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IMMUNOHISTOCHEMICAL INVESTIGATION OF NEURONAL INJURY IN CEREBRAL CORTEX OF COBRA-ENVENOMED RATS

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ABSTRACT. The immunohistochemical expression of neuron-specific enolase, NSE (a cytoplasmic glycolytic enzyme of the neurons), synaptophysin, SYN (a major membrane glycoprotein of synaptic vesicles), and Bcl-2 (anti-apoptotic protein) were determined in cerebral cortex of rats envenomed with neurotoxic venom from Egyptian cobra. Male rats were intramuscularly (IM) injected with a single injection of either physiological saline solution or ½ LD_{50} or LD_{50} of cobra venom and sacrificed 24, 48, or 72 hr after envenoming. Formalin-fixed paraffin sections were immunohistochemically studied by avidin-biotin-peroxidase complex method. Neuron histological structure and isolation of genomic DNA were also detected. The results showed a dose and time-dependent increase in NSE and SYN immunoreactivity in cerebral cortex of envenomed rats except in 72 hr high dose envenoming, where decreased SYN was observed. On the other hand, low dose venom induced high Bcl-2 expression 24 hr after envenoming, while the high dose decreased Bcl-2 protein expression. Temporal and spatial Bcl-2 expression was accompanied by DNA fragmentation in cerebral cortex of all envenomed rats, although no serious histological alterations were noticed. These results suggest that cobra venom may lead to neuronal injury and impairment of axonal transport as ascertained by alterations in NSE and SYN immunoreactivity. It could also indicate that venom alters the molecular machinery of apoptosis by inhibiting Bcl-2 expression; however, some vulnerable cells have the ability to overcome this by increasing Bcl-2 protein. These immunohistochemical investigations can be used as tools for detecting neuronal abnormalities even before the occurrence of any histological alterations in case of cerebral cortex neurotoxicity.

KEY WORDS: immunohistochemistry, neuron specific enolase, synaptophysin, Bcl-2, cerebral cortex, cobra snake venom.

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INTRODUCTION

Neurons are considered the most important components in the nervous system; astrocytes are supportive structure elements in the nervous system (2). The neuron-astrocyte interaction is important in the maintenance of brain homeostasis and is vital for neuronal survival following brain injury (34). Our previous study (11) indicated reactive astrocytosis in cerebral cortex of rats envenomed with cobra venom. Astrocytosis is considered to be related to neuronal abnormalities rather than solely a repair event following brain injury (4).

Detection of brain injury needs some immunohistochemistry techniques including NSE and other antibodies (35). Nogami *et al*. (28) revealed that anti-NSE immunostaining of neurons could be useful in evaluating neuronal damage in the brain injury region. Ding *et al.* (5) added that reduced NSE immunoreactivity reflects neuron loss. However, Kim and Suhr (18) found that tumor cells were strongly positive for NSE and SYN immunohistochemical expression. SYN is a major membrane glycoprotein of synaptic vesicles that is ubiquitously expressed in all neurons (21). It provides a useful tissue marker of synaptogenesis during normal conditions and may provide clues to cerebral pathogenesis (33). Most studies have assessed SYN as a measure of synaptic integrity (22).

Expression of the cell death regulatory protein, Bcl-2, has also been suggested to evaluate the mechanisms of neuronal cell death (9). Bcl-2 inhibits neuronal apoptosis during normal brain development as well as that induced by cytotoxic drugs or growth factor deprivation (29). Apoptotic neuronal cell death is accompanied by endonucleosomal DNA cleavage and differential expression of the anti-apoptotic protein Bcl-2 (36). Benjelloun *et al*. (3) added that cell survival was assessed by Bcl-2 expression and cell death was demonstrated by DNA fragmentation.

The purpose of this study was to investigate the established mechanisms of neuronal injury and apoptosis and their relevance to cell death induced by an environmental neurotoxin such as cobra venom. This will be achieved by detecting immunoexpression of selected neuron-biomarkers (NSE, SYN, and Bcl-2) in cerebral cortex of envenomed rats.

MATERIALS AND METHODS

Snake venom

Lyophilized venom of the Egyptian cobra (*Naja haje*) snake was reconstituted in saline solution. Two doses were used in this study:

1) ½ LD_{50} (0.0125 μg cobra venom/ g body weight); and

2) LD_{50} (0.025 μg cobra venom/ g body weight).

LD_{50} dose was used according to Rahmy and Hemmaid (30).

Experimental animals

Male healthy rats (*Rattus rattus*) weighing 200 ± 10 g were kept in laboratory conditions and provided with water and food. The rats were divided into 3 groups:

1. Control group. Eight normal rats intramuscularly (IM) injected with 0.1 ml saline solution, sacrificed 72 hr after injection.

2. Low dose envenomed group. Rats IM injected with 0.1 ml saline containing ½ LD_{50} dose of cobra venom. The rats were subdivided into three subgroups (10 rats each) sacrificed 24, 48, 72 hr after venom injection, respectively.

