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Extracellular signal–regulated kinase 1/2 is involved in a tamoxifen neuroprotective effect in a lateral fluid percussion injury rat model

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ABSTRACT

Background: The aim of the present study was to determine whether tamoxifen (TMX) causes attenuation of traumatic brain injury (TBI) induced by fluid percussion injury.

Materials and methods: Immediately after the onset of fluid percussion TBI, anesthetized male Sprague-Dawley rats were divided into three major groups and intraperitoneally administered the vehicle solution (1 mL/kg), TMX (1 mg/kg), or TMX (1 mg/kg) plus the extracellular signal–regulated kinase 1/2 antagonist SL327 (30 mg/kg). Another group of rats were used as sham-operated controls. The functional outcomes, such as motor outcomes, were evaluated using an incline plane. The cellular infarction volume was evaluated by triphenyltetrazolium chloride staining. Neuronal loss, apoptosis, and p-ERK1/2 and Bcl2 expression in neuronal cortex cells were evaluated by immunofluorescence methods. All the parameters were assessed on day 4 after injury.

Results: Compared with the sham-operated controls, the TBI-induced motor deficits and cerebral infarction after TBI were significantly attenuated by TMX therapy. The TBI-induced neuronal loss and apoptosis were also significantly reduced by TMX therapy. The numbers of Bcl2- and phospho-ERK1/2-positive neuronal cells in the ischemic cortex after TBI were significantly increased by TMX therapy. These TMX effects were significantly blocked by SL327 administration.

Conclusions: Our results suggest that intravenous injection of TMX may ameliorate TBI in rats by increasing neuronal p-ERK1/2 expression, which might lead to an increase in neuronal Bcl2 expression and a decrease in neuronal apoptosis and cell infarction volume, and it might represent one mechanism by which functional recovery occurred. TMX may be a promising TBI treatment strategy.

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1. Introduction

Traumatic brain injury (TBI) is a major global public health concern. Despite adequate treatment, traumatic head injury commonly causes neuronal loss and apoptosis, leading to long-term neurologic deficits [1–3]. In TBI, apoptosis has been demonstrated to commonly occur in the perilesioned area as a result of secondary brain insults in animal and human studies [4,5]. These events are referred to as the secondary injury mechanism. Therefore, preventing cell apoptosis post-TBI may be an important therapeutic strategy.

Estrogen, a ligand of estrogen receptor, activates signaling cascades in healthy neurons, enhances the biochemical, genomic, and morphologic mechanisms of memory and proactively induces mechanisms of protection against neurodegenerative insults [6]. Estrogen (E2) activation of extracellular signal-regulated kinase 1/2 (ERK1/2) may elicit mechanisms of neuroprotection, as treatment with mitogen-activated protein kinases inhibitors reduces the neuroprotective effects of estrogens [7,8]. However, the application of E2 as a neuroprotectant in humans presents numerous limitations, mainly because of the endocrine actions of the molecule on peripheral tissues, including estrogen-dependent tumors. The possibility of using selective estrogen receptor modulators (SERMs) to exert E2-like neuroprotective actions in the brain has emerged as an alternative to E2 [9].

Tamoxifen (TMX) is a triphenylethylene derivative, non-steroidal first-generation SERM [10]. TMX is brain-blood barrier permeable, and its concentration has been reported to be much higher in the brain than in the serum [11,12]. Its metabolite, 4-hydroxy-tamoxifen, has a shorter half-life but binds to estrogen receptor with a binding affinity 20–30 times greater than that of TMX and equivalent to that of E2 [13,14]. TMX has been demonstrated to be a neuroprotectant for spinal cord injury [14], cerebral ischemia injury [15–17], irradiation-induced brain injury [18], and methamphetamine-induced toxicity [19]. These results imply that TMX may have estrogenic neuroprotective actions similar to those of E2 in the brain.

Furthermore, administration of TMX to ovariectomized rats increases the expression of Bcl2 and decreases the expression of Bax in the hippocampus [20]. Treatment with TMX in cultured hippocampal neurons increases the expression of the antiapoptotic protein Bcl2, an outcome that has been linked to estrogen's neuroprotective effects [8]. These findings introduce the possibility that TMX may have beneficial effects on TBI-induced cortex cell apoptosis and subsequent neuronal protective effects.

Currently, whether TMX has a similar protective effect on TBI remains to be investigated. In the present study, we chose TMX specifically because it is a SERM, it is blood-brain barrier permeable, and it has clear pharmacokinetic activity in the central nervous system (CNS). TMX has been found to be neuroprotective in both transient and permanent experimental ischemic stroke and spinal cord injury. However, it remains unknown whether this agent displays a similar beneficial effect after TBI and what its underlying mechanisms are. In the present study, we have applied SL327 [21], a

brain-penetrating selective inhibitor of ERK kinase, which was demonstrated to be able to selectively inhibit ERK activation in the brain following systematic administration [22], to investigate the role of TMX in neuroprotection after TBI using a fluid percussion cerebral injury model in rats.

In this article, we investigated whether TMX would activate a pERK1/2 and Bcl2 response, reduce neuronal cell apoptosis, decrease neuronal loss, and ameliorate impaired motor function after adult rat TBI. The results provide evidence that TMX might constitute an effective therapeutic neuroprotectant for TBI.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats weighing 290 ± 16 g were used in the experiments. The animals were kept under a 12/12-h light/dark cycle and allowed free access to food and water. The Chi Mei Medical Centre Animal Care and Use Committee approved all the experimental procedures, which conformed to the National Institute of Health guidelines, including minimizing discomfort to the animals during surgery and during the recovery period. At the end of the experiments, 72 h after TBI, the experimental rats were killed with an overdose of urethane for special stain.

