Free Radical Research, July 2009; 43(7): 658–667

informa
healthcare

The protective effect of alpha lipoic acid against traumatic brain injury in rats

HALE Z. TOKLU[1], TAYFUN HAKAN[2], NECAT BİBER[2], SEYHUN SOLAKOĞLU[3], AYLİZ VELIOĞLU ÖĞÜNÇ[4], & GÖKSEL ŞENER[1]

[1]*Marmara University, School of Pharmacy, Department of Pharmacology, Istanbul, Turkey,* [2]*Haydarpasa Numune Teaching and Research Hospital, Department of Neurosurgery, Istanbul, Turkey,* [3]*Istanbul University, School of Medicine, Department of Histology & Embriyology, Istanbul, Turkey, and* [4]*Marmara University, Vocational School of Health Related Sciences, Istanbul, Turkey*

(Received 18 February 2009; revised 17 March 2009)

Abstract

Traumatic brain injury (TBI) was induced by a weight-drop device using 300 g–1 m weight-height impact. The study groups were: control, alpha-lipoic acid (LA) (100 mg/kg, po), TBI, and TBI + LA (100 mg/kg, po). Forty-eight hours after the injury, neurological scores were measured and brain samples were taken for histological examination or determination of thiobarbituric acid reactive substances (TBARS) and glutathione (GSH) levels, myeloperoxidase (MPO) and Na^+-K^+ ATPase activities, whereas cytokines (TNF-α, IL-1β) were determined in blood. Brain oedema was evaluated by wet–dry weight method and blood–brain barrier (BBB) permeability was evaluated by Evans Blue (EB) extravasation. As a result, neurological scores mildly increased in trauma groups. Moreover, TBI caused a significant decrease in brain GSH and Na^+-K^+ATPase activity, which was accompanied with significant increases in TBARS level, MPO activity and plasma proinflammatory cytokines. LA treatment reversed all these biochemical indices as well as histopathological alterations. TBI also caused a significant increase in brain water content and EB extravasation which were partially reversed by LA treatment. These findings suggest that LA exerts neuroprotection by preserving BBB permeability and by reducing brain oedema probably by its anti-inflammatory and antioxidant properties in the TBI model.

Keywords: *Alpha lipoic acid, brain injury, closed head, trauma, blood–brain barrier, oedema*

Introduction

Traumatic brain injury (TBI) is associated with costly health problems and high mortality and morbidity in previously healthy populations. Although TBI represents a significant public health problem in the world, there are currently no treatments that improve clinical outcome measures [1,2]. TBI is classified as primary and secondary injuries. Primary injuries result directly from the traumatic event, while secondary injuries result from delayed processes associated with the trauma such as transient increases in excitatory neurotransmitter efflux [3] resulting in excitotoxicity, ionic imbalance, ATP depletion, proteolysis and oxidative stress [4].

Oxidative stress, an imbalance between oxidants and antioxidants, plays a critical role in the development of secondary injuries following TBI [5]. The brain is highly sensitive to stress-induced neurodegeneration because of its high content of polyunsaturated fatty acids, which are particularly vulnerable to free radical attacks and lipid peroxidation. It is well known that enhanced production of reactive oxygen (ROS)/nitrogen species (RNS) cause oxidative/nitrosative stress leading to damage in lipids, proteins and

Correspondence: Goksel Sener, PhD, Professor of Pharmacology, Marmara University, School of Pharmacy, Tibbiye Cad. 34668 İstanbul, Turkey. Tel: 00 90 216 414 29 62. Fax: 00 90 216 345 29 52. Email: gsener@marmara.edu.tr

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760902988843

nucleic acids [6–8]. On the other hand, antioxidants (enzymatic and/or non-enzymatic) can protect the brain against oxidative damage through scavenging ROS/RNS, inhibiting of ROS/RNS formation and binding metal ions needed for catalysis of ROS generation [9].

There is evidence that progressive pathophysiological changes occur after trauma, resulting in a reduction in brain blood flow and a decrease in tissue oxygen levels leading to ischaemia, subsequent secondary injury and brain oedema. It is documented that failure of the Na^+, K^+-ATPase pump is a common feature in central nervous system (CNS) pathologies related to ischemic conditions and TBI, since the pump is highly sensitive to oxidative stress [10–12]. The activity of Na^+, K^+-ATPase, a membrane-bound enzyme required for cellular transport, is very sensitive to free radical reactions and lipid peroxidation. Reductions in this activity can indicate membrane damage indirectly. Thus Na^+, K^+-ATPase is clearly down-regulated under low O_2 conditions which in turn triggers brain oedema and leads to BBB breakdown [13]. Lima et al. [10] have shown that Na^+, K^+-ATPase activity impairment was closely related to spatial learning deficits and oxidative stress. In summary, cerebral oedema, BBB permeability, MPO activity and oxidative damage play important roles and are the major parts in the secondary brain injury following TBI.

Lipoic acid is a dithiol antioxidant that is an important co-factor in pyruvate dehydrogenase and α-ketoglutarate dehydrogenase in the mitochondria [14,15]. There is a large body of evidence confirming the beneficial effect of LA in the treatment of many diseases, including diabetes, atherosclerosis, degenerative processes in neurons, diseases of joints or AIDS. In the last decade, some researchers have witnessed a surge of interest in the pharmacological properties of lipoic acid [16]. Lipoic acid and its reduced form, dihydrolipoic acid (DHLA), are capable of quenching ROS and RNS such as hydroxyl radicals, peroxyl radicals, superoxide, hypochlorous acid and peroxynitrite, thus they prevent singlet oxygen-induced DNA damage, exhibit chelating activity, reduce lipid peroxidation and increase intracellular glutathione levels [17]. Furthermore, reports emphasize that LA is found to be protective against cyclophosphamide- or adriamycin-induced toxicities in rats [18,19]. Recently, we have shown that LA protected hepatic and renal tissues against ischaemia/reperfusion-induced oxidative damage through its antioxidant effects [20,21].

In the light of these findings, we aimed to investigate whether and to what extent LA would provide protection against trauma-induced oxidative brain injury.

Materials and methods

Animals

Wistar albino rats (300–350 g) were housed in an air-conditioned room with 12-h light and dark cycles, where the temperature ($23 \pm 2^{\circ}C$) and relative humidity (65–70%) were kept constant. All experimental protocols were approved by the Marmara University School of Medicine Animal Care and Use Committee.

Rats were seperated randomly into four experimental groups, with 16 rats (six for blood–brain barrier and oedema evaluation; six for biochemical analysis in the brain tissue and blood, four for histological evaluation) in each group: control (vehicle), LA (100 mg/kg, po), trauma and trauma + LA (100 mg/kg, po). LA was dissolved in %10 DMSO–corn oil, and administered through an intragastric tube once daily for 2 days which began 30 min after the induction of trauma. The active ingredient LA (99% purity, (R)-α-lipoic acid) was supplied by Mikrogen Pharmaceuticals (Istanbul, Turkey).

