Combination therapy with melatonin and dexamethasone in a mouse model of traumatic brain injury

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Abstract

Traumatic brain injury (TBI) is a major cause of preventable death and morbidity in young adults. This complex condition is characterized by a significant blood–brain barrier leakage that stems from cerebral ischemia, inflammation, and redox imbalances in the traumatic penumbra of the injured brain. Recovery of function after TBI is partly through neuronal plasticity. In order to test whether combination therapy with melatonin and dexamethasone (DEX) might improve functional recovery, a controlled cortical impact (CCI) was performed in adult mice, acting as a model of TBI. Once trauma has occurred, combating these exacerbations is the keystone of an effective TBI therapy. The therapy with melatonin (10 mg/kg) and DEX (0.025 mg/kg) is able to reduce edema and brain infarctions as evidenced by decreased 2,3,5-triphenyltetrazolium chloride staining across the brain sections. Melatonin- and DEX-mediated improvements in tissue histology shown by the reduction in lesion size and an improvement in apoptosis level further support the efficacy of combination therapy. The combination therapy also blocked the infiltration of astrocytes and reduced CCI-mediated oxidative stress. In addition, we have also clearly demonstrated that the combination therapy significantly ameliorated neurological scores. Taken together, our results clearly indicate that combination therapy with melatonin and DEX presents beneficial synergistic effects, and we consider it an avenue for further development of novel combination therapeutic agents in the treatment of TBI that are more effective than a single effector molecule.

Key Words
▶ melatonin
▶ dexamethasone
▶ TBI
▶ inflammation
▶ cell death
▶ motor function

Introduction

Traumatic brain injury (TBI) is an insult to the brain from the application of an external physical force that leads to temporary or permanent structural and functional impairment of the brain. TBI is a leading cause of injury-related death and disability (Hyder et al. 2007). Approximately 1.7 million people sustain a TBI in the USA annually and 53,000 of them die from TBI-related injuries (Coronado et al. 2011). In TBI survivors, neuropsychiatric abnormalities, such as cognitive deficits and emotional and behavioral problems, are common and contribute substantially to post-TBI disabilities (Jennekens et al. 2010).
Brain damage following traumatic injury is a result of direct (primary injury) and indirect (secondary or delayed injury) mechanisms. The secondary injury mechanism involves the initiation of an acute inflammatory response, including the breakdown of the blood–brain barrier (BBB), brain edema, the infiltration of peripheral blood cells, and the activation of resident immunocompetent cells, as well as the release of numerous immune mediators such as interleukins and chemotactic factors (Stahel et al. 2000), damaging also the other organs (Hang et al. 2005a, b, Jin et al. 2008). Another significant role in the pathology of TBI is played by oxidative stress (Gilgun-Sherki et al. 2002, Ansari et al. 2008). The most commonly occurring cellular free radical is superoxide radical (O$_2^-$), which is produced when an oxygen molecule gains one electron from another substance. Excess O$_2^-$ leads to tissue damage by promoting hydroxyl radical (OH$^-$) formation through hydrogen peroxide (H$_2$O$_2$), and by combining with nitric oxide (NO) to form peroxynitrite (ONOO$^-$), a powerful oxidant formed from superoxide and NO that can damage a wide array of molecules in cells (Afanas’ev 2007). It has been generally assumed that mitochondria are the major source of O$_2^-$ following brain injury (Lambert & Brand 2009).

Glucocorticoids (GCs) are commonly used to treat allergic, inflammatory, and autoimmune diseases due to their anti-inflammatory and immunosuppressive effects (Jeanneteau et al. 2008). GC receptors (GRs) mediate the effects of high levels of endogenous GCs, such as corticosterone (Cort) and cortisol, and also bind synthetic steroids, such as prednisolone, dexamethasone (DEX), or β-methasone (McEwen 1987). GCs act through many molecular mechanisms including non-genomic and genomic signals. In particular, mechanisms including enzyme activation, such as GR-associated src kinase and phospholipase, and Ca$^{2+}$ mobilization lead to the interaction of GCs with the GR. Following the GC and GR interaction, the GR is activated, translocates to the nucleus, and modulates gene transcription either by stimulation or inhibition. However, although GCs are potent anti-inflammatory drugs, their clinical effects are often transitory, disease recurs on tapering the drug, and chronic use of GCs is accompanied by serious side effects and dependence (Cuzzocrea et al. 2008). GCs exert a wide spectrum of influences on developing organs, including the lung and brain. Brain sensitivity to GCs begins in embryonic life, as the GR is expressed in fetal neurons (McEwen 1987) and is maintained in several adult brain regions, including the hippocampus and cerebellar cortex (Ozawa 2005). It is important to highlight that GCs can also protect against neurodegeneration (Nichols et al. 2005), suggesting that GCs are capable of exerting adaptive effects that prevent neural injury caused by overaggressive cellular defense mechanisms. In this regard, we have recently demonstrated that a combination therapy with a superoxide dismutase (SOD) mimetic or entanercept with DEX respectively exerts a beneficial effect in an arthritis model reducing the dose; on the other hand, it reduces the degree of spinal cord inflammation, ameliorating the recovery of limb function. These results show the reduction of the side effects related to the use of steroids for the management of chronic diseases (Cuzzocrea et al. 2005, Genovese et al. 2007b).

