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Systemic Administration of Zn^{+2} During Reperfusion Phase of Transient Cerebral Ischemia Protects Rat Hippocampus Against Iron–Catalyzed Post-Ischemic Injury

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SUMMARY

1. The present study was designed to test the protective role of intravenous Zn^{+2}against iron–catalyzed reperfusion injury in the hippocampus of ischemic rats.
2. To achieve this goal, total of 100 adult male Wistar albino rats were allotted randomly into 5 groups. Rats in the first group were subjected to surgery (sham-operated) without induction of cerebral ischemia and injected with normal saline (iv). The second group of rats were injected with 6 mg/kg $ZnCl_2$ (iv). In the third group, rats were subjected to cerebral ischemia for 60 min. Animals in the fourth group were subjected to cerebral ischemia for 60 min followed by 8 h reperfusion. In the fifth group, rats were subjected to cerebral ischemia for 60 min followed by 8 h reperfusion and injected with a single intravenous dose of $ZnCl_2$ (6 mg/kg) during the first 5 minutes of reperfusion period . After reperfusion, animals were sacrificed where brains were dissected out on ice and the hippocampi from each animal were isolated and used for analysis.
3. Cerebral ischemia induced an increase of iron content, lipidic peroxidation and apoptosis and metallothionein (MT) expression in the hippocampus. These effects were significantly increased in the hippocampus of ischemic rats subjected to 8 h reperfusion compared to ischemic non reperfused rats. Intravenous administration of $ZnCl_2$ decreased the accumulation of iron, the lipidic peroxidation and apoptosis produced by the reperfusion but increased the level of MT.
4. Data from this study suggest that after 1 h of ischemia there is an increase of BBB permeability and this allows penetration of intravenously injected $ZnCl_2$ which can induce expression of brain metallothionein, increase the antioxidant capacity and diminish iron–catalyzed lipid peroxidation and apoptosis. This may give new insights to improve the outcome of stroke patients.

Key words: Ischemia/Reperfusion, Zn^{+2}, Iron, Metallotionein, Lipid peroxidation , Apoptosis.

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INTRODUCTION

Despite of numerous defenses, the brain is vulnerable to oxidative stress resulting from ischemia/reperfusion (IR).[1] During reperfusion there is an incresse in free radical production due to contact of oxygenated blood with weakness of lipid membranes by ischemia.[2] The contribution of components of reactive oxygen species (ROS) such as superoxide radical, hydroxyl radical, hydrogen peroxide, nitric oxide and peroxynitrite in producing neuronal loss after IR injury is considered to be substantial.[3,4,5] Production of

oxygen radicals during reperfusion following ischemia is accompanied by vasodilatation and abolition of endothelium-dependent responses with increased blood-brain barrier (BBB) permeability.[6] It has been found that reperfusion can induce injury to the blood-brain barrier after middle cerebral artery occlusion in rats.[7] Recent studies suggest that apoptotic cell death occurs *in vivo* in cerebral ischemia/reperfusion models.[8,9]

Numerous studies have demonstrated that both copper and iron play a critical role in the initiation and propagation of lipid peroxidation (LPO).[10] Metal-catalyzed formation of $^{\cdot}OH$ can result in the abstraction of a hydrogen from an unsaturated fatty acid leading to lipid radical formation. This can initiate a propagative cascade of cyclical reactions leading eventually to the repetitive formation of short-chain alkanes and lipid acid aldehydes resulting in the destruction of lipid bilayers.[10] Iron is a very important metal for normal regulation of various metabolic pathways.[1] Disrupted iron homeostasis has been linked to a number of neurodegenerative states[11] including Alzheimer's disease.[12,13], Parkinson's disease[14,15,16], stroke[17], multiple sclerosis[18] and Friedreich's ataxia.[19,20] It was found that treatment with iron chelators such as deferoxamine has been associated with reduced lipid peroxidation, improved post-ischemic vasoreactivity, cerebral perfusion and ATP recovery.[21,22,23] Recent developments in genetics, including the finding of mutations in the pantothenate kinase gene and ferritin light chain gene, have demonstrated a direct relationship between the presence of a mutation in the iron-regulatory pathways and iron deposition in the brain resulting in neurodegeneration .[1]

