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Effects of acrylonitrile on the pathological morphology and apoptosis of neurons in rats

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ABSTRACT. This study aimed to evaluate the effects of acrylonitrile (ACN) on neuronal morphology and apoptosis in rats. An ACN solution was administered to Wistar rats by gavage at doses of 0, 5, 10, or 20 mg/kg, 5 days a week for 13 weeks. The morphology of neurons and the presence of apoptosis was examined by light and electron microscope, DNA electrophoresis, immunohistochemistry, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling. Significant vacuolation and the widening of the interspaces around blood vessels were observed in the groups that received the highest dose. Disordered myelin sheaths, malformed neuronal nuclei, and chromatin condensation at the periphery of the nucleus that formed crescents were also observed in the treated rats. The number of apoptotic neurons was significantly decreased ($P < 0.05$) in the treated groups (5 mg/kg group: 1.5 ± 1.22 apoptotic neurons/slide; 10 mg/kg group: 2.5 ± 1.05 apoptotic neurons/slide; 20 mg/kg group: 2.34 ± 1.21 apoptotic neurons/slide) compared to the control group (4.5 ± 1.52

apoptotic neurons/slide). The number of Bcl-2-positive neurons and the levels of staining were increased in the treated rats compared to those of the control group. These results suggested that ACN may induce serious morphological changes in rat neurons and inhibit neuronal apoptosis in rats.

Key words: Acrylonitrile; Neuron morphology; Apoptosis; Brain neuron; Rat

INTRODUCTION

Oral, skin, or vapor inhalation exposure to acrylonitrile (ACN, CH_2=CH-CN, CAS No. 107-13-1) is generally recognized as highly toxic. It is widely used in plastics, elastomers, synthetic fibers, synthetic rubber, and synthetic resin production as an intermediate (Leonard et al., 1999). ACN has been shown to be a carcinogen and a teratogen in animals, and its chronic inhalation or oral administration has been shown to induce tumors in the brain, forestomach, and Zymbal's gland (Al-Abbasi, 2012; Hamdy et al., 2012; Kolenda-Roberts et al., 2013). ACN was classified as a possible human carcinogen (Group 2B) by the International Agency for Research on Cancer in 1999. Although the pathogenetic mechanism of the carcinogenesis is still unclear, an imbalance of apoptosis has been confirmed to be involved, and this has become a recent topic of research interest (Krantic et al., 2007; Watcharasit et al., 2010; Abo-Salem et al., 2011). Many studies have investigated biological markers, such as Ki-67, p53, Bcl-2, and Cox-2, that can be used to classify carcinoma, atypical hyperplasia, and/or benign hyperplasia (Apostolou et al., 2013). Bcl-2 is an anti-apoptotic protein that belongs to the Bcl family and that interacts with Bax, which is a pro-apoptotic protein, and the ratio of the expression of Bax and Bcl-2 determines whether apoptosis will occur (Weyhenmeyer et al., 2012). In addition, it has been well documented that the Bcl-2 family is involved in tumorigenesis and is overexpressed in many kinds of carcinoma, and it has been implicated in the development of radio- and chemo-resistance (Thomas et al., 2013), such as in head and neck squamous cell carcinomas (Lin et al., 2013).

In this study, the carcinogenetic effects of ACN on brain neurons were assessed by examining proliferation and apoptosis. Cell proliferation was detected with proliferating cell nuclear antigen (PCNA) because the phosphorylation of the Tyrosine 211 in PCNA has been shown to be coincident with pronounced cancer cell proliferation (Yu et al., 2013). Apoptosis was detected by terminal-deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) and apoptotic DNA laddering (Kaptaner and Kankaya, 2013). Bcl-2 and p53 protein were also examined in order to determine the relationship between morphology and apoptotic proteins.

MATERIAL AND METHODS

Animal care and treatment

In total, 48 Wistar rats (140-180 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The rats were acclimatized to the animal facility for 1 week with a 12-h light/dark cycle and free access to food and water. The rats were randomly divided into four groups with 12 rats (six male and six female) in each group. Aqueous dosing solutions were freshly prepared in glass bottles and used immediately in order to prevent possible polymerization. The four groups of rats were administered a solution of ACN with a reported purity

of 99.99% (Shanghai SSS Reagent Co., Ltd., Shanghai, China) by gavage at doses of 0, 5, 10, or 20 mg/kg, 5 days a week for 13 weeks. After the 13 weeks of treatment, the brains of the rats were obtained while they were under anesthesia with ether. This study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Jinshan Hospital, Fudan University.

