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Riluzole prevents morphine-induced apoptosis in rat cerebral cortex

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Abstract:
Neuronal apoptosis has been shown to be associated with the development of tolerance to morphine. In the present study, we investigated the effect of intracerebroventricular (*icv*) administration of an inhibitor of glutamate release, riluzole, on morphine-induced apoptosis in the rat cerebral cortex. Various groups of rats received either morphine (intraperitoneally, *ip*) and vehicle (*icv*) or morphine (*ip*) and different doses of riluzole (*icv*) once per day for 8 days. An *in situ* terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method was used as an apoptosis assay. Levels of the anti-apoptotic factors Bcl-2 and HSP70 and the pro-apoptotic agent caspase-3 were evaluated by immunoblotting. The glutamate concentration in the cerebral cortex was measured by high performance liquid chromatography (HPLC). The results showed that *icv* administration of riluzole decreased the number of apoptotic cells in the cerebral cortex compared with the control group, which was treated with morphine (*ip*) and 1% Tween 80 in 0.9% normal saline (*icv*). The levels of the anti-apoptotic proteins Bcl-2 and HSP70 were higher in the riluzole groups than in the control. Furthermore, co-administration of riluzole with morphine significantly decreased caspase-3 protein levels and glutamate content of the cerebral cortex compared with the control. In conclusion, we found that *icv* administration of riluzole attenuates morphine-induced apoptosis in the cerebral cortex after the development of morphine tolerance.

Key words:
apoptosis, cerebral cortex, glutamate, morphine, riluzole

Introduction

Although opioid drugs such as morphine are indispensable in the clinical management of pain, their repeated use can lead to the development of analgesic tolerance and physical dependence. Opioid tolerance manifests as a loss of agonist potency and as a shift of the dose-response curve to the right. Many neurotransmitter systems are implicated in the development of morphine tolerance elicited by prolonged morphine exposure. During the past decades, many studies have focused on the roles of excitatory amino acid receptors in the development of tolerance to the antinociceptive action of morphine. Numerous studies have

demonstrated that a variety of N-methyl-D-aspartate (NMDA) receptor antagonists and blockers have the ability to inhibit the development of opiate tolerance and dependence [2, 12, 14, 15, 33]. Other studies have shown that activation of NMDARs can lead to neurotoxicity under many circumstances [26, 27]. For instance, peripheral nerve injury has been shown to activate spinal cord NMDARs, which results in not only intractable neuropathic pain but also neuronal cell death by means of apoptosis [20, 35]. In addition, it has been shown that, *in vivo*, neuronal apoptosis occurs in the rat's spinal cord and dorsal raphe nucleus after chronic morphine treatment [7, 17, 21]. Our more recent studies demonstrated that prolonged morphine administration induces up-regulation of pro-apoptotic elements such as caspase-3 and down-regulation of the anti-apoptotic factors Bcl-2 and HSP70 in the rat cerebral cortex and spinal cord [16, 17]. Importantly, up-regulation of caspase-3 and Bax was inhibited when morphine was co-administered with the noncompetitive NMDAR antagonist MK-801, thereby supporting a link between NMDAR activation and intracellular changes in caspase-3 and Bax in response to prolonged morphine administration [21]. One portion of our recently published finding, which is reproduced in Figure 1, indicated that riluzole (2-amino-6-[trifluoromethoxy]benzothiazole), an antiglutamatergic agent, decreases the development of tolerance, shifting the first day of established tolerance from the 8th day in the control group (treated intraperitoneally, *ip*, with morphine and 1% Tween 80, *icv*) to the 13th day [13]. Riluzole interferes with responses mediated by excitatory amino acids, even though it does not interact with any known binding sites on the NMDA, kainate or AMPA glutamate receptors [9]. The neuroprotective effect of riluzole, which has been shown both *in vivo* and in *vitro*, is believed to be beneficial in various neurodegenerative diseases and amelioration of trauma and stroke [1, 10]. In the present study, we were interested in investigating the ef-