The past 20 years has accumulated a large amount of information on the potential role of zinc metal as a cellular antioxidant.[10] In a biochemical system, the earliest report was the observation that zinc antagonized iron-mediated, xanthine/xanthine oxidase–induced peroxidation of erythrocyte membranes.[24] The cardioprotective effects of zinc in several *in vitro* and *in vivo* models of cardiac ischemic injury has been reported.[10] In a recent study, it has been reported that Zn^{2+}can induce expression of brain and liver metallothionein (MT) in chick embryo with subsequent protection against platinum group metal (PGM) toxicity.[25] The authors explained that MT has an important role as a tolerance mechanism against PGM toxicity due to its metal binding capacity. Also, it has been reported that Zn-mediated MT up-regulation in cultured murine macrophage-like J774 cells results in lysosomal stabilization and decreased apoptosis following oxidative stress through an iron binding mechanism.[26] The purpose of the present study was to evaluate the potential protective value of Zn^{2+}administration to ischemic rats early during reperfusion phase against reperfusion–induced neuronal injury in rat hippocampus. Also, the study might provide new therapeutic targets to improve the outcome of stroke patients.

METHODS

Animals and treatments:

Adult male Wistar albino rats weighing 180-200 g were kept under standard laboratory conditions (12 h light/dark cycle at 25±2°C) with free access to food and water. Animals were allotted randomly into five groups (20/group). The first is the control group in which animals were anesthetized and subjected to surgery (sham-operated) without induction of cerebral ischemia and injected with normal saline (iv) with no treatment. The Second group is sham-operated group in which rats were injected with 6 mg/kg $Zncl_2$ (iv) without induction of cerebral ischemia. The third group is the ischemic group where rats were subjected to cerebral ischemia by ligation of the right middle cerebral artery (MCA) and bilateral common carotids for 60 min. The fourth group was ischemic–reperfused group in which ischemic rats were subjected to 8 h reperfusion. The fifth group consisted of rats

exposed to 1 h ischemia followed by 8 h reperfusion and injected with a single intravenous dose of $Zncl_2$ (6 mg/kg) during the first 5 minutes of reperfusion period (8 h). Animals were then sacrificed where brains were dissected out on ice, the two hippocampi from each animal were isolated and used for immunohistochemical detection of MT, evaluation of tissue iron content, flowcytometric analysis for apoptosis and extent of lipid peroxidation. From each group 5 rats were used for immunohistochemical detection of MT in brain sections, another 5 animals for flowcytometric analysis of apoptosis in the hippocampus and the remaining 10 animals were used for determination of tissue iron content and LPO in hippocampal homogenate.

Brain ischemia/reperfusion:

Rats were anesthetized using pentobarbital (50mg/kg, i.p) then subjected to cerebral ischemia where ligation of the right middle cerebral artery (MCA) and bilateral common carotid arteries (CCAs) was performed using methods described previously by Chen et al.[27] and Wang et al.[28] The right MCA was ligated with 10-0 suture. The ligature was removed after 60 min of ischemia to allow reperfusion. Body temperature was monitored and maintained at 37°C throughout the period of surgery and recovery.

Preparation of free hippocampal cells:

Dissociation of hippocampal tissue was achieved according to the method described by Sameto.[29] In brief, hippocampi from each animal was dissected out, cut into small pieces and suspended in10 ml of sterile saline solution (12.38 mMol NaCl, 5.4 mMol KCl, 1.1 mMol Na_2HPO4, 1.1 mMol KH_2PO4, 22 mMol glucose and 0.9 $CaCl_2$) containing 0.25% v/v trypsin in a petri dish. The suspension was then aspirated using 10 ml glass pipette 2-3 times and transferred to screw capped Erlenmeyer flask and shaked on a rotary shaker (80 rpm for 15 min at 37 ^oC). Trypsinization was then halted by addition of 10 ml of serum supplemental medium (SSM) containing 10% v/v fetal bovine serum (FBS) where the disrupted tissue then gently aspirated by 10 ml glass pipette 3 times. The aspirated suspension was carefully filtered through Nitex 210 then Nitex 130 filters (TetKo, Inc. Elmeford, New York) 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 and the viable cells were counted using hemocytometer and trypan blue 0.4% diluted in phosohate buffered saline containing 1mM EDTA (1:16). These cells were used for flowcytometric analysis of apoptosis.