2.2. Traumatic brain injury

The animals were anesthetized with an intraperitoneal (i.p.) administration of a mixture of ketamine (44 mg/kg, intramuscularly [i.m.]; Nankuang Pharmaceutical, Taiwan), atropine (0.062633 mg/kg, i.m.; Sintong Chemical Ind Co, Taiwan), and xylazine (6.77 mg/kg, i.m.; Bayer, Leverkusen, Germany). A craniectomy (2 mm in radius) 4 mm from the bregma and 3 mm from sagittal sutures in the right parietal cortex was performed using a stereotaxic frame. After craniectomy and implantation of an injury cannula, the fluid percussion device (VCU Biomedical Engineering, Richmond, VA) was connected to the animal via a Luer-loc fitting, and the brain was injured with a 2.0–2.2 atm, 25 ms percussion. This injury produces moderately severe brain trauma, as originally described by McIntosh *et al.* [23]. A transient hypertensive response, apnea, and seizure were observed immediately following the fluid percussion injury (FPI) and were used as the criteria for separating the animals into TBI or TBI + treatment groups.

2.3. Treatment intervention

The rats were randomly divided into four major groups: sham-operated ($n = 6$), treated with dimethyl sulfoxide (DMSO) vehicle (4%, i.p., K42088831, vehicle; Merck, Darmstadt, Germany); TBI control + vehicle-treated; TBI + TMX-treated (1 mg/kg, T5648, SERM; Sigma-Aldrich, Shanghai, China) ($n = 6$), and TBI + SL327 (30 mg/kg; Axon 1122, ERK1/2 antagonist; Axon, Groningen, Netherlands) + TMX-treated ($n = 6$). The dosage and time course injection of TMX were

immediately after injury, a second injection 24 h later, and a third injection 48 h later. One dose of SL327 (30 mg/kg) was injected i.p. at 30 min before TBI. Another three control groups, for example, Sham + TMX, Sham + TMX + SL327, and TBI + SL327 (without TMX) were performed also.

All the tests were performed with the investigators blinded to the study groups, which were revealed only at the end of analyses. The animals used for histologic or behavioral studies were provided food and water *ad libitum* throughout the study.

2.4. Cerebral infarction assay

The triphenyltetrazolium chloride (TTC) staining procedures followed those described elsewhere [24]. All 32 animals were killed on day 3 after FPI. Under deep anesthesia (sodium pentobarbital, 100 mg/kg, i.p.), the animals were perfused intracardiac with saline. The brain tissue was then removed, immersed in cold saline for 5 min, and sliced into 2.0-mm sections. The brain slices were incubated in 2% TTC dissolved in phosphate-buffered saline (PBS) for 30 min at 37°C and then transferred to 5% formaldehyde solution for fixation. The volume of infarction, as revealed by negative TTC stains indicating dehydrogenase-deficient tissue, was measured in each slice and summed using computerized planimetry (PC-based Image Tools software; Media Cybernetics, Inc). The volume of infarction was calculated as 2 mm (thickness of the slice) \times [sum of the infarction area in all brain slices (mm²)].

2.5. Neuronal number, ERK1/2, Bcl2, and estrogen receptor α expression in neuronal cells in the cortex using immunofluorescence assay

Adjacent 50- μ m sections corresponding to coronal coordinates 0.20–0.70 mm anterior to the bregma were incubated in 2 mol/L HCl for 30 min, rinsed in 0.1 mol/L boric acid (pH 8.5) for 3 min at room temperature, and then incubated with primary antibodies in PBS containing 0.5% normal bovine serum at 4°C overnight. After being washed in PBS, the sections were incubated with secondary antibodies for 1 h at room temperature. The following antibodies were used in this study: monoclonal mouse antineuronal-specific nuclear protein (Neu-N, MAB377; Millipore Corporation, Billerica, MA) antibody in 1:100 dilution detected with Alexa-Fluor 568 anti-mouse (IgG) antibody (A11031; Invitrogen) at a 1:400 dilution, rabbit anti-Bcl2 protein (ab136285; Abcam) antibody at a 1:100 to 1:300 dilution detected with Alexa-Fluor, 488 anti-rabbit (IgG) antibody (A11034; Invitrogen) in 1:400 dilution, and mouse anti-pERK protein (ab50011; Abcam) antibody at a 1:200 dilution detected with Alexa-Fluor 488 anti-rabbit (IgG) antibody (A11031; Invitrogen) at a 1:400 dilution.

The sections were then washed in PBS/0.6% Tx-100 and were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG in 1% BSA/PBS/0.1% Tx-100 for 60 min. They were mounted with anti-fade mounting media. The number of labeled cells was calculated in five coronal sections from each rat and expressed as the mean number of cells per section. For negative coronal sections, all procedures were performed in the same manner without the primary antibodies.

2.6. Neuronal apoptotic assay in the neuronal cells in the cortex using immunofluorescence staining

On day 4, apoptotic cells were identified by staining with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) [25]. These procedures were conducted as described previously [26]. The number of TUNEL-positive cells was calculated in five coronal sections from each rat in the samples and summed using computerized planimetry (PC-based Image Tools software). The following antibodies were used in this study: monoclonal mouse anti-Neu-N antibody (Neu-N, MAB377; Millipore) at a 1:100 dilution detected with Alexa-Fluor 568 anti-mouse (IgG) antibody (A11031; Invitrogen) at a 1:400 dilution.

2.7. Motor function test

An inclined plane was used to measure limb strength. The animals were placed facing right and then left, perpendicular to the slope of a 20 \times 20-cm buffer ribbed surface of an inclined plane starting at a 55° angle [27]. The angle was increased or decreased in 5° increments to determine the maximal angle at which an animal could hold to the plane. The data for each day were the means of left- and right-side maximal angles. Motor deficit measurements were conducted on day 4.

