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Therapeutic effects of chrysin in a rat model of traumatic brain injury: A behavioral, biochemical, and histological study

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ABSTRACT

Aims: Oxidative stress and apoptosis have major roles in the progression of traumatic brain injury (TBI)-associated motor and cognitive deficits. The present study was aimed to elucidate the putative effects of chrysin, a natural flavonoid compound, against TBI-induced motor and cognitive dysfunctions and possible involved mechanisms.

Main methods: Chrysin (25, 50 or 100 mg/kg) was orally administered to rats starting immediately following TBI induction by Marmarou's weight-drop technique and continuously for 3 or 14 days. Neurological functions, motor coordination, learning and memory performances, histological changes, cell apoptosis, expression of pro- and anti-apoptotic proteins, and oxidative status were assayed at scheduled time points after experimental TBI.

Key findings: The results indicated that treatment with chrysin improved learning and memory disabilities in passive avoidance task, and ameliorated motor coordination impairment in rotarod test after TBI. These beneficial effects were accompanied by increased the concentrations of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione (GSH), decreased malondialdehyde (MDA) content, prevented neuronal loss, diminished apoptotic index, elevated the expression of anti-apoptotic Bcl-2 protein, and reduced the expression of pro-apoptotic Bax protein in the cerebral cortex and hippocampus tissues.

Significance: Our findings suggest that both anti-oxidative and anti-apoptotic properties of chrysin (especially in the dose of 100 mg/kg) are possible mechanisms that improve cognitive/motor deficits and prevent neuronal cell death after TBI.

1. Introduction

Traumatic brain injury (TBI) is one of the most serious causes of neurological disability in children and young adults in the many developed and developing countries [1,2]. TBI affects over 1.7 million people every year in the United States [3], and represents a "silent epidemic" [4,5].

The pathophysiology of TBI is classified into primary and secondary phases. The primary phase is caused by the impact itself [6]. The secondary phase begins immediately after the impact and continues for hours to days after the insult. It triggers complex pathological processes, including inflammatory responses, oxidative stress, neuronal apoptosis, mitochondrial malfunction, altered neurogenesis, and dysfunction of cell signaling pathways [2,7]. Although many efforts have been done to develop effective treatments for TBI, there has been no definitive accepted treatment for the improvement of neurobehavioral deficits to date [8].

Oxidative stress plays a key role in the pathophysiology of the secondary phase of TBI, which can lead to neuronal apoptosis [9] and subsequently, may trigger the development of neurobehavioral deficits [10–12]. Superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO^-) are the main of reactive oxygen species (ROS), which leads to neuronal damage through protein oxidation, peroxidation of membrane lipids, and DNA destruction. Enzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and non-enzymatic antioxidants, such as glutathione (GSH) play an important role against ROS productions and oxidative stress. SOD is considered as a first-line cellular defense against ROS

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productions which it causes conversion of O_2^- (highly reactive and toxic) to H_2O_2 (less reactive and toxic). GPx catalyzes H_2O_2 utilizing reduced GSH, and CAT catabolizes H_2O_2 to molecular oxygen and water [13,14]. It is well known that brain is highly susceptible to oxidative damage due to its high polyunsaturated lipid content, immense oxygen consumption, high metabolic rate, and low concentration of anti-oxidative enzymes [15,16].

TBI-induced apoptosis could be offered as another possible mechanism of neuronal cell death, which characterized by DNA fragmentation [17], and may finally lead to TBI-induced neurological and behavioral dysfunctions. It was documented that the Bcl-2 family of proteins regulates the intrinsic apoptosis signaling pathway through balancing of pro-apoptotic (e.g., Bax and Bak) and anti-apoptotic (e.g., Bcl-2 and Bcl-XL) products [18].

Chrysin (5,7-dihydroxyflavone), a natural flavonoid, plentifully is found in various natural products such as propolis, honey, blue passion flower (*Passiflora caerulea*), vegetables, and fruits [19]. Chrysin has a wide range of biological effects, including antioxidant [20], anti-inflammatory [21], neuroprotective [22], anti-apoptotic [23] and cognitive-improving effects [24]. Furthermore, chrysin has been known as a natural ligand for peroxisome proliferator activated receptor gamma (PPAR-γ), which regulates oxidative stress and inflammation in the central nervous system (CNS) [25,26].

To sum up, the aim of the present study was to investigate the efficacy of oral administration of chrysin on neurological functions, motor coordination, and cognitive performances and also determined whether any of these protective effects were modulated through anti-oxidative and anti-apoptotic properties in both cerebral cortex and hippocampus regions of closed head weight drop rat model.

2. Material and methods

2.1. Chemicals and drugs

The chemicals and drugs used in this experiment were the following: Chrysin, 3,3-diaminobenzidine (DAB), proteinase K (Sigma-Aldrich Co., USA); Catalase (CAT), glutathione peroxidase (GPx), glutathione (GSH), superoxide dismutase (SOD), malondialdehyde (MDA) (ZellBio Co., Germany); Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA); Phosphate-buffered saline (PBS), protease inhibitor cocktail (Roche, Basel, Switzerland); Bcl-2-associated X protein (Bax), B-cell leukemia-2 (Bcl-2), m-IgGκ BP-HRP secondary antibody (Santa Cruz Biotechnology, Inc., CA, USA); Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) (In Situ Cell Death Detection kit, POD, Roche; Germany); Hydrogen peroxide (H_2O_2), sodium hydroxide (NaOH) (Merck Co., Germany); Ketamine, and xylazine (Alfasan Chemical Co, Woerden-Netherlands). All the other chemicals utilized in this experiment were of the analytical grade. Chrysin was freshly prepared by dissolving in PBS containing 3% (v/v) NaOH (0.01 M) as a vehicle and administrated to animal orally using a gavage needle.

2.2. Animals and experimental design

This experimental study was performed in accordance with Ethics Committee guidelines (Ethic code: IR.AJUMS.REC.1396.264) of Ahvaz Jundishapur University of Medical Sciences (Ahvaz, Iran). Adult male Wistar rats, weighing 250–300 g, were housed in an air-conditioned room at 22 ± 2 °C with humidity (50–60%) and a 12 h light/12 h dark cycle with access to food and water ad libitum. Following two weeks of acclimatization, the animals were randomly divided into six groups with 18 in each, as following:

Group I: Sham (no TBI injury + vehicle).
Group II: TBI (TBI injury + vehicle).
Group III: TBI + Ch_{25} (TBI injury + Chrysin 25 mg/kg/day).
Group IV: TBI + Ch_{50} (TBI injury + Chrysin 50 mg/kg/day).
Group V: TBI + Ch_{100} (TBI injury + Chrysin 100 mg/kg/day).
Group VI: Sham + Ch_{100} (no TBI injury + Chrysin 100 mg/kg/day) as positive control.