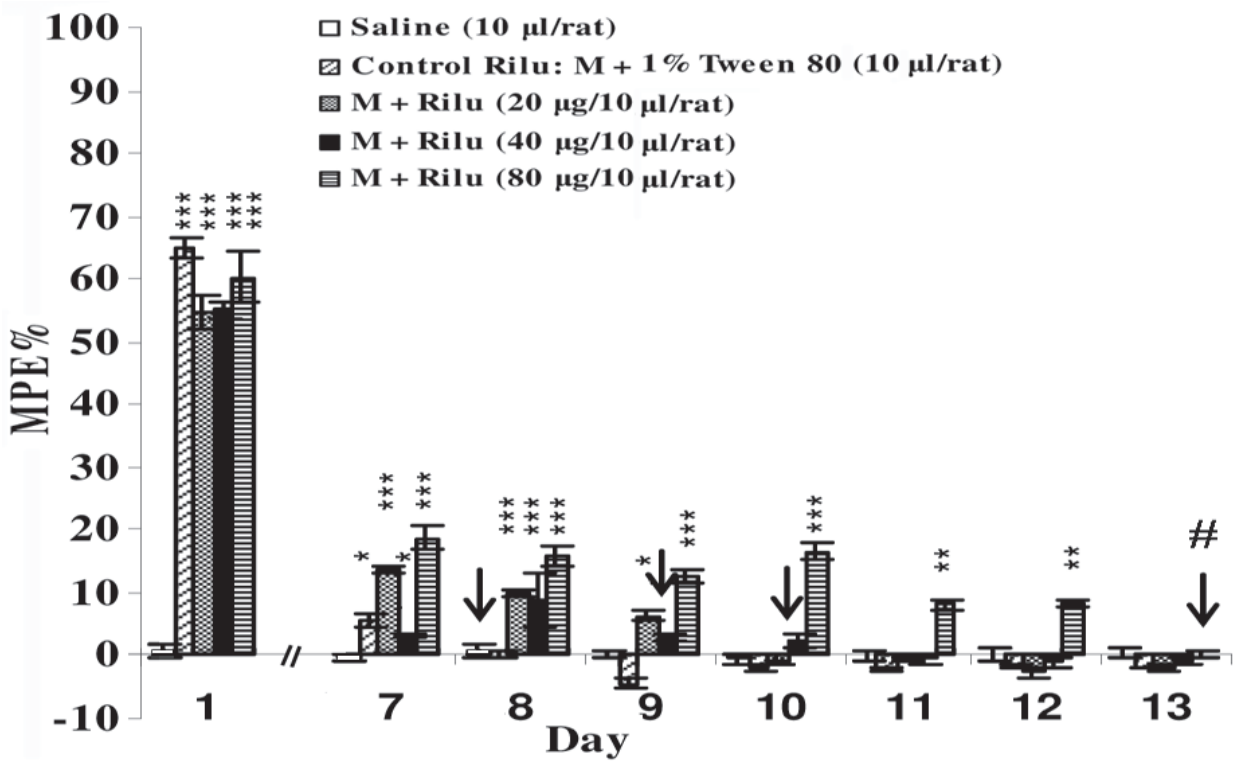

Fig. 1. Effect of daily *icv* injection of riluzole (20, 40 and 80 µg/10 µl/rat) on tolerance to the analgesic effect of morphine. Each bar represents the mean of %MPE (maximal possible effect) ± SEM for 8 rats. A Student's *t*-test was used to compare the statistical differences between each treatment and saline group. A one-way ANOVA followed by Tukey test was used to analyze the statistical significances between the treated and control groups. A p-value of less than 0.05 was considered to be significant in all analyses. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ when compared with saline group. # $p < 0.001$ when compared with control (M + 1% Tween 80). The arrow represents the first day of morphine tolerance. M = Morphine, Rilu = Riluzole [13]

fect of intracerebroventricular (*icv*) administration of riluzole on morphine-induced apoptosis in the rat cerebral cortex after the development of tolerance to the analgesic effects of morphine.

Materials and Methods

Drug treatment

Morphine sulfate (Temad Co., Iran; 10 mg/kg, *ip*) was dissolved in sterile 0.9% normal saline and injected *ip* using 1-ml insulin syringes. Riluzole (Sigma-Aldrich, Inc.; 20, 40 and 80 μg/10 μl/rat) was dissolved in 1% Tween 80 in sterile 0.9% normal saline and infused *icv* using a Hamilton syringe. In addition, two groups of animals received saline and vehicle or riluzole (80 μg/10 μl/rat) alone. The volume of infusion was 10 μl at a rate of 10 μl/min for each rat.

Animals

Male Wistar rats weighing 250–300 g were kept in a temperature-controlled room (24 ± 0.5°C) and were maintained on a 12-h light/dark cycle (light on at 08:00 h) with free access to food and water. All experiments were executed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85–23, revised 1985) and were approved by the research and ethics committee of the Tabriz University of Medical Sciences. In total, 72 rats were used (6 rats per group for TUNEL and western blot analysis).

Icv cannula implantation

Rats were anesthetized with sodium pentobarbital (50 mg/kg, *ip*; Merck, Germany) and were stereotaxically implanted with a stainless steel guide cannula (23 gauge) into the lateral cerebral ventricle with coordinates of –0.8 mm posterior, –1.3 mm midline to lateral and 3.5 mm ventral with respect to bregma [25].

A stainless steel guide (30 gauge) was placed into the guide cannula as a dummy cannula to maintain patency. After surgery, a recovery period of 7 days was allowed before the commencement of the central and systemic injections. Morphine and riluzole injections were started at day 8 after cannula implantation.

Tissue preparation

For the *in situ* TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assay, all animals (n = 6) received the same treatment regimen such as the behavioral groups in our previous study [13]. On the 9th day (one day after tolerance completion in the control group), 2 h after the last dose of vehicle or treatment, the animals were euthanized with pentobarbital and perfused with phosphate buffered saline (PBS). Cerebral cortexes were immediately dissected and fixed in 10% (v/v) formaldehyde (Merck, Germany; pH adjusted to 7.0 with NaOH) for 3 days. The tissues were then embedded in paraffin. To evaluate a possible association between morphine tolerance and apoptosis, we used a group of rats that received morphine along with riluzole at a dose of 80 μg/10 μl/rat, which was the most effective dose of riluzole for inhibition of morphine tolerance. One day after tolerance completion in this group (on day 14th) and 2 h after the last dose of vehicle or treatment, the cerebral cortexes of the rats were removed and prepared as described above.

Detection of apoptotic cells

After fixation and paraffin embedding, sections (3 μm) were cut with a microtome (Leitz, Germany). For the TUNEL assay, an *in situ* Cell Death Detection kit (Roche Applied Science, Cat # 11 684 817 910) was used. After deparaffinization, the tissue sections were stained according to the manufacturer's instructions. Briefly, deparaffinized and rehydrated sections were pretreated with proteinase K (Roche, Germany) for 30 min at 37°C. Then, the sections were exposed to the TUNEL reaction mixture, which contains terminal deoxynucleotidyl transferase and nucleotides including fluorescein isothiocyanate-labeled dUTP (37°C). After 60 min, an anti-fluorescein peroxidase (POD)-linked antibody was added, followed by incubation for 30 min at 37°C. Finally, the reaction product was visualized by 3,3-diaminobenzidine tetrahydrochloride (DAB) incubation for 15 min at RT, and the slides were then counterstained with methylene blue. A subpopulation of apoptotic cells, scattered throughout the tissue section, was intensely stained (brown) by the TUNEL treatment; their numbers were analyzed using a Zeiss Axiovert 100 light microscope (100× objective) over 30 fields.