2.8. Statistical analysis

The results are expressed as the means \pm standard errors of the means for *n* experiments. A two-way analysis of variance for repeated measurements (in the same animals) was used for factorial experiments, whereas Dunnett test was used for post hoc multiple comparisons among means. A value of *P* < 0.05 was considered to indicate a significant difference.

3. Results

3.1. TMX (1 or 5 mg/kg/per day \times 3 d, i.p.) significantly reduced TBI-induced cerebral infarction volume

In the preliminary experiments to determine the effective dosage of TMX in TBI rats compared with that in the sham and TBI controls, the TBI-induced infarction volume (136 ± 5 mm³) was significantly decreased by TMX treatment with 1 mg/kg i.p. (65 ± 7 mm³) (***P* < 0.001) and treatment with 5 mg/kg i.p. (113 ± 6 mm³) (***P* < 0.05) (Fig. 1). Thus, the choice of therapeutic dosage of 1 mg/kg of TMX was based on this preliminary experiment.

3.2. TMX (1 mg/kg/per day \times 3 d, i.p.) significantly attenuated TBI-induced cerebral infarction volume

On day 4, the TTC-stained sections were significantly increased in the infarcted area of vehicle-treated TBI rats compared with those of the sham controls (136 ± 5 versus 0, ***P* < 0.001, respectively). The TBI-induced infarction volume was also significantly reduced by TMX treatment (1 mg/kg, i.p. for three consecutive days) (136 ± 5 versus 65 ± 7 mm³, respectively, ***P* < 0.001). However, the beneficial effects of

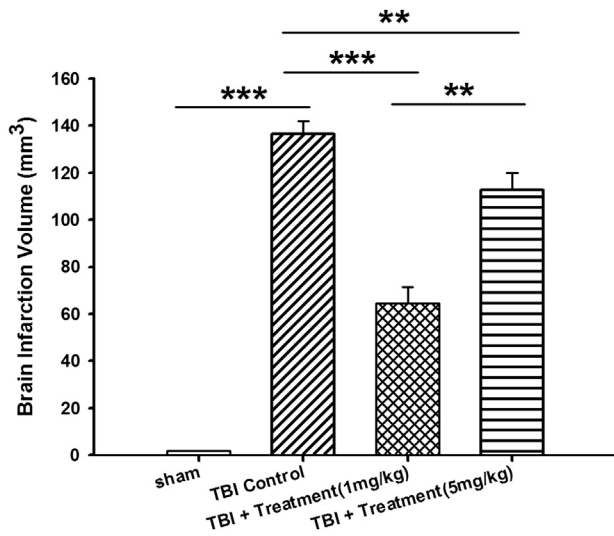


Fig. 1 – Effects of TMX (1 or 5 mg/kg i.p.) treatment on TBI-induced cerebral infarction volume. ** $P < 0.01$, * $P < 0.001$.**

TMX were significantly reversed by SL327 treatment (65 ± 7 versus 161 ± 21 mm³, *** $P < 0.001$) (Fig. 2).

To examine the effects of drug, we tested the Sham + TMX, Sham + TMX + SL327, and TBI + SL327 (without TMX) as control groups. These results showed no significant difference in infarction volume among sham, Sham + TMX and Sham + TMX + SL327 groups. However, TBI + SL327 (without TMX) group revealed the most significant infarction volume among all groups (Fig. 2).

Next, we evaluated the number of neuronal loss, apoptosis, p-ERK expression, and Bcl2 expression in ischemia cortex of neuronal cells and tested the possibility that TMX might have neuroprotective effects after TBI by evaluating the four major groups, for example, Sham, TBI + control, TBI + TMX, and TBI + TMX + SL327.

3.3. TMX significantly attenuated TBI-induced neuronal loss in the cortex

In the Neu-N-stained assay on day 4, positive neuronal cells in the ischemia cortex of vehicle-treated rats were significantly decreased compared with those in the sham controls (63 ± 2 versus 127 ± 6 , respectively, *** $P < 0.001$). The TBI-induced decrease in the number of neuronal-positive cells in the ischemia cortex was significantly improved by TMX treatment (1 mg/kg, i.p. \times 3 d) (63 ± 2 versus 75 ± 3 , respectively, * $P < 0.05$). However, the beneficial effects of TMX were significantly reversed by SL327 treatment (75 ± 3 versus 31 ± 2 , *** $P < 0.001$; Fig. 3).

3.4. TMX treatment significantly attenuated TBI-induced neuronal apoptosis in the cortex

In the TUNEL plus Neu-N-stained assay on day 4, the number of positive neuronal apoptotic cells in the ischemia cortex of vehicle-treated rats was significantly increased compared

with that in the sham controls (57 ± 3 versus 0, respectively, *** $P < 0.001$). The FPI-induced increase in the number of neuronal apoptotic positive cells was significantly improved by TMX treatment, 1 mg/kg, i.p. \times 3 d (57 ± 3 versus 32 ± 3 , *** $P < 0.001$). However, the beneficial effects of TMX were significantly reversed by SL327 treatment (32 ± 3 versus 58 ± 3 , *** $P < 0.0015$; Fig. 4).

3.5. TMX significantly increased neuronal p-ERK1/2 expression in the cortex after TBI

On day 4, the positive neuronal p-ERK1/2 expression of cells in the ischemia cortex of vehicle-treated rats was significantly decreased compared with those in the sham controls (120 ± 4 versus 72 ± 2 , respectively, *** $P < 0.001$). The TBI-induced decrease in the number of p-ERK1/2 expression-positive neuronal cells was significantly improved by TMX treatment (1 mg/kg, i.p. \times 3 d, 72 ± 2 versus 99 ± 3 , *** $P < 0.001$). However, the beneficial effects of TMX were significantly reversed in the TMX plus SL327-treated group (99 ± 3 versus 47 ± 1 , *** $P < 0.001$; Fig. 5).