Chrysin (25, 50 or 100 mg/kg) or vehicle was immediately administrated after TBI induction and continuously for 3 or 14 days by oral gavage once daily. The Sham and Sham + Ch_{100} groups underwent preparatory procedures for brain trauma but were not exposed to brain trauma and received either vehicle or chrysin for 3 or 14 consecutive days. The time line of treatment schedule and protocol design is illustrated in Fig. 1.

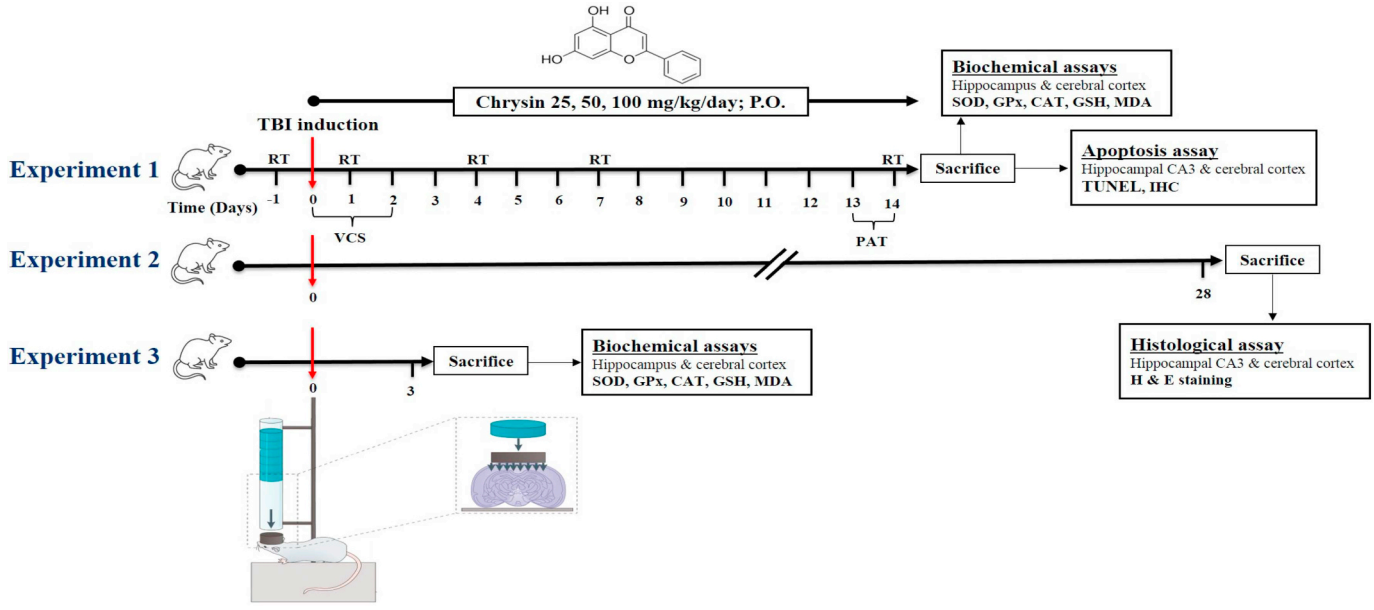

Fig. 1. A schematic diagram of the study design and treatment schedule. RT: Rotarod Test; TBI: Traumatic brain injury; VSC: Veterinary coma scale; P.O.: Per oral; PAT: Passive avoidance test; SOD: Superoxide dismutase; GPx: Glutathione peroxidase; CAT: Catalase; GSH: Glutathione; MDA: Malondialdehyde; TUNEL: Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling; IHC: Immunohistochemistry; H & E staining: Hematoxylin and eosin staining.

2.3. Induction of diffuse TBI

Induction of closed head weight drop model was done by the modified Marmarou's method recommended by Farbood et al. [27,28], using a TBI device made by the Ahvaz Physiology Research Center, Jundishapur University of Medical Sciences, Iran. In brief, after general anesthesia by ketamine-xylazine mixture (50/5 mg/kg, IP), the animal was intubated, then a weight (150 g) was dropped through a 2 m height plexiglass guide tube onto a steel disk fixed to the head of the subject. Subsequently, the animal was immediately connected to a mechanical ventilator (Ugo Basile, Varese, Italy) and the intra-tracheal tube was disconnected as soon as spontaneous breathing recovered.

2.4. Assessment of neurobehavioral functions

2.4.1. Neurological functions

Neurological functions were assessed using veterinary coma scale (VCS) as described previously [29]. VCS is a composite of motor, visual, and respiratory responses that has a scoring range from 3 to 15 (Table 1). The severity of the head injury was classified based on the VCS score into normal (15), mild (13–15), moderate (9–12), severe (3–8), and maximal impairment (3). The VCS was assayed at times −1 (1 h before TBI), 0 (just after TBI), 1, 4, 24, and 48 h after TBI.

2.4.2. Rotarod test

To evaluate motor coordination an accelerating rotarod test (Borj Sanat Azma Co, Tehran-Iran) from 5 to 40 rpm over a time course of 10 min [30] was carried out at −1 (one day before TBI), 1, 4, 7, and 14 days after TBI. Briefly, the animal was placed on a slowly rotating rod (7 cm in diameter), and the latency to falling was documented. On each session day, the rat was given three successive trials separated by an inter-trial interval of 45 min and the average time (in seconds) spent on the rotating rod was calculated for analysis.

2.4.3. Passive avoidance task (PAT)

PAT was performed in order to evaluate the effects of chrysin on passive avoidance learning and memory functions on 13th and 14th days after TBI induction. Details of the experimental apparatus and general procedure were basically described by other researchers [31,32]. The apparatus (Borj Sanat Azma Co, Tehran-Iran) was composed of two equal light and dark chambers, which are separated by a mobile guillotine door. The floors were made of stainless steel rods (3 mm in diameter, spaced 10 mm apart), but the floor of the dark chamber was connected to a shock generator. On habituation day, each rat was placed in the lit chamber of PAT facing away from the door and 10 s later, the guillotine door was opened. The door was locked as the rat entered the dark chamber with four paws, it was returned into its home cage. Acquisition phase was conducted 30 min after the habituation trial. In this phase entrance latency to the dark chamber was recorded as initial latency (IL) from the time the door was lifted, then guillotine door was closed and a mild foot shock was applied to it (0.3 mA, 75 V, 50 Hz) for 3 s. The rat was retained in the lit chamber and received an electrical shock each time they reentered the dark compartment. Termination of the training was when a rat remained in the lighted chamber for 120 consecutive seconds. On the retention phase that given 24 h after the acquisition phase, each animal was again placed into the lighted chamber and 10 s later, the guillotine door was raised. Consequently, step-through latency (STL) was scored as a measure of retention performance. If the animal avoided entering into the dark chamber within 300 s, the retention test was terminated and a ceiling score of 300 s was assigned. No electric shock was delivered in the retention test. Short latency illustrated poorer cognition.

