Research Paper

Metallothionein induction reduces caspase-3 activity and TNFα levels with preservation of cognitive function and intact hippocampal neurons in carmustine-treated rats

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Hippocampal integrity is essential for cognitive functions. On the other hand, induction of metallothionein (MT) by ZnSO4 and its role in neuroprotection has been documented. The present study aimed to explore the effect of MT induction on carmustine (BCNU)-induced hippocampal cognitive dysfunction in rats. A total of 60 male Wistar albino rats were randomly divided into four groups (15/group): The control group injected with single doses of normal saline (i.c.v) followed 24 h later by BCNU solvent (i.v). The second group administered ZnSO4 (0.1 μmol/10 μl normal saline, i.c.v, once) then BCNU solvent (i.v) after 24 h. Third group received BCNU (20 mg/kg, i.v, once) 24 h after injection with normal saline (i.c.v). Fourth group received a single dose of ZnSO4 (0.1 μmol/10 μl normal saline, i.c.v) then BCNU (20 mg/kg, i.v, once) after 24 h. The obtained data revealed that BCNU administration resulted in deterioration of learning and short-term memory (STM), as measured by using radial arm water maze, accompanied with decreased hippocampal glutathione reductase (GR) activity and reduced glutathione (GSH) content. Also, BCNU administration increased serum tumor necrosis factor-alpha (TNFα), hippocampal MT and malondialdehyde (MDA) contents as well as caspase-3 activity in addition to histological alterations. ZnSO4 pretreatment counteracted BCNU-induced inhibition of GR and depletion of GSH and resulted in significant reduction in the levels of MDA and TNFα as well as the activity of caspase-3. The histological features were improved in hippocampus of rats treated with ZnSO4 + BCNU compared to only BCNU-treated animals. In conclusion, MT induction halts BCNU-induced hippocampal toxicity as it prevented GR inhibition and GSH depletion and counteracted the increased levels of TNFα, MDA and caspase-3 activity with subsequent preservation of cognition.

Introduction

It is increasingly apparent that neurological disorders including disruption of brain functions associated with the systemic delivery of chemotherapeutic agents used for treatment of central nervous system (CNS) and non-CNS tumors.1,2 There is a growing evidence that standard-dose chemotherapy can impact cognitive function.3 Previous studies suggested that 18% of all breast cancer patients receiving standard-dose chemotherapy show cognitive defects on post-treatment evaluation,4 and such problems were reported in more than 30% of patients examined two years after treatment with high-dose chemotherapy.5,6 In addition, Vardy and Tannock7 have reported impaired cognitive functions during treatment of solid brain tumors in animal models and in humans. It has been concluded that administration of methotrexate and 5-FU in combination induced impairments in tests of spatial memory and other cognitive tasks in mice.8 Also, a single dose of cyclophosphamide produced a transient acute memory impairment in mice.9 Moreover, Adriamycin has been demonstrated to increase oxidative stress in the brain, which may lead to cell dysfunction or cell death and thus contribute to cognitive dysfunction.10 Furthermore, it has been reported that long-term exposure to 13-cis-retinoic acid, which is often used in chemotherapy regimens, was associated with decreased neurogenesis and cell proliferation in the hippocampus and subventricular zone and impaired spatial learning and memory in young and adult mice.11

Vardy et al.12 reported that the etiology of cognitive impairment after chemotherapy remains unknown although a number of mechanisms have been postulated.13 Candidate mechanisms include: direct neurotoxic effects (e.g., injury to neurons or surrounding cells, altered neurotransmitter levels),14 oxidative stress and DNA damage,15 immune dysregulation and/or release of cytokines,13,16 and blood clotting in small CNS vessels.12

Carmustine (BCNU) is an alkylation agent used in the treatment of brain tumors, myeloma, and both Hodgkin and non-Hodgkin lymphoma.17 The ability of BCNU to cross blood-brain barrier (BBB) may explain its neurotoxicity.18 Studies on the incidence and the mechanisms of cognitive dysfunction after BCNU administration are rare. Among these studies, Dietrich et al.17 explained...
that the cognitive dysfunction induced by the three widely used chemotherapeutic agents (carmustine, cisplatin and cytosine arabinoside) is due to their toxic effects on CNS progenitor cells and non-dividing oligodendrocytes in vitro.19 They also found that these agents caused increased cell death and decreased cell division in the subventricular zone, the dentate gyrus of the hippocampus and the corpus callosum in mice.