3. High dose envenomed group. Rats IM injected with 0.1 ml saline containing LD_{50} dose of cobra venom. The rats were subdivided into three subgroups (10 rats each) sacrificed 24, 48, 72 hr after venom injection, respectively.

Tissue sampling

Five rats from the control group and from each envenomed subgroup were anesthetized by chloroform. Each rat was transcardially perfused with 100 ml saline phosphate buffer (0.1 M, pH 7.6) followed by 400 ml of 10% neutral buffered (0.1 M phosphate buffer) formalin fixative solution. Brains were removed from each rat then fixed in the same fixative for 8 hr, followed by overnight washing in running tap water. The anterior part of the brain samples were dehydrated, cleared, and then infiltrated and embedded in paraffin wax. Paraffin blocks were serially sectioned to produce 5μ-thick sections of cerebral cortex. Serial sections of cerebral cortex were used for histological and immunohistochemical studies.

The rest of non-perfused rats from control group and each envenomed subgroup were also anesthetized by chloroform. Brains were removed from each rat and stored at -80˚C to be used for DNA extraction.

Histological studies

Paraffin sections were stained with hematoxylin and eosin for regular histological investigation of control and envenomed tissues.

Immunohistochemical studies

Paraffin sections of perfused brains from control and envenomed groups were used for detecting NSE, SYN, and Bcl-2 immunoreactivity. The sections were de-waxed and incubated for 1hr at room temperature in 0.3% hydrogen peroxide in phosphate-buffered saline, pH 7.6 (PBS). The slides were washed 3 times (10 min. each) in the same buffer to quench endogenous peroxidase activity. They were incubated for 16 hr at 4 °C in PBS containing 2% normal goat serum (NGS) and 0.5% triton X-100, and then washed again in PBS at room temperature. This was followed by overnight incubation at 4°C with the primary monoclonal antibody (Mouse anti-EMA, ICN, Costa Mesa, CA, USA and Sigma Chem. Comp.) of anti-NSE, anti-SYN, or anti-Bcl-2 then washed 3 time in PBS-2% NGS. The primary antibodies were bounded by a rat adsorbed biotinylated anti-mouse secondary antibody (1,200, Vector Labs, Burlingame, CA, USA), in PBS for 1hr at room temperature. Slides were incubated in avidin-biotin complex linked to peroxidase (ABC Kit, Vector Labs, Burlingame, CA, USA). Peroxidase was seen with 0.03% diaminobenzidine hydrochloride and 0.005% hydrogen peroxide in 0.1 M Tris buffer. All sections were counter stained with hematoxylin dehydrated, cleared, and mounted in Canada balsam. Sections from all groups were simultaneously processed in the same tray.

Isolation of genomic DNA

DNA was extracted from 1g of brain tissue (from each group) frozen by liquid nitrogen and ground to a fine powder according to Sambrook *et al*. (31). Extracted DNA samples were fractionated by 1% Agarose gel electrophoresis. Twenty µl were loaded for each sample at a power supply of 60 V for 3 h. After electrophoresis run, the gel was photographed on a UV transilluminator at 312 nm wavelength using a Polaroid MP-4 land camera.

RESULTS

Histological neuron detection

Cerebral cortex of control rats showed normal neurons with polygonal to round cell bodies. The nuclei were large with condensed chromatin in comparison with those of surrounding support cells. Control neurons also contained dense basophilic cytoplasm (Figure. 1). No serious histological alterations were recorded following envenoming with low or high dose venom except for the appearance of more rounded neurons characterized by dispersed chromatin and prominent nucleoli which reflect a high level of protein (enzyme) synthesis (Figure 2).

NSE immunohistochemical expression

Cerebral cortex of control rats showed positive NSE reactivity represented by the neuron cytoplasm (Figure 3). Similar reactivity was recorded in cerebral cortex of rats envenomed in 24 hr low dose envenoming. A time dependent increase in neuron NSE reactivity was recorded 48 hr (Figure 4) and 72 hr (Figure 5) after envenoming. Another form of time-dependent increase in NSE reactivity was shown by neurons in cerebral cortex of high dose envenomed rats (Figures 6, 7, and 8).

SYN immunohistochemical expression

SYN reactivity was represented in a form of coarsely fine beaded reactivity at the neuron surface in cerebral cortex of control rats. Reactive granules were also scattered in the intercellular matrix between the neurons (Figure 9). Twenty-four hours after low dose envenoming, SYN reactivity was more or less similar to that of the control group. Forty-eight hours (Figure 10) and 72 hr (Figure 11) after envenoming with the same dose, a time-dependent increase in SYN immunoexpression was noticed in a form of dense bands at the periphery of the neurons. Twenty-four hr after high dose envenoming (Figure 12), SYN reactivity was increased in comparison to controls. Dense reactive bands were highly accumulated around the neurons and within the space between them 48 hr after high dose envenoming (Figure 13). However, SYN reactivity was decreased 72 hr after envenoming at this dose (Figure 14).