Many drugs, such as palmitoylethanolamide (Ahmad et al. 2012), have been reported to reduce inflammation in the CNS, of which great importance has been placed on melatonin (Mesenge et al. 1998, Esposito & Cuzzocrea 2010).

Melatonin (N-acetyl-5-methoxytryptamine), the main product of the pineal gland, has been shown to exert neuroprotection in models of brain and spinal cord trauma (Kaptanoglu et al. 2000, Genovese et al. 2005), cerebral ischemia (Borlongan et al. 2000), and excitotoxicity (Cabrera et al. 2000). It is synthesized from the amino acid tryptophan or is formed as the major metabolic end product of serotonin in the pineal gland. It has strong antioxidant and free-radical-reducing effects, thereby detoxifying reactive oxygen products (Dundar et al. 2005, Di Bella & Gualano 2006). Additionally, it also inhibits the pro-oxidative enzyme NO synthase upon stimulating glutathione peroxidase, SOD, and G-6-P dihydrogenase. Since melatonin is a lipophilic enzyme, it does not need a specific binding site or a receptor on the cell membrane (Ismailoglu et al. 2012). Because acute oxidative stress is commonly involved in the progression of secondary neuronal tissue injury in these models, the neuroprotective effects of melatonin have been attributed by some authors to its activity as an antioxidant (Mesenge et al. 1998, Cabrera et al. 2000, Kaptanoglu et al. 2000). However, later studies have failed to confirm the activity of melatonin as a potent direct chain-breaking antioxidant and have suggested that in some circumstances, it can function as a weak preventive antioxidant, presumably by acting as a weak metal ion chelator (Livrea et al. 1997, Antunes et al. 1999, Fowler et al. 2003).

Previous experimental models such as pleurisy, spinal cord injury, and intracerebral hemorrhage have shown a reduction in the degree of secondary damage after combined therapy with melatonin and DEX, strongly suggesting the possibility of reducing the dose and side
Effects of steroid treatment for inflammatory conditions (Crisafulli et al. 2006, Genovese et al. 2007a, Li et al. 2009). In light of preceding in vivo studies and this previous observation, in the present paper, we investigated the effects of combination therapy with melatonin and DEX in a mouse model of TBI. In particular, we investigated the effect of combination therapy on i) behavioral test, ii) the evaluation of infarction (by 2,3,5-triphenyltetrazolium chloride (TTC) staining), iii) metalloproteinase expression, iv) apoptosis protein expression, v) iNOS expression, and vi) brain damage (histology).

Materials and methods

Animals

Male CD1 mice (25–30 g; Harlan Nossan, Milan, Italy), aged 10–12 weeks, were used throughout the study. Mice were housed in individual cages (five per cage) and maintained under a 12 h light:12 h darkness cycle at 21 ± 1 °C and 50 ± 5% humidity. A standard laboratory diet and tap water were available ad libitum. Animal care was in compliance with the Italian regulations on the protection of animals used for experimental and other scientific purposes (D M 116192) as well as with the EEC regulations (O J: of E C L 358/1 12/18/1986).