Apoptotic rate assay by annexin V-FITC staining:

Annexin, an FITC conjugate of the phosphatidylserine (PS)-binding protein, is capable of detecting PS externalization in the early apoptotic cells. We calculated the apoptotic rate of hippocampal neurons with flowcytometry as described by Liu et al.[31] according to the above principle. Dissociated neuronal cells were centrifuged (1000 *g*, 5 min). The pellets were washed twice with 0.1 mol/L PBS, and then an aliquot of PBS-suspended cells containing 1×10^5 cells was transferred to a second set of tubes. The cells were centrifuged again and pellets resuspended in 100 µl binding buffer from the apoptosis detection kit (R&D company, USA). Next, annexin V-FITC solution (5 µl) was added from the kit and stored in the dark at room temperature for 10 min. Following this, propidium iodide (PI) solution (5 µl) was also added from the kit. The annexin V-FITC/PI-stained cells were analyzed by using Scalibur flow cytometer (BD Co., USA) at once. The experiment was repeated five times.

Tissue iron assay:

Hippocampal iron content was determined according to the method described by Weinfeld et al.[30] Briefly, a hippocampus lobe from each animal was homogenized, and then 1 mL of 8.5 mol/L HCl was added. Samples were hydrolyzed at 90℃ for 60 minutes. After cooling, 2 mL of 20% trichloroacetic acid was added to precipitate proteins, and supernatant was collected after centrifuge. The supernatant was run through an acid-washed filter, and the precipitate was washed with 1 mL of 4.25 mol/L HCl plus 20% trichloroacetic acid (1:1). The supernatant was collected, and 4 mL of 1 mol/L sodium citrate was added. pH was adjusted to 3.1 and the final volume was completed to 25 mL. Total tissue iron content was assayed by a spectrophotometer at 562 nm with ferrozine as the color reagent. The concentration of iron is calculated from a standard calibration curve as ng/mg protein.

Immunohistochemical examination for brain Metallothionein:

Expression of brain MT was examined by immunohistochemical staining with monoclonal mouse anti-MT antibody (Dako,U.S.A) and vectastain ABC kit (Vector Laboratories, U.S.A). Brain tissues were dissected into sections (30 *um* thickness) using microtome. After deparaffinization and redehydration, the slides were subjected to quenching endogenous peroxidase activity using 3% H_2O_2 for 10 minutes at room temperature. Nonspecific binding sites were blocked by 5% normal rabbit serum for 1 hour then washed with PBS buffer for 10 minutes. Sections were incubated with primary antibodies (diluted 1:500 in 10mM PBS) overnight at 4°C then washed with PBS buffer for 10 minutes. The sections were then incubated with biotinylated rabbit anti-mouse IgG for 1 hour then washed with PBS buffer for 10 minutes followed by addition of vectastain ABC reagent and incubation for 1 h where the sections then washed with PBS buffer. The substrate solution in the kit, diaminobenzedine, was added where the color produced within 2-7 minutes. The stained sections were washed under tap water, air dried and photographed under light microscope (X 40).

Determination of the extent of lipid peroxidation:

Measurement of hioppocampal lipid peroxides was carried out colorimetrically using thiobarbitueic acid as described by Uchiyama and Mihara[32] where determined lipid peroxides expressed as Malondialdehyde (MDA) concentration (nmol/mg protein). One hippocampus lobe from each animal in all groups was isolated, weighed, and homogenized separately in 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA to obtain a 5% homogenate. In brief, in a teteflon-stoppered test tube 2.5 ml of 20% acetic acid and 1 ml thiobarbitueic acid solution of were added to the tissue homogenate and allowed for boiling at 100 ^{o}C for 30 minutes in a water bath. After cooling under tap water to room temperature 4 ml *n*- butanol was added and shaken vigorously and centrifuged at 600×g for 5 min. Absorbance of the upper colored layer was measured at 532 nm and the concentration of MDA was expressed in terms of nmol /g protein.

