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*Full Length Research Paper*

# Carnosine and cyclosporine A alleviate brain damage after traumatic brain injury in rats

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**The present study investigates the effects of carnosine and/or cyclosporine A (CyA) treatment against traumatic brain injury (TBI) in immature rats. Traumatized rats received carnosine [(200 mg/kg/day, in pre-and post-treatment (i.p.)] for 7 consecutive days following TBI. CyA (20 mg/kg, i.p.) was administrated 15 min and 24 h after TBI. The results revealed that TBI caused sever brain injury indicated by increased nucleotide hydrolysis which was ensured by pronounced increase in ectonucleotidases, NTPDases (ATP and ADP hydrolysis) and 5'-nucleotidase (AMP hydrolysis) in traumatized rats compared with normal animals. TBI also causes elevation of glycolytic enzymatic activities as lactate dehydrogenase (LDH) and phosphoglucoisomerase (PGI) in rats' brains. In addition TBI pronouncedly reduced the activities of antioxidant enzymes glutathione reductase (GR) and catalase (CAT) in brain tissue as compared to normal animals. Injection of carnosine and/or CyA significantly modulates the altered enzymatic activities. In conclusion, the present data may suggest the beneficial effect of carnosine and/or CyA in protection of brain tissues from disorders induced by traumatic injury.**

**Key words:** Carnosine, cyclosporine A, glutathione reductase, traumatic brain injury (TBI).

## INTRODUCTION

Traumatic brain injury (TBI) is associated with costly health problems and high mortality and morbidity in previously healthy populations. Despite advances in research and improved neurological intensive care in recent years, the clinical outcome of severely head-injured patients is still poor. Posttraumatic brain damage is determined by a combination of primary and secondary insults. Primary damage results from mechanical forces applied to the skull and brain at the time of impact, leading to focal or diffuse brain injury patterns. In contrast to the primary insult, secondary brain injuries evolve over time. These injuries are characterized by a complex cascade of molecular and biochemical events that lead to neuroinflammation, brain edema, and delayed neuronal death (Beauchamp et al., 2008).

The studied mechanisms of neuronal damage as secondary insults after TBI are oxidative/nitrosative stress and inflammation. The brain is highly sensitive to stress-induced neurodegeneration because of its high content of polyunsaturated fatty acids (PUFAs), which are particularly vulnerable to free radical attacks and lipid peroxidation (LPO). Within minutes following a traumatic event, a dramatic increase in free radicals saturates endogenous scavenging mechanisms leading to the breakdown of membrane lipids, essential proteins, and DNA ultimately leading to cell death (Hall et al., 2010).

A close relationship exists between the degree of

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oxidative stress and different disorders (Doughari et al., 2012), including the pathogenesis of TBI (Shao et al., 2006). Oxidative stress-related cascades resulting from TBI have been implicated in altered antioxidant defense systems (Ansari et al., 2008), cytoskeletal damage, mitochondrial dysfunction (Singh et al., 2007) increases in extracellular potassium and the indiscriminate release of glutamate (Katayama and Becker, 1990),and altered signal transduction (Sullivan et al., 1998; Bayir et al., 2007). Induction of anaerobic glycolysis and increase in brain glucose utilization are compensatory mechanisms for energy production after TBI (Kawamata et al., 1992; Chen et al., 2000). In addition, some studies have reported that during brain traumatic injury, increase in the concentrations of extracellular ATP and adenosine occurred (Robertson et al., 2001; Pearson et al., 2003). Alterations in activities of enzymes involved in nucleotide hydrolysis, namely ectonucleotidases such as NTPDases and 5' nucleotidase also been reported in brain injury (Nedeljkovic et al., 2006). ATP is recognized as a neurotransmitter in the peripheral, as well as in the central nervous system (Burnstock and Williams, 2000). In physiological situations, extracellular ATP exists at low concentrations; however, in pathological conditions, large quantities of extracellular ATP may cause cell death (Inoue, 2002).

