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Glutamate Excitotoxicity; a Possible Mechanism for Apoptosis of Motoneurons in Adult Mouse Spinal Cord Slices

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In experiments on mice spinal cord slices, it was found that intense apoptosis of motoneurons develops in these slices rather rapidly (6 hours). This process can be significantly suppressed by treatment with the NMDA glutamate receptor antagonist MK-801 (50 μM) and AMPA/kainite non-NMDA glutamate receptor antagonist CNQX (100 μM). Such treatment considerably increased the percentage of viable motoneurons in the ventral horns of the spinal cord. The results obtained confirm the statement that glutamate excitotoxicity could be a significant possible factor responsible for apoptosis of motoneurons under conditions of spinal cord impairment.

Keywords: glutamate excitotoxicity, glutamate receptor antagonists, apoptosis, oxidative stress, spinal cord slices.

INTRODUCTION

Degeneration of neurons is a tragic reality following spinal cord injuries (SCIs) [1] and neurodegenerative disorders [2], e.g., in amyotrophic lateral sclerosis (ALS). Despite extensive researches, the exact mechanisms responsible for neuronal death are poorly known, and there is no universally accepted treatment for the respective pathologies. Therefore, studies of the mechanisms responsible for neuronal degeneration can help to develop an effective strategy for promoting neuronal survival and, consequently, axonal regeneration.

Apoptosis considerably contributes to cellular damage after traumatic SCIs in humans [2] and rats [3]. This process might also be responsible for neuronal cell death following SCI modeling *in vitro*. In this context, Casha et al. in a model of SCI [4] observed intense neuronal apoptosis in spinal cord slices exposed to a weight-drop injury. Also, we have shown apoptosis in adult mouse spinal cord motoneurons within an early time interval of slice culturing [5]. Several signaling pathways, in particular calpain- [6] and calcium-dependent proteases-related ones, have been proposed to explain the mechanism(s) responsible for apoptosis of motor neurons in adult spinal cord slices. Since elevated cytosolic calcium has been reported following SCI [7] and in neurodegenerative diseases [8], it can be assumed that apoptosis is induced as a result of the uncontrolled entry of calcium into these neurons, resulting in abnormally elevated intracellular calcium levels. In accordance with this, our results showed that blocking of voltage-gated calcium channels and/or Na^+/Ca^{2+} exchangers could delay apoptosis of motoneurons in cultured slices [9].

Abnormal functioning of glutamate receptors may be a possible cause for excessive cytosolic calcium levels. Glutamate is a major endogenous excitatory amino acid neurotransmitter in the mammalian CNS, which interacts with the glutamate receptors [10]. There are two known ionotropic glutamate receptors, including NMDA (N-methyl-D-aspartate) receptors, i.e., directly gated channels that allow the influx of cations (most notably, including calcium),and AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid)/kainate receptors (non-NMDA), which pass both Na^+ and K^+ ions [10, 11]. However, some evidence shows that AMPA/kainate receptors are also permeable to calcium [12]. Overstimulation of the ionotropic glutamate receptors (resulting in the so-called glutamate excitotoxicity) may lead to

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neuronal and glial cell death [13]. The former can be alleviated by glutamate receptor antagonists (neuroprotection). Glutamate-induced neurotoxicity is involved in neuronal death in ischemic stroke [14], neurodegenerative diseases [15], and SCI [7]. Based on a calcium-induced neuronal death hypothesis, it is likely that ionotropic glutamate receptors are involved in apoptosis of motor neurons in cultured spinal cord slices. Therefore, the initial approach was to block these receptors, which can be a possible way to delay apoptosis in these neurons. Accordingly, Van Westerlaak et al. [16], by application of ionotropic glutamate receptor antagonists, prevented apoptosis of motor neurons in rat spinal cord slices provoked by an ionotropic glutamate receptor agonist (malonate). Whether or not excitotoxicity also induces apoptosis in unprovoked adult spinal cord slices has hitherto not been studied in any detail. Therefore, our study was designed to investigate if inhibition of the ionotropic glutamate receptors can enhance the viability of cultured spinal cord slices and delay apoptosis of motor neurons in these slices.

