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GRANT NUMBER DAMD17-97-1-7354

TITLE: Pyridostigmine-Induced Neurodegeneration: Role of Neuronal Apoptosis

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REPORT DATE: October 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*

2. REPORT DATE
October 1998

3. REPORT TYPE AND DATES COVERED
Annual (25 Sep 97 - 24 Sep 98)

4. TITLE AND SUBTITLE
Pyridostigmine-Induced Neurodegeneration: Role of Neuronal Apoptis

5. FUNDING NUMBERS
DAMD17-97-1-7354

6. AUTHOR(S)
Isom, Gary E., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

19990330 136

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Although pyridostigmine is a highly charged molecule, some reports suggest it can penetrate into the brain. To determine whether the drug can cause neurotoxic damage centrally, pyridostigmine was injected into rats, the animals were sacrificed at intervals after drug administration and brains examined histologically. Using TUNEL staining and electron microscopy, apoptotic brain cell destruction was noted in cerebral cortex and at higher doses, cell damage was also noted in striatum and hippocampus. Pretreatment with atropine prevented pyridostigmine-induced brain cell apoptosis, showing the involvement of muscarinic receptors. However, antioxidants did not block pyridostigmine-induced apoptosis suggesting that oxidative mechanisms are not involved. Even up to 30 days after injection of pyridostigmine, apoptotic cell death was still evident in rat cortex. Therefore the cell death process initiated by physostigmine continues long after termination of drug treatment. These observations are important because they implicate physostigmine as a causative factor in the Gulf War Syndrome.

14. SUBJECT TERMS
Gulf War

15. NUMBER OF PAGES
27

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Gary E. Dee 22 Oct 1998

PI - Signature Date

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INTRODUCTION

Pyridostigmine has been used clinically for nearly 50 years for myasthenia gravis, yet recent work shows this drug can disrupt peripheral nerves, both functionally and morphologically. Pyridostigmine induces withdrawal of nerve terminal branches from rat diaphragm and causes structural alterations in mitochondria in these nerves (Hudson *et al.*, 1986). Repeated administration of pyridostigmine over a period of 20 days decreased strength of rat skeletal muscle contraction, attributed to decreased neurotransmitter release (Anderson & Chamberlan, 1988). Thus, peripheral nerves are susceptable to damage by pyridostigmine.

Because of its quaternary structure, pyridostigmine would not be expected to cross the blood brain barrier to act on the CNS (Birtley *et al.*, 1966). However, Loewenstein and Lichtenstein *et al.* (1995) suggested that pyridostigmine can enter the brain since they observed that an Israeli soldier experienced severe CNS-mediated symptoms following pyridostigmine treatment. Furthermore, Friedman *et al.* (1996) reported that in mice exposed to a stress protocol (forced swim) only 1/100th of the usual dose of pyridostigmine was required to decrease brain acetylcholineesterase (AchE) activity by 50%. They also noted increased brain levels of c-fos oncogene and AchE mRNA in these stress conditions following pyridostigmine treatment. It appears that under select conditions, pyridostigmine can undergo distribution to the brain despite its charged chemical structure (Sapolsky, 1998).

The present study was undertaken to evaluate the potential of pyridostigmine to produce apoptotic cell death in the rat brain following subacute exposure. Brain sections were examined 3 hrs after dosing (twice daily for 4 days), and at 5, 10, 20 and 30 days after the last pyridostigmine injection, to detect any continued neurotoxicity.

BODY

EXPERIMENTAL METHODS

Animal treatment

All experimental procedures were carried out under protocols approved by the Animal Care Committee of Purdue University and in accordance with the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Male Sprague-Dawley rats, weighing 200-250g were used (Harlan Sprague Dawley, Indianapolis, IN). To evaluate the acute neurotoxicity, rats were treated with pyridostigmine bromide (Sigma Chemical Co., St. Louis, MO) at doses of 0.25, 0.5, 1.0, 1.5 or 1.85 mg/kg (ip), twice daily for 4 days (4 rats per treatment group). Rats of the control group were given an equal volume of saline (vehicle). Three hours after the last injection, rats were anesthetized with pentobarbital (50 mg/kg, ip) and 2 ml of blood was collected from the pulmonary vein. Transcardial perfusion with 50 ml of saline was followed by perfusion with 150 ml of freshly prepared fixative (for histological analysis with 4% paraformaldehyde in PBS; for EM analysis with TRUMPS solution: 2% paraformaldehyde, 2.5% glutaraldehyde in PBS). Brains were removed and immersed in fixative. To detect delayed toxicity, rats were given pyridostigmine (ip) 1.85 mg/kg twice daily for 4 days. At 5, 10, 20 and 30 days after the last injection of pyridostigmine, brains were harvested and fixed as described above. In other studies, rats were pretreated with atropine (25 mg/kg, ip), N-t-butyl-α-phenyl-nitrone (PBN, 32 mg/kg, ip) or Nω-nitro-D-arginine-methyl ester (L-NAME, 50 mg/kg, ip) before treatment with pyridostigmine.

Measurement of serum cholinesterase (ChE) activity

Serum was separated by centrifugation and ChE activity was determined by the method of Ellman *et al.* (1961) in which butyrylthiocholine (BTC) was used as the substrate. ChE hydrolyzes BTC to yield thiocholine which in turn reacts with 5,5-dithiobis-2-nitrobenzoic acid to form 5-thio-2-nitrobenzoate which has an absorbance maximum at 405 nm. The rate of change in absorbance at 405 nm is directly proportional to ChE activity.

Detection of DNA fragments by TUNEL staining

The terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end-labeling (TUNEL) technique was performed on paraffin-embedded brain sections as previously described (Gavrieli *et al.*, 1992; Nitatori *et al.*, 1995) (Apotag *in situ* apoptosis detection kit; Oncor, Gaithersburg, MD). Briefly, after deparaffinizing by washing with xylene and ethanol followed by digesting protein by proteinase K (20 μg/ml) for 20 min at room temperature, brain sections were preincubated in equilibration buffer containing 0.1 M potassium cacodylate (pH 7.2), 2 mM $CoCl_2$, and 0.2 mM dithiothreitol for 10 min at room temperature and then incubated in TUNEL reaction mixture (containing 200 mM postassium cacodylate (pH 7.2), 4 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 30 μM biotin-16-dUTP, and 300 U/ml TdT) in a humidified chamber at 37°C for 1 hr. After incubating in stop/wash buffer for 10 min, the elongated digoxigenin-labeled DNA fragments were visualized using anti-digoxigenin peroxidase antibody solution followed by staining with DAB/H_2O_2 (0.2 mg/ml diaminobenzidine tetrachloride and 0.005% H_2O_2 in PBS, pH 7.4).