Metallothioneins (MTs) are a family of low molecular weight cysteine-rich, protein that are associated with transition metal cations including Zn, Cd, Cu and Hg.20 Previous studies have indicated the role of MT in protecting cells, tissues and organisms from environmental toxicant-induced damage.21,22 Three isoforms of metallothioneins (MT-I, MT-II and MT-III) are expressed in the central nervous system.23 Previous studies reported that brain MT-I/II are upregulated by different stressful stimuli including psychogenic stress,24 administration of bacterial endotoxin,25 glutamate analogues,26 metal toxicity27 and stroke/ischemia.28 In particular MT I and II could protect neuroglial cells from oxidative damage.29 The authors conclude that lack of this protection has been found to potentiate excitotoxicity and neurodegeneration in the hippocampus.

A common feature seen in a number of studies is that MT-I, II reduces delayed cerebral damage and cell death, as well as promotes brain repair, angiogenesis and functional recovery.30 Interestingly, MT-I, II are shown to act directly as neurotrophic agents after treatment of neurons with exogenous MT-I, II proteins, which enhance neuronal survival and axonal outgrowth in both cortical, hippocampal and dopaminergic cultures.31,32

Regulation of metallothionein gene expression by zinc has been documented.33 Free zinc induces the accumulation of large amount of zinc-containing metallothioneins.34 A number of studies have suggested that zinc status affects metallothionein concentration and its mRNA synthesis in the various tissues of growing and adult rats and mice.35 It has been reported that metallothionein induced by zinc exhibits protective effects on the cardiac apoptosis of doxorubicin,36 anthracycline-induced cardiac toxicity37 and adriamycin-induced myocardial injury.38 Increased MT expression in some tissues after in vivo treatment with DNA alkylating agents has been reported.39 It was found that rat hepatocytes with elevated MT levels are resistant to N-methyl-N'-nitro-nitrosoguanidine cytotoxicity.40 Also, overexpressed human MT-IIA gene protects Chinese hamster ovary cells from killing by alkylating agents.41 Moreover, treatment of tumor-bearing mice with ZnSO4 increased hepatic, renal and tumor MT and protected them from cytotoxic actions of cisplatin.35

However few studies have been carried out to explore the role of MT in neurocognitive functions.41,42 The relationship between MT induction and cognition deficits by chemotherapeutic agents is still not clear. The current study aimed to address the effect and mechanism of centrally-induced MT on the impact of BCNU on learning and memory in rats.

Results

Brain metallothionein (MT). Data in Figure 1 explains that injection of ZnSO4 markedly increased hippocampal MT content (53%) when compared to the corresponding control group. Also, administration of BCNU significantly increased MT level in the hippocampus of control rats by an extent of 29% but still at a lower significant level in comparison with ZnSO4-treated group. The group of rats pretreated with ZnSO4 then 24 hours later with BCNU show marked increases in the level of hippocampal MT content up to110%, 36.5% and 61% if compared to the control, ZnSO4-treated or BCNU-treated groups respectively (Fig. 1).

Immunohistochemical staining of MT in brain sections by using specific antibodies revealed that the level of brain MT was obviously increased in the tested groups of rats in the order of ZnSO4-pretreated-BCNU-treated (D) >ZnSO4-treated (B) >BCNU-treated (C) >control (A) groups (Fig. 2).

Learning and memory. Behavioral tests in the present study show that there is direct correlation between the good learning and short-term memory (STM) of rats on one hand and the number of learning trials on the other hand where the number of errors were decreased as the number of trials increased in all tested groups (Fig. 3). BCNU administration deteriorated both learning and memory when compared to the corresponding control groups. However, ZnSO4 administration before BCNU obviously improved both learning and memory (Fig. 3).

Statistical analysis of data (Fig. 4) explain that the group of rats injected with BCNU showed a markedly impaired learning behavior by the extents of 32% from the control group and 39% from ZnSO4-treated group. Administration of ZnSO4 before BCNU normalized the decreased learning behavior (Fig. 4). Also, BCNU administration significantly reduced STM in rats to be 31% and 35% of control and ZnSO4-treated rats respectively (Fig. 5). Rats pretreated with ZnSO4 then BCNU showed significant improvement in STM if compared to rats treated with BCNU without ZnSO4 preadministration as it was increased to reach 81% of the control group and 85% of ZnSO4-treated rats (Fig. 5).