No specific pharmacological therapy is currently available for the preventions of the development of secondary brain injuries, and most therapeutic strategies have failed in translation from “bench to bedside. However, A number of new potential therapeutic targets, including natural products, have been identified that may enable prevention of the onset or reduction of the extent of many oxidative stress injuries including secondary brain injury (Gao et al., 2011; Feng et al.,2012) .

The neuropeptide carnosine (β-Alanyl-l-histidine), first identified nearly a century ago, occurs in innervated tissues including the animal and human brain at concentrations up to 20 mM, and can easily enter the central nervous system from the periphery (Crush, 1970; Gariballa and Sinclair, 2000). There are many theories about its biological functions, such as anti-inflammatory, free radical scavenger (Hipkiss, 2005), immunomodulory and wound healing activities (Nagai and Suda, 1986). Carnosine protects brain neurons (Boldyrev et al., 2004; Shen et al., 2007a) and non-neuronal cells against oxidative injury (Kang et al., 2002).

Cyclosporine A is a highly potent immunosuppressive drug, largely used in solid organ transplantation for the prevention of acute rejection, or for the treatment of various auto-immune diseases (Halloran, 2004). Alessandri et al. (2002) reported that in animals administered CyA following acute, severe TBI reduces the amount of damaged tissue when given following the event. The exact mechanism of action responsible for neuroprotection remains unclear. CyA inhibits mitochondrial dysfunction in the central nervous system (CNS) and prevents calcium efflux. By interfering with calcium release from mitochondria, the secondary cascade of events leading to persistent damage within the CNS is presumed to be interrupted (Sullivan et al., 2000).

The present investigation was designed to elucidate the possible pharmacological action of carnosine and cyclosporine A administration against brain traumatic injury in immature rats.

## MATERIALS AND METHODS

### Chemicals

All chemicals used were of high analytical grade, product of Sigma and Merck companies.

### Animals

In this study, male Wistar albino rats weighing 40 to 60 g and forty days old were used. The rats were obtained from Experimental Animal Care Center, College of Pharmacy, King Saud University. Animals have been kept in special cages, and maintained on a constant 12 h light/12 h dark cycle with air conditioning and temperature ranging 20 to 22°C and humidity (60%). Rats were fed with standard rat pellet chow with free access to tap water *ad libitum* for one week before the experiment. Animal utilization protocols were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the King Saud University, College of Pharmacy.

### Traumatic brain injury (TBI) model

In the the present study, a modification of a well-described closed head trauma model in rats was used. To avoid neuroprotective effects of anesthetic agents (such as halothane or barbiturates), the rats were anesthetized with ether. Rats were laid on a warmed blanket, and heat monitoring was performed with a rectal probe to keep the body temperature at 37.5°C. The contusing device consisted of a hollow plastic tube 60 cm long, 5 mm wide, and perforated at 1-cm intervals to prevent air compression. The device was kept vertical to the surface of the intact skull and guided a falling weight onto the parietal convexity (3 mm anterior and 2 mm lateral to the lambda) (Öztürk et al., 2008). TBI was induced via dropping an object weighing 40 g through the previously mentioned contusing device to produce brain contusion. Following traumatic head injury (TBI), all rats received 100% oxygen until arousal to decrease mortality in experimental groups.

### Experimental groups and treatment

Rats were randomly allocated into five groups (each having seven rats):

G1: Control normal group.
G2: Trauma group: underwent TBI.
G3: Trauma/carnosine treated group: rats underwent TBI and treated interpertonially with carnosine; G4: Trauma/CyA treated group: rats underwent TBI and treated interpertonially with CyA.
G5: Trauma/carnosine+CyA treated group: rats underwent TBI and treated interpertonially with combination of carnosine and CyA.

