 24 hr after brain injury. This suggests that after a brain insult, there is a significant relationship between NOS activity and the apoptotic changes with time ($p < 0.001$).

There are important therapeutic implications for programmed cell death in cerebral ischemia, trauma, and neurodegenerative disorders. If the neuronal death is strictly necrotic, then the cells have a passive role and death is mediated solely by extracellular toxins. In this case, therapeutic approaches have to be directed against the extracellular events that lead to cell death. However, if the cells contribute to their death by apoptotic processes, then one might be able to rescue the neurons from death by intervening in the cell death pathways. The results indicate that NO plays an important role in the pathophysiological process of moderate weight-drop diffuse brain injury. A temporal response of nNOS and iNOS activity was demonstrated in the histopathologically vulnerable brain. In addition, the NO pathway appears to represent an important target for treating TBI and nNOS and/or iNOS inhibitor or the NO precursor may decrease tissue injury.

REFERENCES

1. Bredt DS, Ferris CD, Snyder SH. Nitric oxide synthase regulatory sites; Phosphorylation by cyclic AMP-dependent protein kinase, protein kinase, and calcium /calmodulin protein kinase; identification of flavin and calmodulin binding sites. J Biol Chem 1992;16:10976-82.
2. Ignarro L, Murad F. Nitric oxide. Biochemistry, molecular biology, and therapeutic implication. 1st ed. San Diego: Academic Press; 1995.
3. Knowles RG, Palacios M, Palmer RMJ, Moncada S. Formation of nitric oxide from L-arginine in the Central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. Proc Natl Acad Sci USA 1989;86:5159-62.
4. Palmer RMJ, Ashton DS, Mocada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature Lett 1988;333:664-6.
5. Griffith OW, Stuehr DJ. Nitric oxide synthases: Properties and catalytic mechanism. Annu Rev Physiol 1995;57:707-36.
6. Marletta MA. Nitric oxide: biosynthesis and biological significance. Trends Biochem Sci 1991;14:488-92.
7. Moncada S, Palmer RMJ, Higg EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991;43:109-34.
8. Wada K, Chatzipanteli K, Busto R, Dietrich D. Role of nitric oxide in traumatic brain injury in the rat. J Neurosurg 1998;89:807-18.
9. Dalkara T, Moskowitz MA. Compex role of nitric oxide in cerebral ischemia. Brain Pathol 1994;4:49-57.
10. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. Am J Physiol 1996;271:C1424-C37.
11. Dawson VL, Dawson TM. Nitric oxide neurotoxicity. J Chem Neuroanat 1996;10:179-90.

12. Mesnge C, Verrecchia C, Allix M, Boulu RR, Plotkine M. Reduction of the neurological deficit in mice with traumatic brain injury by nitric oxide synthase inhibitors. J Neurotrauma 1996;13:11-6.
13. Dalkara T, Yoshida T, Irikura K, Moskowitz A. Dual role of nitric oxide in focal cerebral ischemia. Neuropharmacology 1994;33:1447-52.
14. Chao CC, Hu S, Molitor TW, Shaskan EG, Peterson PK. Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. J Immunol 1992;149:2736-41.
15. Fleming I, Gray GA, Schott C, Stoclet JC. Inducible but constitutive production of nitric oxide by vascular smooth muscle cells. Eur J Pharmacol 1991;200:375-6.
16. Iadecola C, Xu X, Zhang F, El-Fakahany EE, Ross ME. Marked Induction of calcium-independent nitic oxide synthase activity after focal cerebral ischemia. J Cereb Blood Flow Metab 1995;15:52-9.
17. Linnik MD, Miller JA, Sprinkle-Cavallo J, Mason PJ, Thompson FY, Montgomery LR, et al. Apoptotic DNA fragmentation in the rat cerebral cortex induced by permanent middle cerebral artery occlusion. Mol Brain Res 1995;32:116-24.
18. MacManus JP, Buchan AM, Hill IE, Rasequinha I, Preston E. Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. Neurosci Lett 1993;164:89-92.
19. Buttery LDK, Evans TJ, Springall DR, Carpenter A, Cohen J, Polak JM. Immunochemical location of inducible nitric oxide synthase in edotoxin-treated rats. Lab Invest 1994;71:755-64.
20. Marmarou A, Foda MA, vanden Brink W, Campbell J, Kita II, Demetradou K. A new model of diffuse brain injury in rats. J Neurosurg 1994;80:291-300.
21. Park CO. the effects of methylprednisolone on prevention of brain edema after experimental moderate diffuse brain injury in rats. Yonsei Med J 1998;39:395-403.
22. Park CS, Baek HM, Chung WG, Lee KH, Ryu SD, Cha YN. Suppression of Flavin-containing monooxygenase by overproduced nitric oxide in rat liver. Mol Pharmacol 1999;56:507-14.
23. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119:493-503.
24. Dawson TM, Dawson VL, Snyder SH. A novel neuronal messenger in brain: the free radical, nitric oxide. Ann Neurol 1992;32:297-311.
25. Knowles RG, Salter M, Brooks SL, Moncada S. Anti-inflammatory glucocorticoid inhibit the induction by endotoxin of nitric oxide synthase in the lung, liver and aorta of the rat. Biochem Biophys Res Commun 1990; 172:1042-8.
26. Murphy S, Simmons MI, Agullo I, Garcia A, Feinstein DI, Galea E, et al. Synthesis of nitric oxide in CNS glial cells. Trends Neurosci 1993;16:323-8.
27. Gross SS, Wolin MS. Nitric oxide: Pathophysiological mechanism. Annu Rev Physiol 1995;57:737-69.
28. Kwon NS, Stuehr DJ, Nathan CF. Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide. J Exp Med 1991;174:761-7.
29. Zhang ZG, Chopp M, Gautam S, Zaloga C, Zhang RL, Schmidt HH, et al. Upregulation of neuronal nitric oxide synthase and mRNA, and selective sparing of nitric oxide synthase-containing neurones after focal cerebral ischemia in rat. Brain Res 1994;654:85-95.
30. Coert BA, Anderson RE, Meyer FB. A comparative study of the effects of two nitric oxide synthase inhibitors and two nitric oxide donors on temporary focal cerebral ischemia in the Wistar rat. J Neurosurg 1999; 90:332-8.
31. Conti AC, Raghupathi R, Trojanowski JQ, Mclntosh T. Experimental brain injury induces regionally distinct apoptosis during the acute and delayed post-traumatic period. J Neurosci 1998;18:5663-72.
32. Gerschenson LE, Rottelo RJ. Apoptosis: a different type of cell death. FASEB J 1992;6:2450-5.
33. Schwartz LM, Smith SW, Jones MEE, Osborne BA. Do all programmed cell deaths occur via apoptosis? Proc Natl Acad Sci USA 1993;90:980-4.
34. Samles SD, Dubinsky JM. Aurintricarboxylic acid protects hippocampal neurons from glutamate excitotoxicity *in vitro*. J Neurochem 1993;61:382-5.
35. Roberts-Lewis JM, Marcy VR, Zhao Y, Vaught JL, Siman R, Lewis HE. Aurintricarboxylic acid protects hippocampal neurons from induced toxicity *in vivo*. J Neurochem 1993;61:378-81.