Glutathione reductase (GR) activity and reduced glutathione (GSH) content. The present data explain that the activity of GR (Fig. 6) and the level of GSH (Fig. 7) in rat hippocampus was aggressively reduced by administration of BCNU to the extents of 49% and 53% of the control group; and 47% and 51% of ZnSO4-treated rats

Figure 1. Effect of ZnSO4 and/or carmustine (BCNU) on the level of hippocampal metallothionein (MT) in rats. Data are presented as mean ± SEM (n = 10) and analyzed using Graph Pad Instat software by one way ANOVA followed by Tukey Kramer post-test. ZnSO4 was injected as a single dose (0.1 μmol/10 μl saline, i.c.v). BCNU was injected in a single dose (20 mg/kg i.v). Animals were sacrificed after three weeks where MT was measured in hippocampal homogenates. (a–c): Indicate significant differences from control, BCNU-treated and ZnSO4-pretreated-BCNU-treated groups respectively at p < 0.05.
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Figure 2. A photomicrograph for immunohistochemical staining of metallothionein (MT) in hippocampal sections from control (A), ZnSO₄-treated (B), BCNU-treated (C) and ZnSO₄-pretreated-BCNU-treated rats (D) shows increased MT level in the order of ZnSO₄-pretreated-BCNU-treated (D) > ZnSO₄-treated (B) > BCNU-treated (C) > control (A). ZnSO₄ was injected as a single dose (0.1 μmol/10 μl saline, i.c.v). BCNU was injected in a single dose (20 mg/kg i.v). Staining of MT in hippocampal sections was carried out by using MT-specific antibodies and ABC detection kit.

Figure 3. Effect of single doses of ZnSO₄ and/or carmustine (BCNU) on learning and short-term memory (STM) in rats. Raw data are presented as mean values (n = 15). ZnSO₄ was injected as a single dose (0.1 μmol/10 μl saline, i.c.v). BCNU was injected in a single dose (20 mg/kg i.v). Learning and short-term memory (STM) were assessed by using radial arm water maze where the number of errors for each group was calculated in 12 trials to indicate learning behaviour. After 30 min from learning phase STM for each group was assessed by measuring the number of errors.

Figure 4. Effect of ZnSO₄ and/or carmustine (BCNU) on learning of rats. Data are presented as mean ± SEM (n = 10) and analyzed using Graph Pad Instat software by one way ANOVA followed by Tukey Kramer post-test. ZnSO₄ was injected as a single dose (0.1 μmol/10 μl saline, i.c.v). BCNU was injected in a single dose (20 mg/kg i.v). Learning was assessed by using radial arm water maze where the number of errors for each group was calculated in 12 trials to indicate learning behaviour. (a–c): Indicate significant differences from control, BCNU-treated and ZnSO₄-pretreated-BCNU-treated groups respectively at p < 0.05.

respectively. Treatment of rats with ZnSO₄ before BCNU resulted in marked increases in the activity of GR (to the extents 76% and 72% of the control and ZnSO₄-treated groups respectively) and GSH content (to the extents of 73% and 71% of the control and ZnSO₄-treated groups respectively).

Malondialdehyde (MDA). In Figure 8 MDA showed 2.5 fold increase in the hippocampus BCNU-treated animals when compared to either control or ZnSO₄-treated groups. In the group of rats preinjected with ZnSO₄ then BCNU there was up to 60% reduction in the level of MDA compared to BCNU-treated rats but still at a higher level of significance in comparison with either control or ZnSO₄-treated animals.

TNFα. Evaluation of serum TNFα revealed that BCNU produced significant increases in the level of TNFα up to 44% and 39% when...
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compared with control or ZnSO4-treated animals respectively. On the other hand, this level showed a marked reduction (15%) in ZnSO4-pretreated-BCNU-treated rats in comparison with only BCNU-treated animals although it was still at a significant higher level if compared to control or ZnSO4-treated groups (Fig. 9).

Caspase-3 enzyme activity. Figure 10 explains that the activity of caspase-3 enzyme was markedly increased in the hippocampus of rats treated with BCNU compared to either control (by 31%) or ZnSO4-pretreated (by 34%) animals. In ZnSO4-pretreated-BCNU-treated rats, the activity of caspase-3 was significantly decreased to the extents of 12% and 15% from of the control and ZnSO4-treated groups respectively.

Histological examination. Figure 11 explains that administration of BCNU resulted in massive neuronal damage manifested as an apparent decrease (45%) in the number of normal-appearing neurons in the middle hippocampus CA1 in which most pyramidal cells died (Fig. 11C) compared with either control (Fig. 11A) or ZnSO4-pretreated animals (Fig. 11B). Animals pretreated with ZnSO4 and 24 hours later with BCNU (Fig. 11D) showed an obvious increase (30%) in the number of intact neurons as compared to BCNU-treated animals. A photomicrograph of rat brain sections stained with Hx & E (X 40) shows CA1 region of the hippocampus from control (A), ZnSO4-pretreated (B), BCNU-treated rats (C) and ZnSO4-pretreated-BCNU-treated rats (D).
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Discussion

Impaired cognitive functions after administration of chemotherapeutic agents have been reported in animal models and in humans.\textsuperscript{7} The present data revealed that injection of BCNU into rats resulted in impairment of learning and STM. This effect is in consistence with the previous study of Reiriz et al.\textsuperscript{9} who reported that a single dose of cyclophosphamide produced a transient acute memory impairment in mice. Also, it was found that adriamycin produced cognitive dysfunction in experimental animals.\textsuperscript{10} In addition, exposure to 13-cis-retinoic acid, impaired spatial learning and memory in mice.\textsuperscript{11} Moreover, the present data are in close agreement with the study of Dietrich et al.\textsuperscript{17} who reported cognitive dysfunction after administration of carmustine, cisplatin and cytosine arabinoside in mice.