Western blot analysis of Bcl-2, HSP70 and caspase-3

For western blotting, rats (n = 6) were rapidly (< 1 min) sacrificed, and the cerebral cortex segments were removed, cleaned and frozen in liquid nitrogen. Subsequently, the tissue samples were homogenized in lysis buffer (500 mM Tris-HCl, pH 7.4, 150 mM NaCl, EDTA 0.5 mM, N-octyl β-D-glucopyranoside 1.5% w/v) containing a complete protease inhibitor cocktail tablet (Roche Cat # 04 693 132 001). The protein quantification for each loading lane was estimated by a protein assay with absorbance at 280 nm. Lysates (20 μg protein) were resolved by SDS-PAGE (12%) for Bcl-2 and HSP70 and by SDS-PAGE (15%) for caspase-3. Next, the lysates were transferred to polyvinylidene difluoride (PVDF) filters (Millipore, Bedford, MA, USA), which were blocked with 5% milk and incubated overnight at 4°C with the following primary antibodies, all of which were purchased from Abcam: caspase-3 (18-20 kDa), 1:500, Cat # ab2302; Bcl-2 (26-29 kDa), 1:1000, Cat # ab16904; HSP 70 (70 kDa), 1:5000, Cat # ab6535; and β-actin (42 kDa), 1:1000, Cat # 8226. Subsequently, PVDF membranes were incubated for 1 h at room temperature with an HRP-conjugated secondary antibody (1:10,000). The blots were then visualized with an enhanced chemiluminiscence (ECL) detection kit/system for 1 min and were exposed to Hyperfilm (Roche) for 30 s to 5 min. Finally, the developed films were scanned, and the density of immunoreactive bands was measured using ImageJ software and normalized to the internal control bands. β-Actin was used as a loading control. For western blotting, differences in the image density were analyzed by a one-way analysis of variance (ANOVA) (multiple groups) followed by a Tukey test.

High performance liquid chromatography (HPLC) analysis of glutamate

To determine the levels of the amino acid neurotransmitter glutamate, HPLC was employed. Tissue samples from rats (n = 6) were homogenized, and protein determination was performed. Subsequently, the homogenates were spun at 4°C for 10 min at 10,000 × g. The chromatograph was a KNAUER (Berlin, Germany) HPLC instrument. The system included a quaternary pump, a RF-551 fluorescence detector (FLD) and an autosampler (Spark, Triatlon), which was controlled by Chromgate software. The analytical column used was a reverse phase Hypersil ODS column (250 × 4.0 mm, 5 μm particle size) at room temperature. For the chromatographic separation, the mobile phases consisted of 8% acetonitrile in 12.5 mM phosphate buffer, pH 7.2, with an o-phthalaldehyde (OPA) derivative of glutamate eluted at a gradient flow rate of 1 ml/min for 8 min and 2 ml/min for 12 min, and the detection was carried out with 330 nm and 460 nm as the excitation and emission wavelengths, respectively. Glutamic acid was used as a standard. Solutions of glutamic acid (0.75, 1.5, 3, 6 and 12 μg/ml) were injected into the HPLC instrument, and a calibration curve was plotted. A 2.5% solution of supernatant in distilled water was used for derivatization and determination of glutamate. The level of glutamate was calculated by comparing the peak areas with those of the standards, and the values were expressed as μmol/100 mg protein [23].

Data analysis

The histological data (cell counting) from cerebral cortex sections were averaged and are expressed as the means ± SEM. Data from the western blots are expressed as % of control (morphine, *ip*, and 1% Tween 80, *icv*), and data from HPLC are expressed as the means ± SEM. Student's *t*-test was used to compare the means of two groups, and a one-way analysis of variance (ANOVA) followed by Tukey test was used to compare the means of multiple treatment groups; p-values of less than 0.05 were considered to be significant.

Results

The presence of apoptotic cells was evidenced by TUNEL staining

The TUNEL method was used to identify apoptotic cells. In the control group (morphine, *ip*, and 1% Tween 80 in 0.9% normal saline, *icv*), the number of TUNEL-positive cells significantly ($p < 0.001$) increased in cerebral cortex in comparison with vehicle-treated animals (Fig. 2), thereby indicating that there was an increased basal level of apoptosis in morphine-treated animals. The average number of TUNEL-positive cells in the cerebral cortex was significantly reduced in the riluzole groups (40 and 80 μg/10 μl/rat)