3.6. TMX significantly increased neuronal Bcl2 expression in the cortex after TBI

On day 4, the number of Neu-N plus Bcl2 double-positive cells was significantly reduced in the ischemia cortex of vehicle-treated rats compared with those in the sham controls (58 ± 3 versus 83 ± 3 , respectively, *** $P < 0.001$). The TBI-induced decrease in the number of Bcl2 expression-positive neuronal cells was significantly mitigated by TMX treatment compared with the sham controls (1 mg/kg, i.p. \times 3 d, 58 ± 3 versus 70 ± 3 , respectively, * $P < 0.05$). However, the beneficial effects of TMX were significantly reversed in the TMX plus SL327-treated group (70 ± 3 versus 39 ± 4 , *** $P < 0.001$; Fig. 6).

3.7. TMX significantly attenuated TBI-induced motor deficits

The maximal grip angle of vehicle-treated rats 3 d after FPI injury was significantly decreased compared with sham-operated controls ($46^\circ \pm 0^\circ$ versus $56^\circ \pm 1^\circ$, respectively, *** $P < 0.001$). The FPI-induced motor dysfunction was significantly improved by TMX ($46^\circ \pm 0^\circ$ versus $52^\circ \pm 0^\circ$, *** $P < 0.001$), but the beneficial effects of TMX were significantly reversed by TMX plus SL327-treated group ($52^\circ \pm 0^\circ$ versus $44^\circ \pm 3^\circ$, * $P < 0.05$; Fig. 7).

4. Discussion

4.1. Summary of the present study

In the present study, TMX was administered at a dose of 1 mg/kg for three consecutive days to counteract the cell damage, neuronal apoptosis in the ischemia cortex, and neurologic deficits induced by TBI in rats via exertion of its selective estrogen receptor modulation ability. To our knowledge, this is the first study to present neuroprotective effects using TMX injection in traumatic CNS injury and demonstrate that the

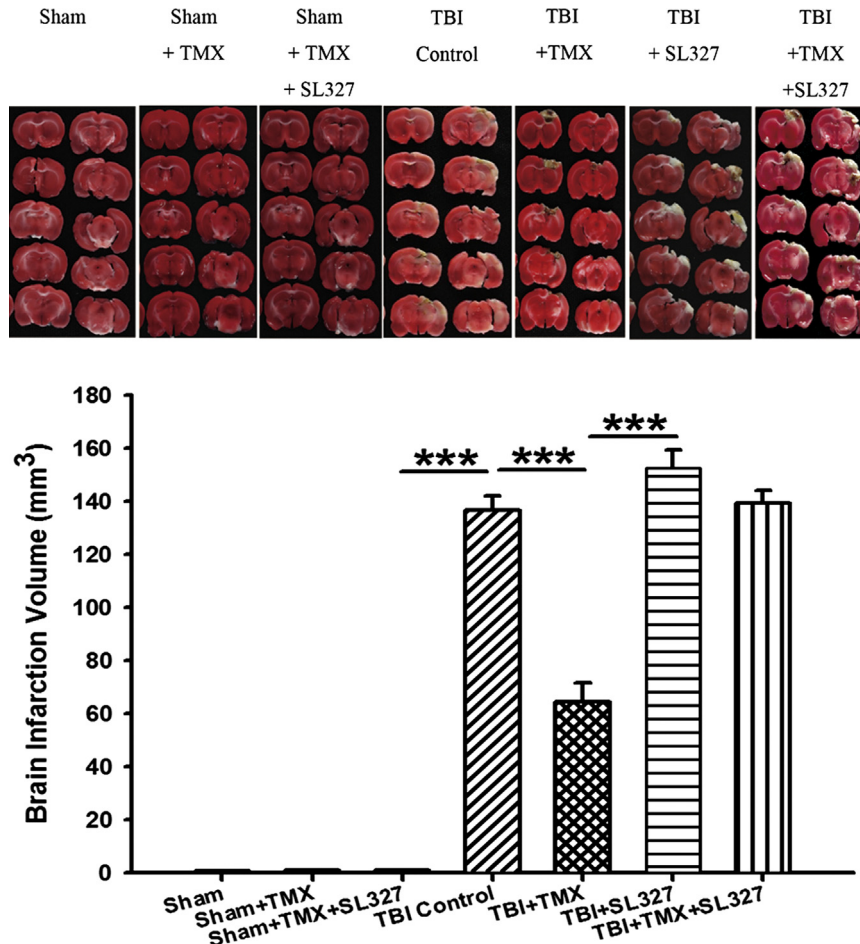


Fig. 2 – Effects of TMX (1 mg/kg, i.p.) treatment on the TBI-induced cerebral infarction volume. ***P < 0.001.

activation of ERK1/2 might be involved in the TMX-induced antineuronal apoptosis effect. These data can extend the current knowledge of TMX in traumatic CNS injuries and will hopefully serve as a foundation for future studies on the importance of SERM therapy in TBI.

4.2. The dosage and time course determination

In present study, we chose the end point for our study that was 72 h after TBI because of the motor dysfunction that persists from 72 h to 1 year after severe lateral fluid percussion injury [28]. In our previous study, using an FPI rat model, we demonstrated TBI-induced infarction volume was significantly increased when compared with a sham-group at 72 h after TBI. However, treatment with magnolol [29], secretome from normoxia culture medium [30], or agmatine [31] immediately after TBI could significantly attenuate TBI-induced infarction volume at day 4 post-TBI. In the present study, we found that when TMX was administered, the drug could also significantly counteract the infarction volume after injury, although a 3-day observation is a short period.