Pathological brain examination

The formalin-fixed, paraffin wax-embedded brain samples were sectioned at 5μm and stained with hematoxylin-eosin. The histological diagnoses were determined by two independent pathologists. The brain of each rat was examined by light microscope, and four of the six brains in each sex of each group were examined by electron microscope. The neurons in the hippocampus were examined.

Test of neuron apoptosis

Four of the rat brains in each group were examined for neuronal apoptosis with the TUNEL method (Roche Diagnostics GmbH, Mannheim, Germany). Six slides of each rat brain were examined in order to determine the amount and distribution of apoptotic neurons.

DNA agarose gel electrophoresis

DNA was extracted from the rat brains (Sangong Biotech Co., Ltd., Shanghai, China), and DNA ladders were examined with agarose gel electrophoresis.

Immunohistochemical assays of Bcl-2, PCNA, and p53 protein

Bcl-2 protein, PCNA, and p53 protein were analyzed with immunohistochemistry (Wuhan Boster Biological Technology., LTD., Wuhan, China). The thickness of the slides was 10 μm, and the staining intensities were scored semiquantitatively with the following scores: negative = 0; sandy beige = 1; brown = 3; dark brown = 5. The percentages of positive cells were scored with the following scores: negative = 0; <25% = 1; 25-50% = 2; 50-75% = 3; or >75% = 4.

Statistical analysis

The differences among the groups were analyzed with the one-way ANOVA method, which was followed by the Duncan test, with SPSS 13.0 (IBM Corporation, Armonk, NY, USA). The treatment groups were considered to be statistically different with P values less than 0.05.

RESULTS

Pathological changes

The results from the light microscopic observations are shown in Table 1, Figure 1, and

Figure 2. The neurons in the brains in the 5-mg/kg ACN group were almost normal, with only occasional vacuolation. Significant vacuolation and widening of the interspaces around the brain vessels were observed in the neurons in the 10-mg/kg group, and these changes were more marked in the neurons of the 20-mg/kg group. The neurons in the control group were normal.

Table 1. Effects of acrylonitrile on pathological changes in rat brains (N = 12 in each group).

Dose (mg/kg)	Gender	Hemorrhage	Edema	Degeneration	Necrosis
0	♂	-	-	±	-
	♀	-	±	±	-
5	♂	-	-	±	-
	♀	-	-	±	-
10	♂	-	±	+	-
	♀	-	±	+	-
20	♂	-	±	+	-
	♀	-	+	+-++	-

(-) = Normal; ± = local pathological changes occurred occasionally; + = slight pathological changes, <1/3 field of vision; ++ = moderate pathological changes, <1/2 field of vision.

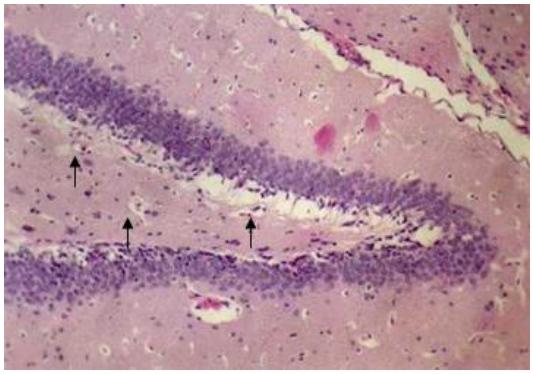

Figure 1. Vacuolation in neurons (indicated by arrows) in the hippocampus. Acrylonitrile: 10 mg/kg, hematoxylin and eosin stain, 150X.

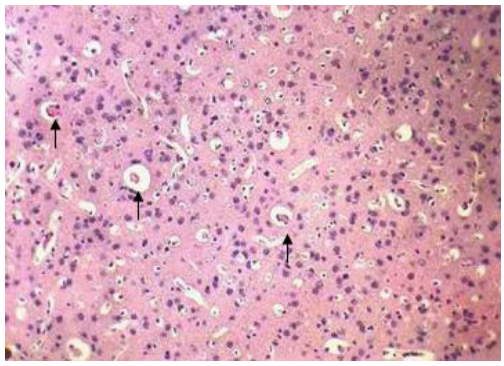

Figure 2. Widening of the interspace around the brain vessels (indicated by arrows). Acrylonitrile, 10 mg/kg, hematoxylin and eosin stain, 150X.