METHODS

Preparation of organotypic spinal cord slices and the treatments used. In this experimental study, adult female Balb/c mice (23–25 g) purchased from the Pasteur Institute (Tehran, Iran) were used. The animals were housed in plastic cages at 20°C under a 12-h light/dark cycle with water and food *ad libitum*. The animals were deeply anesthetized by an i.p. injection of sodium pentobarbital (60 mg/kg) and subsequently killed by a heart puncture. The spinal cord was dissected and placed in ice-cold phosphate-buffered saline (PBS, pH 7.4). The thoracic region of the spinal cord was then sliced transversally into 400 μm-thick sections using a McIlwain tissue chopper (Stoelting, USA). The slices were subsequently placed in four-well sterile plastic plates where each well contained 450 μl of the medium composed of a mixture of 50% minimum essential medium, 25% Hanks balanced salt solution, 25% horse serum, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 6 g/l glucose, and 1% penicillin-streptomycin (pH 7.3–7.4). The slices were divided into four groups (n = 6 in each): (i) freshly prepared slices (0 h), which were immediately fixed; (ii) control slices cultured for 6 h in the medium; (iii) slices treated with an NMDA glutamate receptor antagonist, MK-801 (Sigma, USA, 50 μM), and (iv) slices treated with an AMPA/kainate non-NMDA glutamate receptor antagonist, CNQX (Sigma, USA, 100 μM). The control and treated slices were then incubated for 6 h at 37°C in a humidified atmosphere (air with 5% CO_2). MK-801 and CNQX were prepared as stock solutions in dimethylsulfoxide (DMSO) and stored in aliquots at –20°C. These aliquots were directly added to the medium. The control group received a corresponding amount of DMSO. To find out an effective concentration, effects of different concentrations (10, 25, 50, and 100 μM of MK-801 and 25, 50, 100, and 150 μM of CNQX) were tested with respect to apoptosis of motoneurons of spinal cord slices. Our results revealed that 50 and 100 μM of MK-801 and CNQX are rather effective concentrations, respectively.

Fixation and sectioning. The freshly prepared and cultured slices were fixed in Stefanini's fixative (2% paraformaldehyde and 0.2% picric acid in 0.1 M PBS, pH 7.2) for at least 2 h. The fixed slices were washed in PBS (3×5 min) and incubated overnight in 20% sucrose in PBS at 4°C. Then, the slices were cut into 10 μm-thick sections using a cryostat (Leica, Germany); the obtained sections were collected and mounted on poly-L-lysine-coated glass slides.

Assessment of cellular viability. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, USA) staining was used to assess the slice viability. MTT was dissolved in PBS as a 5 mg/ml stock solution. The freshly prepared slices and cultured ones were transferred into four-well sterile plastic plates, where each well contained 450 μl of the culturing medium (four slices in each well). For the assay, 50 μl of the stock solution was added to the medium, and the cells were incubated at 37°C for 20 min. The slices were then photographed using a bright-field microscope.

Assessment of apoptosis. To study morphological manifestations of apoptosis, a combination of propidium iodide (PI, Sigma, USA, 10 μg/ml in PBS, 15 min at room temperature) and Hoechst 33342 (Sigma, USA, 10 μg/ml in PBS, 1.0 min at room temperature) was used. The cryostat sections were washed in PBS (3×5 minutes), mounted in glycerol/PBS (1:1), and coverslipped. Digital photographs were taken with an Olympus camera attached to an Olympus fluorescence microscope (Olympus Optical Co. Ltd., Japan) using the appropriate excitation and emission filters. The percentage of viable motor neurons was estimated by counting 12 randomly

selected ventral horns from each experiment.

Statistical analysis. Results are expressed as means ± s.d.. The statistical significances of inter-group differences were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's *post-hoc* test. In all cases, $P < 0.05$ was considered significant.

RESULTS

Ionotropic glutamate receptor antagonists increased the cell viability in slices. In freshly prepared slices (0 h), intense MTT staining was observed in both white and gray matters (Fig. 1A). In slices cultured for 6 h (control), staining in the white matter and ventral horns was noticeably less pronounced (B). Applications (exposure 6 h) of 50 μM MK-801 (C) or 100 μM CNQX (D) to the culture considerably increased manifestations of the slice viability compared to the control.