In the preliminary experiments, we determined and compared the treatment effects of TMX at doses of 1 and 5 mg/kg in the first 3 days after TBI. A single dose of 5 [16] and 1 mg/

kg/d for 7 d [15] had therapeutic effects in a rat ischemia model. However, treatment with TMX for three consecutive days during the acute stage after TBI has not been investigated. In our study, the results indicate that TMX significantly attenuated TBI-induced cerebral infarction volume when treatment with 1 or 5 mg/kg for three consecutive days was applied. Based on the result of infarction volume, higher dose of TMX tested (5 mg/kg) was less effective than the 1 mg/kg dose. The possible mechanism maybe due to 1 mg/kg/d doses of TMX, which are in the therapeutic range (0.8 and 2.4 mg/kg/d), could dramatically reduce infarct of the affected cerebral hemisphere as previous reported in an ischemia model [17]. Our results demonstrate the therapeutic effects of this compound when administered during the acute stage in a TBI rat model, which explored in clinical setting.

4.3. Neuroprotective effects of TMX injection after TBI

Cell death resulting from apoptosis is a well-known secondary injury mechanism [3] that commonly occurs in the perilesioned areas in animal and human studies [4]. In the present study, we found that the number of TUNEL-positive cells counted in the cerebral cortex surrounding the primary injury site were significantly increased on day 4 TBI but were reduced

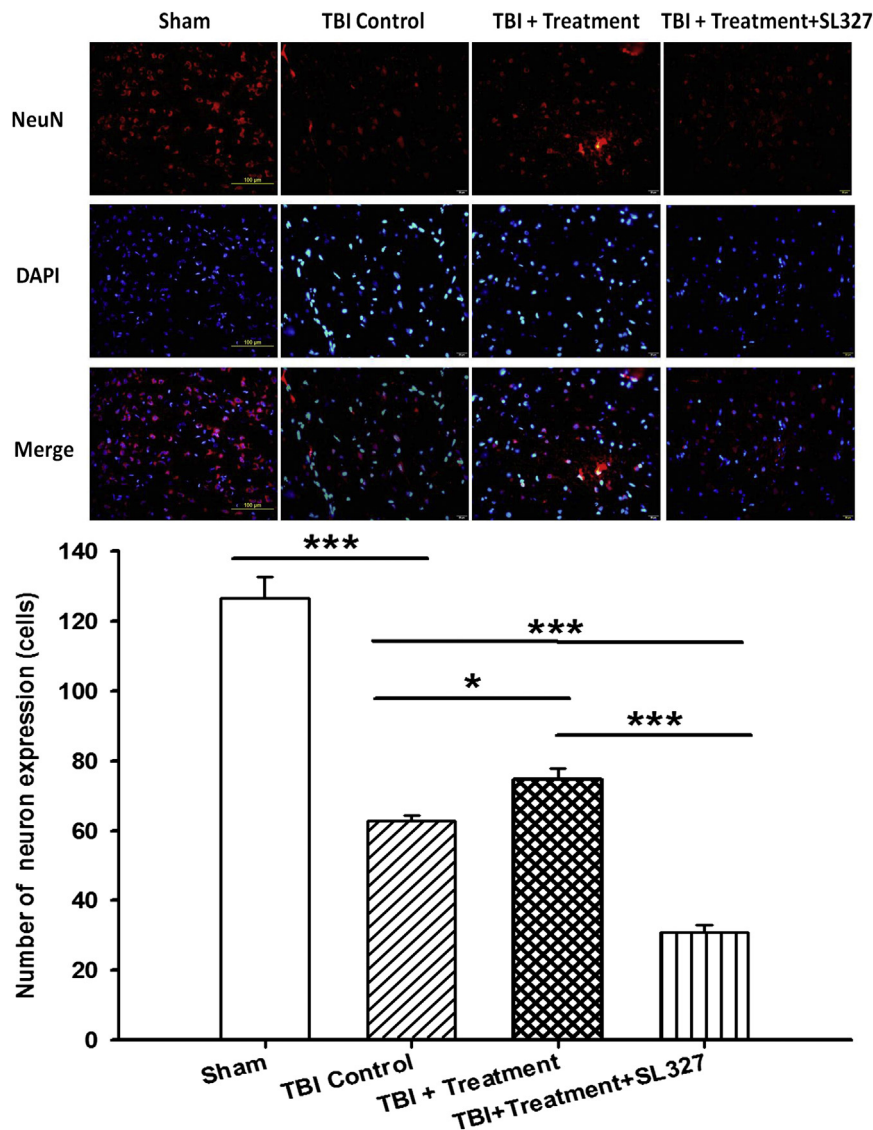


Fig. 3 – Effects of TMX on the TBI-induced neuronal loss in the cortex. The top panels depict representative positive Neu-N, DAPI staining for one sham rat, one TBI rat, one TBI + TMX treatment rat, and one TBI + TMX + SL327 treatment rat. * $P < 0.05$, *** $P < 0.001$. DAPI = 4',6-diamidino-2-phenylindole.

with TMX treatment. These results also suggest that the protective effects of TMX may be at least partly due to attenuation of apoptotic activity of brain tissue after TBI. These results are consistent with several previous models, such as irradiation-induced brain injury [18] and spinal cord injury [15]. Thus, these results further corroborate TMX therapy as a promising strategy after TBI.

4.4. ERK1/2 and Bcl2 are involved in TMX neuroprotective mechanisms

In the present data, TMX therapy reduces cell apoptosis, neuronal loss, and improves motor recovery from TBI in rats. To investigate the mechanism by which TMX induces functional recovery after TBI, p-ERK1/2 and Bcl2 expression in the injured cortex neuron was examined.

Our study demonstrated that TMX-induced antiapoptosis via pERK1/2 activation, and these patterns of pERK immunoreactivity and TMX-induced anti-apoptosis in cerebral cortical neurons were attenuated by pretreatment with the selective MEK inhibitor SL327. These findings are suggestive of the potential molecular mechanisms involved in the protective effect of p-ERK1/2 activation and expression. These results also support the hypothesis that activation of pERK1/2 may be a neuroprotective factor involved in preventing apoptosis after TBI.