Diffuse brain injury

The widely used diffuse brain injury model of Marmarou et al. [22] was used. Briefly, a device which works by dropping a constant weight from a specific height was used. Rats were placed on a 5 cm foam rubber platform and a 300 g weight was dropped from a 1 m height which induces a mild trauma, as shown by Ucar et al. [23]. For the induction of trauma the scalp of each of the anaesthetized (100 mg/kg ketamine + 1 mg/kg chlorpromazine) rat was shaved, a midline incision was performed and the periosteum was retracted. The metallic disc was fixed to the central portion of skull by using bonewax. The animals were placed in a prone position on a foam bed. The lower end of the steel tube was then positioned directly above the helmet (2 mm thick steel disc). The injury was delivered by dropping the freely falling steel 300 g weight from a 1 m height. An inflexible rope was tied to the weight to prevent repeated impacts. At the end of the study (48 hours after trauma induction) neurological examination was performed and half of the rats were decapitated to obtain blood and tissue samples, while EB assay was performed for BBB permeability in the remainders.

Neurological examination

The neurological examination scores were conducted according to Bederson's modified neurological examination test [24,25]. We used a 20-point neuro-score to assess motor and behavioural deficits. Briefly, the consciousness, performance in a smooth climbing platform, extremity tonus, walking and postural reflexes, circling and response to the nociceptive

stimuli were assessed. For walking and posture rats were allowed to move about freely on the floor and were observed. In the circling test the rats were held gently by the tail, suspended 1 m above the floor, and observed for forelimb flexion. Normal rats extend both forelimbs toward the floor. The rotation degree and time was measured. Finally the response to the nociceptive stimuli was assessed by tail-immersion test in 56°C water. All behavioural tests were conducted by a 'blinded' investigator. The sequence of testing animals by a given task was randomized for the animals. Twelve animals from each group were subjected to neurological examination. Afterwards, half of them were used for the biochemical analysis in blood and brain and the other half were used for the EB extravasation experiments.

EB assay for BBB permeability

To evaluate the BBB integrity, EB dye was used as a marker of albumin extravasation [25,26]. Briefly, EB dye (2% in saline, 4 ml/kg) was injected via the jugular vein of the anaesthesized (100 mg/kg ketamine + 1 mg/kg chlorpromazine) rats 48 h after the induction of trauma and it was allowed to remain in circulation for 30 min. At the end of experiments, the chest was opened and rats were perfused transcardially with 250 ml of saline at a pressure of 110 mm Hg for ~15 min until the fluid from the right atrium became colourless. After decapitation, the brain was removed and cerebral cortex was isolated. Then, each cortex was weighed for quantitative measurement of EB-albumin extravasation. Brain samples were homogenized in 2.5 ml phosphate-buffered saline and mixed by vortexing for 2 min after the addition of 2.5 ml of 60% trichloroacetic acid (TCA), to precipitate the protein. Samples were cooled and then centrifuged for 30 min at 1000 *g*. The supernatant was measured at 620 nm for absorbance of EB using a spectrophotometer (Shimadzu UV1208, Japan). EB was expressed as μg/mg of brain tissue against a standard curve.

Brain water content

Brain oedema was evaluated by the drying-weighing method based on the measurement of the water content of brain [25,26]. The whole brain was weighed and then dried for 48 h at 100°C, afterwards re-weighed. The percentage of water was calculated according to the following formula: $\%H_2O = [(\text{wet weight} - \text{dry weight})/\text{wet weight}] \times 100$.

Plasma TNF-α and IL-1β levels

Plasma TNF-α and IL-1β were quantified according to the manufacturer's instructions and guidelines using enzyme-linked immunosorbent assay (ELISA) kits (Biosource International, Nivelles, Belgium).

Biochemical analysis in the brain tissue

Chemiluminescence (CL) assay. To assess the role of reactive oxygen species in trauma-induced brain damage, luminol and lucigenin chemiluminescences were measured as indicators of radical formation. Lucigenin (bis-N-methylacridiniumnitrate) and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were obtained from Sigma (St Louis, MO). Measurements were made at room temperature using a Junior LB 9509 luminometer (EG&G Berthold, Germany). Cerebral cortex tissues were put into vials containing PBS-HEPES buffer (0.5 M PBS containing 20 mM HEPES, pH 7.2). ROS were quantitated after the addition of enhancers such as lucigenin or luminol for a final concentration of 0.2 mM. Luminol detects a group of reactive species, i.e. $^{\cdot}OH$, H_2O_2, HOCl radicals and lucigenin is selective for $O^{-}{}_2$ [27,28]. Counts were obtained at 1 min intervals and the results were given as the area under curve (AUC) for a counting period of 5 min. Counts was corrected for wet tissue weight (rlu/mg tissue).

TBARS and GSH assays. Tissue samples were homogenized with ice-cold trichloracetic acid (1 g tissue plus 10 ml 10% TCA) in an Ika Ultra Turrax (Janke & Kunkel T25, Staufen, Germany) tissue homogenizer. The malonedialdehyde (MDA) levels were assayed for products of lipid peroxidation by monitoring TBARS formation as described previously [29]. Lipid peroxidation is expressed in terms of MDA equivalents using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and the results are expressed as nmol TBARS/g wet tissue. Glutathione measurements were performed using a modification of the Ellman procedure [30]. Briefly, after centrifugation at 2000 g for 10 min, 0.5 ml of supernatant was added to 2 ml of 0.3 mol/l $Na_2HPO_4.2H_2O$ solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg/ml 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. Glutathione levels were calculated using an extinction coefficient of $1.36 \times 10^5\ M^{-1}\ cm^{-1}$. The results are expressed in μmol GSH/g wet tissue.

MPO activity. MPO activity in tissues was measured by a procedure similar to that described by Hillegass et al. [31]. Samples brain tissues were homogenized in 50 mM potassium phosphate buffer (PB), with pH 6.0, and centrifuged at 41 400 g for 10 min. The pellets were then suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw-cycles, with sonication between cycles, the samples were centrifuged at 41 400 g for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM PB, o-dianisidine and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the

amount of MPO present that caused a change in absorbance, measured at 460 nm for 3 min. MPO activity was expressed as U/g wet tissue.

Na^+-K^+-ATPase activity. Measurement of Na^+-K^+-ATPase activity is based on the measurement of inorganic phosphate released by ATP hydrolysis during incubation of homogenates with an appropriate medium containing 3 mM ATP as a substrate. The total ATPase activity was determined in the presence of 100 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 30 mM Tris HCl (pH 7.4), while the Mg^{2+}-ATPase activity was determined in the presence of 1 mM ouabain. The difference between the total and the Mg^{2+}-ATPase activities was taken as a measure of the Na^+-K^+-ATPase activity [32,33]. The reaction was initiated with the addition of the homogenate (0.1 ml) and a 5-min pre-incubation period at 37°C was allowed. Following the addition of Na_2ATP and a 10 min re-incubation period, the reaction was terminated by the addition of ice-cold 6% perchloric acid. The mixture was then centrifuged at 3500 g and Pi in the supernatant fraction was determined by the method of Fiske and Subbarrow [34]. The specific activity of the enzyme was expressed as nmol Pi mg^{-1} protein h^{-1}.