The relationship between the integrity of hippocampus and normal learning and memory pattern has been documented.\textsuperscript{17} The present study showed that BCNU induced oxidative stress in the hippocampus of rats as indicted by aggressive inhibition of GR enzyme activity.

Figure 9. Effect of ZnSO\textsubscript{4} and/or carmustine (BCNU) on the level of reduced tumour necrosis factor-alpha (TNF\textalpha) in the serum of rats. Data are presented as mean \(\pm\) SEM (n = 10) and analyzed using Graph Pad Instat software by one way ANOVA followed by Tukey Kramer post-test. ZnSO\textsubscript{4} was injected as a single dose (0.1 \(\mu\)mol/10 \(\mu\)l saline, i.c.v). BCNU was injected in a single dose (20 mg/kg i.v). TNF\textalpha level was assessed in the serum at the end of the experiment period (3 weeks). (a-c): Indicate significant differences from control, BCNU-treated and ZnSO\textsubscript{4}-pretreated-BCNU-treated groups respectively at \(p < 0.05\).

Figure 10. Effect of ZnSO\textsubscript{4} and/or carmustine (BCNU) on the activity of caspase-3 enzyme in the hippocampus of rats. Data are presented as mean \(\pm\) SEM (n = 10) and analyzed using Graph Pad Instat software by one way ANOVA followed by Tukey Kramer post-test. ZnSO\textsubscript{4} was injected as a single dose (0.1 \(\mu\)mol/10 \(\mu\)l saline, i.c.v). BCNU was injected in a single dose (20 mg/kg i.v). Caspase-3 activity was assessed in hippocampal homogenates at the end of the experiment period (3 weeks). (a-c): Indicate significant differences from control, BCNU-treated and ZnSO\textsubscript{4}-pretreated-BCNU-treated groups respectively at \(p < 0.05\).

Figure 11. A photomicrograph of rat brain sections stained with Hx & E (X 40) shows CA1 region of the hippocampus from control (A), ZnSO\textsubscript{4}-treated (B), BCNU-treated rats (C) and ZnSO\textsubscript{4}-pretreated-BCNU-treated rats (D) explains that administration of BCNU resulted in massive neuronal damage manifested as a decrease (45%) in the number of normal-appearing neurons (as calculated as mean number/10 fields) in the middle hippocampus CA1 in which most pyramidal cells died (C) compared with either control (A) or ZnSO\textsubscript{4}-treated animals (B). Animals pretreated with ZnSO\textsubscript{4} and 24 hours later with BCNU (D) shows an obvious increase (30%) in the number of intact neurons as compared to BCNU-treated animals. ZnSO\textsubscript{4} was injected as a single dose (0.1 \(\mu\)mol/10 \(\mu\)l saline, i.c.v). BCNU was injected in a single dose (20 mg/kg i.v).
activity, reduction of GSH content and increased hippocampal content of MDA in comparison with the control groups. In addition, serum TNFα and the activity of caspase-3 showed significant higher hippocampal levels in BCNU-treated animals compared to corresponding control values. Moreover, microscopic examination of hippocampal sections showed deteriorated histological features in rats treated with BCNU if compared to control groups. These data are in harmony with the previous investigations that reported BCNU as a potent GR inhibitor and GSH depletor in several tissues.53,54 It has been explained that depletion of GSH, as a consequence of GR inhibition will increase cellular oxidative damage.45