Inhibition of apoptosis of motoneurons by ionotropic glutamate receptor antagonists. In spinal cord sections, motoneurons were identified by their morphological characteristics (large cell bodies and large nuclei) and location (ventral horns). In freshly excised slices (0 h), the motoneurons looked intact. They had comparatively large somata, large nuclei, and the expected distribution of nuclear chromatin with no signs of apoptosis (Fig. 2A). In contrast, a noticeable proportion of motoneurons in slices cultured for 6 h (control) displayed clear morphological signs of apoptosis, including changes in the form of the nuclei and chromatin condensation (B), as compared with motoneurons of the former group (0 h). Applications of 50 μM MK-801 (C) or 100 μM CNQX (D) for 6 h effectively inhibited changes in the nuclei and chromatin condensation in motoneurons. Also, MK-801 and CNQX significantly increased the percentage of viable motoneurons in the ventral horns after 6 h as compared with the pattern seen in the control group (Fig. 3).

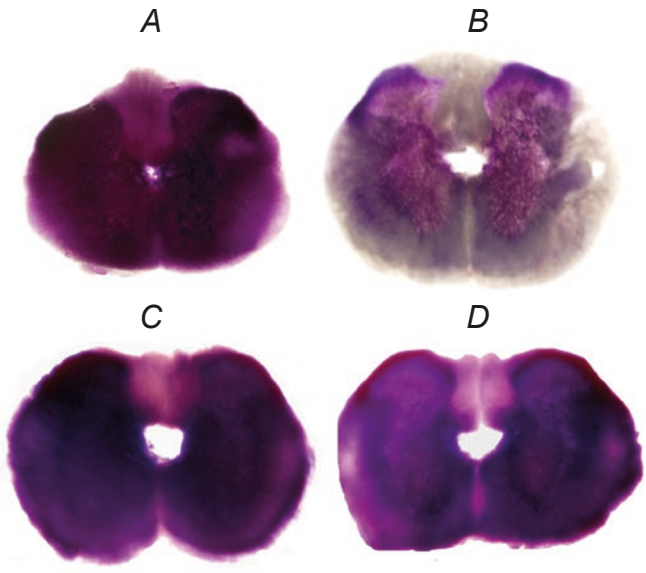


Fig. 1. MTT staining for assessing the spinal cord slice viability. A) A freshly prepared slice (0 h); B) a slice cultured for 6 h (control), C and D) slices subjected to administration of 50 μM MK-801 (C) and 100 μM CNQX (D) for 6 h. As can be seen, applications of an NMDA glutamate receptor antagonist (C) and of an AMPA/kainate (non-NMDA) glutamate receptor antagonist (D) considerably increase the slice viability. Magnification 40×.

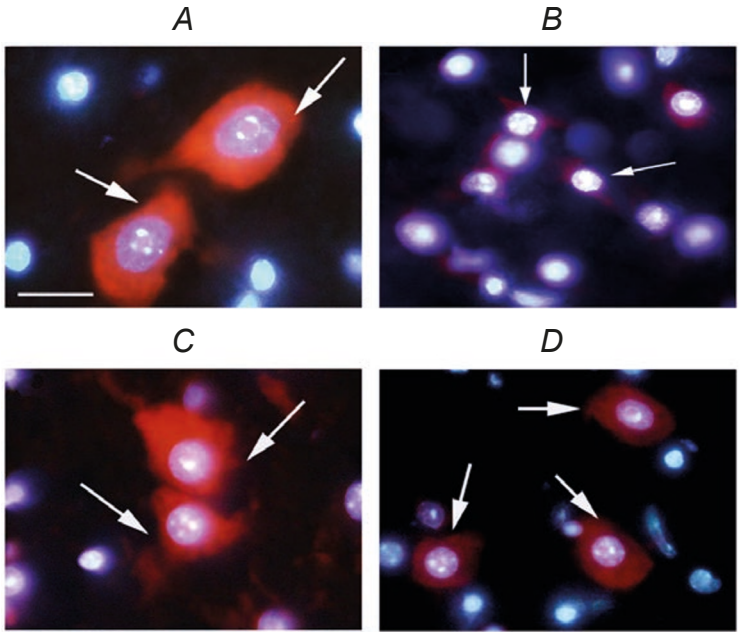
DISCUSSION

In this our study, organotypic spinal cord slices from adult mice were used to investigate the effect of glutamate receptor antagonists on cultured slices. Such slices are representative as models of an injured spinal cord [17] and could be used for evaluation of the neuronal survival and death [18] and also for that of motoneuron regeneration [19].