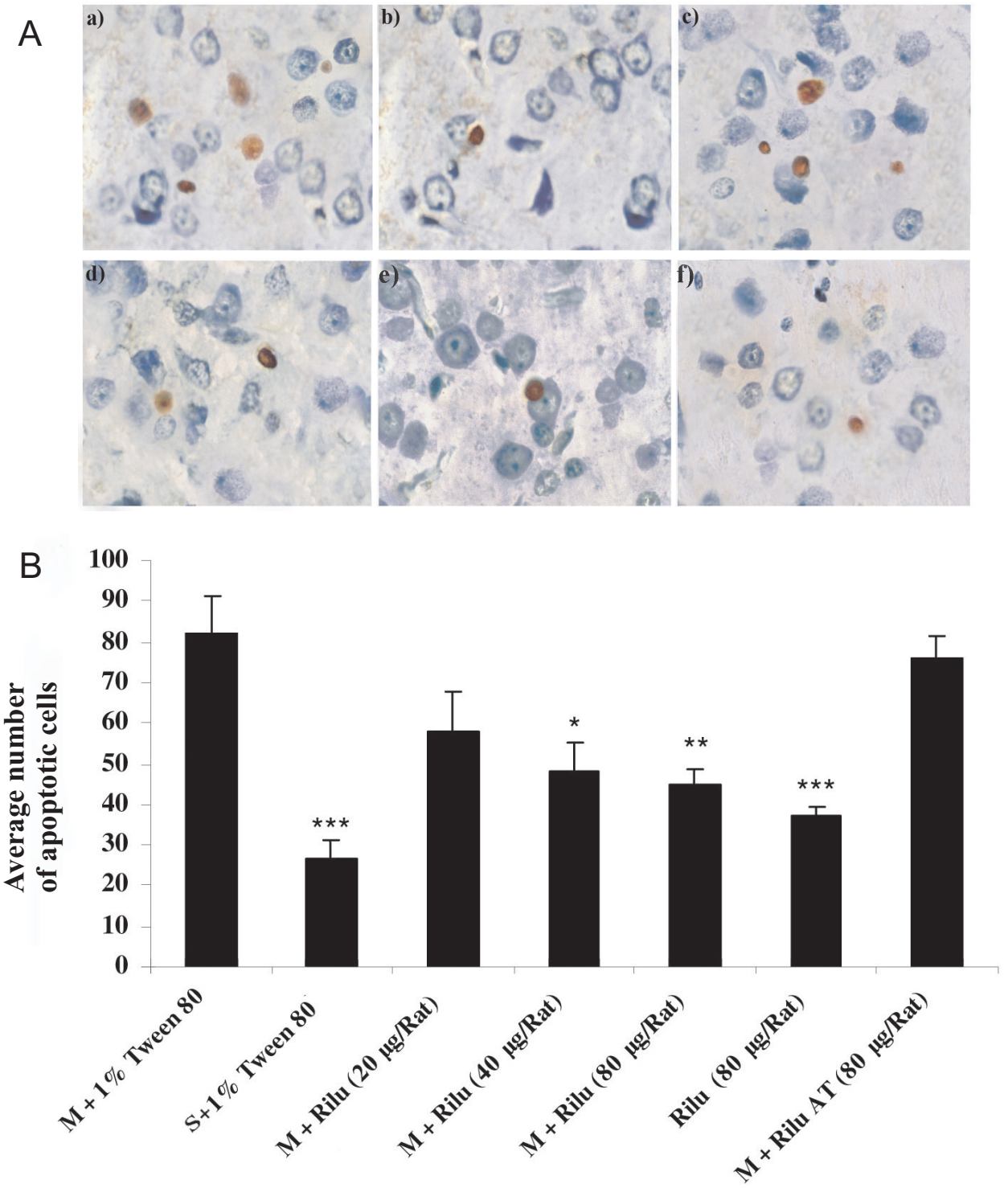

Fig. 2 (**A**) Tissue sections from rat cerebral cortex were prepared and assayed with an In Situ Cell Death Detection Kit, POD. Slides were counterstained with methylene blue. Representative photos illustrate the subpopulation of apoptotic cells, which are scattered throughout the tissue section and were intensely stained (brown) by the TUNEL treatment. Slides were analyzed with a light microscope (100 × objectives). (**A**) a: Morphine + 1% Tween 80, b: Saline + 1% Tween 80, c: Morphine + Riluzole (20 µg/10 µl/rat), d: Morphine + Riluzole (40 µg/10 µl/rat), e: Morphine + Riluzole (80 µg/10 µl/rat), f: Riluzole (80 µg/10 µl/rat). (**B**) Quantification of apoptotic cells. The data represent the mean ± SEM number of apoptotic (TUNEL-positive) cells in 30 fields, which were counted at a magnification of 100× with a light microscope. A one-way ANOVA followed by Tukey test was used to analyze the statistical significances. A p-value of less than 0.05 was considered to be significant for all analyses,* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ when compared with the control group (M + Tween 80 1%). S = Saline, M = Morphine, Rilu = Riluzole, AT = After tolerance

compared with those in the control group (morphine, *ip*, and 1% Tween 80, *icv*). There were significant differences in the number of TUNEL-positive cells between animals that had received vehicle or riluzole without morphine *versus* those that received morphine ($p < 0.001$). Animals treated with the higher dose of riluzole (80 μg/10 μl/rat) had fewer TUNEL-positive cells than animals treated with the lower doses. The group that received morphine and riluzole (80 μg/10 μl/rat) for 13 days had developed tolerance; they showed an increase in the number of apoptotic cells comparable to that seen in control conditions (morphine, *ip*, and 1% Tween 80, *icv*).