Activation of the pERK1/2 pathway may be associated with both cell survival and death depending on the stimuli and cell types involved, and the balance among the intensity and duration of pro- versus antiapoptotic signals transmitted by ERK1/2 determines whether a cell survives or undergoes apoptosis [32]. Our present study results were consistent with

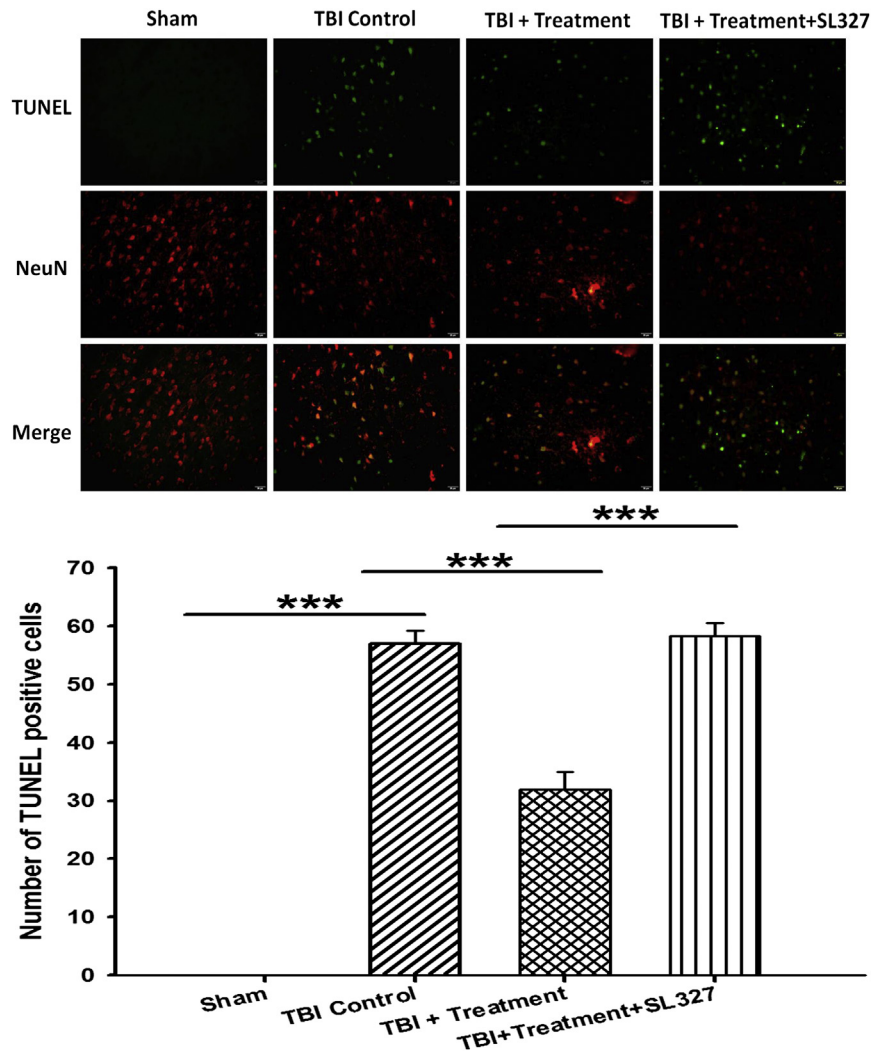


Fig. 4 – Effects of TMX treatment on the FPI-induced neuronal apoptosis in the cortex on day 4 after TBI. The top panels depict representative positive Neu-N and TUNEL staining for one sham rat, one TBI rat, one TBI + TMX treatment rat, and one TBI + TMX + SL327 treatment rat. *P < 0.001.**

previous findings in that the upregulation of the ERK1/2 pathway is associated with cell survival and neuroprotection, such as with nicotine-mediated neuroprotection in spinal cord neurons [33], erythropoietin-mediated neuroprotection in retinal neuron culture [34], and cyclic AMP affecting noradrenaline on dopaminergic neurons [35]. In contrast, pERK activation may play an important role in inducing cell apoptosis, such as in cisplatin-induced human cervical carcinoma HeLa cell apoptosis [36], TMX-induced rat C6 glioma apoptosis [37], pERK-activated and pEKR-mediated cell cycle arrest and apoptosis after DNA damage [38], and activation of pERK in a middle cerebral artery occlusion model induces cell death in ischemia penumbra [15].

pERK1/2-mediated survival signaling has been proposed to be mediated through the activation of the RSK-Creb-Bcl2 (p90 ribosomal S6 kinase—cyclic AMP response element-binding protein—B-cell lymphoma-2) pathway, which promotes cell survival through transcriptional upregulation of antiapoptotic

Bcl2 proteins [39,40]. In the present study, we found TMX significantly increased TBI-induced neuronal Bcl2 expression, but this was reversed by SL327 treatment in the injured cortex. It could also decrease neuronal apoptosis and finally improve motor function. We believe that increased levels of pERK1/2 and Bcl2 in the injured cortex, resulting in a decrease in neuronal apoptosis, are one mechanism by which functional recovery might occur. In addition, the pERK1/2 expression in the astrocytes, oligodendrocytes, and microglia and the relationship between p-ERK1/2 expression in those cells and inducing cell apoptosis or survival with/without TMX treatment after TBI needs to be clarified in the future.