Total protein assay:

Total protein was determined according to the method of Lowery et al.[33] In brief, 1.0 ml of alkaline copper solution (prepared by mixining 50 ml of 2 % Na_2CO_3 in 0.10 N NaOH with 1.0 ml of 0.50 % CuS04.5 H_20 in 1 % sodium tartrate) was added to a test tube containing 0.20 ml of 10% hippocampal 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 H_2O before use) was added 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 either mean±SD (for % of apoptosis) or mean±SE (for other parameters). Experimental data were analyzed with ANOVA (non parametric for % of apoptosis) followed by Tuckey Kramer post ANOVA test at $P<0.05$.

RESULTS

Analysis of hippocampal cell apoptosis

Figure (1) shows annexin V-FITC staining for hippocampal neurons. Compared with both normal control and control $ZnCl_2$-treated group, the three tested groups showed higher apoptotic rate. In the group of rats exposed to ischemia for 1 h (C), cell population is shifted partially to apoptotic cell region (R_2) and this shift is larger when compared with either control rats (A) or control rats treated with $ZnCl_2$ (B). This shift is clearly increased in neurons from rats exposed to ischemia for 1 h plus 8 h reperfusion (D) if compared to A or B or C. The shift of cell population in neurons from rats exposed to ischemia for 1 h then treated with $ZnCl_2$ during the first 5 min of 8 h reperfusion (E) towards R_2 is lower than in D but still higher than A and B.

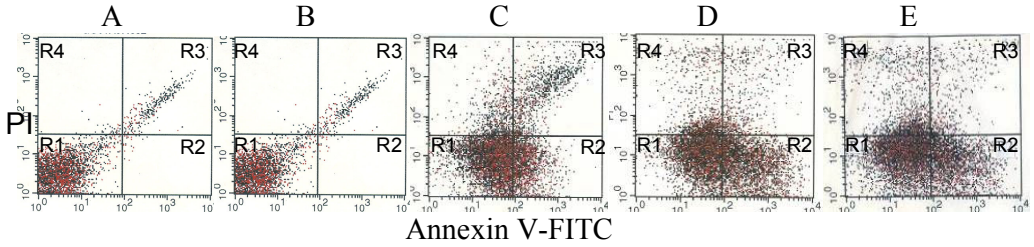

Figure 1: *Annexin V-FITC staining for hippocampal neurons. The different labeling regions in this quadrant represented the different cell population. R_1: vital cells (PI-negative/annexin V-negative); R_2: apoptotic cells (PI-negative/annexin V-positive); R_3: dead cells (PI-positive/annexin V-positive); R_4: fragments of injured cells (PI-positive/annexin V-negative. A: Neurons from control rats, B: Neurons from control rats treated with 6 mg/kg $ZnCl_2$ (iv), C: Neurons from rats exposed to ischemia for 1 h, D: Neurons from rats exposed to ischemia for 1 h plus 8 h reperfusion.; E: Neurons from rats exposed to ischemia for 1 h then treated with 6 mg/kg $ZnCl_2$ (iv) during the first 5 min of 8 h reperfusion.*

The data in figure (2) explains that, percentage of apoptosis of neurons in the control and control group treated with 6 mg/kg $ZnCl_2$ (iv) accounted for 3.00 ± 1.83 and 3.75 ± 2.22 respectively. When neurons were subjected to ischemia for 60 min, the percentage of apoptosis in the hippocampal cells was significantly elevated to 16.00 ± 3.00. Reperfusion of the ischemic brains (for 8 h) lead to a marked increase in the percentage of hippocampal cell apoptosis (35.75 ± 7.50) on comparison with control and control-$ZnCl_2$-treated or 60 minutes ischemic rats. Intravenous injection of $ZnCl_2$ (6 mg/kg, iv) during the first 5 minutes of reperfusion period resulted in a significant reduction in the percentage of apoptosis in the hippocampus of rats to be 23.75 ± 5.68 when compared to the ischemic–reperfused animals. Also, our data show that the percentage of apoptosis in the hippocampus in the group of animals injected with $ZnCl_2$ (6 mg/kg, iv) during the first 5 minutes of reperfusion period is not significantly different from that in the ischemic non

reperfused animals but still at higher significant when compared to either control or control-$ZnCl_2$ treated groups.