Electron microscope observations

An electron microscopic photomicrograph is shown in Figure 3. The neurons in the brains of the 5-mg/kg group were almost normal, and malformed nuclei were seen only occasionally. Disordered myelin sheaths, malformed neuronal nuclei, and chromatin condensation in the periphery of the nucleus that formed crescents were all observed in the rat brains in the 10-mg/kg group. Apoptotic bodies were seen occasionally in this group. There were obviously malformed nuclei in the neurons, with chromatin concentrations that collected at the edge of nuclei in the 20-mg/kg group. The neurons in the control group were normal.

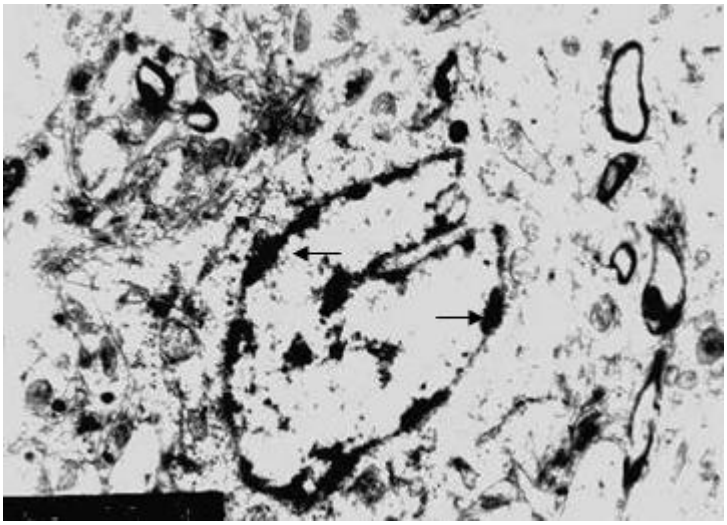

Figure 3. Neurons (indicated by arrows) with malformed nuclei and condensed chromatin at the periphery of the nucleus. Acrylonitrile, 20 mg/kg, H800 Electronmicroscope (EMS), 9000X.

Apoptotic cells

Brownish-yellow stain particles were observed in TUNEL-positive cells with the light microscope. The results are shown in Table 2 and Figure 4. The mean numbers of apoptotic cells were 1.50 ± 1.22 cells/slide in the 5-mg/kg group, 2.50 ± 1.05 cells/slide in the 10-mg/kg group, and 2.34 ± 1.21 cells/slide in the 20-mg/kg group. The numbers of apoptotic cells in these treated groups were significantly less ($P < 0.05$) than that of the control group (4.50 ± 1.52 cells/slide).

DNA ladder observations

No DNA ladder was observed with agarose gel electrophoresis in the treated or control groups.

Immunohistochemical assays of Bcl-2, PCNA, and p53 protein

There were more Bcl-2-positive cells in the treated groups, and the staining intensities

of the apoptotic cells in the treated groups were greater than that of the control group, but there were no significant differences in the numbers of apoptotic cells and the staining intensities among the three treated groups. The stain particles were dispersed in the nuclei, and the Bcl-2-positive cells were dispersed in the rat brains. There was no significant expression of PCNA or p53 protein in the rat brains in any of the treated or control groups.

Table 2. Effects of acrylonitrile on the inhibition of neuronal apoptosis.

Dose (mg/kg)	Total No. of apoptotic cells	No. of slides	No. of apoptotic cells per slide	P
0	27	6	4.50 ± 1.52	
5	9	6	1.50 ± 1.22	<0.01
10	15	6	2.50 ± 1.05	<0.05
20	14	6	2.34 ± 1.21	<0.01