The increased extent of lipid peroxidation and apoptosis (as indicated by increased caspase-3 activity) in the hippocampus as well as the level of serum TNFα after BCNU administration in the present study is supported by previous studies on various tissues. In this respect, it was found that incubation of BCNU with rat brain slices for two hours produced marked depletion of GSH (up to 70%) accompanied with increased extent of lipid peroxides.46 Also, BCNU was found to inhibit the activity of GR and promoted the oxidative damage in the nigrostriatal system.47 In addition, BCNU could inhibit GR, depleted GSH and induced apoptosis in PC12 cells.48 It has been reported that BCNU administration, as one of common breast cancer regimen, resulted in apparent inhibition of lung GR activity and GSH content with increased levels of TNFα and lipid peroxidation with marked histological alterations.49 Moreover, the interrelation between oxidative stress and release of inflammatory cytokines including TNFα and subsequent tissue damage has been proved.46 GSH depletion was reported to be associated with augmentation of an oxidative stress-mediated proinflammatory state in alveolar epithelial cells by an oxidative stress-dependent mechanism.51 Recently, it has been concluded that BCNU induced lung fibrosis in rats, an effect mediated by increased oxidative stress and induction of the proinflammatory cytokine TNFα.52 On the other hand, Ali-Osman et al.53 explained that, BCNU metabolites are very reactive electrophiles capable of depleting GSH and inducing apoptosis. It was also stated that the cognitive dysfunction induced by the three widely used chemotherapy agents (carmustine, cisplatin and cytosine arabinoside) is due to their toxic effects on CNS progenitor cells and nondividing oligodendrocytes of in vitro.19 In addition, BCNU was found to increased cell death and decreased cell division in the hippocampus of mice.17 Since neurotoxicity, DNA damage, Oxidative stress and release of cytokines were reported as mechanisms of impairment of cognitive functions after chemotherapy;12 it could be suggested that BCNU administration resulted in hippocampal toxicity in the form of apoptosis, increased level of lipid peroxidation and histological alterations with subsequent impairment of learning and memory.

The involvement of MT in protection against various stress insults has been reported.33,39,54 Immunohistochemical and biochemical techniques in the present study showed that BCNU administration markedly increased the level of MT in the hippocampus of rats. Also, i.c.v injection of ZnSO4 into either control animals or 24 hours before administration of BCNU resulted in significant increase in the level of MT in the hippocampus of both groups in the order of BCNU-ZnSO4−pretreated >control. It is generally accepted that the expression of MT-I/II proteins is highly inducible in response to a range of stimuli, including metals, hormones, cytokines, oxidative agents, inflammation and stress.22 The induced MT in response to BCNU administration is in harmony with the reported role of MT in resistance to alkylating agents during treatment of cancers such as bladder cancer55 and esophageal cancer.56 Also, recent studies strongly suggested a significant role of MT during neurodegenerative diseases and in response to brain injury.57 In addition, Schroder et al.59 have considered that tissue induction of MT as a mechanism of resistance to oxidative stress produced by alkylating agents. Therefore, the increased level of MT in BCNU-treated animals may represents an acute phase response to act against increased oxidative stress produced by BCNU.

It has been stated that, metal-induced synthesis of MT is mediated by activation of metal response element-binding transcription factor (MTF-1), a zinc-sensitive trans-acting factor.57,58 The increased level of MT after ZnSO4 injection in the present work is in close agreement with the early study of Ebadi and Swanson59 who found that i.c.v injection of zinc induced brain MT. In addition, several studies have suggested that zinc status affects metallothionein concentration and its mRNA synthesis in the various tissues of growing and adult rats and mice.60 Recently, it has concluded that administration of Zn2+ (i.v) during reperfusion phase of cerebral ischemia could pass BBB and induced hippocampal MT.28 Although MT has been documented to protect against neuronal injury,61 its role in neurobehavioral function have not been well characterized.57 Behavioral tests in the present study showed marked potentiation of learning and memory in rats pretreated with ZnSO4 (i.c.v), 24 hours before BCNU, compared to those without ZnSO4 preadministration. These data may explain the decisive role of MT induction in the improvement of cognition during BCNU administration. In this respect, West et al.57 believed that alterations in MT function could alter neural function in ways that would impair behavioral function. The authors explained that MTs play a significant role in zinc homeostasis in the hippocampus which is essential in maintenance of normal cognitive functions. Also, it was concluded that the hippocampus, which plays a central role in cognitive functions such as memory depends on correct physiological actions of zinc, which is heavily concentrated in the hippocampal mossy fiber system.61 In addition, MT-I/II were found to protect against the adverse effects of both zinc under and overload.62 Moreover, metallothionein-null mice showed learning deficits in passive avoidance and water maze escape learning.63