2.5. Biochemical assay

Six rats in each group were deeply and irreversibly anesthetized with an overdose of mixed ketamine-xylazine 3 or 14 days post-TBI. Their brains were isolated, cerebral cortex and hippocampi tissues were rapidly dissected on ice, washed by PBS, and stored at −80 °C until further use. The frozen brain tissues were thawed, homogenized (100 mg tissue per 1 ml of cold PBS, pH 7.4, plus protease inhibitor cocktail) and centrifuged at 10000 ×g for 20 min at 4 °C. Subsequently, the clear supernatants were divided into aliquots and frozen immediately at −80 °C until analysis. Total protein concentrations of the samples were evaluated according to the manufacturer's protocols using a Bio-Rad protein assay kit. The values of the studied oxidative stress markers including SOD, GPx, CAT, GSH, and MDA in both cerebral cortex and hippocampus tissues were analyzed by commercial ELISA kits according to the manufacturer's instructions. These concentrations were normalized to protein content per sample.

2.6. Histological assay

2.6.1. Hematoxylin and eosin (H & E) staining

In this set of studies, we assessed the protective effects of chrysin on TBI for neuron abundance in both cerebral cortex and hippocampal CA3 histologically 28 days after TBI, to allow time for any neuronal loss to develop. Three animals of each group were irreversibly anesthetized and transcardially perfused with PBS containing 5% formalin. After decapitation, their brains were taken out, fixed by 10% paraformaldehyde solution (for 72 h), embedded in paraffin and cut in 5 μm thick. In the next step, tissue sections were stained by H & E. Six slides per animal were selected, then intact neurons (normal cells) from the cerebral cortex and hippocampal cornu ammonis 3 (CA3) regions were assessed under a light microscope (Olympus PX 50 F3 model, Japan).

2.6.2. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay

TUNEL staining was done according to the manufacturer's protocol to

Table 1
Veterinary coma scale (VCS).

Task	Description	Description points
Motor function	Straight walk (normal rat)	8
	Mildly confused and drowsy with purposeful movements	7
	Lethargic or unconscious state, powerless to stand, but maintains sternal recumbency	6
	Lethargic, withdraws to foot-pinch, and pay attention to visual stimuli by lifting the head; no sternal recumbency	5
	Withdraws to foot-pinch	4
	Spontaneous moving	3
	Extensor posturing (to stimuli or spontaneous)	2
	No responsiveness to stimuli	1
Eye response	Normal animal	4
	Open on stimulation	3
	Normal eyelid reflexes	2
	No eyelid response to sensory stimuli	1
Respiration	Normal animal	3
	Ataxic	2
	Apneic	1

the paraffin sections. Following deparaffinization by standard methods, samples were treated with 20 µg/ml proteinase K for 20 min, and immersed in 0.3% H2O2 in methanol for 10 min to block endogenous peroxidase activity. Thereafter, the samples were incubated in the TUNEL reaction mixture in the dark for 60 min at 37 °C, followed by PBS washes. Following 3× PBS wash, the slides were incubated for 30 min with secondary anti-fluorescein-POD-conjugate. Subsequently, the samples were stained with DAB chromogen substrate in the dark for 5 min at 25 °C [33]. The required images were taken under a light microscope.

A cell was TUNEL-positive when the nuclear staining was brown, intense, and homogenous. Apoptotic index was calculated as TUNEL-positive cells / total number of cells × 100 [34]. Apoptotic index in the cerebral cortex and hippocampal CA3 areas were measured (six sections per animal and three animals per group were analyzed).

2.6.3. Immunohistochemistry (IHC) assay

After deparaffinization and incubation in citrate buffer solution (98 °C) with pH 6.0 for 15 min, the tissue sections were blocked with 10% goat serum in PBS for 60 min. Thereafter, the sections were incubated overnight with monoclonal antibody against Bax or Bcl-2 (dilution 1:50) in a humidity chamber at 4 °C. The tissue sections were treated with m-IgGκ BP-HRP as secondary antibody (dilution 1:100) in PBS plus 10% goat serum for 2 h at room temperature. Next, they were incubated with a DAB which was used as chromogen substrate for 5 min. Finally, the tissue sections were counterstained with hematoxylin and observed by light microscopy. Six immunohistochemical slides per animal were blindly assayed using H-score. The calculation of H-score for each section was found as follows: H-score = ΣPi (i + 1), where "i" is the intensity of staining (0 = negative, 1 = low, 2 = medium and 3 = high) and "Pi" is the percentage of stained cells for each intensity (0 to 100%) [35].

2.7. Statistical analysis

All data analyses were done using Statistical Package of Social Sciences (SPSS) software version 16 (SPSS; Chicago, IL, USA). Data for the VCS values and motor coordination in the rotarod test were analyzed by repeated measurements analysis of variance (RM-ANOVA), and significant differences between individual groups were assessed using one-way ANOVA followed by Tukey's *post-hoc* test. Other data was analyzed by one-way ANOVA and represented as mean ± standard error of mean (SEM). For all comparisons, p-values of < 0.05 was assigned as statistically significant.

3. Results

3.1. Assessment of neurobehavioral functions

3.1.1. Chrysin does not improve neurological deficits

Neurological functions were evaluated using VCS at −1 (before TBI), 0 (just after TBI), 1, 4, 24 and 48 h post-TBI. A repeated measures ANOVA analysis of the VCS values showed the overall significant effects of the treatment ($F_{5, 30} = 325.528$; $p < 0.001$), day ($F_{7, 210} = 751.289$; $p < 0.001$) and day × treatment interaction ($F_{35, 210} = 37.042$; $p < 0.001$). The following Tukey's post hoc test revealed the VCS values for the TBI group was significantly decreased compared to that for the Sham group at 0, 1, 4 and 24 h post-TBI ($p < 0.001$ for each comparison, Fig. 2A), suggesting obvious neurological deficits in the rats with TBI.

The VCS values increased with time in the TBI and the TBI + Ch (25, 50 or 100 mg/kg) groups due to spontaneous recovery. Chrysin consumption effectively did not improve the neurological deficits in traumatic animals.