Controlled cortical impact experimental TBI

TBI was induced in mice (n = 10 per group) by a controlled cortical impactor (CCI). Mice were anesthetized under i.p. ketamine + xylazine (2.6/0.16 mg/kg body weight respectively). A craniotomy was made in the right hemisphere encompassing bregma and lambda and between the sagittal suture and the coronal ridge with a micro motor hand piece and drill (UGO Basile S.R.L., Comerio VA, Italy). The resulting bone flap was removed, and the craniotomy enlarged further with cranial rongeurs. A cortical contusion was produced on the exposed cortex using a controlled impactor device (Impact One Stereotaxic impactor) for CCI (myNeurolab.com, Richmond). Briefly, the impacting shaft was extended, and the impact tip was centered and lowered over the craniotomy site until it touched the dura mater. Then, the rod was retracted and the impact tip was advanced further to produce a brain injury of moderate severity in mice (tip diameter, 4 mm; cortical contusion depth, 3 mm; impact velocity, 1.5 m/s). Immediately after the injury, the skin incision was closed with nylon sutures, and 2% lidocaine jelly was applied to the lesion site to minimize any possible discomfort.

Experimental groups

Mice were randomly allocated into the following groups:

1. Sham + vehicle group: mice were subjected to identical surgical procedures except for TBI shock and were kept under anesthesia for the duration of the experiment (n = 10).

2. TBI + vehicle group: mice were subjected to TBI and received the vehicle for melatonin (1% ethanol, v/v) and for DEX (saline i.p. bolus) 1 and 6 h after brain trauma (n = 10).

3. DEX group: same as the TBI + vehicle group, but were administered DEX (0.025 mg/kg i.p. bolus) 1 and 6 h after brain trauma (n = 10).

4. MEL group: same as the TBI + vehicle group, but were administered melatonin (10 mg/kg i.p. bolus) 1 and 6 h after brain trauma (n = 10).

5. MEL + DEX group: same as the TBI + vehicle group, but were injected with melatonin (10 mg/kg i.p.) and DEX (0.025 mg/kg i.p. bolus) 1 and 6 h after brain trauma (n = 10).

The doses of melatonin (10 mg/kg, i.p.) and DEX (0.025 mg/kg i.p.) used here were based on previous in vivo studies (Crisafulli et al. 2006, Genovese et al. 2007a). The half-lives of melatonin and DEX are 27 and 200 min respectively (Cevc & Blume 2004, Venegas et al. 2012). Several recent results have illustrated the importance of initiating therapeutic interventions as soon as possible following TBI, preferably within 4 h post-injury, to achieve the best possible neuroprotective effect (Sullivan et al. 2011).

Behavioral testing: rotarod test

The rotarod treadmill (Accuscan, Inc., Columbus, OH, USA) provided a motor balance and coordination assessment. Each animal was placed in a neutral position on a cylinder (3 and 1 cm diameter for rats and mice respectively), then the rod was rotated with the speed accelerated linearly from 0 to 12000 g within 60 s, and the time spent on the rotarod was recorded automatically. The maximum score given to an animal was fixed to 60. For testing, the animals were subjected to three trials and the average score on these three trials was used as the individual rotarod score. The elevated body swing test (EBST) provided a motor asymmetry parameter and involved handling the animal by its tail and recording the direction of the biased body swings. The EBST consisted of 20 trials with the number of swings ipsilateral to the lesion.
and contralateral to the injured hemisphere being recorded and expressed as a percentage to determine the biased swing activity (Zohar et al. 2011).

Evaluation of infarction using TTC staining

To evaluate the infarct, the TTC staining technique was used. Briefly, mice were killed by decapitation at 24 h after TBI. The brains were quickly removed and placed in ice-cold saline for 5 min. Six serial sections from each brain were cut at 2 mm intervals from the frontal pole using a rodent brain matrix (ASI Instrument, Inc., Warren, MI, USA). The sections were incubated in 2% TTC saline solution for 30 min at 37 °C. The stained brain sections were stored in 10% formalin and refrigerated at 4 °C for further processing and storage.

Histological examination

Tissue segments containing the lesion (1 cm on each side of the lesion) were paraffin embedded and cut into 5 mm-thick sections. Sections were then deparaffinized with xylene, and then stained with hematoxylin and eosin. All sections were studied using an Axiosvision Zeiss microscope (Milan, Italy).