The obtained data revealed that pretreatment of rats with ZnSO4 (i.c.v) before BCNU conferred protection of the hippocampus against BCNU-induced damage as the levels of caspase-3 and MDA were markedly reduced but the contents of MT and GSH as well as the activity of GR in the hippocampus were significantly higher if compared to only BCNU-treated rats. Also, serum TNFα showed apparent reduction in rats preinjected with ZnSO4. This protection was further confirmed by histological examination. These data are in line with the previous studies in which neuronal injury was reported to be implicated in cognitive dysfunction.17 On the other hand, MT-I, II were shown to enhance neuronal survival and axonal outgrowth in both cortical, hippocampal and dopaminergic cultures.30,32 In addition, MT was found to protect against neuronal damage via its powerful antioxidant and antiapoptotic properties.9,64 Moreover, MT counteracted oxidative stress and apoptosis in animals treated with DNA alkylating agents.39 Furthermore, treatment of
tumor-bearing mice with ZnSO₄ increased hepatic, renal and tumor MT and protected them from cytotoxic actions of cisplatin. It is important to note that MT-I, II are powerful scavengers of free radicals, likely as a function of their cysteine residues. In addition, it was concluded that MT induction creates a new pool of thiols in the cell cytosol that can attenuate damaging effects of GSH depletors in rat hepatocytes. Further studies indicated that the released Zn²⁺ from MT might suppress free radical-mediated damage by its ability to induce the metalloenzyme superoxide dismutase or to stabilize the cell membrane function. In addition, it has been shown MT induced by zinc exhibited protective effects from doxorubicin-induced myocardial apoptosis and adriamycin-induced myocardial injury by inhibition of oxidative stress-mediated mitochondrial cytochrome c release and caspase-3. On the other hand, the oxidative stress-dependent pathway for increased levels of TNFα and other cytokines and their implication in neuronal injury has been reported.

Methods

Animals. Adult male Wistar albino rats weighing 150–180 g were obtained from the Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The animals were housed in metabolic cages under standard laboratory conditions (12 h light/dark cycle at 25 ± 2°C) with free access to pulverized standard rat pellet food and tap water.

Experimental protocol. A total of 60 male Wistar albino rats were randomly divided into four groups (15/group): The first group served as a control group in which the animals received a single intracerebroventricular (i.c.v) dose of normal saline (10 μl) followed 24 h later with an i.v injection of BCNU solvent (normal saline containing 10% ethanol). The second group administered a single dose of ZnSO₄ (0.1 μmol/10 μl normal saline, i.c.v) followed 24 h later with an injection of BCNU solvent (i.v). Rats in the third group received a single dose of BCNU (20 mg/kg, i.v) 24 h after an injection (i.c.v) of normal saline (10 μl). Fourth group received a single dose of ZnSO₄ (0.1 μmol/10 μl normal saline, i.c.v) followed 24 h later with an injection of BCNU (20 mg/kg, i.v). After three weeks, animals in all groups were subjected to behavioral tests for learning and short-term (STM) memory using radial arm water maze then anaesthetized with ether and sacrificed where five rats from each group were used for histological analysis. The remaining ten rats were used for biochemical analysis where BCNU toxicity was evaluated by measuring MDA (as a marker for lipid peroxidation), caspase-3 activity (as a marker for apoptosis) as well as the possible mechanisms for this toxicity by measuring GR activity and GSH content in the hippocampus as well as serum TNFα level. The ability of MT to counteract BCNU-induced hippocampal toxicity was evaluated by measuring the previous parameters after MT induction by ZnSO₄. In each group, blood samples from each animal were collected separately, centrifuged and used for determination of TNFα. The two lobes of hippocampus in each animal were rapidly dissected out on ice, washed with saline and cut into small pieces and used for biochemical analysis. Hippocampal pieces from each animal in each group were homogenized and used for determination of the activity of hippocampal glutathione reductase (GR) and the contents of MT, reduced glutathione (GSH), malondialdehyde (MDA) as well as total protein. Another portion of piece was dissociated into free cells by trypsinization where the activity of caspase-3 was assessed in the cell lysate.

Intracerebroventricular (i.c.v) injection of ZnSO₄. Rats were anaesthetized by administration of sodium pentobarbital (60 mg/kg, i.p) then placed gently in a stereotaxic apparatus with the tooth bar 5 mm above the ear bar. Sagittal incision was made and the scalp was exposed. By using brain map and stereotaxic coordinates, the bony calcaneum was penetrated with dental drill to allow access into the brain. ZnSO₄ solution was slowly infused using Hamilton microsyringe where the syringe was left in place for 5 min. After removal of the syringe, the wounds were sutured.