3.1.2. Chrysin improves motor coordination test

Rotarod test was mainly designed to calculate motor coordination. As depict in Fig. 2B, results of the rotarod test showed that there were alterations in motor coordination of animals before and after TBI. Repeated measure ANOVA analysis of the latency to fall from the rotarod displayed significant effect of the treatment ($F_{5, 30} = 30.240$; $p < 0.001$) and day ($F_{4, 88} = 75.972$; $p < 0.001$). However, there was no significant effect of day × treatment interaction ($F_{20, 88} = 1.183$; $p > 0.05$) for the fall latency from the rotarod.

The following Tukey's post hoc test revealed the TBI group spent the

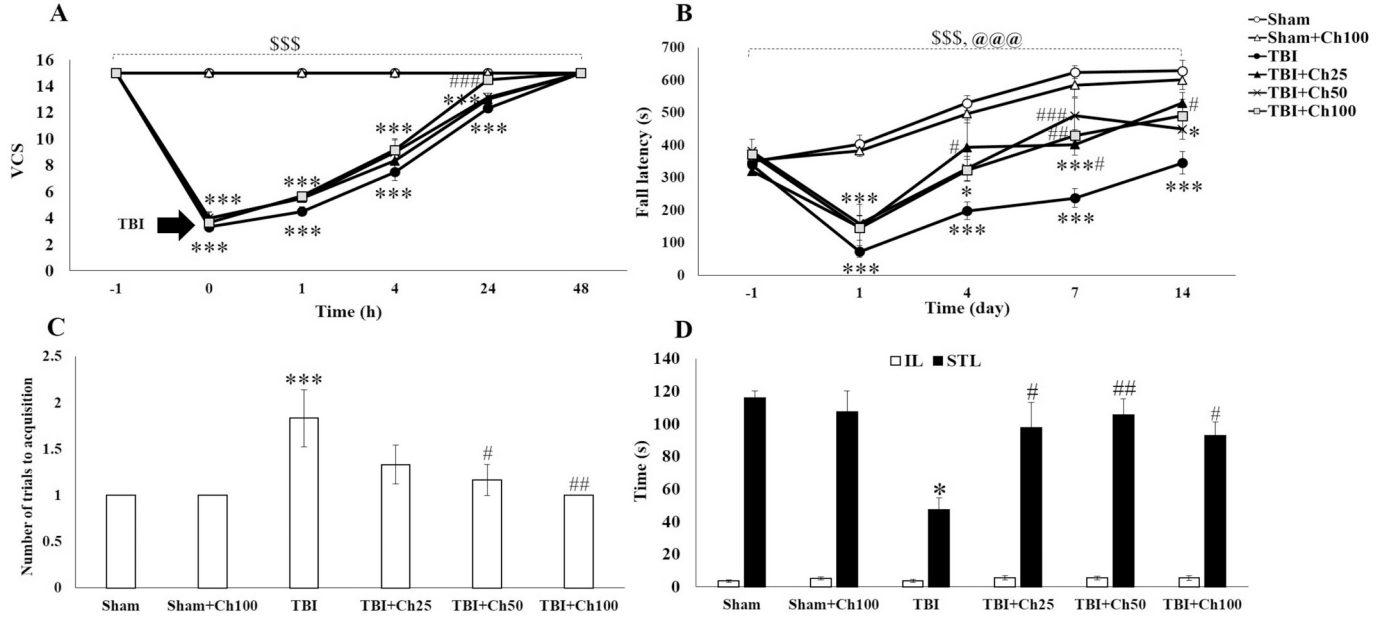

Fig. 2. Effects of chrysin (25, 50 or 100 mg/kg/day; P.O., for 14 consecutive days) on neurological signs by veterinary coma scale (VCS) (A), motor coordination in the rotarod test (B), number of trials to acquisition (C), initial latency (IL) and step-through latency (STL) of PAL (D) in normal and traumatic rats. Each bar represents mean ± SEM of six animals per group. $^{\$\$\$}p < 0.001$ indicates the significant difference in the area below the curve vs. the Sham group; $^{@@@}p < 0.001$ indicates the significant difference in the area below the curve vs. the TBI + Ch (25, 50 or 100 mg/kg) groups; *p < 0.05, **p < 0.01, ***p < 0.001 vs. the Sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the TBI group.

least amount of time on fall latency from the rotarod in comparison to the Sham group ($p < 0.001$ for each day tested). A progressive and significant increase in the fall latency from the rotarod was found after chrysin consumption compared to the TBI group from the 4th day until the end of the study.

3.1.3. Chrysin improves learning and memory deficits

As illustrated in Fig. 2C, the number of acquisition trials was significantly enhanced in the TBI group as compared with the other groups ($F_{5, 30} = 4.383$; $p < 0.01$). Chrysin administration (25, 50 or 100 mg/kg) decreased it in a dose-dependent manner ($p > 0.05$, $p < 0.05$, $p < 0.01$, respectively).

The results of the animals' performance in the acquisition phase of PAL showed that the IL was similar in all experimental groups ($F_{5, 30} = 0.659$; $p > 0.05$). In the retention phase, the STL in the TBI group was markedly less than that in the Sham group ($F_{5, 30} = 5.579$; $p < 0.01$). Chrysin treatment at doses of 25, 50 and 100 mg/kg significantly improved the STL in the traumatic animals ($p < 0.05$,