**Table 1.** Effect of carnosine and/or cyclosporine A treatment on the level of ATP- hydrolyzing enzymes in brain tissues of traumatized rats

| Parameter | 5'-nucleotidase | NTPDase (ADP) | NTPDase (ATP) |
|---|---|---|---|
| Control | 0.36 ± 0.031 | 0.44 ± 0.026 | 0.71 ± 0.066 |
| TBI | 0.72 ± 0.034[a] | 0.85 ± 0.03[a] | 1.66 ± 0.13[a] |
| TBI/Car | 0.40 ± 0.015[bc] | 0.55 ± 0.03[bc] | 0.85 ± 0.03[bc] |
| TBI/CyA | 0.53 ± 0.02[ac] | 0.66 ± 0.025[ac] | 1.07 ± 0.025[ac] |
| TBI/Car+CyA | 0.36 ± 0.022[nc] | 0.46 ± 0.036[nc] | 0.68 ± 0.05[nc] |

Data are expressed as mean± SD of 6 rats in each group. 5'-nucleotidase, NTPDase(ADP) and NTPDase(ATP) enzyme activities are expressed as µmol Pi released/min/mg protein. [a]*P*< 0.0001, [b]*P*< 0.05 compared with normal control group. [c]*P* < 0.0001 when compared with TBI untreated group. n=non-significant when compared with normal group, using ANOVA followed by Bonferroni as post ANOVA test.

Control and traumatic groups received only physiological saline as a vehicle control. Carnosine was administered (200 mg/kg/day, i.p.) for 7 consecutive days following TBI (Boldyrev et al., 1999). Cy A (20mg/kg, i.p.) was administrated 15 min and 24 h after TBI (Colley et al., 2010).

After 7 days post TBI, all animals were fasted overnight and sacrificed under ether anesthesia. The brains from different animal groups were immediately removed weighed and washed using chilled saline solution.

Brains were minced and homogenized in ten volumes of ice-cold Tris–HCl buffer (50 mM, pH 7.4) using a homogenizer (Ultra Turrax IKAT18 Basic homogenizer, Germany). The homogenates were centrifuged for 15 min at 5000 rpm. The supernatant were used for biochemical enzyme analysis.

### Biochemical analysis

NTPDase–like activity (E.C.3.6.1.5 apyrase, ATP diphospho-hydrolase, ecto/CD39) were assessed by measuring the levels of ATP, ADP hydrolysis (Battastini et al., 1991) and 5`- Nucleotidase (E.C.3.1.3.5,CD37) by measuring the level AMP hydrolysis (Heymann et al., 1984). The activities of these enzymes were calculated through measuring the inorganic phosphate (Pi) (Chan et al., 1986). Enzyme activities are expressed as µmol Pi released/min/mg protein. LDH activity was evaluated in a reaction mixture containing tris buffer (50 Mm, pH, 7.5), sodium pyruvate (0.6 mM) and NADH (0.18 mM).

The rate of Nicotinamide adenine dinucleotide (NADH) consumption is determined at 340 nm and is directly proportional to the LDH activity (Bergmeyer, 1975). Phosphoglucoisomerase (PGI) activity was measured in a reaction medium containing tris-HCl buffer (0.2M, pH 7.4), fructose-6-phosphate (5 mM), $MgCl_2$ (10 mM), NADP (0.2 mM). The increase in extinction at 340 due to nicotinamide adenine dinucleotide phosphate (NADPH) production was recorded (Wu and Racker, 1959). Catalase (CAT) was determined by monitoring the decomposition of hydrogen peroxide as described by Aebi (1984). Glutathione reductase (GR) activity was measured by the modified method of Erden and Bor (1984). The reaction mixture contained the following in the final concentration: 4.1 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 5.7 mM ethylenediaminetetraacetate (EDTA), 60 mM KCl, 2.6 IU GSSG and 0.2 mM of NADPH in final reaction volume of 1 ml. The reaction was started by the addition of tissue extract containing approximately 100 µg of protein. The decrease in absorbance was monitored at 340 nm.

### Statistical analysis

Data were analyzed by comparing values for different treatment groups with the values for individual controls. Results are expressed as mean ± S.D. The significant differences among values were analyzed using analysis of variance (one-way ANOVA) coupled with Bonferroni as post ANOVA test.