Western blot analysis

Western blot was performed in the traumatic penumbra area from the ipsilateral injured brain and also in a similar area from the control and/or contralateral tissues using antibodies as described earlier. Cytosolic and nuclear extracts were prepared as described previously (Bethea et al. 1998) with slight modifications. Brain tissues from each mouse were suspended in extraction buffer A containing 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 0.15 mM pepstatin A, 20 mM leupeptin, and 1 mM sodium orthovanadate, homogenized at the highest setting for 2 min, and centrifuged at 1000 g for 10 min at 4 °C. Supernatants represented the cytosolic fraction. Pellets, containing enriched nuclei, were resuspended in buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 0.2 mM leupeptin, and 0.2 mM sodium orthovanadate. After centrifugation at 15 000 g for 30 min at 4 °C, supernatants containing the nuclear protein were stored at −80 °C for further analysis. The levels of BAX, BCL-2, MMP-2, MMP-9, and iNOS were quantified in the cytosolic fraction from brain tissue collected at 24 h after TBI. The filters were blocked with 1 × PBS and 5% (w/v) non-fat dried milk (PM) for 40 min at room temperature and subsequently probed with specific Abs anti-Bax (1:500; Santa Cruz Biotechnology), anti-iNOS (1:200; BD Transduction Laboratory, San Diego, CA, USA), and anti-Bcl-2 (1:500; Santa Cruz Biotechnology) at 4 °C overnight. Furthermore, the filters were probed with anti-MMP-2 antibody (1:500; Chemicon International, Temecula, CA, USA) and anti-MMP-9 antibody (1:500; Calbiochem, Darmstadt, Germany) for 2 h at room temperature in 1× PBS, 5% (w/v) non-fat dried milk, and 0.1% Tween-20 (PMT). Membranes were incubated with a peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of proteic lysates, they were also incubated in the presence of the antibody against β-actin (1:5000; Santa Cruz Biotechnology). The relative expression of the protein bands of Bax (≈23 kDa), Bcl-2 (≈29 kDa), iNOS (≈130 kDa), MMP-2 (≈72 kDa), and MMP-9 (≈92 kDa) was quantified by densitometry scanning of the X-ray films with a GS-700 Imaging Densitometer (GS-700; Bio-Rad Laboratories) and a computer program (Molecular Analyst; IBM, Helar Division, Messina, Italy).

Statistical analysis

Data are expressed as the mean ± S.E.M. of n observations. For the in vivo studies, n represents the number of animals studied. In the experiments involving histology, values are representative of at least three experiments performed on different experimental days. A P value of <0.05 was considered as significant. Data were analyzed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons.

Results

Behavioral testing

To investigate the relationship between neurological deficits in the setting of TBI, we used the rotarod test, which is considered as the most sensitive vestibulomotor to assess motor function. At 24 h after TBI, mice were subjected to the EBST. CCI-injured mice displayed a range of impairments in locomotor tasks, as shown in Fig. 1. The melatonin (10 mg/kg) or DEX (0.025 mg/kg) treatment improved, in part, latency compared with the TBI+vehicle treatment (Fig. 1). We demonstrated that
Combination therapy with melatonin (10 mg/kg) and DEX (0.025 mg/kg) appreciably ameliorated latency compared with the TBI vehicle treatment (Fig. 1).

Histological examination

Histological examination of the brain sections of TBI-induced mice, at the level of the perilesional area, stained 24 h after injury, taken from the saline-treated animals (data not shown), revealed significant tissue damage such as prominent and thickened blood vessels, ischemic changes, and gliosis in the brain parenchyma (Fig. 2A). The melatonin (10 mg/kg) or DEX (0.025 mg/kg) treatment did not attenuate the development of acute inflammation at 24 h after TBI (Fig. 2B and C respectively); on the contrary, we showed that the combination therapy with melatonin (10 mg/kg) and DEX (0.025 mg/kg) significantly reduced the degree of brain injury (Fig. 2D).

Brain infarctions following TBI

Concerning the overall brain injury, the measurement of brain infarctions is a standard method to evaluate ischemic injury after stroke. To evaluate the effect of melatonin, DEX, and their combination on brain infarctions in TBI, we performed TTC staining (Fig. 3A).
The infarct area (Fig. 3B) and infarct volume (Fig. 3C) were significantly reduced after the combined therapy with melatonin and DEX. There was also a slight improvement in the treatment with individual drugs (Fig. 3).