Bcl-2 immunohistochemical expression

The control animals showed an evident expression of Bcl-2 protein located in all cell cytoplasmic components, but the nuclei were not immunostained (Figure 15). A rapid increase of Bcl-2 immunoreactivity was observed in cerebral cortex of low dose rats 24 hr after envenoming (Figure 16). This increase was reduced by 50% 48 hr after envenoming; however, the expression was still more pronounced than that of the control animals. Nevertheless, Bcl-2 immunoreactivity was progressively decreased 72 hr after envenoming to a level less than control animals. The diminished level of Bcl-2 expression included decreased number of immunostained cells and lower intensity of immunoreactivity (Figure 17).

A loss of Bcl-2 immunoreactivity was also observed within the cerebral cortex 24 hr after high dose envenoming (Figure 18). Decline of Bcl-2 expression extended throughout the first 48 hr to a level lower than that of the control. However, only a few neuronal cells with intense Bcl-2 reactivity were noticed in certain areas of the cerebral cortex 24 hr and 48 hr after envenoming (Figure 19). This enhanced Bcl-2 immunoreactivity was not seen 72 hr after high dose envenoming. It seems likely that Bcl-2 protein expression is markedly inhibited after this time (Figure 20).

Genomic DNA isolation

Treatment with cobra venom induced DNA fragmentation in samples extracted from cerebral cortex of envenomed rats (Figure 21). This fragmentation was clearly evident when compared with DNA extracted from control animals and was represented by a single band in lane 1. Application of low dose induced a time-dependent DNA fragmentation. This was observed at 24 hr (lane 2), increased at 48 hr (lane 3), and markedly noticeable 72hr after envenoming (lane 4). A similar pattern of time-dependent DNA fragmentation was also seen in high dose envenoming (lanes 5, 6, and 7). In comparison, high dose was more effective in inducing DNA fragmentation than the low dose.

DISCUSSION

Snake venoms are not only limited to envenoming victims, but they are also proving to be valuable research tools and diagnostic agents. In this study, cobra venom, which is known to contain a variety of postsynaptic and presynaptic neurotoxins (20), was used to induce neurotoxic models to investigate NSE, NYS, and Bcl-2 immunoreactivity during neurotoxicity.

The results indicated a dose and time-dependent increase in NSE and SYN immunoexpression in cerebral cortex of envenomed rats. NSE is a biochemical diagnostic marker, which reflects pathogenic processes in the brain and is used to identify ongoing neuronal degeneration (38). The presence of NSE immunoreactivity indicates no evidence of neuronal cell loss (26). However, NSE overexpression could be correlated with overall histological evidence of damage (32). In this way, Woertgen *et al*. (40) proved a close relationship between NSE release and the severity of traumatic brain injury in a cortical impact model.

On the other hand, SYN is a synaptic protein involved in regulating transmitter release and synaptic plasticity (39). It is a very reliable presynaptic marker (24). SYN accumulation in a form of disclosed large coarse pericellular reactivity indicates abnormal axon transport (8). Increased SYN immnuoreactivity is usually associated with impaired synaptic function, resulting in cognitive deficits (19). This impairment could be due to a possible blockage of neuron receptors by common postsynaptic cobra toxins (41) so that synaptic vesicles are accumulated around neurons. This was confirmed by Apel *et al*. (1) who reported that the long chain neurotoxin alpha-cobratoxin do not affect the release of acetylcholine, but effect a block at the post-synaptic nicotinic receptors.

On the contrary, decreased SYN immunoreactivity 72 hours after high dose envenoming could be considered as a late-stage phenomenon, while its increase occurs at an early stage of injury (25). Decreased SYN is suggestive of synaptic pathology (7) and may occur as tissue degeneration progresses (37). It also reflects either post-transcriptional abnormalities of SYN or a diminished number of axonal projections of neurons of cerebral cortex (10).

This disturbance in NSE and SYN immunoreactivity could indicate possible neuronal abnormalities although this was not proved by routine histological techniques. However,

neuronal abnormalities could be indicated by immunoexpression of the anti-apoptotic protein Bcl-2, which can determine whether a neuron survives or dies (23). In this way, Offen *et al* (29) explained that Bcl-2 overexpression in neurons provides protection against neurotoxins, while neurons deficient in Bcl-2 are more susceptible to neurotoxins. Jayanthi *et al*. (17) added that neuronal cells that overexpress Bcl-2 protein are protected against apoptosis. Thus, the increase in Bcl-2 24 hr after low dose envenoming is consistent with a protective role of neurons against apoptosis. However, the decreased Bcl-2 immunoexpression after 48 and 72 hr low dose envenoming and after high dose may signal neuronal vulnerability to pro-apoptotic stimuli and neuronal atrophy (16). Nevertheless, some vulnerable cells have the ability to overcome apoptosis by increasing Bcl-2 protein at certain stages of envenoming. Decrease in Bcl-2 protein is known to occur prior to apoptosis and could be considered as an important step in pro-apoptotic response to neurotoxicity (12). Therefore, it is likely that Bcl-2 reduction contributes to apoptosis and neuronal cell death (6). Niu *et al*. (27) explained that apoptosis is induced in neural cells by Bcl-2 down regulation and that the rate of apoptosis is inversely correlated with Bcl-2 expression.