Changes in the Bcl-2, HSP 70 and caspase-3 protein content of the cerebral cortex

Our results show that after the development of morphine tolerance, levels of the anti-apoptotic factors Bcl-2 ($p < 0.01$) and HSP70 ($p < 0.001$) decreased significantly, while expression of the pro-apoptotic protein caspase-3 increased in the cerebral cortex (Figs. 3–5). All three doses of riluzole (20, 40 and 80 μg/10 μl/rat) increased the amount of HSP70 significantly ($p < 0.001$) while only the highest dose of riluzole (80 μg/10 μl/rat) was able to significantly attenuate the morphine-induced down-regulation of Bcl-2 ($p < 0.01$). In addition, the results show that riluzole,

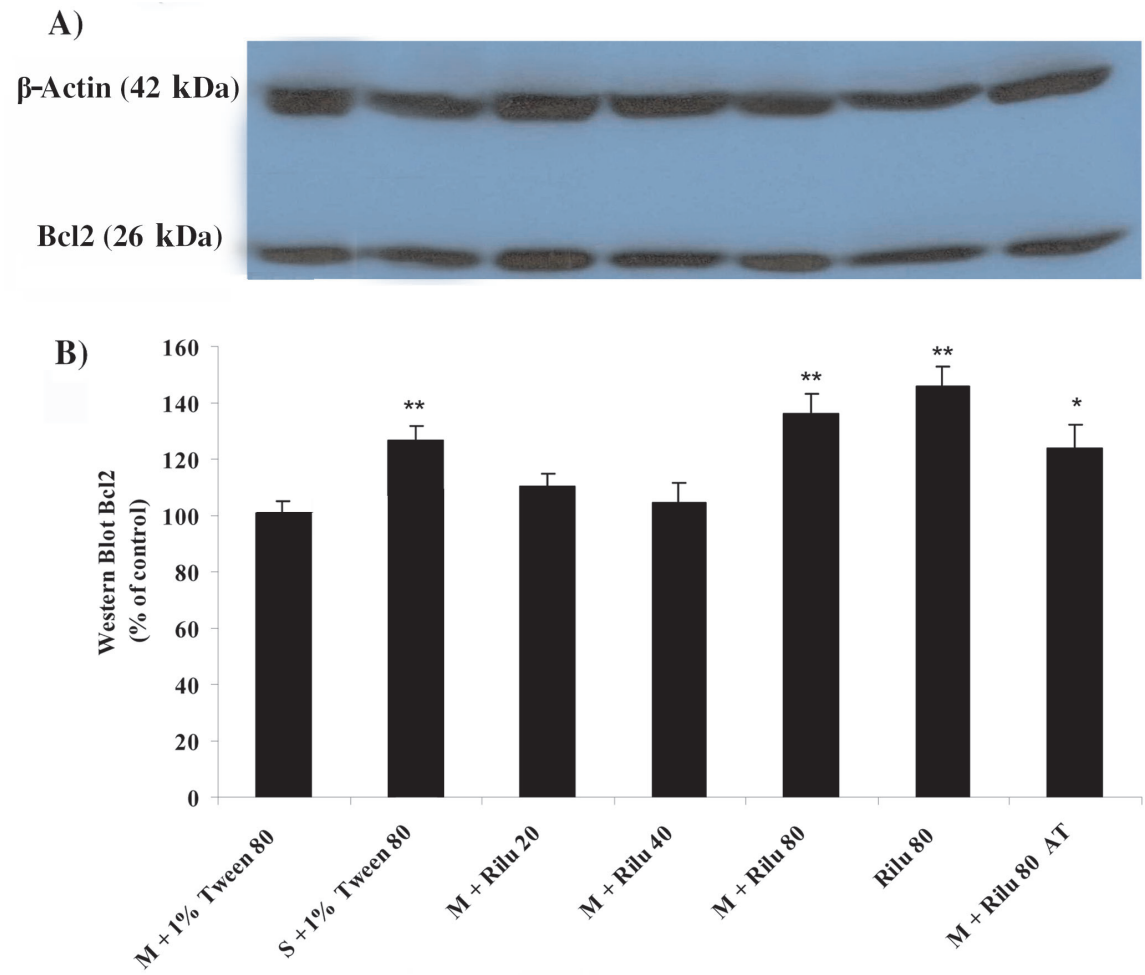

Fig. 3. Effect of *icv* riluzole (20, 40 and 80 μg/10 μl/rat) on morphine-induced changes in intracellular Bcl-2 levels (n = 5 rats per group for separate sample collections). (**A**) Representative autoradiographs of western blots illustrate a down-regulation in the levels of Bcl-2 protein (26 kDa) in rats receiving morphine (M + 1% Tween 80) daily for 9 days compared with the corresponding S + 1% Tween 80 group. Riluzole (80 μg/10 μl/rat) increased the amount of Bcl-2 protein in combination with morphine. (**B**) The statistical analysis showed differences among the various groups in the gray density obtained for the bands on the Bcl-2 western blot. A one-way ANOVA followed by Tukey test were used to analyze the statistical significances. A p-value of less than 0.05 was considered to be significant for all analyses, * $p < 0.05$, ** $p < 0.01$ compared with the corresponding control group (morphine, *ip*, and 1% Tween 80, *icv*). S = Saline, M = Morphine, Rilu = Riluzole, AT = After tolerance