Detection of apoptosis using fluorescence

For fluorescence immunohistochemical detection and quantitation of apoptosis, rat brain sections were made as before. After deparaffinizing and hydrating the sections, a commercial kit for *In Situ* Cell Death Detection (Boehringer Mannheim) was used. Briefly, specimens were rinsed with PBS and covered with a reaction mixture containing terminal deoxynucleotidyl transferase A(TdT) and fluoresein-deoxyuridine triphosphate (dUTP). Sections were incubated in a humidified chamber for 1 hr at 37°C. Reactions were terminated by rinsing the sections with PBS and then sections were mounted with glass coverslips using permount and fluorescence was observed using a fluorescent microscope. Positive controls were obtained by pretreating brain sections with 10 μg/ml DNase at 37°C for 5 min.

Electron microscopic (EM) analysis of apoptotic cells

Brains were fixed and harvested as before and approximately 1 mm cubes were removed from cortex, striatum, hippocampus and substantia nigra. The cubes were postfixed in 2% OsO_4 overnight and then dehydrated in an ethanol series. Pieces were embedded in Epon 8-10, cut into 60-90 μm sections using a microtome and mounted on grids. Sections were stained for 30 min in 2% uranyl acetate and examined by transmission electron microscopy at a magnification of 6,500-10,000. Apoptotic cells were characterized by chromatin margination to nuclear membrane, chromatin clumping and shrinkage of cell cytoplasm.

Electrophoretic detection of DNA fragmentation

To confirm DNA fragmentation we used gel electrophoresis to detect DNA laddering. DNA was isolated from fresh rat brains (cortex, striatum and hippocampus) using the method described by Thomaidou *et al.* (1997). Briefly, the tissue was homogenized in extraction buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 0.5% SDS) containing 50 μg/ml RNase I. After incubation for 1 hr at 37°C, 100 μg/ml proteinase K was added, and the samples were left at 50°C for 3 hr. The DNA was extracted with phenol/chloroform (24:1) and precipitated overnight in absolute alcohol containing 0.3 M sodium acetate at 20°C. After centrifugation, the pellet was washed in 70% ethanol and resuspended in buffer (0.1 M Tris-HCl, pH 8.0, and 10 mM EDTA). DNA samples, about 2.5 μg each, were separated electrophoretically on 1.5% agarose gels containing ethidium bromide (0.4 μg/ml) and viewed with UV transillumination.

RESULTS

Effect of pyridostigmine on AChE

Pyridostigmine (0.25-1.85 mg/kg ip twice daily for 4 days) significantly decreased serum AChE at all dose levels (Fig. 1). When the dosage of pyridostigmine was increased to 1.85 mg/kg, the serum AChE activity was reduced to 62% of control. At time intervals after cessation of pyridostigmine administration (5, 10, 20 and 30 days) serum AChE activity was not different from control (data not shown). This demonstrates AChE activity returns to control levels before day 5 after pyridostigmine administration and pyridostigmine produces no residual inhibition of the enzyme.

Induction of acute apoptosis by pyridostigmine

TUNEL staining is a sensitive method which detects DNA fragmentation *in situ* and is used as a marker for cells undergoing apoptosis (Gavrieli *et al.*, 1992). Three hours after the last injection of pyridostigmine (1.85 mg/kg, ip twice daily for 4 days), 3 of 4 rats exhibited extensive apoptosis in the cortex, striatum and hippocampus (Fig. 2). When the rats received 1.5 mg/kg twice daily for 4 days, 3 of 4 rats again exhibited apoptotic cell death only in cortex and striatum. At 1.0 or 0.5 mg/kg (twice daily for 4 days) only 1 of 4 animals exhibited

TUNEL staining that was limited to the cortex (Fig. 3). The fluorescent TUNEL technique produced similar results (not shown).

Prolonged pyridostigmine-induced apoptosis

Rats were sacrificed 5, 10, 20 and 30 days after the last pyridostigmine treatment (4 days of 1.85 mg/kg pyridostigmine) and the fluorescence-TUNEL procedure was used to detect apoptotic cell death. At each post-treatment time, cortical apoptosis was detected. This cell death was restricted to the cortex (Fig. 4 & 5). It appears that pyridostigmine initiates a programmed cell death process which continues in the cerebral cortex after cessation of treatment. It is important to note that the TUNEL technique only detects active apoptosis, since apoptotic cells are cleared rapidly from the tissue and are not detectable after a few hrs (Bursch *et al.*, 1990).

Electron microscopy

To confirm that apoptosis was induced by pyridostigmine, transmission electron microscopy was used. An increased incidence of ultrastructural changes characteristic of apoptosis (Fig. 6) (chromatin margination to the nuclear membrane, chromatin clumping and cytoplasmic condensation) was observed within 3 hrs after pyridostigmine treatment (1.85 mg/kg, twice daily for 4 days) and at 5, 10, 20 and 30 days following treatment.

The effect of atropine, PBN and L-NAME on pyridostigmine-induced apoptosis

Atropine treatment (25 mg/kg) 30 min before each pyridostigmine dose, blocked the apoptotic response so that cortical and striatal sections did not display TUNEL staining (Fig. 7D & F). These data were quantitated by counting the number of apoptotic figures in the tissue sections and are depicted in Fig. 8; the strong blockade of physostigmine-induced apoptosis by atropine is clearly seen. However, pretreatment of rats with PBN, an antioxidant which can cross the blood brain barrier, and L-NAME, nitric oxide synthase inhibitor, did not alter the neurotoxic response to pyridostigmine (not shown) despite a report that cholinesterase inhibitors increase lipid peroxidation (Yang & Dettbarn, 1996).