Zhang *et al.* demonstrated that TMXs neuroprotection persists after pretreatment with a pure estrogen receptor antagonist, ICI 182,780, in rat focal cerebral ischemia injury [41]. These results imply that the upstream pathway of ERK1/2 activation may not involve estrogen receptors [42]. It is unclear whether the TMX effects observed in our study involve

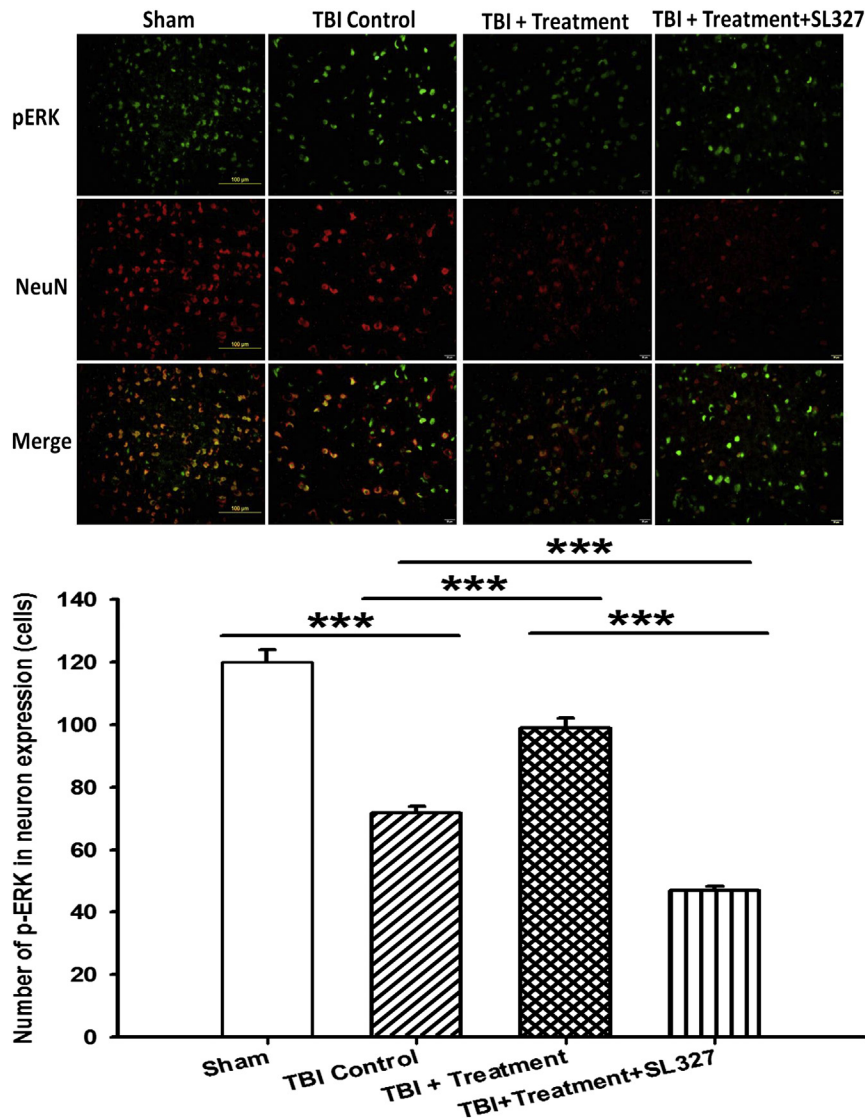


Fig. 5 – Effects of TMX treatment on p-ERK expression in neuronal cells in the cortex (p-ERK plus Neu-N stain assay) on day 4. The top panels depict representative positive Neu-N and p-ERK staining for one sham rat, one TBI rat, one TBI + TMX treatment rat, and one TBI + TMX + SL327 treatment rat. *P < 0.001.**

the activation of the estrogen receptor. To answer this question, whether neuroprotection by TMX is affected by estrogen receptor blockade from ICI 18278 and estrogen receptor expression in neuronal cells should be clarified in the future.

In our previous study, we found that the hippocampal pERK1/2 signal pathway played a critical role in TBI-induced depression. These results suggest that the development of a positive regulator of ERK1/2 may provide a new direction for the treatment of TBI-induced depression [43]. Whether TMX is beneficial in TBI-induced depression is worth evaluation.

4.5. Other TMX effects beyond activating p-ERK1/2 associated neuroprotection

In addition to the activation of p-ERK1/2 expression, TMX therapy can produce free radical scavenging and antioxidant

activity *in vitro* and *in vivo* [15,44,45], inhibit excitatory amino acid release via swelling activated anion channels in swollen astrocytes [46], reduce reactive gliosis after a TBI [47], induce an anti-inflammatory response [48], potentiate the basal activity of presynaptic glycine receptors facilitating glycine release [49], upregulate the expression of the antiapoptotic gene seladin-1 (selective Alzheimer disease indicator-1) in human neuroblasts [50], and depress glutamate release through inhibition of voltage-dependent Ca^{2+} entry and protein kinase C in rat cerebral cortex nerve terminals [51]. Therefore, these aforementioned actions might contribute to the powerful neuroprotection demonstrated by the compound. Thus, we believe that TMX may be a very useful therapy for TBI patients because of its multiple and effective effects. We suggest that the use of TMX in the acute stage of TBI might have clinical benefits in the future.