Metalloproteinase expression after TBI

To evaluate the degradation of extracellular matrix components and edema formation, the expression of MMP-9 and MMP-2 was determined by western blot analysis. A basal level of MMP-2 expression was detected in the tissues from control mice, as shown by the immunoreactive band migrating at ~72 kDa (Fig. 4A; for densitometry analysis, see Fig. 4A1). A significant upregulation of MMP-2 expression was observed in the brain tissues from mice subjected to TBI (Fig. 4A; for densitometry analysis, see Fig. 4A1). Melatonin and DEX reduced MMP-2 expression (Fig. 4A; for densitometry analysis, see Fig. 4A1), but a considerable reduction was seen in the combination therapy. Similarly, a basal level of MMP-9 expression was detected in the tissues from control mice, as shown by the immunoreactive band migrating at ~92 kDa (Fig. 4B; for densitometry analysis, see Fig. 4B1). A significant upregulation of MMP-9 expression was observed in the brain tissues from TBI-induced mice when compared with the controls (Fig. 4B; for densitometry analysis, see Fig. 4B1). The melatonin and DEX treatment also prevented TBI-induced MMP-9 expression;
however, the combination therapy with melatonin and DEX significantly reduced MMP-9 expression (Fig. 4B; for densitometry analysis, see Fig. 4B1).

Modulation of iNOS expression after TBI

To determine the role of NO produced during TBI, iNOS expression was evaluated by western blot analysis. A significant increase in iNOS expression (Fig. 5A) was observed in the brain from mice subjected to TBI. Consequently, the melatonin and DEX treatment prevented TBI-induced iNOS expression (Fig. 5A; for densitometry analysis, see Fig. 5B); on the other hand, a considerable reduction was demonstrated by the combination therapy with melatonin and DEX (Fig. 5A; for densitometry analysis, see Fig. 5B).

Effects of melatonin and DEX on apoptosis in the brain after TBI

To test whether brain damage was associated with apoptosis, 24 h after TBI, the appearance of proteic effectors of canonical mitochondrial apoptosis, such as pro-apoptotic (Bax) proteins and anti-apoptotic (Bcl-2) proteins, was investigated by western blot analysis. The balance of Bax levels was appreciably increased in the brain from mice subjected to TBI (Fig. 6A). On the contrary, the melatonin and DEX treatment prevented TBI-induced Bax expression (Fig. 6A; for densitometry analysis, see Fig. 6A1), but a marked improvement was evident in the combination therapy involving the two drugs. Moreover, in the brain extract of sham mice, a basal level of Bcl-2 was detected (Fig. 6B). In TBI-induced mice, Bcl-2 expression was significantly reduced (Fig. 6B). Melatonin and DEX administration showed respectively an increase in Bcl-2 expression (Fig. 6B; for densitometry analysis, see Fig. 6B1); however, the combination therapy with melatonin and DEX significantly restored the Bcl-2 signal (Fig. 6B; for densitometry analysis, see Fig. 6B1).

Discussion

Animal models of TBI using the CCI technique are physiologically relevant to TBI in humans. CCI reproduces many of the features of brain injuries, including motor
diseases with inflammatory components, including rat model of spinal cord injury (Genovese et al. 2007a). Melatonin (25 and 50 mg/kg) exerts a protective effect in a oxidative exacerbations (Khan et al. 2008). The failure of therapies targeting only neuronal protection is, in part, attributable to the lack of concomitant protection of cerebral blood vessels from the secondary injury of inflammation and accumulating oxidative stress. In this respect, TBI has similar pathophysiology to ischemic stroke. Both complications involve hypoxia, a disrupted BBB, edema, inflammation, neurodegeneration, and neurological deficits. To date, major clinical trials with neuroprotective drugs that targeted TBI amelioration have not been efficacious (Jain 2008). The failure of therapies targeting only neuronal protection is, in part, attributable to the lack of concomitant protection of cerebral blood vessels from the secondary injury of inflammation and accumulating oxidative exacerbations (Khan et al. 2009).

In this regard, we have previously demonstrated that melatonin (25 and 50 mg/kg) exerts a protective effect in a rat model of spinal cord injury (Genovese et al. 2007a) and examined the effect of melatonin on several neurological diseases with inflammatory components, including dementia, Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, stroke, and brain ischemia/reperfusion, but also on traumatic CNS injuries (Esposito & Cuzzocrea 2010). The protective effects of melatonin were not limited to an overall anti-inflammatory effect, but included significant protection of injury, as well as the inhibition of key pro-inflammatory mediators (Reiter et al. 1997). As an antioxidant, melatonin can directly scavenge free radicals while also acting indirectly to increase the expression of endogenous antioxidant enzymes (Reiter et al. 2000), and may be superior to glutathione, mannitol, and vitamin E (Reiter et al. 1997). Additionally, due to its amphiphilic structure, melatonin has no barriers to its distribution and may have the advantages of having a lower-side effect profile and producing fewer pharmacokinetic or pharmacodynamic interactions compared with xenobiotic antioxidants. Therefore, melatonin could be highly effective in protecting the traumatized brain from oxidative damage (Kelso et al. 2011).