Apoptotic changes of neuronal cells were also confirmed by the regional distribution of DNA fragmentation, which coincided with decreased Bcl-2 expression in cerebral cortex of envenomed rats. DNA fragmentation is considered as an early event in neuronal death following brain injury (42). The appearance of increased laddered DNA fragmentation is considered as hallmarks of apoptosis (13). Jan *et al*. (15) explained that cobra venom causes apoptosis in brain tumor cells by activating the pro-apoptotic c-Jun N-terminal kinase signal transduction pathway. It seems that these nuclear DNA changes and the disturbance in Bcl-2 immunoexpression occur earlier than cytoplasmic degeneration and involve a possible apoptotic neuron death mechanism (14).

These immunohistochemical studies could be considered as indicators of the disturbance in neuron functional activities and tools for detecting neuronal pathology even in the absence of obvious histological alterations. It is believed to be useful in cases of patients who survive for a short time after a fatal neurotoxic injury but without obvious structural alterations.

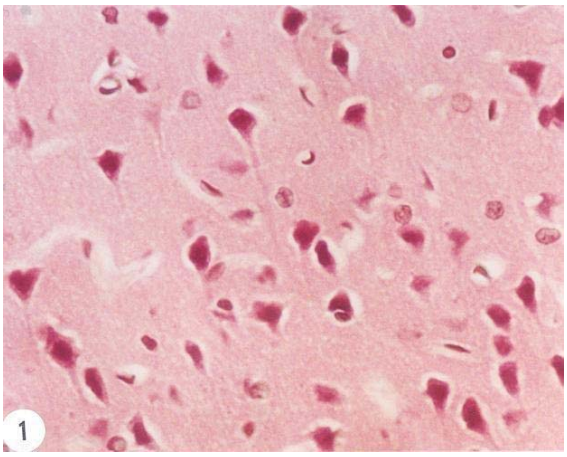

Figure 1. Cerebral cortex of a control rat showing normal neurons mostly with polygonal shaped cyton and dense nuclei. Note a few neurons with round cell bodies (H&E). (X400)

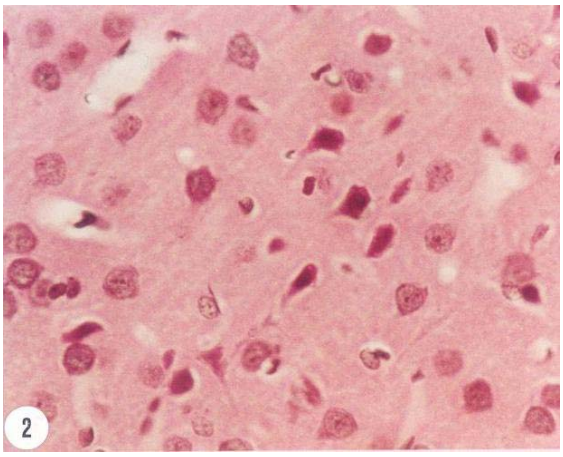

Figure 2. Cerebral cortex of a rat 72 hr after low dose envenoming showing normal neurons. Note increased number of neurons with round cytons, but a few polygonal cell bodies (H&E). (X400)

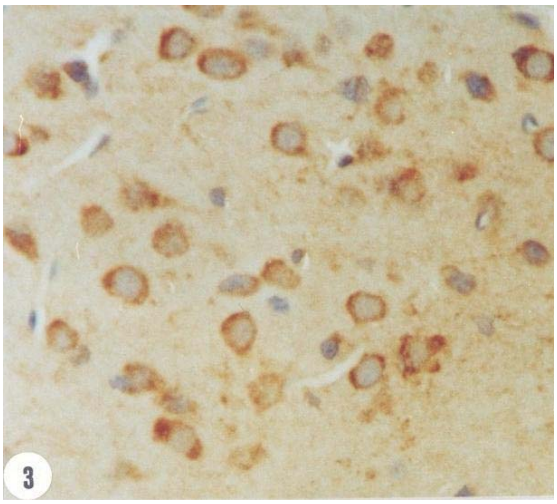

Figure 3. Control cerebral cortex of a rat showing positive NSE immunoreactivity in the neuron cytoplasm. (X400)