DNA laddering

Electrophoresis of DNA taken from brains of pyridostigmine-treated rats did not show distinct nucleosome ladders typical of DNA fragmentation, possibly because the fragmentation of genomic DNA which did occur was below the detection limit of the agarose gel electrophoresis method. Piantadosi *et al* (1997) had similar difficulty in clearly demonstrating laddering in brains of rats treated with carbon monoxide even though apoptosis was easily demonstrated by TUNEL staining.

DISCUSSION

This study demonstrates that pyridostigmine can induce a dose-related apoptosis in which the cortex is the most sensitive brain area. At the higher dose (1.85 mg/kg) apoptotic cells were detected within striatum and hippocampus as well as in cortex. The distribution of apoptotic cells was not even and was more concentrated in certain areas within the cortex, striatum or hippocampus presumably associated with cholinergic innervation. It appears that the apoptotic response to pyridostigmine continues for up to 30 days after exposure to pyridostigmine, even though AChE activity returns to control levels when drug administration ceases.

Atropine, an antagonist of muscarinic receptors, blocked the neurotoxicity of pyridostigmine, which confirms that accumulation of acetylcholine and excessive activation of muscarinic receptors in the brain is a key step in pyridostigmine-induced neuronal apoptosis.

PBN and L-NAME did not reduce neuronal apoptosis induced by pyridostigmine, which suggests that the neurotoxicity of pyridostigmine is not mediated by reactive oxygen species.

In response to intense cholinergic stimulation, cell systems adjust genetically to compensate. Levels of cholinesterase increase and enzymes involved in acetylcholine synthesis decrease. In the short term, these genetic changes appear to be beneficial since they were associated with a quieting of electrical activity which was observed in mouse brain corticohippocampal slices following anticholinesterase treatment (Kaufer *et al.*, 1998). In the long term, however, these genetic changes may cause continued apoptotic neurodegeneration (Beeri *et al.*, 1995).

Programmed cell death continued for 30 days in the rats after termination of pyridostigmine treatment in the present study. This was not due to slow removal of cells dying by apoptosis since such cells are generally removed in a few hours (Bursch *et al.*, 1990). Thus pyridostigmine appears to initiate the cell death process which then continues on for an extended time after termination of pyridostigmine dosing. Most likely, pyridostigmine penetrates into the brain, blocks cholinesterase, to allow acetylcholine accumulation and the intense cholinergic stimulation leads to genetic changes in factors controlling acetylcholine synthesis and also to a delayed neuronal cell death.

Many military personnel involved in the Persian Gulf War have complained of neurological symptoms of unknown etiology. The symptoms include headache, loss of memory, depression, anxiety, cognitive dysfunction and chronic fatigue (The Iowa Persian Gulf Study Group, 1997). Abou-Donia *et al.* (1996, 1996a) have suggested that the combined exposure to pyridostigmine (to protect against nerve gas), DEET (insect repellant) and permethrin (insecticide) or chlorpyrifos (insecticide) contributed to the Gulf War Syndrome.

Individually these agents reportedly showed little toxicity but together they were thought to overwhelm liver and plasma esterases leading to decreased breakdown and increased transport to nervous tissues. Present studies suggest that pyridostigmine alone may be responsible for some of the symptoms of the Gulf War Syndrome.

It is possible that peripheral actions of pyridostigmine may contribute to the harmful effects on the brain. Muscle hyperactivity with lactate formation, ATP exhaustion centrally (Lea *et al.*, 1996; Richter *et al.*, 1996) and depletion of antioxidants may occur to exaggerate any direct action on brain neurons. Thus complex actions of pyridostigmine may be contributing factors in The Gulf War Syndrome.

CONCLUSIONS

1) Despite its charged chemical structure which would be expected to prevent penetration into the brain, pyridostigmine causes apoptotic neural degeneration in rat brain cortex. Higher doses are needed to cause similar degeneration in rat brain striatum and hippocampus.

2) The apoptotic neural damage caused by pyridostigmine in rat brain cortex is a continuing process which persists for at least 30 days after cessation of pyridostigmine administration.

3) Atropine, a muscarinic receptor antagonist, blocks pyridostigmine-induced apoptotic brain injury showing that excessive stimulation of muscarinic receptors is essential to the apoptotic neural degeneration caused by pyridostigmine.

4) Oxidative processes do not appear to play an important role in pyridostigmine-induced brain damage since the antioxidants "PBN" and "L-NAME" do not block the effect.

5) It is possible that some of the symptoms of the Gulf War Syndrome are related to apoptotic brain injury caused by pyridostigmine taken by military personnel as a preventative against nerve gas intoxication.

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FIGURE LEGENDS

Figure 1. Effect of pyridostigmine (0.25-1.85 mg/kg) twice daily for 4 days on AChE activity of rat blood. Three hrs after the last injection of pyridostigmine, blood was collected and serum was separated for detecting AChE activity.

Figure 2. TUNEL-stained paraffin-imbedded sections from brains of rats treated with a high dose of pyridostigmine. (A, C and E) Saline control of cortex, hippocampus and striatum respectively. (B, D and F) Pyridostigmine treated (1.85 mg/kg twice daily for 4 days) cortex, hippocampus and striatum.

Figure 3. TUNEL-stained paraffin-imbedded sections from brains of rats treated with low doses of pyridostigmine. (A and B) pyridostigmine treated (1.0 and 1.5 mg/kg twice daily for 4 days) cortex. (C) Pyridostigmine treated (1.5 mg/kg twice daily for 4 days); striatum. Arrows indicate apoptotic figures.