The radial arm water maze procedure. The radial arm water maze consisted of a black circular water tub with six V-shaped stainless steel structures arranged to form a swimming field of an open central area and six arms. The water temperatures was maintained at 25°C. A goal arm is one that has a black platform submerged 1cm below the water level. Starting from a start arm (any arm other than the goal arm), the rat must find the submerged platform by swimming to the end of the goal arm. Each rat was allowed 12 consecutive training trials (learning phase) followed by a short-term memory (STM) test trial (30 min delay periods). Rats were allowed to take rest for 5 min to avoid exhaustion at the end of the 6th trial. Rats were allowed 1 min per trial to find the submerged platform. A trial ended when the rat located the submerged platform, where it was allowed to stay trial 15 sec on the platform before removal to begin the next trial in a different start arm. If the rat did not find the submerged platform in the goal arm after one min, it was guided the goal arm and allowed to stay on the platform for 15 sec before removal to begin the next trial. A correct selection occurred when the rat swam directly to the goal arm, while error was registered when the rat entered into an arm other than the goal arm. All experiments were done in dimly lit room and the rat had to use cues in the room to spatially memorize the location of the platform. After the last trial the mean number of errors was calculated for each group to indicate the learning behavior.
Histological examination and immunohistochemical staining for brain MT. Rats were anaesthetized with ether and brains were dissected out on ice and fixed in 10% formalin saline solution then embedded in paraffin. The 5 μm sections were stained on glass slides by Hx & E and examined under light microscope for histological alterations in the hippocampus. The mean number of 10 microscopic fields of normally-appeared hippocampal neurons was calculated.

Expression of brain MT was evaluated by immunohistochemical staining of brain tissues using monoclonal mouse anti-MT antibody (Dako, Carpentry, CA, USA) and vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). After deparaffinization, brain sections on glass slides were subjected to quenching of endogenous peroxidase activity using 3% H2O2 for 10 min at room temperature. Non-specific binding sites were blocked by 5% normal rabbit serum for 5 min. The supernatant (200 μl) was added to 0.5 ml of 5% trichloroacetic acid, centrifuged at 1,000 xg for 30 min. The reaction was stopped by addition of 100 μl substrate solution and incubated in dark for 30 min at room temperature. The optical density was then read at 450 nm using a 96-well plate spectrometer (Spectra Max 190, Molecular Devices Corp., Sunnyvale, CA, USA). TNFα level (pg/ml) was calculated from a standard curve and multiplied by the dilution factor.

Determination of hippocampal MT. Hippocampal content of MT was measured by ELISA. Briefly, in a microtiter plate, 150 μl of 10% tissue homogenate from each sample in coating buffer (0.015 M Na2CO3, 0.023 M NaHCO3, 0.02% Na azide in 80 ml H2O, pH 9.8) added to the wells and left at 4°C overnight. The wells were then washed three times with phosphate buffered saline (PBS) solution containing 1% Tween (pH, 7.4). To each well 150 μl of MT primary antibodies (diluted 1:500 in PBS-Tween solution) and incubated on a shaker for 2 h. Anti-mouse Ig G secondary antibodies diluted in PBS-Tween were added (150 μl/well) and the plate was incubated at room temperature for 2 h followed by washing of the wells with PBS-Tween three times. The substrate solution (p-nitrophenol) was added (150 μl/well) and incubated in dark at room temperature for 30 min. The reaction was stopped by addition of 50 μl 3N NaOH solution to each well. The absorbance of produced color was read at 405 nm in ELISA reader where MT concentration (μg/g protein) was calculated from a standard calibration curve of MT protein.

Determination of hippocampal GR activity and GSH content. GR activity was measured by the method of Carlberg and Mannervick. Briefly, 100 μl of 0.2 M sodium phosphate, pH 7.6 (containing 1 mM EDTA), 25 μl 20 mM GSSG and 25 μl 2 mM NADPH were incubated at 30°C for 10 min. 50 μl of 5% hippocampus homogenate sample (in 50 mmol/L phosphate buffer, pH 7.0 containing 0.1 mM EDTA) were added into a reaction cuvette, and the rate of change of absorbance at 340 nm was measured for 3 min. The activity of GR was expressed as mU/mg protein.

Tissue levels of acid soluble thiols, mainly reduced glutathione (GSH), were determined colorimetrically at 412 nm according to Ellman. In brief, 0.5 ml of previously prepared homogenate was added to 0.5 ml of 5% trichloroacetic acid, centrifuged at 1,000 xg for 5 min. The supernatant (200 μl) was added to a tube contains 1,750 μl of 0.1 M potassium phosphate buffer (pH 8) and 50 μl of 5,5 dithiobis-2-nitrobenzoic acid (DTNB) reagent. The tubes were mixed and the developed yellow color was measured against standard curve of reduced glutathione. Protein thiols (protein-SH) were expressed as μmol/g tissue.