Protein assay. The protein concentration of the supernatant was measured by the Lowry et al. [35] method. Briefly, the principle was based on the reaction of copper(II) ion in alkaline solution with protein to form complexes, which react with the Folin-phenol reagent, a mixture of phosphotungstic acid and phosphomolybdic acid in phenol. The product became reduced to molybdenum/tungsten blue and was detected colourimetrically by absorbance at 500 nm.

All biochemical assays are expressed relative to wet weight of brain tissue.

Histological preparation and analysis

Anaesthesized (100 mg/kg ketamine + 1 mg/kg chlorpromazine) rats ($n = 4$ for each group) were perfused transcardiacally with a solution of 2.5% glutaraldehyde, in 0.1 M PBS (pH 7.4) and post-fixed with 1% OsO4, then dehydrated in routine alcohol series and embedded in Epon-812 resin. Sections from retrosplenial granular, retrosplenial agranular and parietal association cortices were cut in coronal planes of the brain tissues with ultra microtome (LKB, Sweden). Semi-thin (1 μm) and thin (500 nm) sections, stained with toluidine blue and 1% lead citrate and uranyl acetate, respectively, were evaluated by light and JEOL-1011 (Japan) electron microscope equipped with MegaView digital imaging and capturing system, and AnalySIS software (Soft Imaging Systems, Germany).

Statistical analysis

Statistical analysis was done using a GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). All data are expressed as means ± SEM. For the biochemical results, groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Neurological examination scores were evaluated by non-parametric Mann Whitney test. Values of $p < 0.05$ were considered as significant.

Results

Mortality rate was 16/21 (76%) in the trauma group and 16/19 (84%) in the lipoic acid group. Deaths occured in the first minutes after the induction of trauma and then no deaths occurred in the next 48 h.

The neurological examination score was significantly higher in the trauma ($p < 0.001$) group when compared with the control. However, in the LA-treated trauma group, although the score was still higher than the control, it was significantly reduced ($p < 0.01$, Figure 1).

The EB content of the brain significantly increased ($p < 0.001$) in the trauma group, indicating the change in BBB integrity. On the other hand LA treatment significantly preserves EB extravasation ($p < 0.01$, Figure 2A). Similarly, increase in brain water content indicating oedema in the trauma group was partially reduced by LA treatment (Figure 2B).

Trauma caused significant increases in serum levels of proinflammatory cytokines ($p < 0.001$), while LA treatment in the trauma group decreased these elevations (Figure 3). Chemiluminescence levels in the brain samples detected by both luminol and lucigenin probes showed significant increases in the trauma group as compared to the CL levels of the control group ($p < 0.05$, Figure 4). On the other hand, LA treatment in the trauma group decreased the elevations in CL values of lucigenin significantly

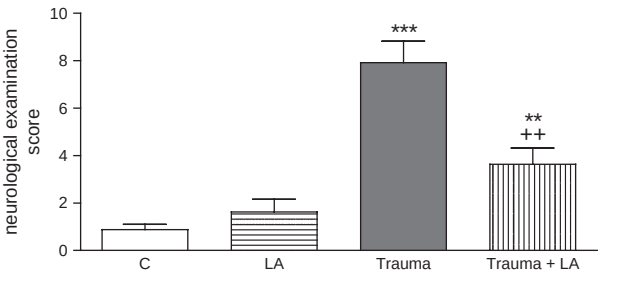

Figure 1. Bederson's modified neurological examination scores at 48 h following TBI. Twelve rats were examined in each group. Statistical analysis was carried out by non-parametric Mann-Whitney test. $^{**}p < 0.01$, $^{***}p < 0.001$ vs control group; $^{++}p < 0.01$ vs trauma group. Values are represented as mean ± SEM.

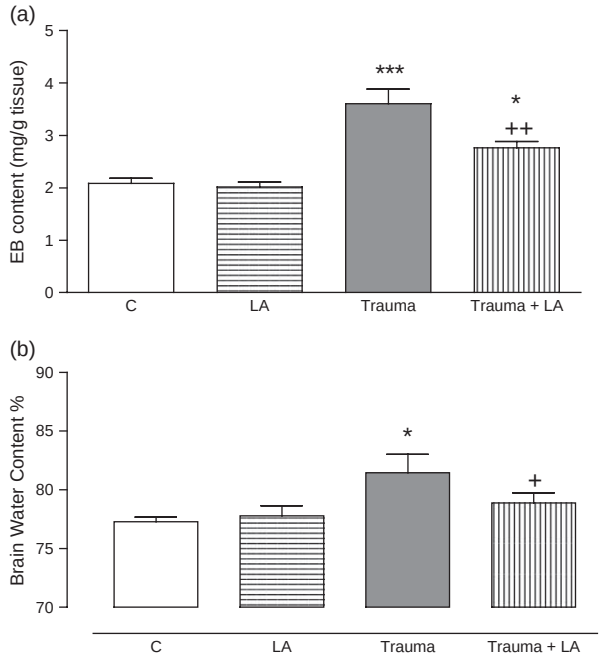

Figure 2. (A) Evans blue (EB) content (an index for blood–brain barrier) and (B) water content of brain tissues of rats in the control, trauma and α-lipoic acid (LA) treated-groups. Each group consists of six rats. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $^{*}p<0.05$, $^{***}p<0.001$ vs control group; $^{++}p<0.01$ vs trauma group. Values are represented as mean $\pm$ SEM.

($p<0.05$–0.01). Furthermore, luminol CL values tended to decrease, however it was not statistically significant. In parallel to the CL data, the brain tissue TBARS content in the control group (35.7 ± 1.4 nmol/g) was elevated by traumatic injury (51.3 ± 3.4 nmol/g, $p<0.001$); however, LA treatment significantly decreased the trauma-induced elevation in brain TBARS level (37.2 ± 0.9 nmol/g, $p<0.001$; Figure 5A). In accordance with that, trauma caused a significant decrease in brain GSH level (0.98 ± 0.1 μmol/g; $p<0.001$) when compared to control group (1.5 ± 0.1 μmol/g), while in the LA-treated trauma group, brain GSH content was found to be preserved (1.3 ± 0.1 μmol/g; $p<0.05$), being not significantly different from that of the control group (Figure 5B).