## RESULTS

Table 1 shows the levels of ATP hydrolyzing enzymes in normal and traumatized animals. The data revealed that, TBI induced stimulation of ectonucleotidases, NTPDase (using ADP as substrate) and NTPDase (using ATP as substrate) as well as 5'- nucleotidase activities in traumatized rats compared to normal healthy animals. Administration of either carnosine or CyA alone or in combination significantly down modulated the levels of the above enzyme activities. Figures 1 and 2 illustrate that, TBI caused elevation in glycolytic enzyme activities (LDH and PGI), while administration of either Car or CyA alone or in combination significantly down-regulated the levels of these enzymes. Our results revealed that TBI pronouncedly reduced the activities of antioxidant enzymes, glutathione reductase (GR) and catalase (CAT), in rats' brain relative to their level in normal animals. Injection of either Car or CyA in traumatized rats effectively restored the reduced enzyme activities. Meanwhile, treatment with the two agents in combination was more effective, as it restored the altered studied enzyme activities (Figures 3 and 4).

## DISCUSSION

Results of the present study showed that TBI induced significant up-regulation of the enzyme chain for the complete hydrolysis of extracellular ATP. These results confirmed significant up-regulation of both NTPDase and ecto 5'-nucleotidase activities in brain tissues of traumatized

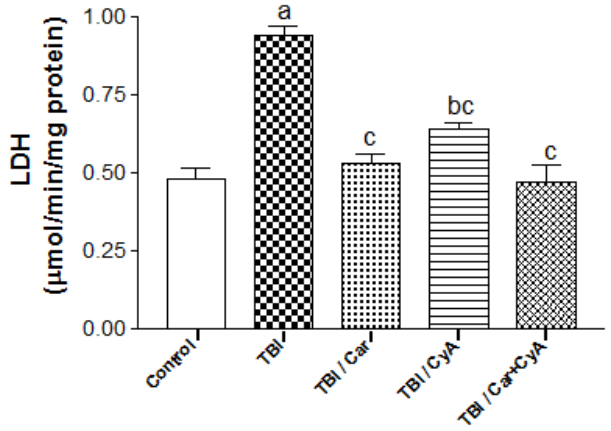


**Figure 1.** Effect of carnosine and/or cyclosporine A treatment on lactate dehydrogenase activity in brain tissues of traumatized rats. [a]*P*< 0.0001, [b]*P*< 0.05 compared with normal control group. [c]*P* < 0.0001 as compared with TBI untreated group, using ANOVA followed by Bonferroni as post ANOVA test.

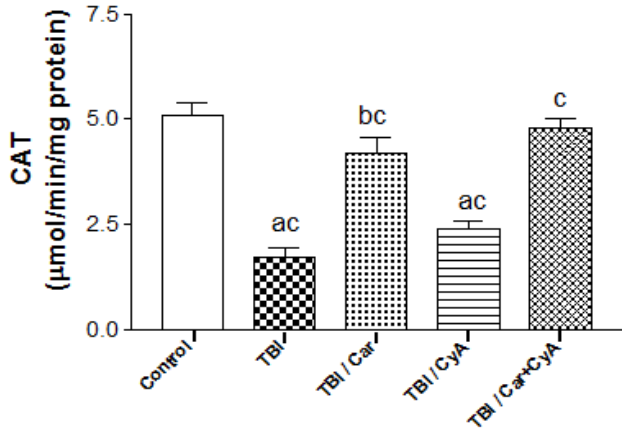


**Figure 3.** Effect of carnosine and/or cyclosporine A treatment on catalase activity in brain tissues of traumatized rats. [a]*P*< 0.0001, [b]*P*< 0.05 compared with normal control group. [c]*P* < 0.0001 when compared with TBI untreated group, using ANOVA followed by Bonferroni as post ANOVA test.