Determination of serum TNFα. Serum TNFα, was assayed by using ELISA kit purchased from R&D Systems (Minneapolis, MN, USA) according to the protocol provided by the manufacturer. Briefly, in a microplate, 50 μl of the assay diluent added to each well. Then 50 μl of the diluted serum sample (2-fold dilution in the calibrator diluent) were added to each well and mixed gently by tapping the plate frame for 1 min then the plate was covered and incubated for 2 h at room temperature. After incubation, each well in the plate was then aspirated and washed five times with the kit washing buffer followed by addition of 100 μl substrate solution and incubated in dark for 30 min at room temperature. The optical density was then read at 450 nm using a 96-well plate spectrometer (Spectra Max 190, Molecular Devices Corp., Sunnyvale, CA, USA). TNFα level (pg/ml) was calculated from a standard curve and multiplied by the dilution factor.

Determination of hippocampal caspase-3 activity. Hippocampal cell suspension was prepared from hippocampal tissue following the method described by Sameto. Briefly, hippocampi from each animal were dissected out, cut into small pieces and suspended in 10 ml sterile saline solution (12.38 mmol/L NaCl, 5.4 mmol/L KCl, 1.1 mmol/L NaH2PO4, 1.1 mmol/L KH2PO4, 22 mM/L glucose and 0.9 CaCl2, containing 0.25% v/v trypsin, in a Petri dish. The suspension was then aspirated two to three times using glass pipette and transferred to a screw-capped Erlenmeyer flask and shaken on a rotary shaker (80 r.p.m. for 15 min at 37°C). Trypsinization was then halted by the addition of 10 mL serum supplemental medium (SSM) containing 10% v/v fetal bovine serum (FBS) and the disrupted tissue was then gently aspirated three times using a 10 ml glass pipette. The aspirated suspension was carefully filtered through NiteX 210 then NiteX 130 filters (TetKo, Elmeford, NY, USA) by gravity. The entire filtrate was then centrifuged at 800 g for 5 min at room temperature and the pellet was resuspended in fresh SSM. Viable cells were counted using a haemocytometer and trypan blue 0.4% diluted in phosphate-buffered saline (PBS) containing 1 mM EDTA (1:16).

Hippocampal caspase-3 activity was measured by using Caspase-3/ CPP32 activity Colorimetric Assay Kit (Biovision Incorporate, USA) according the procedure supplied by the manufacturer. Briefly, the prepared cell suspension was centrifuged. Then cells were resuspended in chilled cell lysis buffer (1–5 x 106 cells/50 μl) and incubated on ice for 10 min, centrifuged for 1 min in a microcentrifuge (10,000 xg). To each sample pellet, 50 μl cell lysis buffer was added followed by addition of 50 μl of 2X reaction buffer (containing 10 mM DTT). 5 μl of the 4 mM DEVD-pNA substrate (200 μM final conc.) was added and to each sample and incubated at 37°C for 2 h. Samples were read at 405-nm in a microtiter plate ELISA reader. The absorbance of the produced color (pNA) from treated animals was compared with control animals to determine the percentage of caspase-3 activity changes.

Determination of hippocampal MDA. Hippocampal lipid peroxides were determined colorimetrically using thiobarbituric acid (TBA), as described by Uchiyama and Mihara, where lipid peroxides...
expressed in terms of MDA concentration (nmol/mg protein). In brief, hippocampal lobes were homogenized separately in 50 mmol/L phosphate buffer (pH 7.0) containing 0.1 mmol/L EDTA to obtain a 5% homogenate. In a Teflon-stoppered test tube, 2.5 ml of 20% acetic acid and 1 ml TBA solution were added to the tissue homogenate and allowed to boil at 100°C for 30 min in a water bath. After cooling to room temperature under tap water, 4 ml n-butanol and samples were added and shaken vigorously and centrifuged at 600 g for 5 min. The absorbance of the upper colored layer was measured at 532 nm and the concentration of MDA was calculated from a standard calibration curve and expressed as nmol/g protein.

**Total protein assay.** Total protein was determined according to the method of Lowry et al. In brief, 1.0 ml of alkaline copper solution (prepared by mixing 50 ml of 2% Na2CO3 in 0.10 N NaOH with 1.0 ml of 0.50% CuSO4 . 5 H2O in 1% sodium tartrate) was added to a test tube containing 0.20 ml of 10% hippocampus homogenate (w/v), mixed well and allowed to stand for 10 min at room temperature. Then, 0.10 ml of Folin’s reagent (phospho-molybdic-phosphotungstic reagent diluted 1:1 in H2O before use) was added to the test tube and thoroughly mixed. After 30 min the optical density was measured against blank at 500 nm. Protein concentration was calculated from a standard calibration curve in which bovine albumin was used in different concentrations.

**Statistical analysis.** Data were presented as mean ± SEM and analyzed using Graph Pad Instat software by one way ANOVA followed by Tukey Kramer post-test at p < 0.05.

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**References**