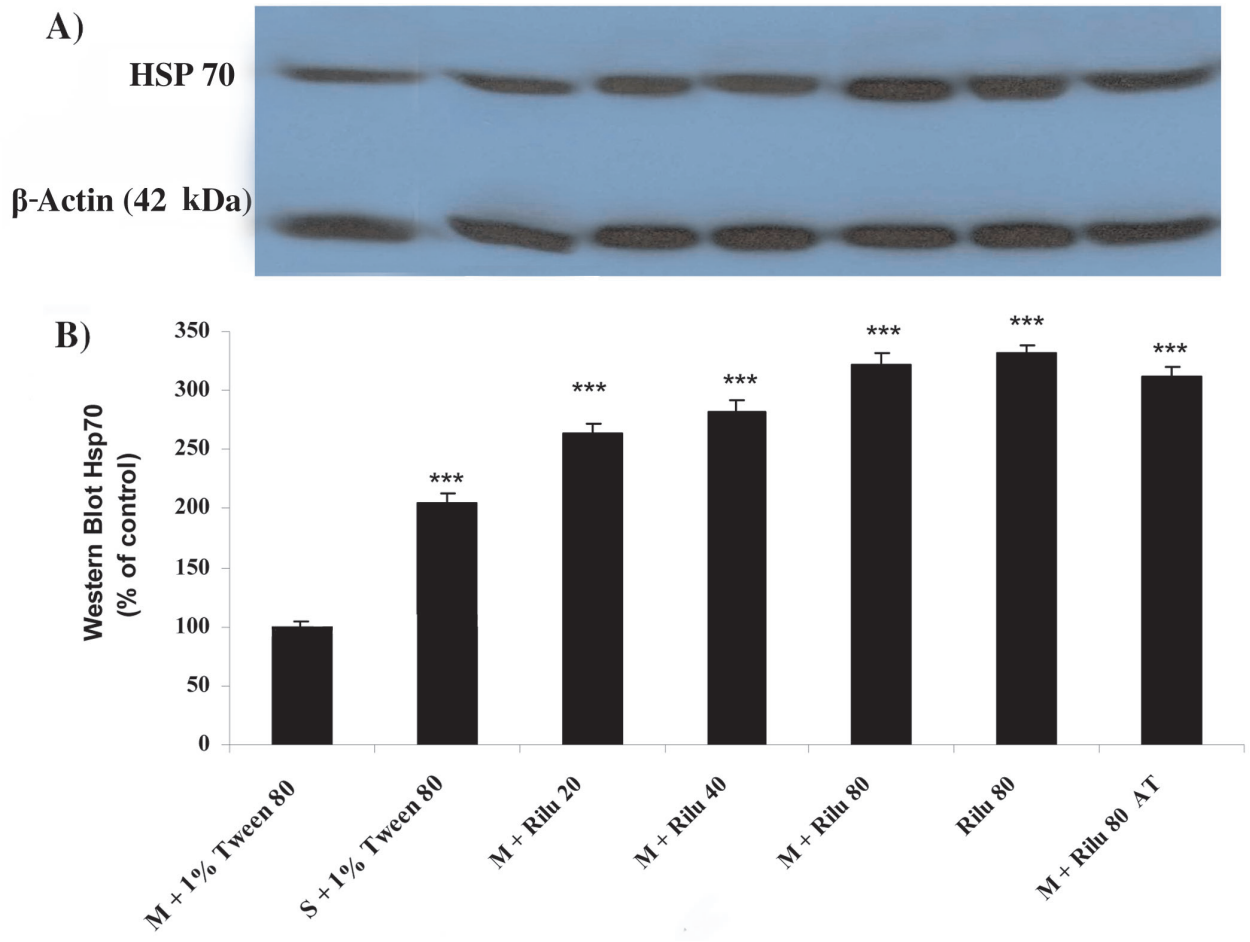

Fig. 4. Effect of *icv* riluzole (20, 40 and 80 μg/10 μl/rat) on morphine-induced changes in intracellular HSP 70 levels (n = 5 rats per group for separate sample collections). (**A**) Representative autoradiographs of western blots illustrate a down-regulation of the levels of HSP 70 protein in rats receiving morphine (M + 1% Tween 80) daily for 9 days compared with the corresponding S + 1% Tween 80 group. Riluzole (40 and 80 μg/10 μl/rat) increased the amount of HSP 70 protein. (**B**) The statistical analysis showed differences in the gray density from the bands on the western blot of HSP 70 among the various groups. A one-way ANOVA followed by Tukey test were used to analyze the statistical significances. A p-value of less than 0.05 was considered to be significant for all analyses, *** $p < 0.001$ compared with the corresponding control group (morphine, *ip*, and 1%Tween 80, *icv*). S = Saline, M = Morphine, Rilu = Riluzole, AT = After tolerance

at doses of 40 and 80 μg/10 μl/rat significantly decreased the levels of the pro-apoptotic factor caspase-3 in the cerebral cortex compared with the control (morphine, *ip*, and 1% Tween 80, *icv*) ($p < 0.05$), as shown in the western blots in Figure 5.

Effect of riluzole on glutamate concentration in the cerebral cortexes of morphine-treated rats

The results presented in Table 1 show that chronic administration of morphine (10 mg/kg, *ip*) for 9 days induced a significant increase in glutamate concentration in the cerebral cortex of morphine-tolerant rats ($p < 0.001$). On the other hand, our findings indicate that *icv* administration of riluzole (40 and 80 μg/10 μl/rat) concomitantly with morphine was able to attenuate this elevation in glutamate concentration ($p < 0.001$).