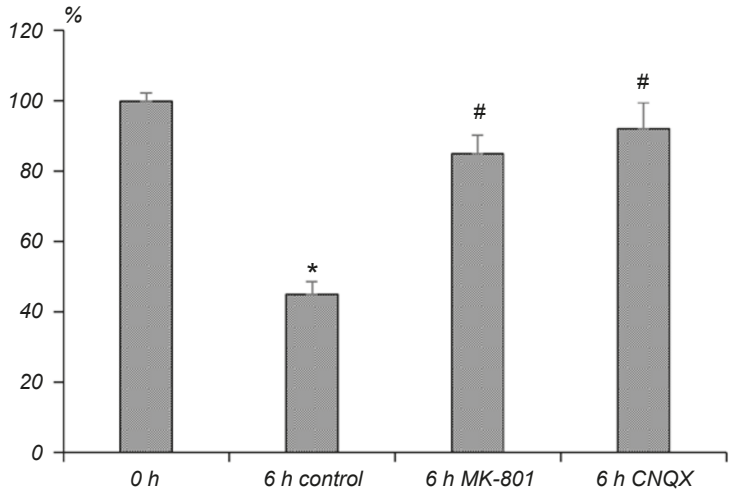
We used the MTT assay to evaluate the mitochondrial integrity in the cultured spinal cord slices. Such assay was chosen over other viability assays, e.g., trypan blue or propidium iodide (PI) [20], which assess the membrane integrity in the regions within slices but provide no information about the mitochondrial or metabolic functions. MTT is reduced by the active mitochondrial enzyme succinate dehydrogenase into insoluble formazan precipitate (purple), which then is accumulated within living cells but not within dead cells [21]. Therefore, the intensity of the purple color is directly related to the number of live cells. The method can also be used to localize the areas of cell death within the slices.

It was demonstrated that cell death induced by SCI is based mostly on apoptosis, as was determined by morphological and biochemical examinations [22]. In agreement with the results of our previous study [5, 23], we confirmed, in part, that morphological features of apoptosis in motoneurons are manifested within an early time interval (6 h) after the beginning of slice culturing.

There is evidence that excessive activation of ionotropic glutamate receptors is a reason for



F i g. 2. Effects of the NMDA glutamate receptor antagonist MK-801 (50 μM) and the AMPA/kainate (non-NMDA) glutamate receptor antagonist CNQX (100 μM) on manifestations of apoptosis of motoneurons in spinal cord slices. A) Motoneurons from freshly prepared slices (0 h); B) motoneurons from slices cultured for 6 h (control), displaying morphological features of apoptosis; C and D) administrations of 50 μM MK-801 (C) or 100 μM CNQX (D) can prevent signs of apoptosis in motoneurons from slices cultured for 6 h. Arrows point out motoneurons. Scale bar 20 μm for all panels.



F i g. 3. Percentage of viable motoneurons in fresh spinal cord slices (0 h), slices cultured for 6 h, and those exposed to the NMDA glutamate receptor antagonist (MK-801, 50 μM) and AMPA/kainate (non-NMDA) glutamate receptor antagonist (CNQX, 100 μM) and also cultured for 6 h. Data are presented as means ± s.d. * Significant intergroup differences ($P < 0.05$) estimated by one-way ANOVA followed by the Tukey's test ($n = 12$ in each group) in comparison with the 0 h group; # significant differences from the 6 h control group.

excitotoxicity leading to neuronal death in stroke [24], neurodegenerative diseases [15], and SCI [7]. In accordance with this, inhibition of these receptors by their antagonist can effectively protect against neuronal death [25]. In addition, blocking of these receptors attenuated manifestations of apoptosis of motor neurons in spinal cord slices subjected to weight-drop injuries [4] or to the action of a glutamate receptor agonist [13]. Therefore, it is reasonable to assume that, in this our study, apoptosis of motoneurons in cultured slices subjected to no additional influences was

due to glutamate-related excitotoxicity. If this hypothesis is true, blocking of ionotropic glutamate receptors will be able, therefore, to attenuate apoptosis of these cells. Interestingly, we showed that administration of NMDA and AMPA/kainate glutamate receptor antagonists not only increases the viability of cultured slices and the percentage of viable motoneurons in these slices, but also delays morphological features of apoptosis in these neurons.