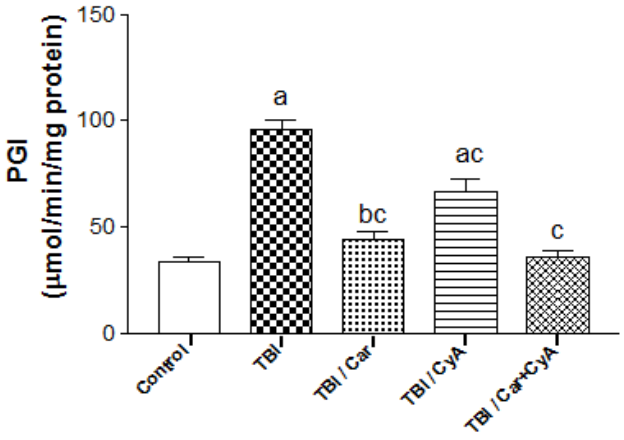


**Figure 2.** Effect of carnosine and/or cyclosporine A treatment on phosphoglucoisomerase activity in brain tissues of traumatized rats. [a]*P*< 0.0001, [b]*P*< 0.05 compared with normal control group. [c]*P* < 0.0001 when compared with TBI untreated group, using ANOVA followed by Bonferroni as post ANOVA test.

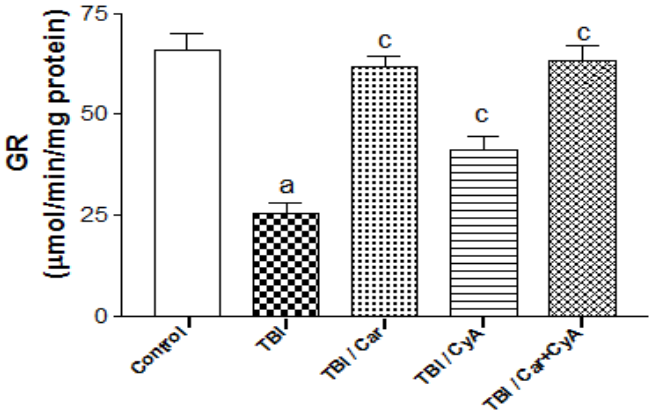


**Figure 4.** Effect of carnosine and/or cyclosporine A treatment on glutathione reductase activity in brain tissues of traumatized rats. [a]*P*< 0.0001 compared with normal control group. [c]*P* < 0.0001 compared with TBI untreated group, using ANOVA followed by Bonferroni as post ANOVA test.

animals. NTPDases hydrolyze the extracellular ATP and ADP in the presence of $Ca^{2+}$ and $Mg^{2+}$, while 5' nucleotidase catalyzes the hydrolysis of AMP, thus playing an important role in adenosine production. These results were confirmed by previous studies in which the up-regulation of ectonucleotidase activities induced by TBI is predominantly promoted by the release of ATP and other adenine nucleotides from damaged cells, and this induce astrocytic proliferation and migration of immune cells, and microglial cells to the injured area (Robertson et al., 2001; Cavaliere et al., 2003; Davalos et al., 2005; Nedeljkovic et al., 2006). By activation of astrocytes and microglia, ectonucleotidases are expressed abundantly from these cells (Wink et al., 2003).

These results have a lot of indication. First, the up-regulation of ectonucleotidases implies that the enzymes play an important role in the control of cellular responses induced by TBI. It is well known that accumulation of ATP during brain tissue injury is cytotoxic and extends a magnitude of cellular damage (Bonan et al., 2000). A major effect of ATP is receptor-mediated elevation of intracellular $Ca^{2+}$ both in neurons and glial cells through

activation of P2X and P2Y receptors, respectively (James and Butt, 2002). Hyperactivation of P2 receptors may thus be as cytotoxic as hyperactivation of glutamate receptors. It has been shown that synaptic glutamate receptor activation leads to neuronal swelling in substrate deficient human brain and in normoxic brain slices (Espanol et al., 1994), and contributes to the initial cell swelling that accompanied anoxic depolarization in neonatal cerebrocortical brain slices (Werth et al., 1998; Brahma et al., 2000). Extracellular adenosine production, on the other hand, may be involved in the protection of injured tissue (Stone, 2005). So, multiple glutamate receptor types seem to be involved in edema formation during ischemia (Brahma et al., 2000). Adenosine inhibits the release of glutamate via presynaptic A1 adenosine receptors (Brambilla et al., 2005), and thus reduces the cytotoxic effects of glutamate. Therefore, increased ectonucleotidase activity following TBI could have an important effect in terminating the function of extracellular ATP, including its cytotoxic effects. However, at the same time the potential increase of brain adenosine level and a lower availability of ATP as an excitatory neurotransmitter affect in particular, the hippocampus synaptosomal fraction, since this region of brain plays a key role in memory and learning (Bruno et al, 2005). Thus, stimulation of brain ectonucleotidases may be related to post-traumatic cognitive disturbances (Alessandri et al., 2002; Colley et al., 2010). Consequently, the enzymatic control of nucleotide levels is important in the process of brain homeostasis.