Myeloperoxidase activity, which is accepted as an indicator of neutrophil infiltration, was significantly higher in the brain tissue of the trauma group (7.8 ± 0.7 U/g, $p<0.05$) than that of the control group (5.8 ± 0.4 U/g; Figure 6A). On the other hand, LA treatment in the trauma group significantly decreased brain tissue MPO level (5.9 ± 0.3, $p<0.05$), which was found to be not different than that of the control group. Na^{+}-K^{+}-ATPase activities in the brain tissue samples were reduced in the trauma group ($p<0.01$), indicating impaired transport function and membrane damage in the tissue (Figure 6B). However,

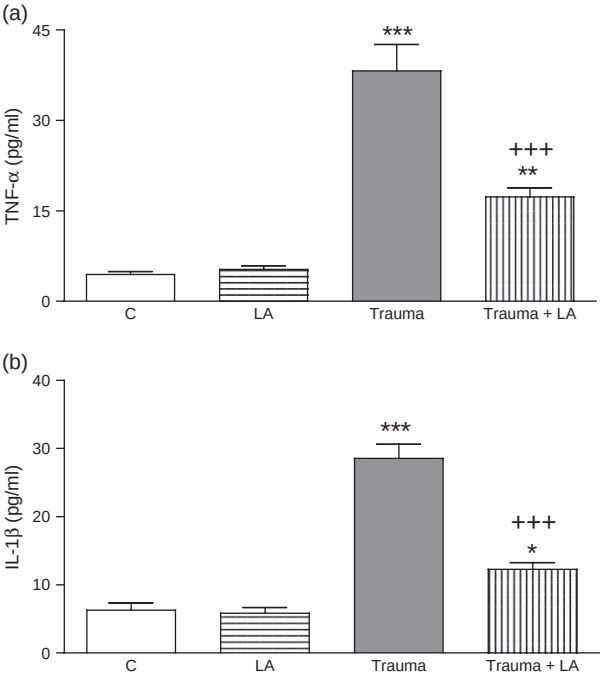

Figure 3. Plasma (A) TNF-α, and (B) IL-1β, levels of control, trauma and α-lipoic acid (LA) treated-groups. Each group consists of six rats. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $^{*}p<0.05$, $^{**}p<0.001$, $^{***}p<0.001$ vs control group; $^{+}p<0.05$, $^{++}p<0.01$ vs trauma group. Values are represented as mean $\pm$ SEM.

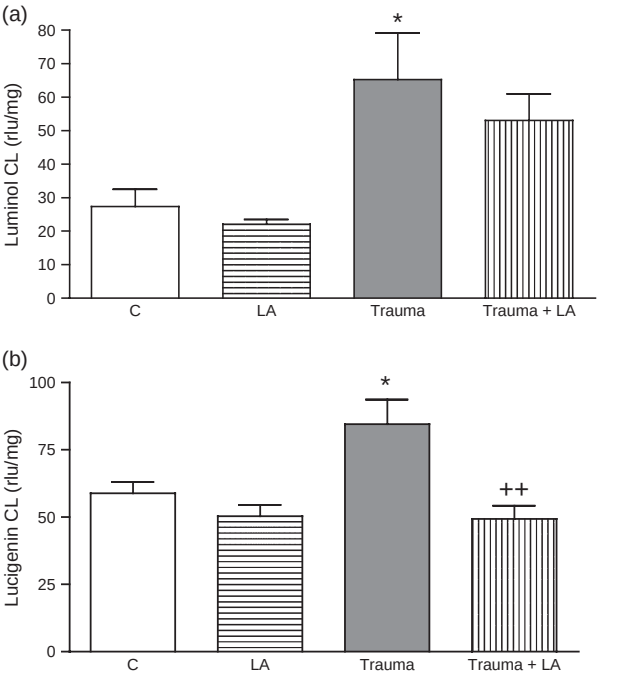

Figure 4. Luminol and lucigenin chemiluminescence values in the brain tissues of control, trauma and α-lipoic acid (LA) treated-groups. Each group consists of six rats. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $^{*}p<0.05$ vs control group; $^{++}p<0.01$ vs trauma group. Values are expressed relative to wet weight of brain tissue and are represented as mean $\pm$ SEM.

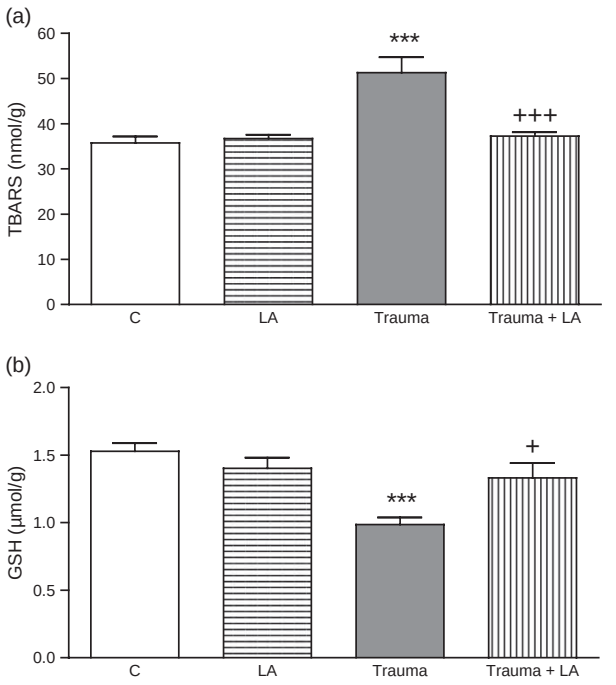

Figure 5. (A) Thiobarbituric acid reactive substance (TBARS) and (B) glutathione (GSH) levels in the brain tissues of control, trauma and α-lipoic acid (LA) treated-groups. Each group consists of six rats. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $^{***}p<0.001$ vs control group; $^{+}p<0.05$; $^{+++}p<0.001$ vs trauma group. Values are expressed relative to wet weight of brain tissue and are represented as mean $\pm$ SEM.

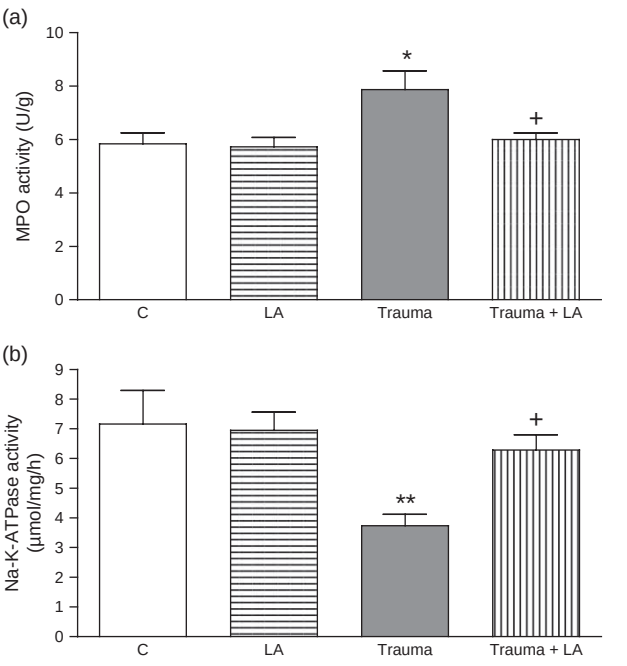

Figure 6. (A) Myeloperoxidase (MPO) and (B) Na^+-K^+ ATPase activities in the brain tissues of control, trauma and α-lipoic acid (LA) treated-groups. Each group consists of six rats. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $^{*}p<0.05$; $^{**}p<0.01$ vs control group; $^{+}p<0.05$ vs trauma group. Values are expressed relative to wet weight of brain tissue and are represented as mean $\pm$ SEM.

in the LA-treated traumatic rats, the measured Na^+-K^+-ATPase activities was not different than those of the control rats.