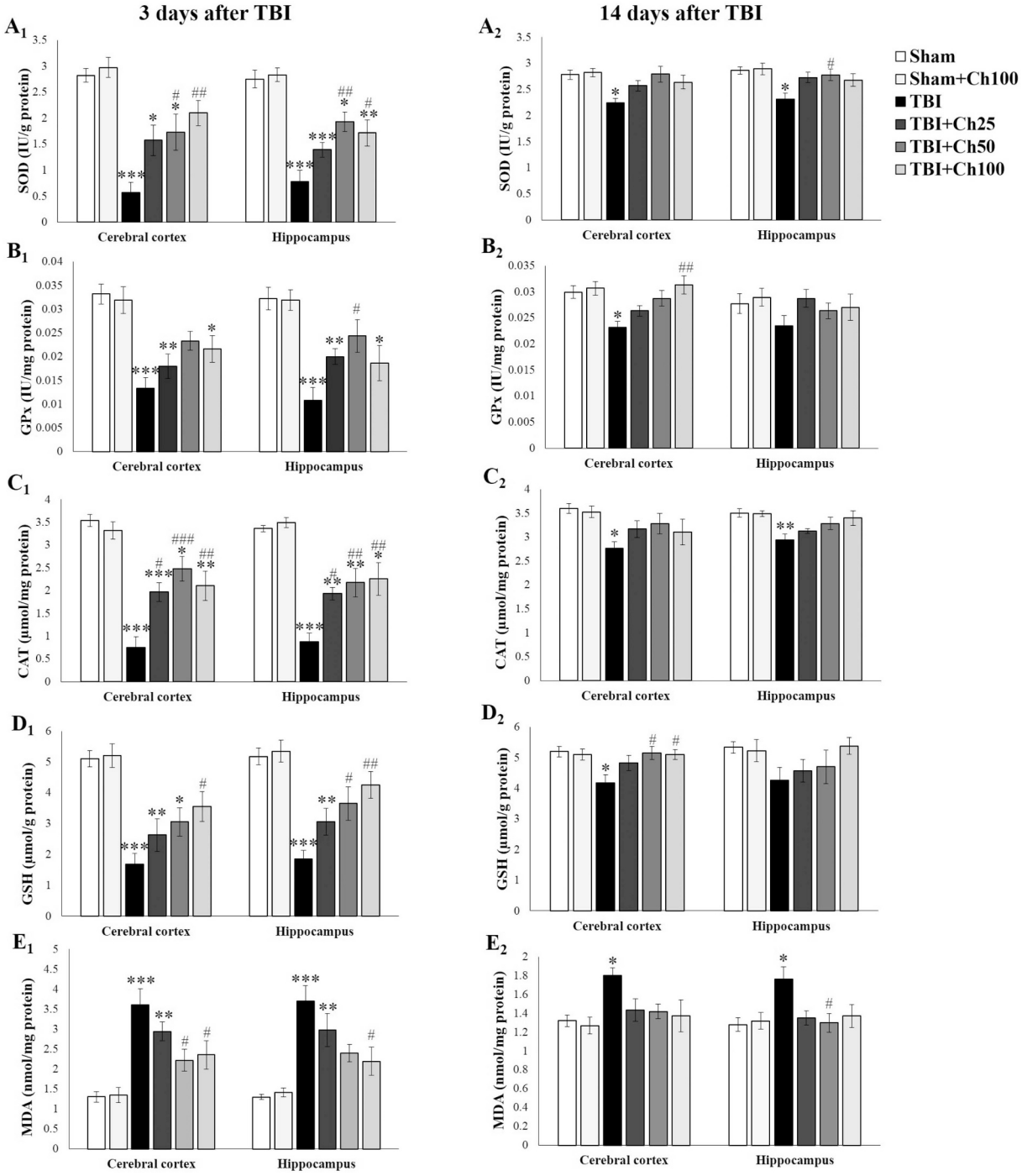

Fig. 3. Effects of chrysin (25, 50 or 100 mg/kg/day; P.O., for 3 or 14 consecutive days) on SOD ($A_{1,2}$), GPx ($B_{1,2}$), CAT ($C_{1,2}$), GSH ($D_{1,2}$), and MDA ($E_{1,2}$) levels in both cerebral cortex and hippocampus tissues of normal and traumatic rats. Each bar represents mean ± SEM of six animals per group. *$p < 0.05$, **$p < 0.01$ vs. the Sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the TBI group.

$p < 0.01$ and $p < 0.05$ respectively, Fig. 2D). These findings indicate that treatment of traumatic animals with chrysin could improve memory deficit in PAT.

3.2. Biochemical results

3.2.1. Chrysin ameliorates oxidative stress in the cerebral cortex and hippocampus tissues

The free radical damage was assessed 3 and 14 days after TBI by lipid peroxidation as determined by MDA level. As represented in Fig. 3E_1 and E_2, the Sham animals did not differ in the MDA level at either 3- or 14-days after TBI. Three days after TBI, there was an elevated MDA level in both cerebral cortex ($F_{5,\ 30} = 9.482$; $p < 0.001$) and hippocampus ($F_{5,\ 30} = 9.167$; $p < 0.001$) of the TBI group when compared to the Sham group. At the 14th day post-TBI, MDA level of traumatic animals was decreased in comparison to the 3th day, however, it was markedly higher than the Sham group ($F_{5,\ 30} = 2.7$; $p < 0.05$ for cerebral cortex, and $F_{5,\ 30} = 3.298$; $p < 0.05$, for hippocampus). In addition, chrysin treatment at doses of 50 and 100 mg/kg to the traumatic animals caused a significant decline in MDA levels in both cerebral cortex ($p < 0.05$, for each comparison) and hippocampus ($p > 0.05$ and $p < 0.05$, respectively) compared to the TBI group on the 3th day, and returned to the Sham group level on the 14th day.

On the 3th day after TBI, decreased activities of free radical scavenging enzymes (SOD, GPx, and CAT) and also non-enzymatic defense system (GSH) were statistically observed in both cerebral cortex and hippocampus of the TBI group when compared to the Sham group [cerebral cortex: ($F_{5,\ 30} = 13.21$; $p < 0.001$ for SOD; Fig. 3A_1), ($F_{5,\ 30} = 10.09$; $p < 0.001$ for GPx; Fig. 3B_1), ($F_{5,\ 30} = 18.70$; $p < 0.001$ for CAT; Fig. 3C_1), and ($F_{5,\ 30} = 11.14$; $p < 0.001$ for GSH; Fig. 3D_1)] and [hippocampus: ($F_{5,\ 30} = 18.02$; $p < 0.001$ for SOD), ($F_{5,\ 30} = 8.931$; $p < 0.001$ for GPx), ($F_{5,\ 30} = 19.10$; $p < 0.001$ for CAT), and ($F_{5,\ 30} = 11.05$; $p < 0.001$ for GSH)]. These reductions were markedly increased by chrysin treatment of traumatic animals (at doses of 50 and 100 mg/kg) as compared to the TBI group in the brain areas examined ($p < 0.05$; Fig. 3A_1–D_1).

The oxidative status was ameliorated with time in the TBI group due to spontaneous recovery compared to the Sham group on the 14th day, however it did not reach Sham levels [cerebral cortex: ($F_{5,\ 30} = 6.006$; $p < 0.05$ for SOD), ($F_{5,\ 30} = 4.794$; $p < 0.05$ for GPx), ($F_{5,\ 30} = 2.818$; $p < 0.05$ for CAT), and ($F_{5,\ 30} = 3.469$; $p < 0.05$ for GSH)] and [hippocampus: ($F_{5,\ 30} = 3.918$; $p < 0.05$ for SOD), ($F_{5,\ 30} = 1.114$; $p > 0.05$ for GPx), ($F_{5,\ 30} = 4.320$; $p < 0.001$ for CAT), and ($F_{5,\ 30} = 1.525$; $p > 0.05$ for GSH)]. Also, after treatment with chrysin, SOD, GPx, and CAT activities and GSH level were all increased and returned to the Sham group levels (Fig. 3A_2–D_2).