Discussion

The purpose of this study was to explore the cellular mechanism of riluzole's effects on tolerance to morphine. Our findings are consistent with the idea that morphine-induced tolerance and apoptosis is attenuated by *icv* administration of riluzole. The cellular

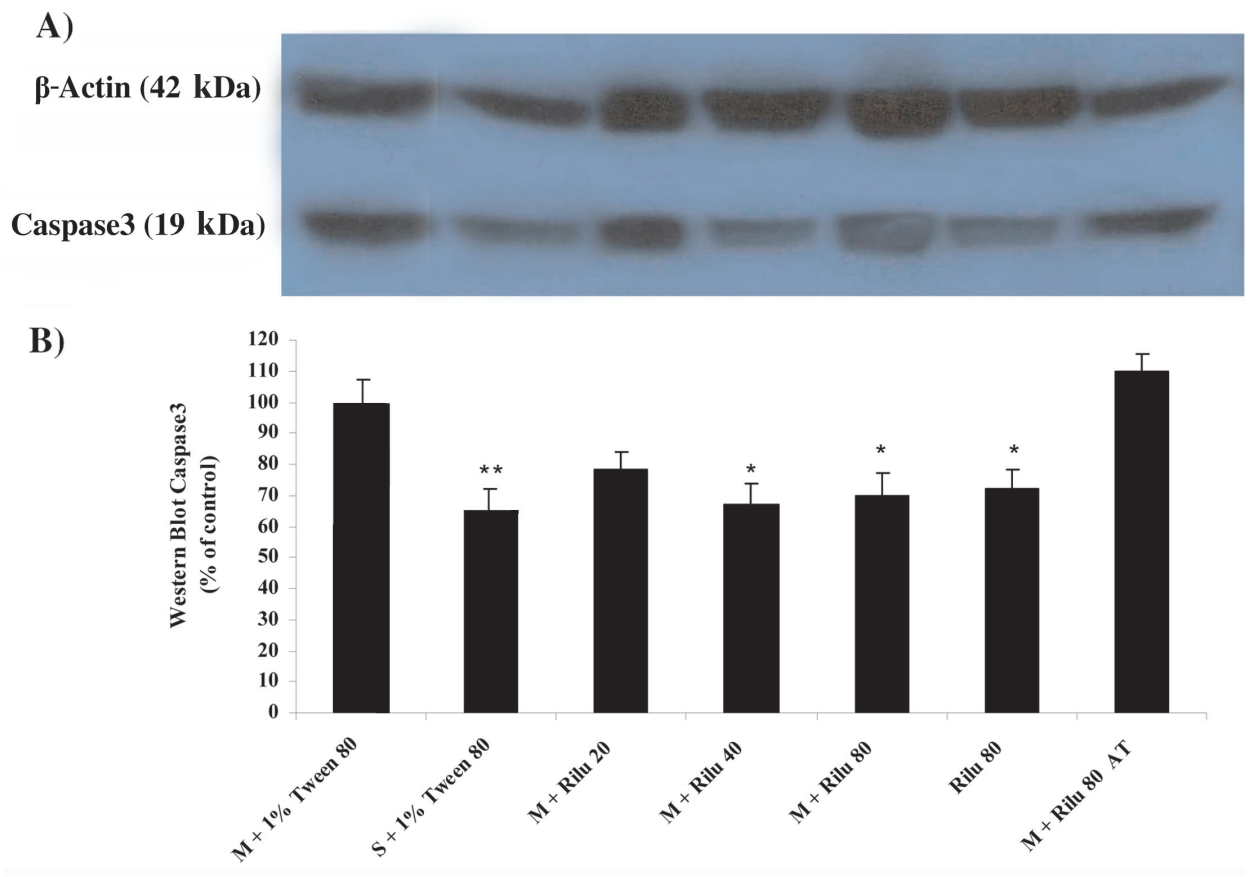

Fig. 5. Effect of *icv* riluzole (20, 40 and 80 μg/10 μl/rat) on morphine-induced changes in intracellular caspase-3 content (n = 5 rats per group for separate sample collections). (**A**) Representative autoradiographs of western blots illustrate up-regulation of the levels of caspase-3 protein (19 kDa) in rats receiving morphine (M + 1% Tween 80) daily for 9 days compared with the corresponding S + 1% Tween 80 group. (**B**) The statistical analysis showed differences in the gray density obtained for the bands on the western blot representing caspase-3 protein among different groups in the cerebral cortex. A one-way ANOVA followed by Tukey test were used to analyze the statistical significances. A p-value of less than 0.05 was considered to be significant for all analyses, * $p < 0.05$ compared with the corresponding control group (morphine, *ip*, and 1% Tween 80, *icv*). S = Saline, M = Morphine, Rilu = Riluzole, AT = After tolerance

Tab. 1. Concentration of glutamate in cerebral cortex of rat

Treatment	Glutamate concentration (umol/100 mg protein)
S + 1% Tween 80	310 ± 9.69***
M +1% Tween 80	478.5 ± 5.2
M + Rilu (20 μg/10 μl/rat)	456.6 ± 9.75
M + Rilu (40 μg/10 μl/rat)	449.8 ± 6.51*
M + Rilu (80 μg/10 μl/rat)	405.71 ± 9.39***
Rilu (80 μg/10 μl/rat)	304.8 ± 7.50***

Each data represents the mean ± SEM for 5 rats. One-way ANOVA followed by Tukey's test was used to analyze the statistical significances. A p-value less than 0.05 was considered to be significant in all analyses, * $p < 0.05$, *** $p < 0.001$ when compared to control (M + 1% Tween 80) group. S = Saline, M = Morphine, Rilu = Riluzole

mechanism underlying the development of morphine tolerance remains controversial. It has been demonstrated that certain addictive drugs, such as morphine, can induce apoptosis in both *in vitro* and *in vivo* studies [7, 17, 28, 30]. More recently, we showed *in vivo* that neuronal apoptosis occurs in the rat spinal cord after chronic morphine treatment and is associated with an elevation in caspase-3 levels and a decrease in the amounts of the anti-apoptotic agents Bcl-2 and HSP70, suggesting that chronic morphine administration may lead to changes within the central nervous system (CNS) [17]. The results of the present study indicate that prolonged exposure to morphine induces apoptotic cell death in the cerebral cortex, which contributes, at least in part, to the behavioral manifestation of morphine tolerance. Our recent study showed