Morphological results

Electron microscopic evaluation revealed that fine structure of neurons and mishmash of profiles of dendritic, axonal and glial processes were intact in both control and LA-treated trauma groups (Figures 7A and D). Also astrocytic foot processes participating in the blood–brain barrier were seen intact both in control and LA-treated trauma groups, although mild swelling was observed in the LA group (Figure 7D). On the other hand, severe swelling as a finding of hydropic degeneration in neuronal cell bodies and neurophils (i.e. in axons, dendrites and glial processes) was observed in the trauma group (Figure 7B). Also, crumbling of neurotubules and loss in mitochondrial cristae were prominent in this group (Figure 7C).

Discussion

The exact molecular mechanisms that lead to TBI are certainly complicated and not completely understood. Consequently, extensive experimental research efforts are currently focusing on this area in order to elucidate the molecular pathophysiology of injury which presumably will aid in designing advanced therapeutic strategies for better protection of the tissues. Antioxidants are the preferential protective agents among these strategies. Thus, in the present study we have investigated the beneficial effects of alpha-lipoic acid, a powerful antioxidant against trauma-induced brain damage.

There is evidence that progressive pathophysiological changes occur after trauma, resulting in a reduction in brain blood flow and a decrease in tissue oxygen levels leading to ischaemia, subsequent second injury and brain oedema. Several clinical and experimental studies have demonstrated that secondary brain injury can be magnified by a number of processes including free radical generation, inflammation, production of adhesion molecules, cytokine and chemokine generation, etc., which are frequently upregulated in response to TBI [36,37]. Increased levels of these molecules within the injured brain, including IL-1β, TNF-α, IL-6 and intercellular adhesion molecule-1, are believed to contribute to the cerebral damage, cell death and BBB dysfunction [38]. Interleukin-1 has been characterized in animal models of TBI as a promoter of neuroinflammation and demonstrated that it activates other proinflammatory factors such as TNF-α [39] and acts synergistically with them. The leaking of proinflammatory mediators to the circulation contributes to the

development of hyperinflammation referred to as systemic inflammatory response syndrome [40]. In agreement with this hypothesis, in our study following trauma, plasma proinflammatory cytokines, TNF-α and IL-1β, were significantly increased. On the other hand it has been demonstrated that LA has a neuroprotective effect in the various neurodegenerative disorders such as cerebral ischaemia-reperfusion, excitotoxic amino acid brain injury, mitochondrial dysfunction, diabetes and diabetic neuropathy, inborn errors of metabolism and other causes of acute or chronic damage to brain or neural tissue [15]. In the current study we have also found that LA administration following TBI could reduce the generation of proinflammatory cytokines which play an important role in the oxidative brain injury.

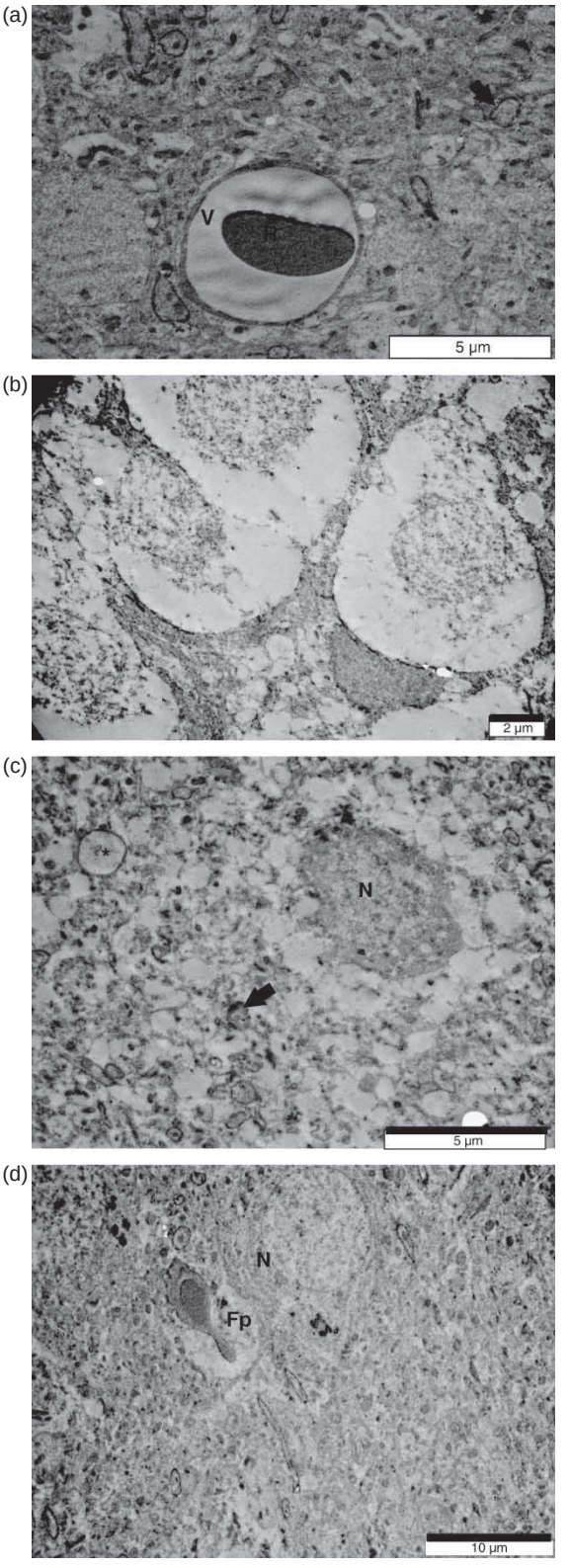

Figure 7 (*Continued*)

Traumatic injury to the brain triggers the accumulation of harmful mediators, including highly toxic ROS, and there is a close relationship between the degree of oxidative stress and the pathogenesis of TBI [41,42]. A significant number of studies now show that LA and its reduced form, DHLA, directly scavenge reactive oxygen species ROS and RNS species and protect cells against a host of insults where oxidative stress is part of the underlying aetiology [43,44]. Accordingly, we evaluated the generation of ROS in the brain tissues by enhanced chemiluminescence (CL) assay using luminol and lucigenin probes. The two CL probes, luminol and lucigenin, differ in selectivity. Luminol detects H_2O_2, OH^-, hypochlorite, peroxynitrite and lipid peroxyl radicals, whereas lucigenin is particularly sensitive to superoxide radical [27,28]. In the present study, increases in luminol- and lucigenin-CL levels support the notion that brain injury induced by trauma involves toxic oxygen metabolites and LA treatment decreases these elevations by its antioxidant action. In accordance with the increases in toxic oxygen metabolites, the brain TBARS level was also significantly increased, indicating the presence of enhanced lipid peroxidation following traumatic injury.