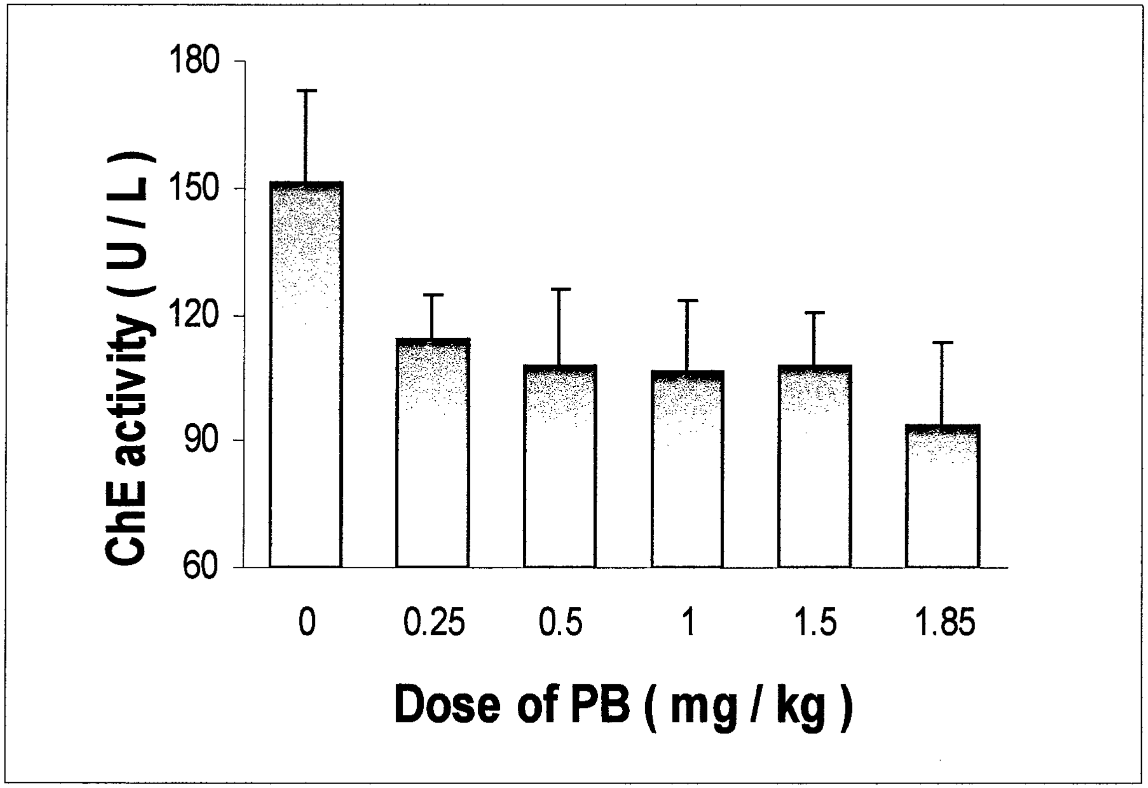
Figure 4. Prolonged effect of pyridostigmine on *in situ* DNA fragmentation detected by TUNEL staining. (A) Normal cortical section (saline), cortical sections after treatment with pyridostigmine (1.85 mg/kg twice daily for 4 days) followed by a drug free period of 5 (B), 10 (C), 20 (D) and 30 (E) days after the last pyridostigmine dose. F = positive control DNAse pretreatment.

Figure 5. Electron micrographs of rat cerebral cortex after 4 days of pyridostigmine administration. (A) Normal cortical cell, (B) pyridostigmine treated (1.85 mg/kg twice daily for 4 days) cortex. Magnification is 6000.

Figure 6. Electron micrographs of rat cerebral cortex 30 days after pyridostigmine administration. (A) Normal cortical cell, (B) apoptotic cortical cell 30 days after last pyridostigmine treatment (1.85 mg/kg, twice daily for 4 days).

Figure 7. Atropine blockade of pyridostigmine-induced apoptosis in rat brain fragmentation detected by TUNEL staining. (A) Normal brain (saline), (B) positive control brain section treated with DNase I, (C and E) the cortical and striatal sections from rats treated with pyridostigmine (1.85 mg/kg twice daily for 4 days), (D and F) cortical and striatal sections from rats pretreated with atropine (30 min pretreated with 25 mg/kg, ip) before pyridostigmine (1.85 mg/kg twice daily for 4 days).

Figure 8. Effect of atropine (AT) on apoptosis in rat brain induced by pyridostigmine (PB). *Indicates significant difference from pyridostigmine alone at the 0.001 level.


ChE activity (U / L)
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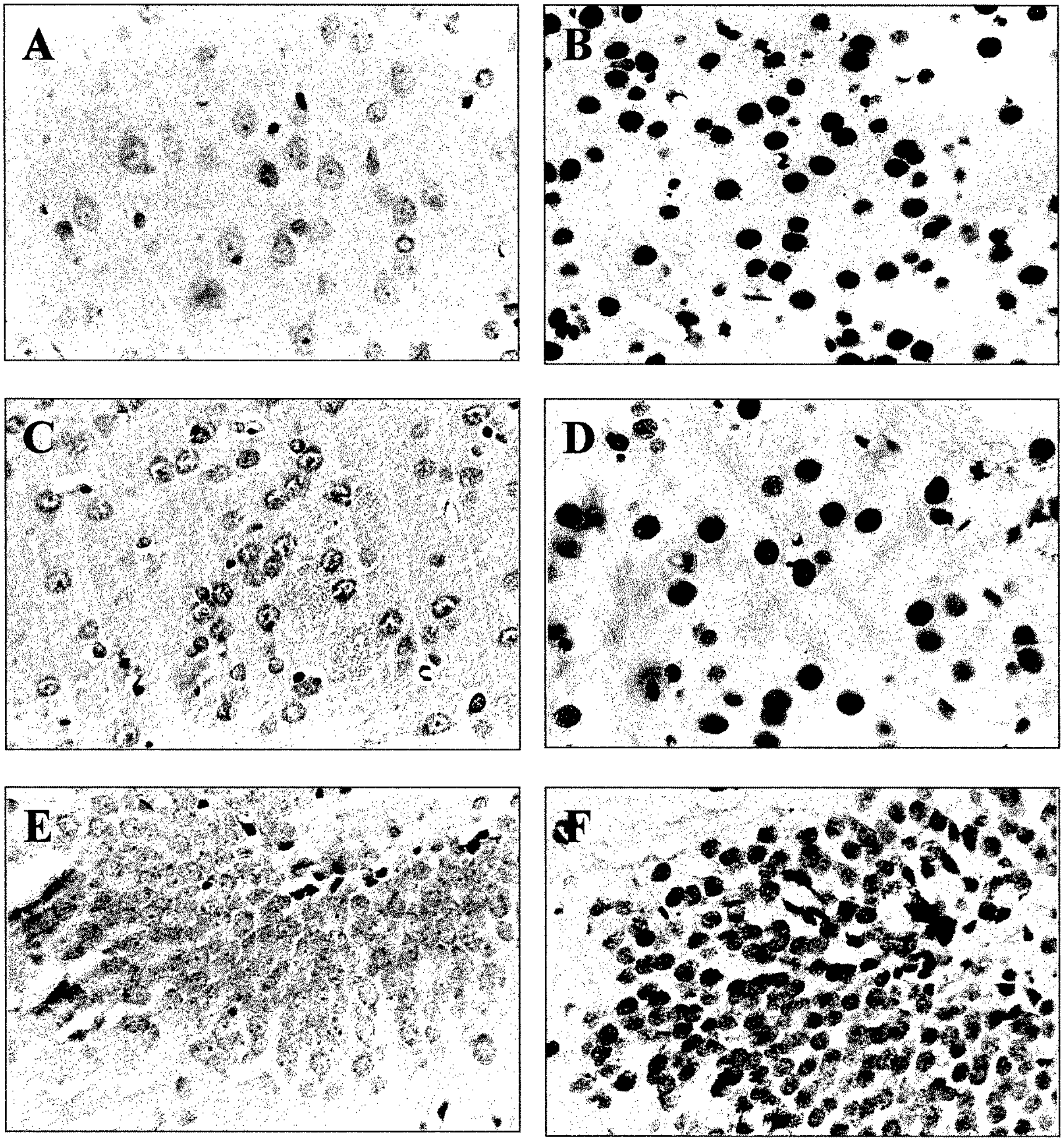
150
120
90
60
0
0.25
0.5
1
1.5
1.85
Dose of PB (mg / kg)