GCs, e.g. DEX, are potent immunosuppressive and anti-inflammatory agents that are used therapeutically in several inflammatory pathologies (Crisafulli et al. 2006). In addition to their peripheral effects, GCs also exert effects in the CNS. GCs have neuroprotective, anticonvulsive, and anxiolytic effects and have been linked to depression, epilepsy, anxiety, and memory loss. Beneficial effects of moderate increases in GCs include the modulation of synaptic plasticity and hippocampal-dependent cognition. The plethora of actions of GCs in the brain suggests communication with neurotrophic signaling systems. Neurotrophin receptors in the CNS promote neuronal survival and synaptic plasticity (Jeanneteau et al. 2008).

This study has provided the first evidence that combination therapy with melatonin (10 mg/kg) and DEX (0.025 mg/kg), used at a dose that is not more effective when administered as a single treatment, attenuated: i) brain infraction, ii) the loss of motor function, iii) the expression of apoptosis proteins, iv) iNOS expression, v) metalloproteinase expression, and vi) the degree of brain injury. Overall, combination therapy with melatonin and DEX resulted in morphometric improvements such as improved tissue morphology, reduced contusion volume, and reduced loss of myelin. These data were confirmed by TTC staining that showed a significant reduction in infarct area and infarct volume after combination therapy with melatonin and DEX in relation to the treatment with single drugs. Thereafter, the degradation of the basal lamina and the activation of metalloproteinases, MMP-9 and MMP-2, were also observed as a result of CNS injury (Esposito et al. 2008, Esposito & Cuzzocrea 2010).
The observed decrease in the expression of MMP-2 and MMP-9 represented protection against the degradation of the basal lamina in the combination therapy group, supporting even more the effectiveness of the synergic treatment with melatonin and DEX.

Among the inflammatory mediators, iNOS induction and the consequent product peroxynitrite have been implicated in TBI. Peroxynitrite is formed via a diffusion-limited reaction of superoxide and NO, which damages biomolecules by nitrotyrosine formation. The expression of iNOS has been found near the necrotic and inflammatory areas mainly in neutrophils/macrophages, where it plays a crucial role in secondary brain damage subsequent to TBI in humans (Orihara et al. 2001), and the inhibition of iNOS protected against injury in TBI animal models (Clark et al. 1996, Singh et al. 2007). In this regard, we observed that co-administration of melatonin with DEX appreciably inhibited the expression of iNOS after the injury, indicating that the combination therapy has the potential to protect the brain against iNOS-mediated neurodegeneration in TBI.

Moreover, the major focus of TBI research should be the protection of neurons from apoptotic cell death by reducing the secondary injury of inflammation and oxidative stress (Mohr et al. 1997). In this study, combination therapy with melatonin and DEX also reduced the pro-apoptotic protein expression of Bax and increased anti-apoptotic protein expression with respect to the treatment with single drugs. Taken together, this result suggests that melatonin with DEX prevents the loss of the anti-apoptotic way and reduces pro-apoptotic pathway activation.

Inflammation, an essential component of TBI, is not only involved in oxidative stress and apoptotic cell death but also hinders the recovery of motor behavioral functions as shown by the rotarod task, which is considered as the most sensitive test to assess motor function in mice. In our study, we demonstrated that the combination therapy with melatonin and DEX significantly improved latency compared with single drug treatments, indicating that combination therapy-mediated mechanisms promote recovery and enhance repair mechanisms.

In conclusion, our findings confirmed that the strategies targeting multiple pro-inflammatory pathways may be more effective than targeting a single effector molecule. We have demonstrated that combination therapy with a potent antioxidant such as melatonin and an anti-inflammatory agent such as DEX
significantly protects against TBI by reducing exacerbation in neurovascular units and chronic inflammation, which are involved in the induction of cell death and neurobehavioral deficits.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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