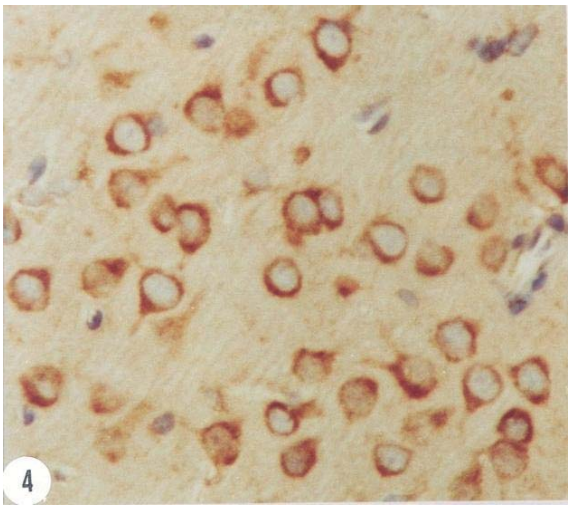

Figure 4. Increased NSE immunoreactivity in neurons 48 hr after low dose envenoming. (X400)

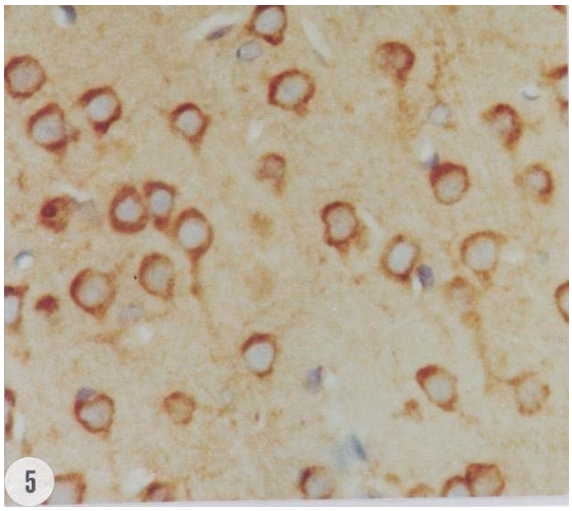

Figure 5. Strong NSE immunoexpression in neurons 72 hr after low dose envenoming. (X400)

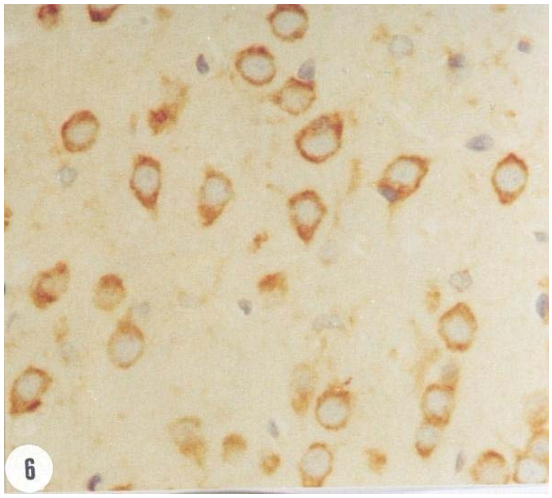

Figure 6. NSE immunoreactivity 24 hr after high dose envenoming. (X400)

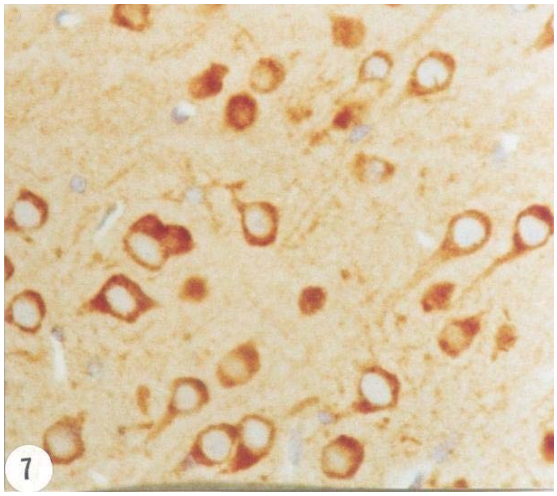

Figure 7. Intense NSE-positive reactivity in neurons 48 hr after high dose envenoming (X400)

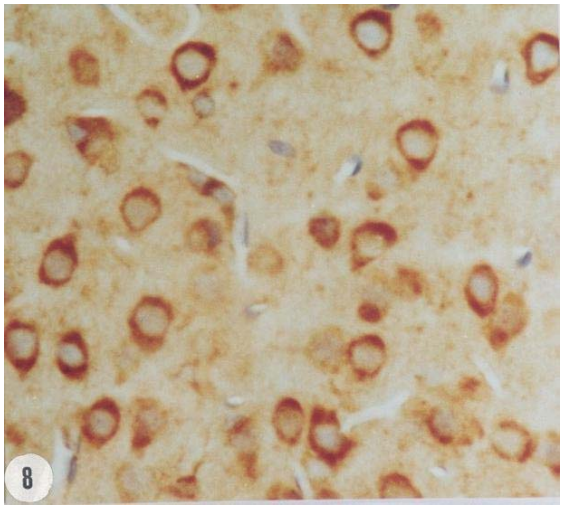

Figure 8. The highest NSE immunoexpression 72 hr after high dose envenoming. (X400)