Figure 1



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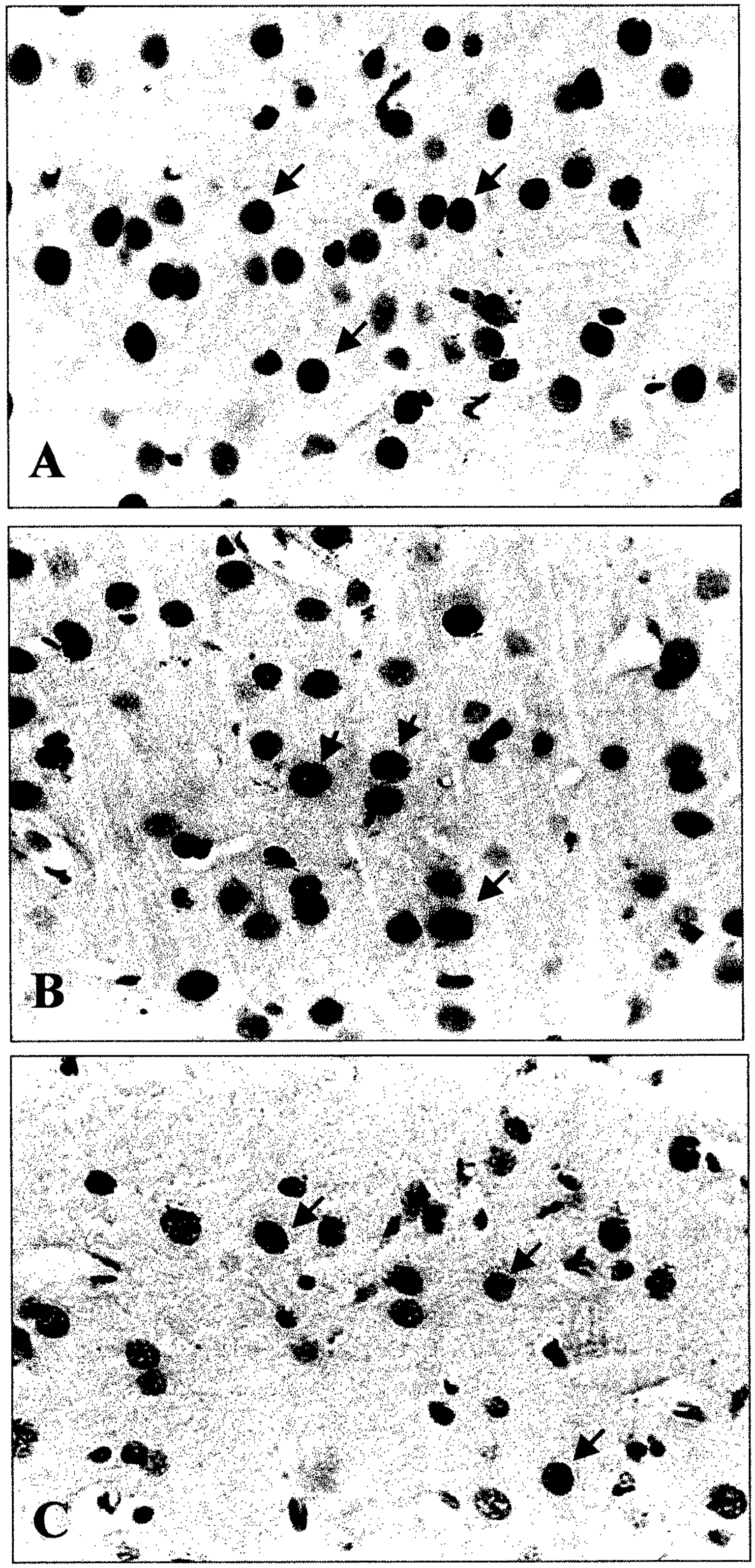