that chronic administration of morphine for 8 days induced tolerance to its analgesic effects, while administration of riluzole (80 μg/10 μl/rat) decreased the development of this tolerance by shifting the first day of established tolerance from the 8th to the 13th day (Fig. 1). The results indicated that there was a significant shift to the right in the dose-response curve as well as an increase in the antinociceptive 50% effective dose (ED_{50}) of morphine for animals who received morphine also compared with those that received morphine and riluzole (80 μg/10 μl/rat). Moreover, administration of riluzole (80 μg/10 μl/rat) alone was without a significant analgesic effect, so this result is not related to morphine-independent analgesic effects of riluzole [13]. Furthermore, it has been demonstrated that both morphine tolerance and the associated neuronal apoptosis share a common cellular mechanism mediated, at least in part, by the NMDAR, because MK-801 blocks both tolerance and apoptosis [21]. Activation of NMDARs, in contrast, initiates intracellular pathways leading to apoptotic cell death. Our results, in agreement with those of others, indicate that chronic morphine administration significantly increases apoptosis in the rat cerebral cortex ($p < 0.001$). On the other hand, co-administration of riluzole delayed the onset of morphine-induced apoptosis and significantly decreased the average number of TUNEL-positive cells ($p < 0.01$). This finding is in line with our recent results concerning the lumbar region of the spinal cord [17]. In addition, we found that the group that received morphine and riluzole (80 μg/10 μl/rat) for 13 days had developed tolerance; they showed an increase in the number of apoptotic cells, as under control conditions. This result indicates that after the completion of tolerance in both the control (morphine, *ip*, and 1% Tween 80, *icv*) and the treated groups, apoptosis had already developed. Other studies have demonstrated that chronic treatment of rats with morphine (to induce tolerant and dependent states) is associated with a remarkable differential modulation of two key proteins involved in the regulation of programmed cell death in the brain; namely, there is an up-regulation of the pro-apoptotic Fas receptor, as well as intracellular pro-apoptotic elements such as caspase-3, combined with an opposing moderate down-regulation of the anti-apoptotic oncoprotein Bcl-2 [4, 21].

Our results in this study were in line with previous reports indicating that morphine treatment can decrease the Bcl-2 content of the brain [4, 16]. Riluzole (80 μg/10 μl/rat), on the other hand, can increase the Bcl-2 level in the cerebral cortex when given in combination with morphine.

Previous studies have demonstrated that HSP70 proteins participate in protein folding and transport, refolding of denatured proteins and cellular protection from apoptosis. HSP70 protects cells from apoptotic/necrotic death, which normally occurs after heat shock, exposure to tumor necrosis factor-α, oxidative stress, ceramide, anti-cancer drugs, radiation or nitric oxide [11, 22]. HSP70 has been reported to inhibit heat shock-induced apoptosis downstream of cytochrome c release but upstream of caspase-3 activation [19]. Our results, in agreement with data from other groups showing that morphine significantly decreased HSP70 levels in rat neurons [8], demonstrated that the HSP70 content of the cerebral cortex fell after morphine chronic administration in morphine tolerant rats. On the other hand, all three doses of riluzole significantly increased the amount of HSP70 ($p < 0.001$). The parallel increases in two anti-apoptotic factors, Bcl-2 and HSP70, represent a possible cellular mechanism for riluzole-mediated neuroprotection. Morphine treatment also increased the caspase-3 content of the cerebral cortex, and the statistical analysis revealed that riluzole (at doses of 40 or 80 μg/10 μl/rat) was able to prevent the morphine-induced increase in caspase-3. Previous studies have demonstrated that chronic morphine administration can increase glutamate release in the CNS [3, 33]. In cerebrocortical nerve terminals, riluzole inhibits glutamate release by reducing Ca^{2+} influx through P/Q type Ca^{2+} channels. Importantly, excessive release and accumulation of glutamate, which is associated with an increase in the level of intracellular calcium, plays an important role in CNS injury and neurodegenerative diseases [34]. It has also been reported that high concentrations of riluzole can attenuate excitatory amino acid receptor activation and decrease the excitability of the postsynaptic cell membrane [5, 6]. In agreement with previous studies, our results showed that morphine increased the level of glutamate in the cerebral cortex, while co-administration of riluzole (40 and 80 μg/10 μl/rat) significantly attenuated the elevation of the glutamate concentration. Although glutamate plays different roles in glutamatergic and GABAergic neurons, and measuring the overall glutamate content in the brain is not specific enough to indicate the presence of glutamate-induced excitotoxicity, these results confirm previous findings and sug-

gest that this decrease in glutamate is a possible factor in the neuroprotective and antiapoptotic effects of riluzole.

Morphine treatment has been demonstrated to lead to oxidative stress in various cells [24, 29] and has been proposed to induce macrophage injury in mice and humans [18]. Riluzole, apart from its role as an antiglutamatergic agent, has been reported to have antioxidant effects and could protect dopaminergic neurons against oxidative stress by reducing lipid peroxidation and ATP consumption [31, 32]. Thus, the antioxidant properties of riluzole may be important for its protection against morphine-induced apoptosis; this idea needs to be further investigated.

In conclusion, we found that *icv* administration of riluzole attenuates morphine-induced apoptosis in the cerebral cortex after the development of tolerance to morphine's analgesic effects. In addition, riluzole decreased the morphine-induced elevation in glutamate concentration in the rat cerebral cortex. However, further studies are required to clarify the other mechanisms underlying riluzole's effects on morphine-induced tolerance.

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