Cellular defenses, such as the GSH system and antioxidant enzymes, protect tissue from the damaging effect of free radicals. GSH is a ubiquitous tripeptide and a major intracellular antioxidant molecule and constitutes an important mechanism against oxidative stress [45].

Several studies have demonstrated that, due to TBI, lipid peroxidation is increased concomitantly as glutathione decreases; indicating oxidative damage [7,46]. Similarly in our study GSH levels were significantly decreased in parallel to the increase in

Figure 7. (A) Blood–brain barrier, neurophils and myelin sheath around axonal processes were seen intact in control group (R = red blood cell, V = blood vessel; arrow = myelin sheath). (B) Hydropic degeneration characterized with severe swelling in perikaryon and dispersion of chromatin material were obvious in trauma group. (C) Neurotubular breakdown in axonal and dendritic processes (*) and loss in mitochondrial cristae (arrows) were prominent in trauma group. (D) Intact neurotubules in neurophils and myelin sheath around axons and mild swelling in astrocytic foot processes (Fp) but in neurons (N) were observed in the LA-treated group.

lipid peroxidation. As LA reduced the oxidative injury on cellular structures, the level of the intracellular antioxidant glutathione, which is otherwise oxidized when inactivating free radicals, was not changed. Thus, it appears that the anti-oxidative effect of LA on lipid peroxidation does not involve the expenditure of tissue GSH stores, but the antioxidant pool is further supported by the action of LA.

Oxidative stress-induced acute inflammatory response plays an important role in several diseases. It has been demonstrated that TBI damages the blood–brain barrier, then the blood cells such as neutrophils and macrophages accumulate in the brain and further sustain the cerebral inflammatory cascade [47]. BBB disruption and brain oedema are common complications after TBI which can lead to deteriorating neurological outcomes. On the other hand inflammation has been reported to play a role in the development of vasogenic brain oedema which occurs as a result of BBB disruption [48,49]. In this study MPO activity, a marker for the infiltration of neutrophils, was assayed for quantitative indication of the presence of inflammation in the brain tissue and showed that, due to oxidative stress following TBI, MPO is increased significantly.

Since myeloperoxidase (MPO) plays a fundamental role in oxidant production by neutrophils it is used for quantitative indication of the presence of inflammation. Thus, the increased MPO activity observed in the current study clearly demonstrates that trauma-induced brain damage is a neutrophil-dependent inflammatory condition. The neutrophils have a role in oxidant injury via the mechanisms that include the activation of NADPH oxidase or MPO enzyme systems. Activated neutrophils release MPO, causing production of large amounts of HOCl, which oxidizes and damages macromolecules, including proteins, lipids, carbohydrates and nucleic acids [8]. Our results are in agreement with similar studies that have found that increased MPO activity plays a role in oxidative stress-induced brain injury [48,50] and the antioxidant drugs prevent the damage by inhibiting neutrophil infiltration [51,52]. Similarly, in the current study, LA through its anti-inflammatory and antioxidant properties, suppressing the MPO enzyme activity, improves neurological outcome and reduces cerebral oedema, BBB permeability, which play important roles and are the major parts in the secondary brain injury following TBI.

It is well known that at least part of the secondary damage to the brain is due to a 'metabolic crisis', whereby the brain's energy metabolism is compromised. Part of this might be due to free radical mediated damage to Na^+, K^+-ATPase, as seen in the present study. Na^+, K^+-ATPase is a crucial enzyme responsible for the generation of the membrane potential, through the active transport of sodium and potassium ions in the CNS, necessary to maintain neuronal excitability and cellular volume control. It is present at high concentrations in the brain cellular membrane, consuming $\sim$40–50% of the ATP generated in this tissue, and is highly responsive to changes in membrane fluidity [53,54]. Inhibition of this activity is found in various neuropathological conditions, including cerebral ischaemia [55], epilepsy [56], neurodegenerative disorders [57] and in secondary oedema due to spinal cord injury [58]. Increasing evidence has demonstrated that Na^+, K^+ ATPase is highly vulnerable to free radical attack [12,57]. In our study TBI induced a significant decrease in enzyme activity, indicating membrane damage and deterioration of membrane fluidity in parallel with an increase in lipid peroxidation. On the other hand, LA by its antioxidant effects reducing lipid peroxidation increased Na^+, K^+-ATPase activity.

Although various reports suggest that LA penetrates the BBB readily [14–16], a recent study has demonstrated that orally administered LA does not cross BBB or an efflux mechanism that pumps LA out may be responsible for the absence of LA in the brain [59]. However, orally given LA has been shown to exert neuroprotection [15]. Consistently our study also showed that oral LA administration protects brain tissue against oxidative stress, reduces oedema formation and reduces blood–brain barrier permeability and thereby preserves neuronal damage [60]. Chng et al. [59] have demonstrated that LA was unable to penetrate in to the brain, suggesting that the antioxidant effect of LA in brain may not be due to its direct effect in the central nervous system but due indirectly through its metabolite dihydrolipoic acid (DHLA). They also suggested that LA can act as an indirect antioxidant by stimulating certain signal transduction pathways to initiate cellular anti-stress response mechanisms [61]. There is evidence indicating that aconitate hydratase (AH), the enzyme that catalyses conversion of citrate into isocitrate, is also vulnarable to free radical attacks. Oxidation of this enzyme by free radicals results in the decrease of the enzyme activity and accumulation of citrate. Makeeva et al. [62] have shown that LA treatment restored the AH enzyme activity and decreased citrate accumulation after experimental myocardial ischemia. On the other hand, lipoamide, a metabolite of LA, is a co-factor for mitochondrial alpha-keto acid dehydrogenase complexes and so giving oral lipoic acid might be expected to boost lipoamide and so help sustain oxidative metabolism. Exogenously supplied lipoic acid is rapidly taken up by cells and reduced to dihydrolipoic acid. The reducing power for this comes from NADH and NADPH, so dosing with lipoic acid has the potential to affect the NADH/NAD^+ ratio and the NADPH/$NADP^+$ ratio [43]. All these results indicate that lipoic acid, besides its own antioxidant potential, also supports the functions of other antioxidant components in the body.