3.3. Histological results

3.3.1. Chrysin prevents neuronal loss in the cerebral cortex and hippocampal CA3 regions

Based on the results shown in Fig. 4A and B, histological changes in the cerebral cortex and hippocampal CA3 areas were assessed by H & E staining. There was no significant difference between the Sham group and the Sham + Ch_{100} group (data not shown). A significant decline was found in the number of intact neurons of the cerebral cortex ($F_{4,\ 10} = 10.27$; $p < 0.001$) and hippocampal CA3 ($F_{4,\ 10} = 26.87$; $p < 0.001$) areas of the TBI group compared to the Sham group, indicating the neuronal loss in traumatic animals. Chrysin treatment of the traumatic rats markedly protected the cerebral cortex at dose of 100 mg/kg ($p < 0.05$) and hippocampal CA3 neurons at doses of 50 ($p < 0.05$), and 100 mg/kg ($p < 0.001$) as compared to the TBI group.

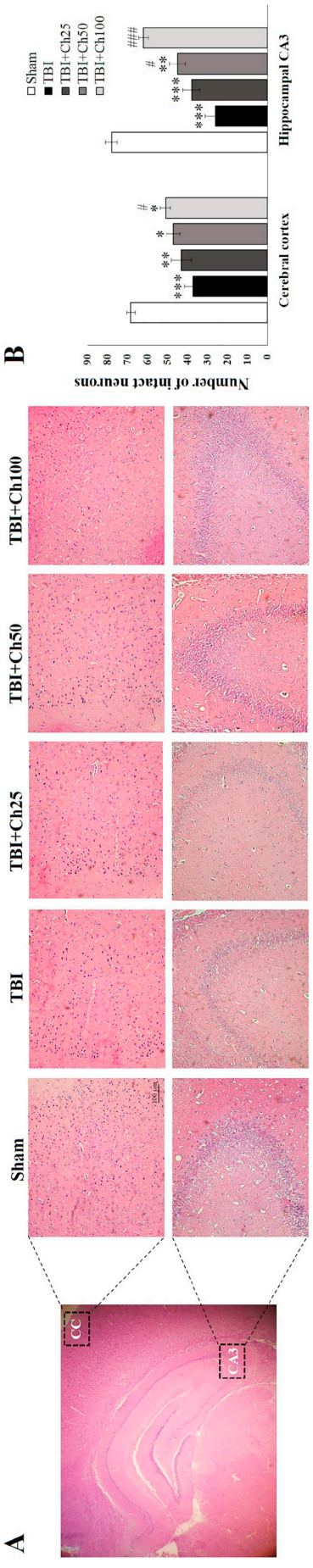

Fig. 4. Effects of chrysin (25, 50 or 100 mg/kg/day; P.O., for 14 consecutive days) on histological changes in the cerebral cortex (CC) and hippocampal CA3 regions (H & E staining) of normal and traumatic rats 28 days after TBI. "A" represents photomicrograph of cerebral cortex (CC) and hippocampal CA3 regions of the rat brain, and "B" includes the quantitative data of the number of intact neurons. Each bar represents mean ± SEM of three animals per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the Sham group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. the TBI group.

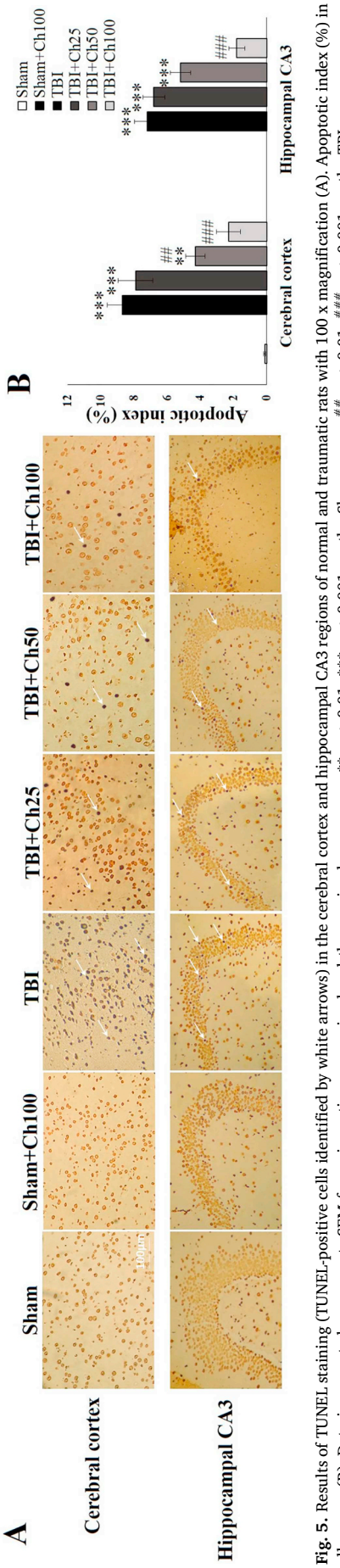

Fig. 5. Results of TUNEL staining (TUNEL-positive cells identified by white arrows) in the cerebral cortex and hippocampal CA3 regions of normal and traumatic rats with 100 x magnification (A). Apoptotic index (%) in all groups (B). Data is presented as mean ± SEM from six sections per animal and three animals per group. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the Sham group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the TBI group.

3.3.2. Chrysin decreases TUNEL-positive cells in the cerebral cortex and hippocampal CA3 regions

The apoptotic cells were identified by TUNEL staining in the brain slides. In the Sham and the Sham + Ch_{100} groups, TUNEL-positive cells were absent or rare in both cerebral cortex and hippocampal CA3 regions (Fig. 5A). Apoptotic index was significantly increased in the cerebral cortex ($F_{5,\ 12} = 31.06$; $p < 0.001$) and hippocampal CA3 ($F_{5,\ 12} = 41.68$; $p < 0.001$) areas of the TBI group compared to the Sham group (Fig. 5B).

Apoptotic index was also increased in the TBI + Ch_{25} and TBI + Ch_{50} groups compared to the Sham group ($p < 0.001$, and $p < 0.01$ for cerebral cortex; $p < 0.001$, and $p < 0.001$ for hippocampal CA3, respectively). But interestingly, there was no significant difference between the TBI + Ch_{100} group versus the Sham group ($p > 0.05$). Both 50 and 100 mg/kg doses of chrysin declined the apoptotic index in the cerebral cortex ($p < 0.01$, and $p < 0.001$, respectively) and hippocampus ($p > 0.05$ and $p < 0.001$, respectively) of traumatic rats as compared to the TBI group.