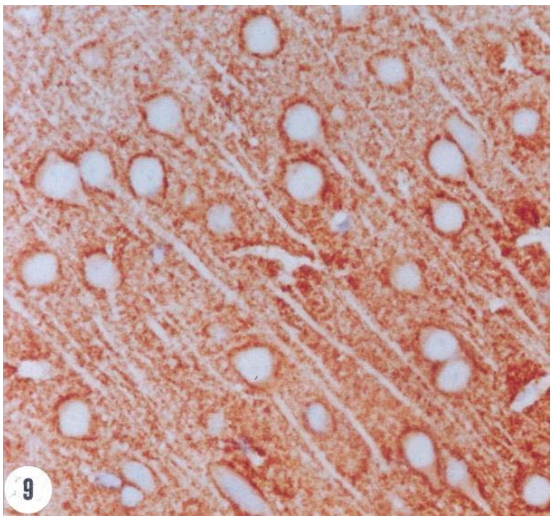

Figure 9. SYN immunoexpression at the periphery of cytons and between neurons of a control cerebral cortex. (X400)

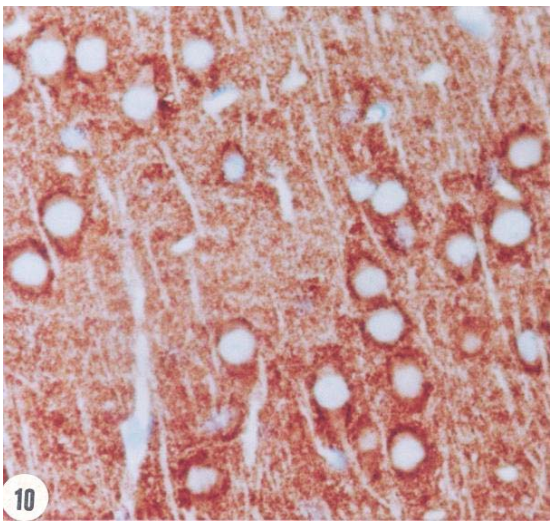

Figure 10. Increased SYN immunoreactivity 48 hr after low dose envenoming. (X400)

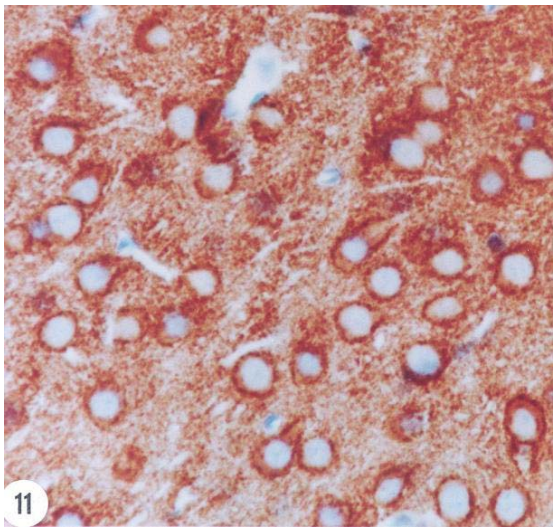

Figure 11. Neurons of cerebral cortex 72 hr after low dose envenoming showing intense SYN reactivity. (X400)

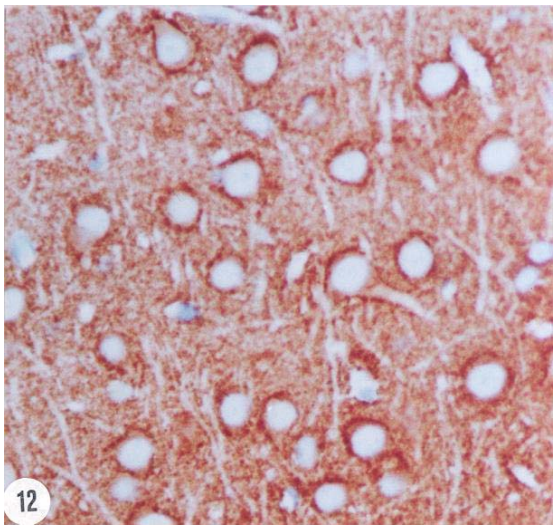

Figure 12. High SYN immunoreactivity 24 hr after high dose envenoming. (X400)