As a conclusion, since LA is known to have various metabolic antioxidant properties it is possible to speculate that it may also be effective against TBI. Although neuroprotective mechanisms of LA have been demonstrated, none of the previous studies focused on cerebral inflammatory response and oxidative damage that might facilitate the development of secondary brain damage following primary trauma. Thus, the present findings have demonstrated for the first time that the neuroprotective effects of LA in TBI-induced oxidative damage may be due to an augmentation of the endogenous antioxidants and the inhibition of lipid peroxidation by maintaining a balance in oxidant–antioxidant status, inhibiting neutrophil infiltration and modulating proinflammatory cytokines, nominating LA as a highly promising agent in avoiding the development of TBI-induced brain damage.

Acknowledgements

The authors are grateful to Mikrogen Pharmaceuticals for supplying alpha lipoic acid.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] McKee JA, Brewer RP, Macy GE, Borel CO, Reynolds JD, Warner DS. Magnesium neuroprotection is limited in humans with acute brain injury. Neurocrit Care 2005;2:342–351.

[2] Roberts I, Schierhout G, Alderson P. Absence of evidence for the effectiveness of five interventions routinely used in the intensive care management of severe head injury: a systematic review. J Neurol Neurosurg Psychiatry 1998;65:729–733.

[3] Biegon A, Fry PA, Paden CM, Alexandrovich A, Tsenter J, Shohami E. Dynamic changes in N-methyl-D-aspartate receptors after closed head injury in mice: implications for treatment of neurological and cognitive deficits. Proc Natl Acad Sci USA 2004;101:5117–5122.

[4] Sullivan PG, Keller JN, Mattson MP, Scheff SW. Traumatic brain injury alters synaptic homeostasis: implications for impaired mitochondrial and transport function. J Neurotrauma 1998;15:789–798.

[5] Shao C, Roberts KN, Markesbery WR, Scheff SW, Lovell MA. Oxidative stress in head trauma in aging. Free Radic Biol Med 2006;41:77–85.

[6] Ansari MA, Ahmad AS, Ahmad M, Salim S, Yousuf S, Ishrat T, Islam F. Selenium protects cerebral ischaemia in rat brain mitochondria. Biol Trace Elem Res 2004;101:73–86.

[7] Ozdemir D, Uysal N, Gonenc S, Acikgoz O, Sonmez A, Topcu A, Ozdemir N, Duman M, Semin I, Ozkan H. Effect of melatonin on brain oxidative damage induced by traumatic brain injury in immature rats. Physiol Res 2005;54:631–637.

[8] Reiter RJ, Tan DX, Manchester LC, Qi W. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species. A review of the evidence. Cell Biochem Biophys 2001;34:237–256.

[9] Ozturk E, Demirbilek S, Kadir-But A, Saricicek V, Gulec M, Akyol O, Ozcan Ersoy M. Antioxidant properties of propofol and erythropoietin after closed head injury in rats. Prog Neuropsychopharmacol Biol Psychiatry 2005;29:922–927.

[10] Lima FD, Souza MA, Furian AF, Rambo LM, Ribeiro LR, Martignoni FV, Hoffmann MS, Fighera MR, Royes LF, Oliveira MS, de Mello CF. Na^+, K^+-ATPase activity impairment after experimental traumatic brain injury: relationship to spatial learning deficits and oxidative stress. Behav Brain Res 2008;193:306–310.

[11] Werner C, Engelhard K. Pathophysiology of traumatic brain injury. Br J Anaesth 2007;99:4–9.

[12] Kurella E, Kukley M, Tyulina O, Dobrota D, Matejovicova M, Mezesova V, Boldyrev A. Kinetic parameters of Na/K-ATPase modified by free radicals in vitro and in vivo. Ann NY Acad Sci 1997;834:661–665.

[13] Unterberg AW, Stover J, Kress B, Kiening KL. Edema and brain trauma. Neuroscience 2004;129:1021–1029.

[14] Packer L, Roy S, Sen CK. Alpha lipoic acid: a metabolic antioxidant and potential redox modulator of transcription. Adv Pharmacol 1997;38:79–101.

[15] Packer L, Tritschler HJ, Wessel K. Neuroprotection by the metabolic antioxidant alpha-lipoic acid. Free Radic Biol Med 1997;22:359–378.

[16] Packer L, Witt EH, Tritschler HJ. α-Lipoic acid as a biological antioxidant. Free Radic Biol Med 1995;19:227–250.

[17] Moini H, Packer L, Saris NE. Antioxidant and prooxidant activities of a-lipoic acid and dihydrolipoic acid. Toxicol Appl Pharmacol 2002;182:84–90.

[18] Prahalathan C, Selvakumar E, Varalakshmi P, Kumarasamy P, Saravanan R. Salubrious effects of lipoic acid against adriamycin-induced clastogenesis and apoptosis in Wistar rat bone marrow cells. Toxicology 2006;222:225–232.

[19] Selvakumar E, Prahalathan C, Sudharsan PT, Varalakshmi P. Protective of lipoic acid on cyclophosphamide-induced testicular toxicity. Clin Chim Acta 2006;367:114–119.

[20] Dulundu E, Ozel Y, Topaloglu U, Sehirli O, Ercan F, Gedik N, Sener G. Alpha-lipoic acid protects against hepatic ischaemia-reperfusion injury in rats. Pharmacology 2007;79:163–170.

[21] Sehirli O, Sener E, Cetinel S, Yüksel M, Gedik N, Sener G. Alpha-lipoic acid protects against renal ischaemia-reperfusion injury in rats. Clin Exp Pharmacol Physiol 2008;35:249–255.

[22] Marmarou A, Foda MA, van den Brink W, Campbell J, Kita H, Demetriadou K. A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics. J Neurosurg 1994;80:291–300.

[23] Ucar T, Tanriover G, Gurer I, Onal MZ, Kazan S. Modified experimental mild traumatic brain injury model. J Trauma 2006;60:558–565.

[24] Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurological examination. Stroke 1986;17:472–476.

[25] Toklu HZ, Keyer Uysal M, Kabasakal L, Sirvanci S, Ercan F, Kaya M. The effects of riluzole on neurological, brain biochemical, and histological changes in early and late term of sepsis in rats. J Surg Res 2009;152:238–248.

[26] Durmaz R, Ertilav K, Akyuz F, Kanbak G, Bildirici K, Tel E. Lazaroid U-74389G attenuates oedema in rat brain subjected to post-ischemic reperfusion injury. J Neurol Sci 2003;215:87–93.

[27] Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. J Clin Invest 1993;91:2546–2551.

[28] Davies GR, Simmonds NJ, Stevens TR, Sheaff MT, Banatvala N, Laurenson IF, Blake DR, Rampton DS. Mucosal reactive oxygen metabolite production in duodenal ulcer disease. Gut 1992;33:1467–1472.