3.3.3. Chrysin decreases the expression of Bax protein and increases the expression of Bcl-2 protein in the cerebral cortex and hippocampal CA3 regions

To evaluate the anti- and pro-apoptotic effects of chrysin in the animal model of TBI, the expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins were assessed in both cerebral cortex and hippocampal CA3 areas 14 days post-TBI. There was a significant elevation in the expression of Bax protein (Fig. 6A and C) and reduction in the expression of Bcl-2 protein (Fig. 6B and D) in the cerebral cortex ($F_{5,\ 12} = 8.489$; $p < 0.05$, for Bax; $F_{5,\ 12} = 34.34$; $p < 0.001$, for Bcl-2) and hippocampal CA3 ($F_{5,\ 12} = 16.39$; $p < 0.001$, for Bax; $F_{5,\ 12} = 8.258$; $p < 0.01$ for Bcl-2) areas of the TBI group compared with the Sham group.

This imbalance of Bcl-2 family (increased Bax and decreased Bcl-2) was also detected in the TBI + Ch_{25}, and TBI + Ch_{50} groups compared to the Sham group in these brain areas. Although, as compared to the TBI group, chrysin treatment of traumatic animals markedly decreased the expression of Bax protein and increased the expression of Bcl-2 protein in both cerebral cortex and hippocampal CA3, especially at the dose of 100 mg/kg.

4. Discussion

The secondary phase of brain injury following TBI related to oxidative stress, inflammation, and neuronal apoptosis, which plays a main role in motor and cognitive deficits [11,36,37]. Accordingly, drugs which can simultaneously adjust various signal pathways may have an important functional role in treating TBI patients. Neuroprotective effects of chrysin, as a natural flavonoid compound, on motor and cognitive functions have been shown in different models of CNS injury [38–42].

The current study assessed the influences of chrysin consumption in a rat model of TBI upon neurological outcomes, motor coordination, cognitive function, oxidative status, histological changes, neuronal apoptosis and expression of apoptotic markers in both cerebral cortex and hippocampus regions post-TBI. The major findings of this study are 1) Chrysin-treated rats had no better performance on the VCS at 0 h to 24 h post-TBI, but had improved performance on learning and memory in PAT and motor coordination in rotarod test compared with the TBI group; 2) At 3th day post-TBI, chrysin increased the concentrations of SOD, GPx, CAT, GSH, and suppressed the elevation of MDA level; However, oxidative status in the rats treated with vehicle or chrysin was reversed to the Sham group levels at 14th day; 3) Chrysin consumption attenuated TBI-induced apoptosis and regulated Bcl-2 family protein expression (decreased Bax and increased Bcl-2) at 14th day, it also could prevent neuronal loss 28 days after TBI.

In the present study, the obvious neurological deficits were

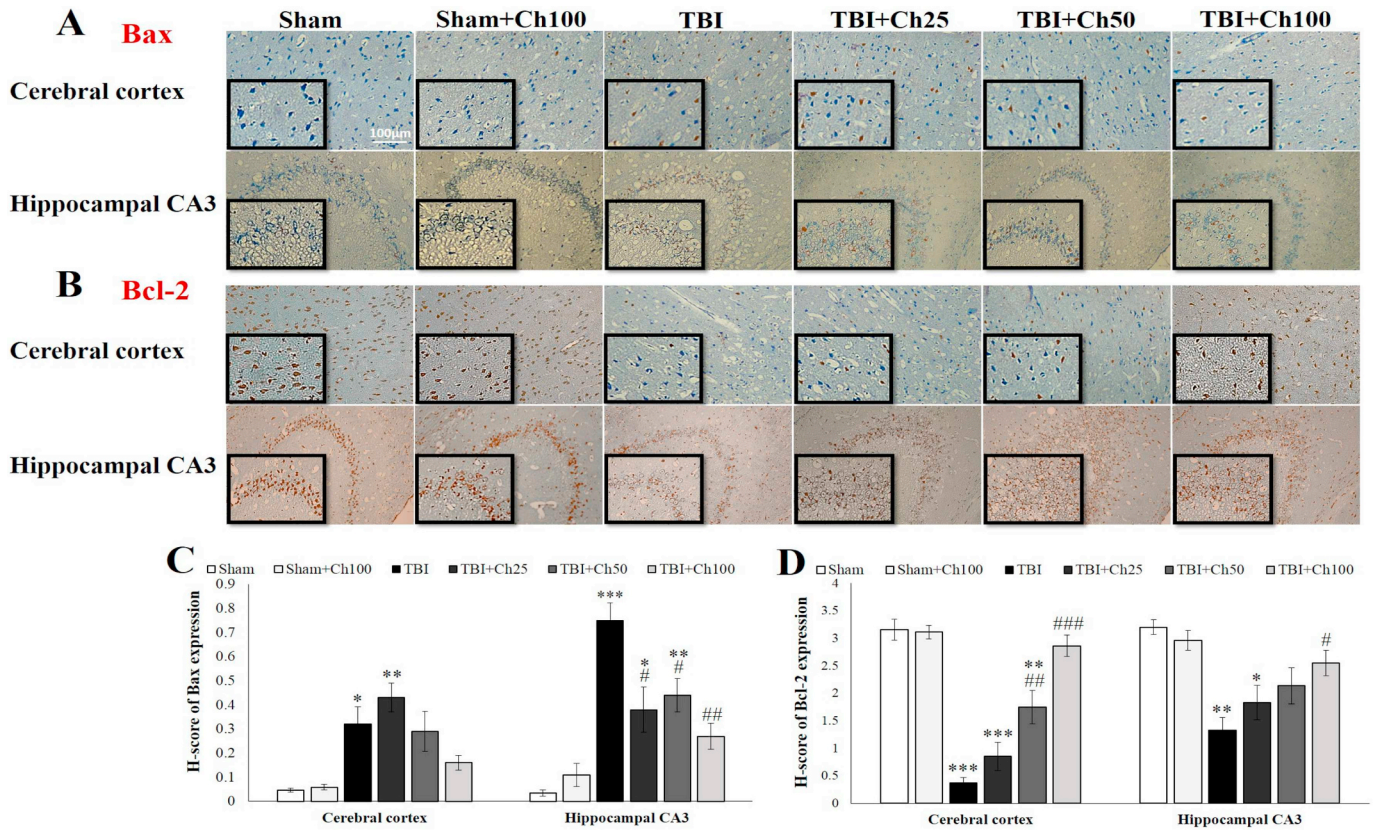

Fig. 6. Results of IHC staining for expressions of Bax (positive expression of Bax was identified by the presence of brown staining in the nucleus and/or cytoplasm; A) and Bcl-2 (positive expression of Bax was identified by the presence of brown staining in the nucleus and/or cytoplasm; B) in the cerebral cortex and hippocampal CA3 regions of normal and traumatic rats (100 x magnification). H-score of Bax (C) and Bcl-2 (D) expressions in all groups. Data is presented as mean ± SEM from six sections per animal and three animals per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. the Sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the TBI group.

observed at 0 to 24 h following TBI induction. Likewise, the traumatic animals had a poor performance in the rotarod test. These results are in agreement with previous studies [27,43,44]. Chrysin treatment of the traumatic animals had no effect on neurological deficits. However, previous studies have demonstrated that chrysin improved neurological functions in different insults such as autoimmune neuritis and spinal cord injury [40,42]. This controversy may be explained by differences in the model of CNS injury, chrysin dose, treatment period, administration route, the severity of TBI, and environmental conditions.