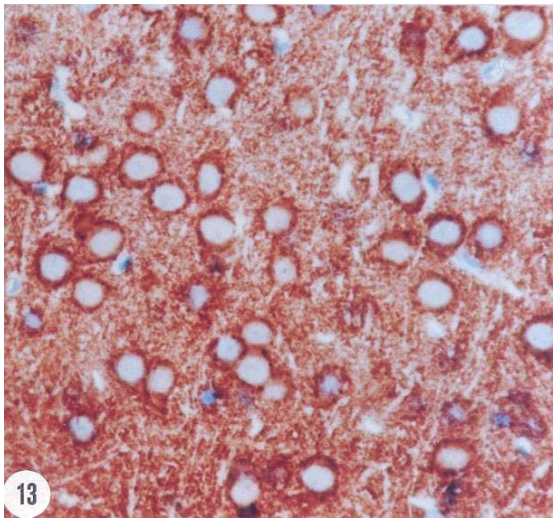

Figure 13. Accumulation of immunoreactive SYN expressed 48 hr after high dose envenoming. (X400)

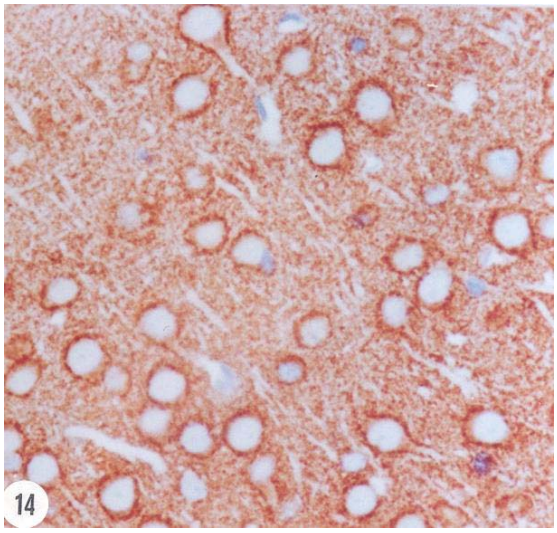

Figure 14. Decreased SYN immunoreactivity at the periphery of cytons 72 hr after high dose envenoming. (X400)

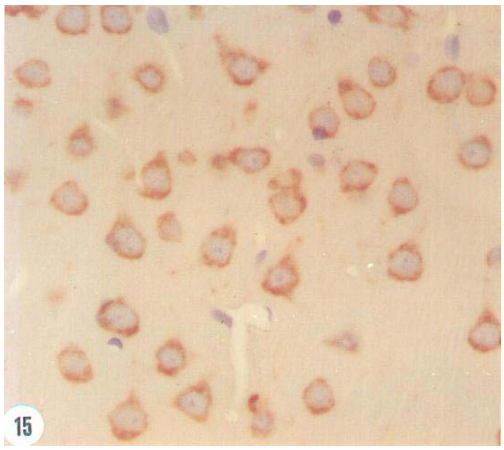

Figure 15. Bcl-2 immunoexpression in the cytoplasm of control neurons. (X400)

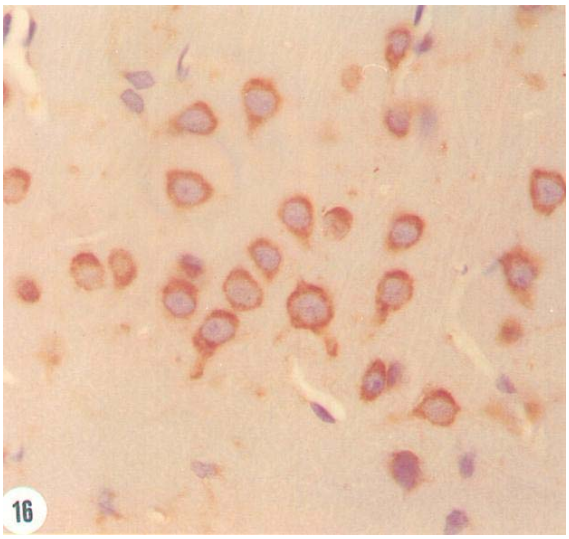

Figure 16. Increased Bcl-2 immunoexpression 24 hr after low dose envenoming. (X400)

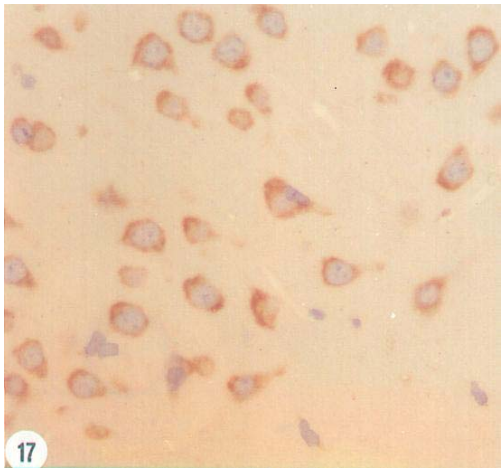

Figure 17. Neurons of cerebral cortex 72 hr after low dose envenoming showing lower Bcl-2 reactivity than that of the previous group. (X400)