Administeration of either Car, CyA or their combination to rats with TBI provides remarkable down-regulation of NTPDase and ecto 5'-nucleotidase activities in the brain tissue, suggesting the abilities of both drugs in alleviating brain injury induced by such enzymes, and may have a beneficial effect in attenuating post-traumatic cognitive disturbances as a consequence event of induced ecto-nucleotidases. Carnosine has protective, antioxidant, and antiapoptotic properties. This endogenous dipeptide is importat in nervous cell defense against brain injury. Carnosine increased the resistance of neuronal membranes to the *in-vitro* induced oxidation and also suppressed the glutamate receptor hyperactivation (Stvolinskii et al., 2003; Shen et al., 2007b; Min et al., 2008).

On the Other hand, diverse outcome measures have demonstrated that CyA treatment attenuate post-traumatic cognitive and motor dysfunction (Riess et al., 2001; Alessandri et al., 2002), and reduce cellular and molecular abnormalities observed in the acute and delayed secondary post-injury phases (Mbye et al., 2009; Mazzeo et al., 2009a; b). Accumulating evidence indicates that, CyA provides neuroprotection in TBI primarily by inhibiting mitochondrial permeability transition pore formation, thus preventing bioenergetics failure and downstream deleterious cascades (Colley et al., 2010).

The current investigation demonstrated that, TBI induced anaerobic glycolysis as indicated by the increase in PGI and LDH activities in rat brains. Previous work showed similar marked hyperglycolysis early after experimental TBI in rats (Yoshino et al., 1999; Statler et al., 2003). Both PGI and LDH are indirectly involved in energy (ATP)-producing machinery. The excessive increase in these enzyme activities may be explained by their essential need for more energy production in defending against brain damage. Some reports stated that early after TBI, cerebral glucose utilization is increased in response to release of ions and excitatory amino acids, such as glutamate from injured cells (Faden et al., 1989; Bergsneider et al., 1997; Yoshino et al., 1999; Statler et al., 2003). While, energy for glutamate uptake by astrocytes is derived from glycolysis (Pellerin and Magistretti, 1994). Some authors suggested that mitochondrial function is depressed after TBI, and that anaerobic glycolysis is therefore facilitated in compensation, this increased glycolysis supports restoration of ionic homeostasis, and generates ECF lactate (Andersen and Marmarou, 1992; Pellerin and Magistretti, 1994).

Administration of the used agents either alone or in combination markedly reversed TBI induced hyperglycolysis. However the combination of the two agents was more effective in down-modulating of the brain glycolytic enzyme activities, PGI and LDH. This suggests the beneficial effect of both agents in either ameliorating brain injury responsible for hyperglycolysis or having important role in energy production by attenuating mitochondrial depression. The dipeptide, carnosine has been showed to increase ATP production by activating oxidative phosphorylation (Churchil et al., 1995), and increase the liberation of ATP in mammalian muscles during anoxic stress (Millar et al., 1993). It also normalized adenylate energy charge (AEC) in the chronic infection (Soliman et al., 2001). On the other hand, it has been shown that, CyA administration following acute and severe TBI, reduced the amount of damaged tissue when it was given following the event (Buki et al., 1999; Okonkwo and Povlishock, 1999; Okonkwo et al., 1999; Alessandri et al., 2002). CyA inhibits mitochondrial dysfunction in the CNS and prevents calcium efflux by interfering with calcium release from mitochondria, which leads to secondary cascade of events that leads to persistent damage within the CNS (Sullivan et al., 1999, 2000, 2005).