An excitotoxic pathway, which is mediated by ionotropic glutamate receptors, may appear a key step in initiation of the apoptosis cascade in the motoneurons under study. Overactivation of ionotropic glutamate receptors by a high level of glutamate can directly increase the influx of cations, in particular of $Ca^{2+}$and Na^{+}, leading to depolarization of the neurons and, consequently, to activation of voltage-gated calcium channels; this allows further influx of Ca^{2+} [26,27]. Such events cause Ca^{2+} overload of the cytosol, which induces dysregulation of Ca^{2+} homeostasis and initiates various apoptotic cascades in motoneurons. Accordingly, we showed that chelation of extracellular Ca^{2+} by ethylene glycol-bis(β-aminoethyl ether)-*N',N',N',N'*-tetraacetic acid (EGTA) [23] and blocking of voltage-gated calcium channels [9] could delay apoptosis of motoneurons in mouse spinal cord slices.

After accumulation of Ca^{2+} and increase in the cytosolic Ca^{2+} levels, a variety of possible signaling cascades can be proposed to explain the cellular and molecular mechanisms responsible for glutamate-induced apoptosis among motoneurons. Calcium overloading can trigger several downstream signaling events, including toxic abnormal enzymatic activation, mitochondrial dysfunction, and excessive generation of free radicals [14]. Activation of calcium-dependent proteases, such as calpains responsible for degradation of cytoskeletal, membrane, and nuclear proteins (lamins in particular), induces cell shrinkage and also chromatin condensation [28]. Accordingly, activation of calpain in apoptotic motoneurons in the spinal cord [6], in particular under conditions of SCI [29], was reported. Additionally, Ca^{2+}-dependent endonucleases are responsible for nuclear changes and DNA fragmentation [30]. These enzymes are major players during neuronal apoptosis [31].

Mitochondria also play an essential role in the maintenance of a low concentration of Ca^{2+} in the cytosol. A transient increase in cytosolic calcium stimulates mitochondrial uptake of calcium [32]. This can, therefore, more or less protect neurons against elevation of the intracellular Ca^{2+} concentration [33]. Under pathological conditions, for instance glutamate excitotoxicity, accumulation of Ca^{2+} in the mitochondria leads to damage of their oxidative phosphorylation and, consequently, to a low energy production [33]. Also, this induces changes in the mitochondrial voltage and opening of the mitochondrial permeability transition pores (MPTPs). This, in turn, results in the release of apoptogenic proteins, including apoptosis-inducing factor (AIF), endonuclease G, and cytochrome c from the mitochondria [32, 34].

A crucial role of oxidative stress is implicated in the pathology of most neurodegenerative diseases [12]. Mitochondrial dysfunction can, therefore, induce neuronal apoptosis by enhancing generation of reactive oxygen species (ROSs). This, in turn, negatively affects cellular lipids, proteins, and nucleic acids [12].

It should be taken into account that apoptosis is a rather complex process that can be triggered by multiple and simultaneously acting signaling pathways. Therefore, excitotoxicity-induced pathological shifts in motoneurons may not be triggered by only those mechanisms, which induce apoptosis in these neurons.

Thus, the possibility that ionotropic glutamate receptor antagonists can increase the viability of slices and delay the development of apoptosis of motoneurons in "unprovoked" mouse spinal cord slices suggests that glutamate excitotoxicity-mediated apoptosis is rather rapidly (within a few hours) induced in motoneurons of these slices. Therefore, this mechanism might also be significant under conditions of SCIs.

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The experiments correspond to the international ethical standards and were approved by the local Ethical Committee of the Arak University.

The authors, H. R. Momeni, M. Jarahzadeh, and E. Farjad, confirm the absence of any conflicts over commercial or financial relations, relations with organizations or individuals that could in any way be related to the study, and also in interrelations between the co-authors.

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