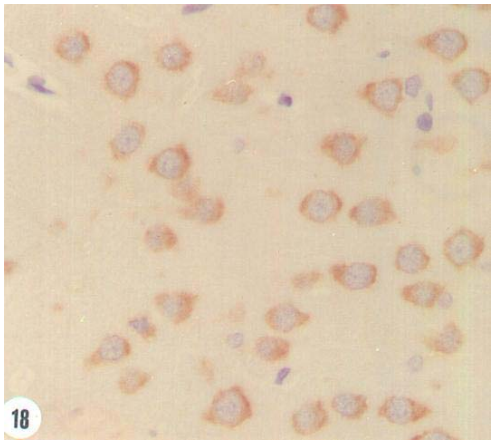

Figure 18. Decreased Bcl-2 reactivity after 24 hr after high dose envenoming. (X400)

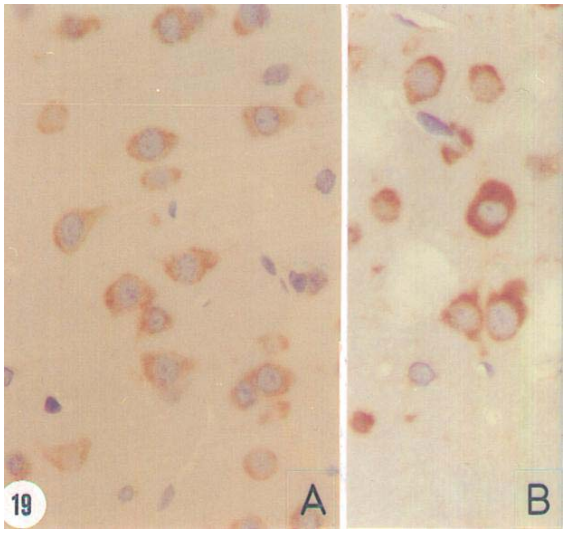

Figure 19. Low Bcl-2 immunoexpression 48 hr after high dose envenoming (Figure A). Note cells with intense reactivity (Figure B). (X400)

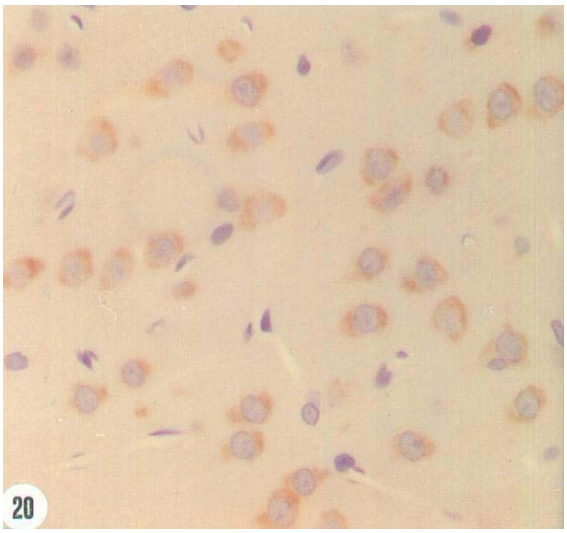

Figure 20. Highly decreased Bcl-2 immunoreactivity 72 hr after high dose envenoming. (X400)

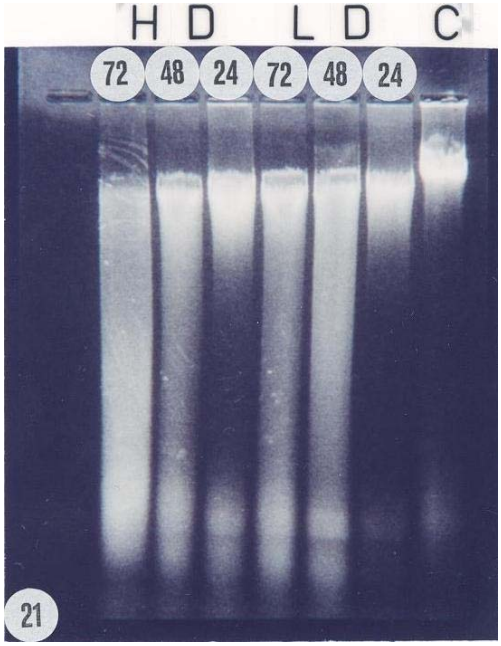

Figure 21. Gel electrophoresis showing isolated genomic DNA of the brain in control and envenomed samples. C: Control; 24L, 48L, 72L: Samples 24, 48, and 72 hr after low dose (LD) envenoming; 24H, 48H, 72H: Samples 24, 48, and 72 hr after high dose (HD) envenoming. Note DNA fragmentation in sample from envenomed rats.

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