[29] Beuge JA, Aust SD. Microsomal lipid peroxidation. Meth Enzymol 1978;53:302–311.
[30] Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. J Lab Clin Med 1963;61:882–888.
[31] Hillegass LM, Griswold DE, Brickson B, Albrightson-Winslow C. Assessment of myeloperoxidase activity in whole rat kidney. J Pharmacol Meth 1990;24:285–295.
[32] Kim YK, Lee SH, Goldinger JM, Hong SK. Effect of ethanol on organic ion transport in rabbit kidney. Toxicol Appl Pharmacol 1986;86:411–420.
[33] Reading HW, Isbir T. The role of cation activated ATPase in transmitter release from the rat iris. Q J Exp Physiol 1980;65:105–116.
[34] Fiske CH, SubbaRow Y. The colorimetric determination of phosphorus. J Biol Chem 1925;66:375–400.
[35] Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurements with the folin phenol reagent. J Biol Chem 1951;193:265–275.
[36] Ansari MA, Roberts KN, Scheff SW. Oxidative stress and modification of synaptic proteins in hippocampus after traumatic brain injury. Free Radic Biol Med 2008;45: 443–452.
[37] Knoblach SM, Fan L, Faden AI. Early neuronal expression of tumour necrosis factor-a after experimental brain injury contributes to neurological impairment. J Neuroimmunol 1999;95:115–125.
[38] Chen G, Shi J, Hu Z, Hang C. Inhibitory effect on cerebral inflammatory response following traumatic brain injury in rats: a potential neuroprotective mechanism of N-acetylcysteine. Mediators Inflamm 2008;716458.
[39] Fan L, Young PR, Barone FC, Feuerstein GZ, Smith DH, McIntosh TK. Experimental brain injury induces differential expression of tumour necrosis factor-alpha mRNA in the CNS. Brain Res Mol Brain Res 1996;36:287–291.
[40] Smrcka M, Mrlian A, Karlsson-Valik J, Klabusay M. The effect of head injury upon the immune system. Bratisl Lek Listy 2007;108:144–148.
[41] Shohami E, Gati I, Beit-Yannai E, Trembovler V, Kohen R. Closed head injury in the rat induces whole body oxidative stress: overall reducing antioxidant profile. J Neurotrauma 1999;16:365–376.
[42] Slemmer JE, Shacka JJ, Sweeney MI, Weber JT. Antioxidants and free radical scavengers for the treatment of stroke, traumatic brain injury and aging. Curr Med Chem 2008;15:404–414.
[43] Biewenga GP, Haenen GR, Bast A. The pharmacology of the antioxidant lipoic acid. Gen Pharmacol 1997;29:315–331.
[44] Petersen Shay K, Moreau RF, Smith EJ, Hagen TM. Is alpha-lipoic acid a scavenger of reactive oxygen species in vivo? Evidence for its initiation of stress signaling pathways that promote endogenous antioxidant capacity. IUBMB Life 2008;60:362–367.
[45] Ross D. Glutathione, free radicals and chemotherapeutic agents. Pharmacol Ther 1998;37:231–249.
[46] Kerman M, Cirak B, Ozguner MF, Dagtekin A, Sutcu R, Altuntas I, Delibas N. Does melatonin protect or treat brain damage from traumatic oxidative stress? Exp Brain Res 2005;163:406–410.
[47] Morganti-Kossmann MC, Satgunaseelan L, Bye N, Kossmann T. Modulation of immune response by head injury. Injury 2007;38:1392–1400.
[48] Darakchiev BJ, Itkis M, Agajanova T, Itkis A, Hariri RJ. Changes of MPO activity and brain water accumulation in traumatic brain injury experiments. Acta Neurochir Suppl 1997;70:98–101.
[49] Feng DF, Zhu ZA, Lu YC. Effects of magnesium sulfate on traumatic brain oedema in rats. Chin J Traumatol 2004;7:148–152.
[50] Scholz M, Cinatl J, Schädel-Höpfner M, Windolf J. Neutrophils and the blood–brain barrier dysfunction after trauma. Med Res Rev 2007;27:401–416.
[51] Sener G, Toklu H, Kapucu C, Ercan F, Erkanli G, Kaçmaz A, Tilki M, Yeğen BC. Melatonin protects against oxidative organ injury in a rat model of sepsis. Surg Today 2005;35: 52–59.
[52] Toklu HZ, Tunali Akbay T, Velioglu-Ogunc A, Ercan F, Gedik N, Keyer-Uysal M, Sener G. Silymarin, the antioxidant component of Silybum marianum, prevents sepsis-induced acute lung and brain injury. J Surg Res 2008;145:214–222.
[53] Erecinska M, Cherian S, Silver IA. Energy metabolism in mammalian brain during development. Prog Neurobiol 2004;73:397–445.
[54] Erecińska M, Silver IA. Ions and energy in mammalian brain. Prog Neurobiol 1994;43:37–71.
[55] Wyse AT, Streck EL, Barros SV, Brusque AM, Zugno AI, Wajner M. Methylmalonate administration decreases Na^+, K^+-ATPase activity in cerebral cortex of rats. Neuroreport 2000;11:2331–2334.
[56] Grisar T. Glial and neuronal Na+-K+ pump in epilepsy. Ann Neurol 1984;16(Suppl):S128–S134.
[57] Yu SP. Na^+, K^+-ATPase: the new face of an old player in pathogenesis and apoptotic/hybrid cell death. Biochem Pharmacol 2003;66:1601–1609.
[58] Yang YB, Piao YJ. Effects of resveratrol on secondary damages after acute spinal cord injury in rats. Acta Pharmacol Sin 2003;24:703–710.
[59] Chng HT, New LS, Neo AH, Goh CW, Browne ER, Chan EC. Distribution study of orally administered lipoic acid in rat brain tissues. Brain Res 2009;1251:80–86.
[60] Arivazhagan P, Shila S, Kumaran S, Panneerselvam C. Effect of DL-alpha-lipoic acid on the status of lipid peroxidation and antioxidant enzymes in various brain regions of aged rats. Exp Gerontol 2002;37:803–811.
[61] Hagen TM, Ingersoll RT, Lykkesfeldt J, Liu J, Wehr CM, Vinarsky V, Bartholomew JC, Ames AB. R)-alpha-lipoic acid-supplemented old rats have improved mitochondrial function, decreased oxidative damage, and increased metabolic rate. FASEB J 1999;3:411–418.
[62] Makeeva AV, Popova TN, Matasova LV, Yama IN. Effects of lipoic acid on citrate content, aconitate hydratase activity, and oxidative status during myocardial ischaemia in rats. Biochemistry (Mosc) 2008;73:76–79.

This paper was first published online on iFirst on 23 May 2009.