Cognitive impairments along with motor disorders are occasionally observed early and may persist beyond one year post-TBI [45–53]. Moreover, learning is disrupted in both humans and animals that have sustained a mild TBI [49,54]. It is well known that motor cortex is important for motor learning [55], whereas the hippocampal CA3 neurons are known to have an important role in memory processes, including contextual memory acquisition and retrieval information [56,57]. The PAT was performed in order to evaluate learning and memory deficits in the rats on 13th and 14th day's post-TBI. The results from the PAT showed TBI induction disrupts the consolidation and memory retrieval, which were depicted as an enhanced the number of acquisition trials and a reduced the STL. Functionally, chrysin improved passive avoidance learning and memory performances and motor coordination in the traumatic rats, which is in conformity with previous studies [20,58,59].

Oxidative stress caused by a misbalance between ROS productions and antioxidant defenses. Under normal physiological conditions, the innate antioxidant defense systems of the brain can certainly neutralize ROS produced by toxic reactions. However, under many pathological situations, brain anti-oxidative defense against ROS productions fails and afterward, neuronal activity and also cognitive performance will be severely affected [60]. The occurrence of oxidative stress following TBI plays a critical role in the development and progression of neurobehavioral deficits [10,61]. The present results suggest that TBI affects the activity of SOD, GPx, CAT, and level of GSH three days after TBI, which are responsible for scavenging free radicals. Indeed, declined SOD and GPx activities and reduction of GSH level following TBI, may be closely related to the enhanced ROS, highly reactive hydroxyl radicals, lipid peroxides and also increased antioxidants consumption [62,63]. These circumstances eventually could lead to aggravating further oxidative stress and eventually cell death [59,64,65]. In addition, amelioration of oxidative status was observed with time in the traumatic animals may be due to spontaneous recovery compared to the normal rats 14 days after TBI. In our study also MDA level (a common indicator of lipid peroxidation and reflects the level of free radical-induced damage) was markedly enhanced three days after TBI, demonstrating that the antioxidant defenses were not able to offset oxidative damage. Chrysin could effectively upregulate values of SOD, GPx, and GSH, and subsequently alleviated oxidative stress in both cerebral cortex and hippocampus tissues of traumatic animals, which might be due to the antioxidative action of chrysin. Therefore, it seems that the improving effect of chrysin against TBI-induced motor/cognitive deficits in the chrysin-treated rats was mediated, at least partially, by the potentiation of antioxidant and free radical scavenging capabilities.

Moreover, it is well-documented that chrysin has PPAR-γ agonist activity, its property is associated with anti-oxidative and anti-inflammatory effects within the CNS [25,26]. Hence, some beneficial effects of chrysin against oxidative stress may be due to its ability to mimic the inhibitory effects of the PPAR-γ ligand on oxidative stress, although the mechanism of its action was not investigated in this study.

In the current study, obtained results of the histological evaluation

exhibited a decreased number of intact neurons in both cerebral cortex and hippocampal CA3 areas of traumatic rats 28 days post TBI. In addition, apoptotic index was increased in these brain areas two weeks after TBI-induction. Consistent with the findings of this work, previous studies have shown that neuronal apoptosis is found in various brain parts of animals in the early hours after TBI-induction, and persisted for up to two weeks [66–69]. In the present study, elevation in the expression of pro-apoptotic Bax protein and reduction in the expression of anti-apoptotic Bcl-2 protein were observed in the brain. Imbalanced Bcl-2 family members induce permeabilization of the outer mitochondrial membrane, release of cytochrome *c* in the cytosol, initiator caspase-9 activation, and subsequently activating caspase-3 can initiate the intrinsic apoptotic pathway causing neuronal cell death [70]. Surprisingly, chrysin treatment could effectively reduce apoptotic cell death possibly through regulation of Bcl-2 family protein expression, which mediated by the mitochondrial apoptotic pathway. Thus, the improvement of TBI-related motor/cognitive disturbances by chrysin may be partly due to its anti-apoptotic activity against TBI-induced neuronal apoptosis.

It has been demonstrated that chrysin ameliorates motor and cognitive functions, by modulation of oxidative status, and suppression of neuronal cell apoptosis via regulation of caspase3, Bax and Bcl-2 [20,41]. In accordance with the mentioned studies, the results of the current study indicate a defensive role of chrysin against TBI-induced motor and cognitive deficits which may be due to its both anti-apoptotic and anti-oxidative properties.

Previous studies have assessed the dose-response relationships of chrysin treatment in different models of brain injury. Some studies illustrated that 100 mg/kg dose of chrysin was the most effective [40,71]. In the other studies, both 25 and 50 mg/kg doses of chrysin provided potent neuroprotection post various brain injuries [22,58,72], and 100 mg/kg dose was found to be ineffective [39]. In the current study, two doses of chrysin (50 and 100 mg/kg doses) were more effective in treating TBI with no obvious adverse effects, and chrysin given at 100 mg/kg led to the most remarkable improvement of cognitive deficits and prevention of neuronal cell death.

5. Conclusion

The results of the present study demonstrated that treatment with chrysin improved TBI-associated motor/cognitive disabilities and prevented neuronal loss, possibly via amelioration of oxidative status (increased concentrations of SOD, CAT, GPx, GSH, and decreased MDA level), and inhibition of apoptosis through regulation of Bcl-2 family protein expression (decreased Bax and increased Bcl-2) in the cerebral cortex and hippocampal CA3 areas. The results suggest that treatment with chrysin by a dose of 100 mg/kg was more effective against TBI-associated neurobehavioral deficits. However, further research is required to investigate the involved mechanisms in details.

Conflict of interest

The authors declare that there is no conflict of interest.

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