Reactive oxygen species (ROS) and nitrogen species (RNS) are widely implicated in the pathogenesis of secondary neuronal damage and apoptotic/necrotic cell death after traumatic or ischemic brain injury (Lee et al., 2003; Danielisová et al., 2007; Hall et al., 2010). Some studies reported that free radicals have a role in cerebral ischemia and trauma. Free oxygen radicals either disrupt the blood–brain barrier or cause brain edema by affecting the neurons (Hall and Braughler, 1993; Santos et al., 2005).

These radical species can also cause extensive damage to biological macromolecules, including peroxidation of membrane polyunsaturated fatty acids (PUFA) (Hall et al., 2004, 2010). PUFA peroxidation ultimately leads to loss of both structural and functional integrity of the cell with generation of toxic aldehydic byproducts, such as acrolein (Uchida et al., 1998), which may further contribute to neuronal cell death by protein and DNA modification (Graham et al., 1978). Neurons death can lead to tissue shrinkage or loss following TBI. In addition, some experiments have demonstrated that nitric oxide (NO) and proinflammatory cytokines released by micro-glial cells, which act as resident macrophage-like cells in the brain, are also responsible for neuronal cell death (Lee et al., 2003). Mitochondrial function is very sensitive to the presence of NO (Clementi et al., 1998) that is a potent inducer of mitochondrial permeability transition (MPT). MPT reflects the formation of proteinaceous pores in the mitochondrial membrane that allow free diffusion of all molecules smaller than 1.5 kDa (Schweizer and Richter, 1994). CAT and GR are antioxidant enzymes that play key role in reducing production of free radicals (Dringen et al., 2005). CAT converts peroxides into nontoxic forms, often with the concomitant oxidation of reduced glutathione (GSH) into the oxidized form (GSSG), and GR recycles GSSG to GSH (Dringen et al., 2005). In line with previous published data, the present study demonstrated diminished antioxidant enzyme activities, CAT and GR in traumatized rat brains (Azbill, et al., 1997; Shao et al., 2006).

The antioxidant enzymes work in concert to protect the cell. Cell death can result if ROS production exceeds the defense capacity (Shao et al., 2006). Drugs that reduce oxidative stress status appear to be a rational choice for the prevention of those neurological disorders. Treatment of traumatized rats with the current agents either alone or in combination successfully up-modulate the decreased antioxidant enzyme activities. Carnosine as well as its combination with CyA showed higher ability against TBI induced oxidative stress, as they were more effective in restoring the antioxidant defense enzymes near their normal levels. The effectiveness of carnosine was mainly related to its antioxidant activity that is capable of counteracting oxidative process and also acting as a free radical scavenger (Boldyrev et al., 2004). Besides it protects nervous cells as was shown in various models (Shen et al., 2007b; Murad et al., 2011), carnosine exhibits a significant antioxidant protecting effect in case of brain damaged induced either by ischemic injury or hypobaric hypoxia (Stvolinskii et al., 2003; Dobrota et al., 2005). Carnosine appears to influence deleterious patho-logical processes in focal cerebral ischemia, decreasing ROS levels and infarct size. It is neuroprotective when administered at time points both before and after the induction of ischemia (Rajanikant et al., 2007; Min et al., 2008). Carnosine also increased the resistance of neuronal membranes to the induced oxidation and suppressed the glutamate receptor hyperactivation (Stvolinskii et al., 2003; Boldyrev et al., 2004; Min et al., 2008). Restoration of antioxidant enzymes by CyA may be related to its direct inhibitory effects on activation of microglia cells, which have a role in inflammatory cytokines and free radical production (Candelario-Jalil et al., 2007; Hailer, 2008). This effect may be beneficial for prevention of neuroinflammation and neurodegeneration induced by TBI. Previous prospective clinical trials have shown that, CyA have a good safety profile when treatments were initiated within the first 12 h post injury in patients with severe head injuries (Mazzeo et al., 2009b).

In conclusion, the present data suggest the ability of carnosine and CyA to protect brain against traumatic injury which may be related to their beneficial effect in maintaining brain homeostasis through modulating brain nucleotide levels, regulating energy metabolism and restoring the antioxidant defense system.

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