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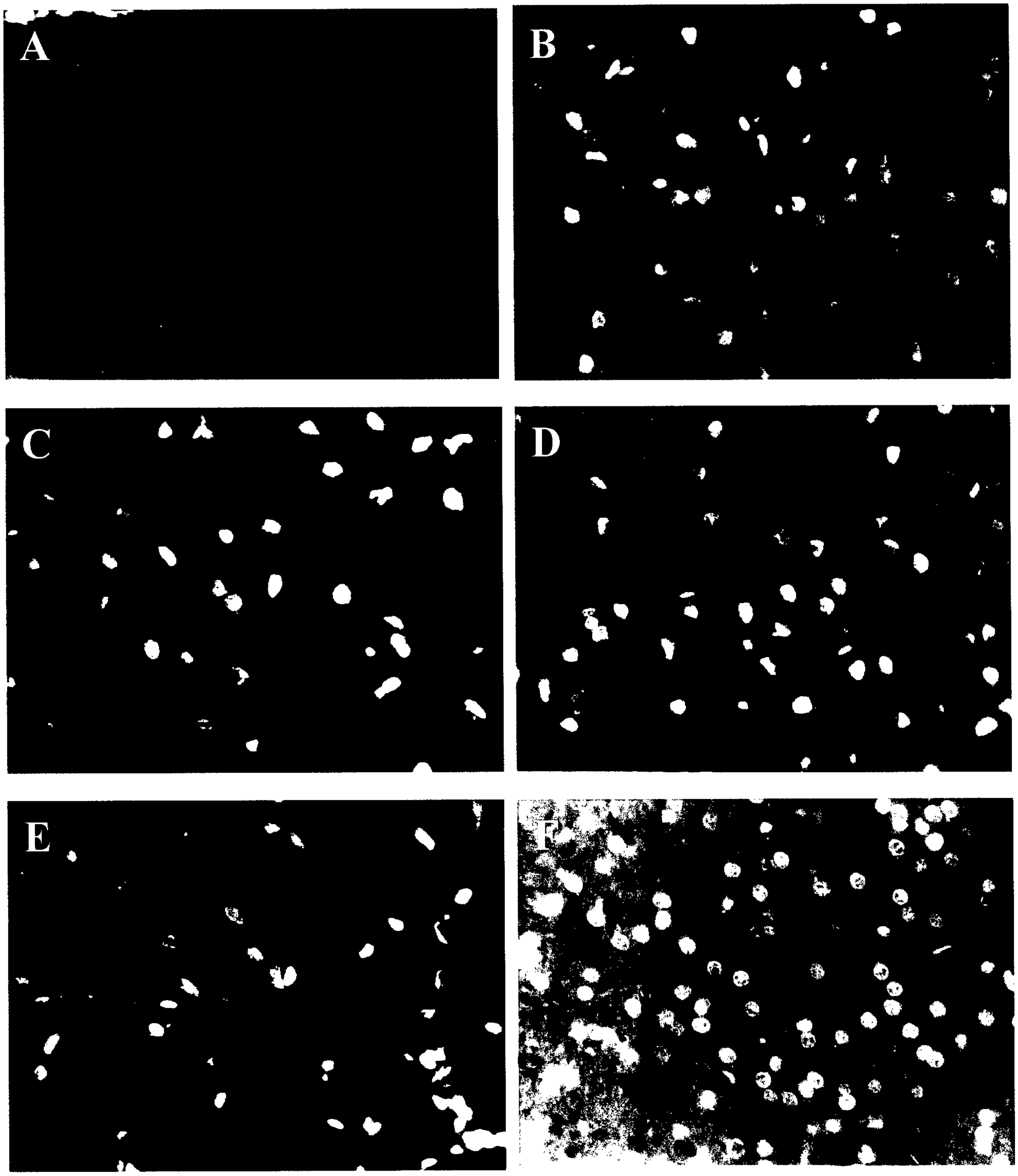

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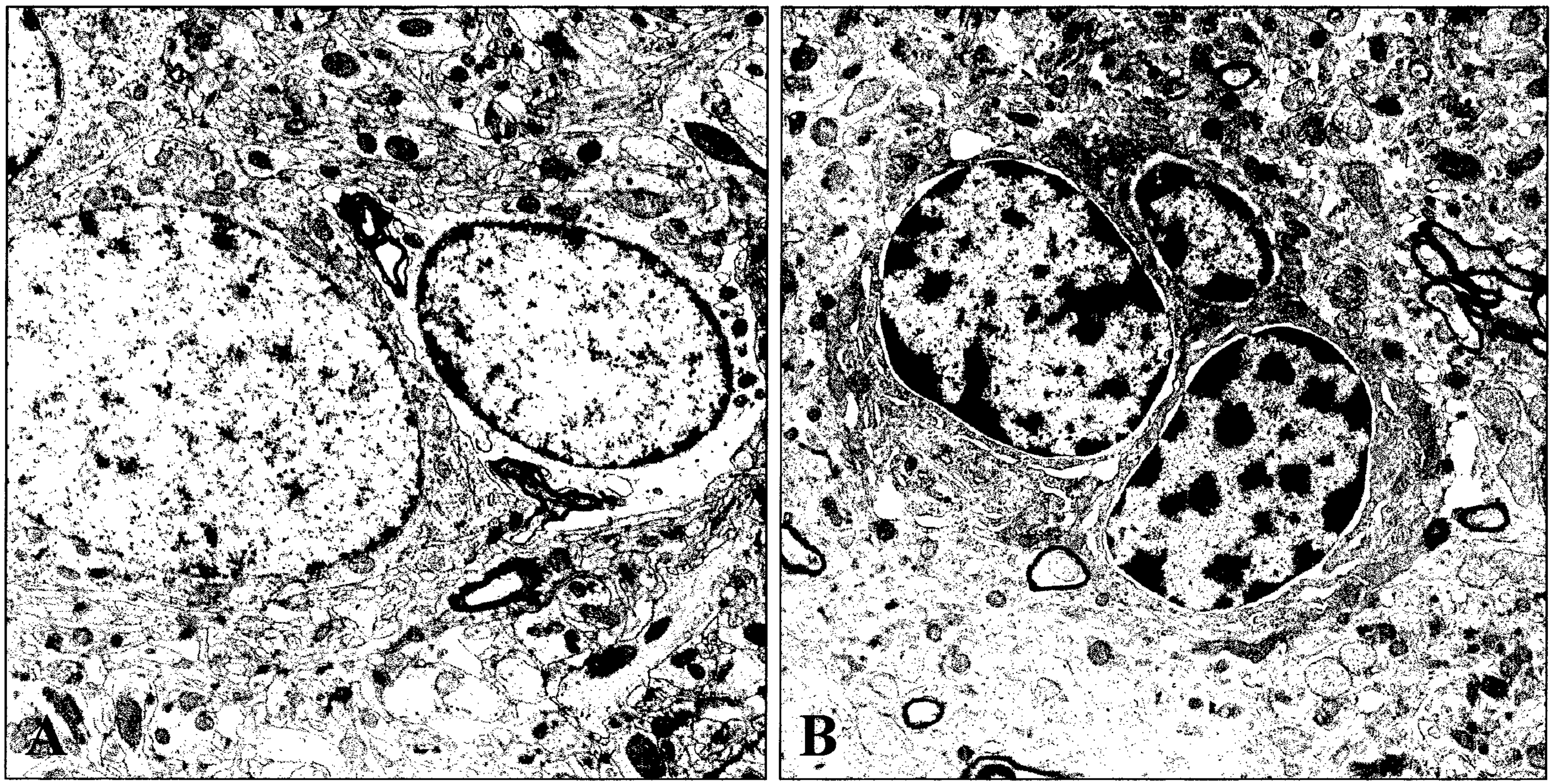