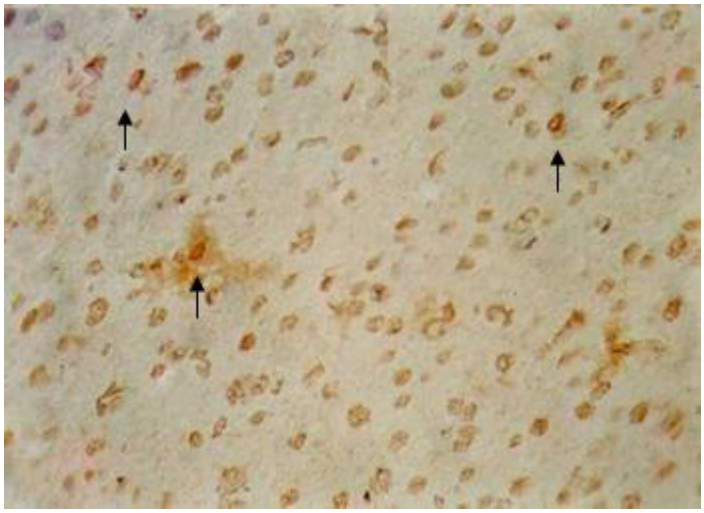

Figure 4. An apoptotic neuron with an annular nucleus. Acrylonitrile = 20 mg/kg, terminal-deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL), 300X.

DISCUSSION

ACN, which is classified as a noxious substance, may suppress spontaneous apoptosis in order to promote tumor growth. Damage to the brain was obvious in the treatment groups. Edema and degeneration were observed with the light microscope, and malformed nuclei, disordered structures of the myelin sheath, and chromatin condensation in the periphery of the nuclei were observed with the electronic microscope. The higher the dose of ACN, the more serious the damage was. The pathological changes that were induced by ACN may possibly indicate tumor growth, and these were consistent with other research results (Al-Abbasi, 2012; Hamdy et al., 2012; Kolenda-Roberts et al., 2013).

Apoptosis is the natural death process of nucleolate cells in which some self-regulatory mechanisms, especially endogenetic DNA endonuclease, are activated (Krantic et al.,

2007; Abo-Salem et al., 2011). Apoptosis is a cell suicide mechanism that enables metazoans to control cell number and eliminate cells that threaten the animal's survival (Watcharasit et al., 2010; Weyhenmeyer et al., 2012). The development of tumors is related not only to the overproliferation of cells, but also to the inhibition of cell apoptosis. In this study, only a few apoptotic bodies were detected in the treatment groups, and this was consistent with the TUNEL results. These findings suggested that the normal cell cycle was destroyed, which is the initial step in tumorigenesis and which indicated that ACN could inhibit the apoptosis of neurons, and this might be related to the carcinogenesis of ACN in animals. However, an *in vitro* experiment has shown that ACN induces apoptosis in SH-SY5Y cells through a mechanism involving the generation of oxidative stress-mediated Bax induction (Watcharasit et al., 2010). The differences in the effects of ACN on apoptosis in the different studies may have been due to different experimental methods and the kinds of the cells that were examined. In this study, which was an *in vivo* experiment, the cells that we chose to examine were neurons, while, in Watcharasit et al. (2010) *in vitro* study, the cells were human neuroblastoma SH-SY5Y cells. However, these differences require further research.

Apoptosis is regulated by the bcl-2 gene family, bax, c-myc, p53, fas/Apo1, the ICE gene family, and other genes. The bcl-2 gene family includes bcl-2, bcl-x, bax, bad, bak, mcl-1, A1, and other genes. The bcl-2 gene is a proto-oncogene, and it was the first gene found to be related to apoptosis (Konstantinidou et al., 2005; Thomas et al., 2013). Bcl-2 gene overexpression has been shown to inhibit apoptosis (Yang et al., 2011; Kim et al., 2012). Bcl-2 gene expression has been shown to have many biological effects. For example, it can prevent or reduce apoptosis that results from radiation, free radicals, chemical pharmaceuticals, and oxidation damage (Tichý, 2006; Adams and Cory, 2007; Zheng et al., 2011). The results of this study showed that bcl-2 gene expression was higher in the treated groups than in the control group, suggesting that apoptosis was inhibited in the treated groups. These findings were consistent with the results of the apoptotic cell tests and the electron microscopic observations.

Because PCNA and p53 protein showed no differences in the different groups, the results suggested there may be no tumor growth in the brain yet as PCNA is a useful marker of the proliferation of cells and p53 is an anti-oncogene.

In summary, the results of this study suggested that ACN may induce serious morphological changes in the neurons of rat brains and that ACN can inhibit the apoptosis of neurons in rats.

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