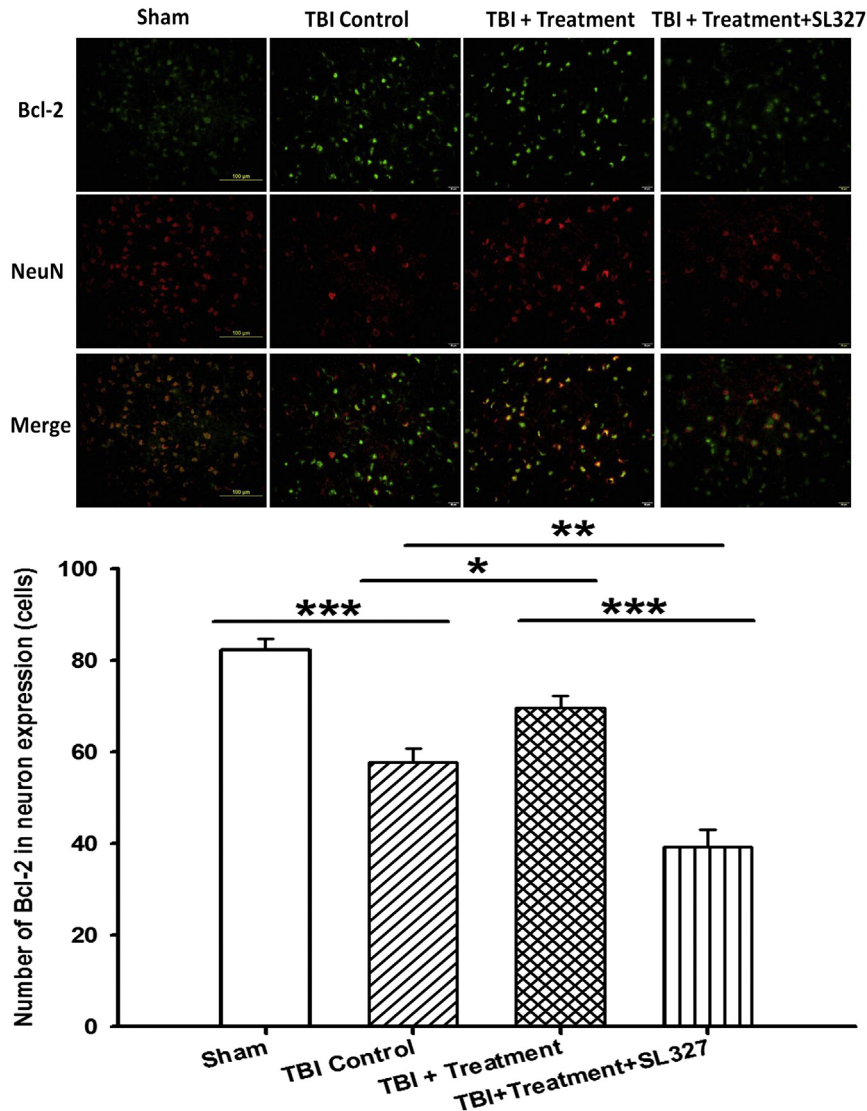


Fig. 6 – Effects of TMX treatment on Bcl2 expression in neuronal cells in the cortex (Bcl2 plus Neu-N stain assay) on day 4. The top panels depict representative positive Neu-N and Bcl2 staining for one sham rat, one TBI rat, one TBI + TMX treatment rat, and one TBI + TMX + SL327 treatment rat. *P < 0.05, **P < 0.01, *P < 0.001.**

4.6. Sex hormone therapy after TBI

The neuroprotective effects of sex hormone such as progesterone and estrogen for TBI have been proven effectively at the bench [7,8,52]. These experimental observations have been extended to clinical trials involving patients with TBI when treated with progesterone [53,54]. However, despite estrogen has accumulated impressive evidence as a neuroprotectant in laboratory studies, translation to patients remains to be shown. TMX, a SERM, treatment in male [14,16] and surgically ovariectomized female rodents [15,17,20] greatly improves spinal cord injury or ischemic brain injury [14–17]. In the present study, we further demonstrate that TMX protects male rats from FPI-induced cerebral infarction and functional deficits. However, whether TMX protects female rats from TBI insult or not is needed to be evaluated in the future.

4.7. Limitations of the present study

Several drawbacks of present study should be considered. First, the study is males only because the potential gender differences in providing TMX as a therapeutic option following TBI. Second, no detailed dose-response effects of TMX extend beyond infarct volume to neuronal loss, apoptosis, pERK1/2, and Bcl2 levels if higher dosage 5 mg/kg injected. Third, only one method (inclined plane test) to evaluate functional outcome and finally demonstrating mechanisms of action with limited means without appropriate controls including Sham + TMX, Sham + TMX + SL327, and TBI + SL327 (without TMX) was performed, which are important for drug treatment trials. Therefore, many more prospective studies are required.

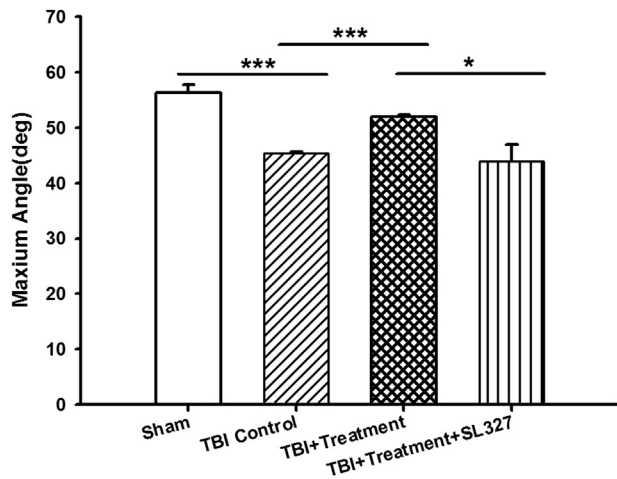


Fig. 7 – Effects of TMX (1 mg/kg, i.p.) treatment on TBI-induced motor deficits evaluated by maximum angle in incline plane grasp on day 4. *P < 0.05, *P < 0.001.**

5. Conclusion

In the acute stage, i.p. injection of TMX administered at a dose of 1 mg/kg for three consecutive days ameliorates TBI in rats by promoting neuronal p-ERK1/2 and Bcl2 expression, reducing neuronal cell loss and apoptosis, which contributes to improve functional outcomes in inclined plane test. TMX may be a promising treatment strategy for TBI.

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Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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