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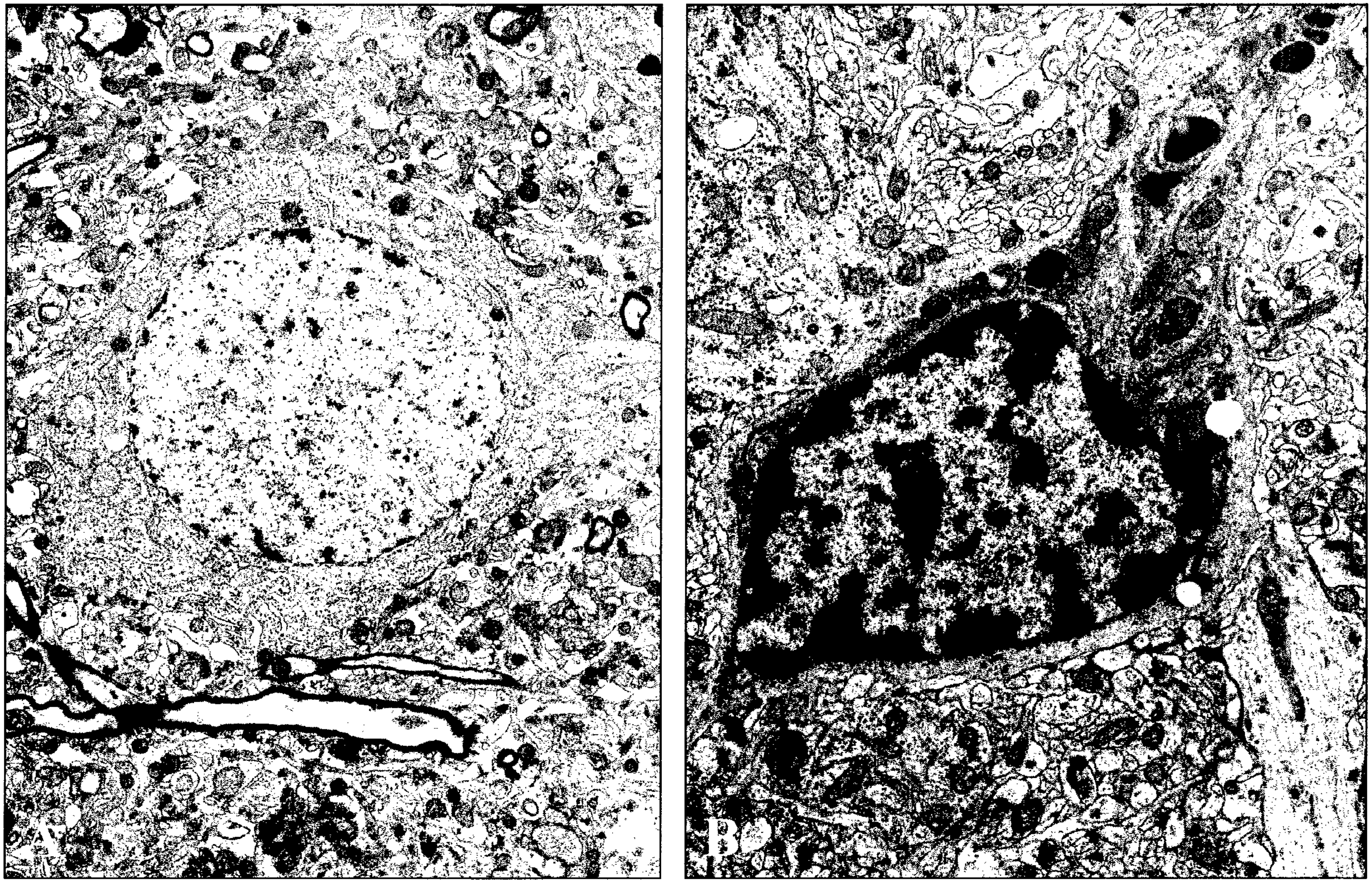

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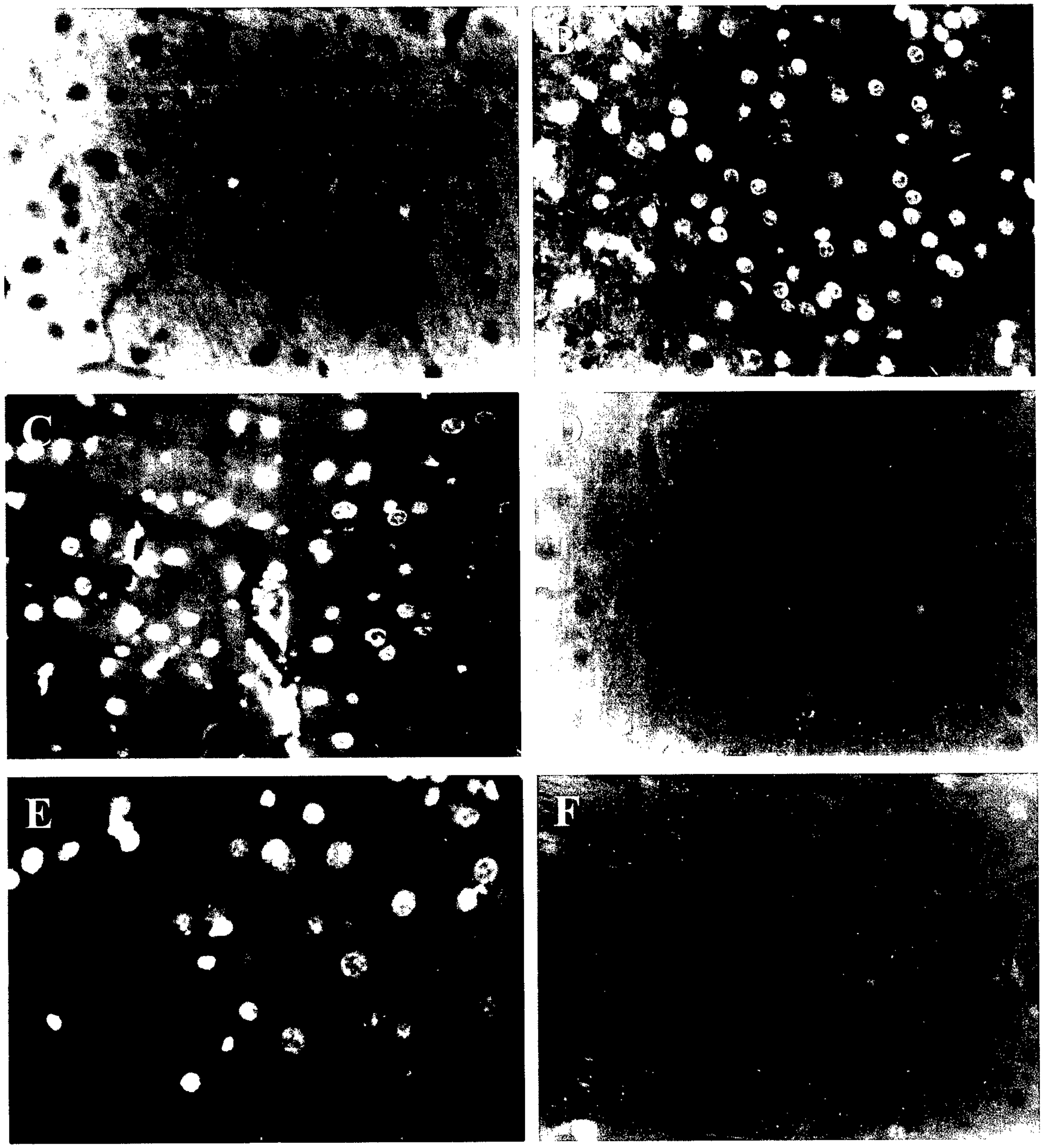

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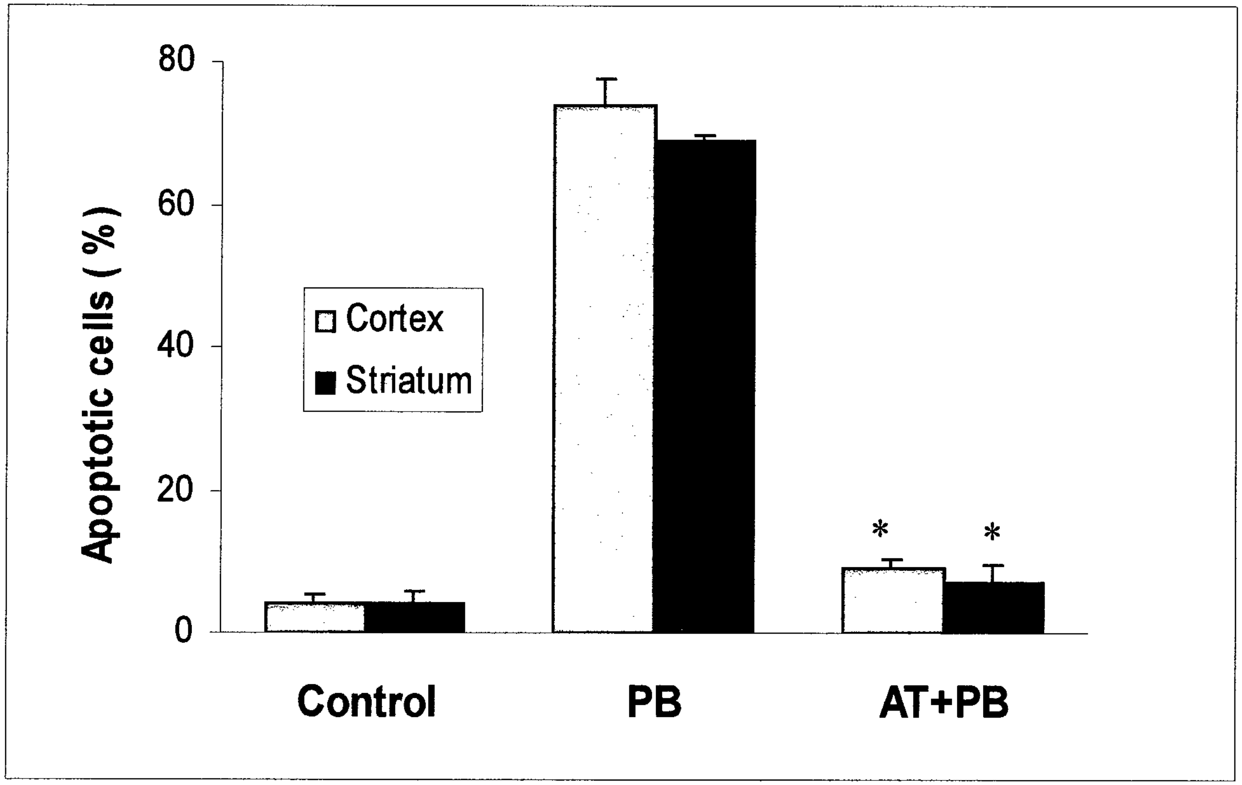
80
60
40
20
0
Apoptotic cells (%)
Cortex
Striatum
*
*
Control
PB
AT+PB

Figure 8