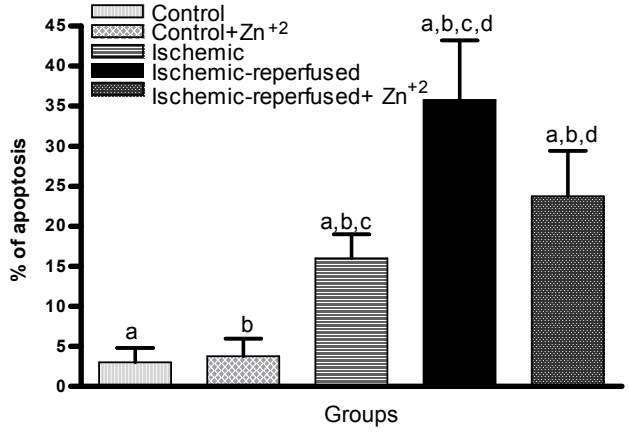

Figure 2: *Percentage of apoptosis in the hippocampal neurons from control, control-$ZnCl_2$-treated, ischemic, ischemic-reperfused and ischemic-reperfused-$ZnCl_2$ treated rats. Data are presented as mean±SD (n=5) and analyzed using Graph Pad Instat soft ware by nonparametric one way ANOVA followed by Tuckey Kramer post ANOVA test where groups having the same symbol (a, b, c, d) are significantly different from each other at P <0.05.*

Tissue iron content

Fig.3 revealed that, hippocampus iron content (ng/mg protin) is significantly increased after exposure of rats to brain ischemia for 60 min (31.50 ± 2.10) compared with either control or control-$ZnCl_2$ treated groups (22.70 ± 1.90 and 23.00 ± 1.68 respectively). Reperfusion of the ischemic rat brains (for 8 h) markedly increased the iron level in the hippocampus (44.00 ± 2.23) upon comparison with ischemic non-reperfused animals or both control and control-$ZnCl_2$ treated groups. Iron in the hippocampus of rats injected with $ZnCl_2$ (6 mg/kg, iv) during the first 5 minutes of reperfusion period is lower than that in ischemic reperfused animals (34.40 ± 1.97) which does not differ from ischemic non-reperfused animals but still higher compared with either control or control- $ZnCl_2$ treated groups.

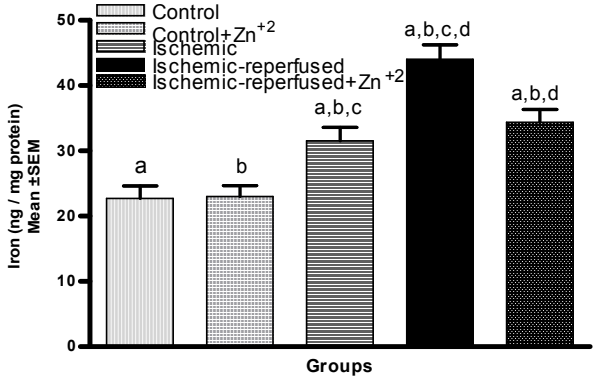

Figure3: *Iron content (ng/mg protein) in the hippocampus of control, control-$ZnCl_2$-treated, ischemic, ischemic-reperfused and ischemic-reperfused-$ZnCl_2$ treated groups of rats. Data are presented as mean ± SEM (n=10) and analyzed using using Graph Pad Instat software by one way ANOVA test followed by Tuckey Kramer post ANOVA test where groups having the same symbol (a, b, c, d) are significantly different from each other at P <0.05*

Brain metallothionein

Immunohistochemical staining to metallothionein (MT) using specific antibodies in the brain tissue (fig.4) shows that intravenous injection of $ZnCl_2$ to control animals (B) resulted in no increase in the stain of hippocampal MT when compared to control rats without $ZnCl_2$ treatment (A). Exposure to brain ischemia for 1 h (C) increased the intensity of the staining to MT than in control rats without or with $ZnCl_2$ treatment (A and B respectively). Reperfusion of ischemic rat brain notably increased the intensity of MT staining (D) more than in than in control rats without or with $ZnCl_2$ treatment and rats exposed to ischemia for 1 h. Rats exposed to ischemia for 1 h then 8 h reperfusion and treated with 6 mg / kg $ZnCl_2$ (iv) during the first 5 min of reperfusion (E) resulted in the highest level of MT staining in the hippocampal sections.

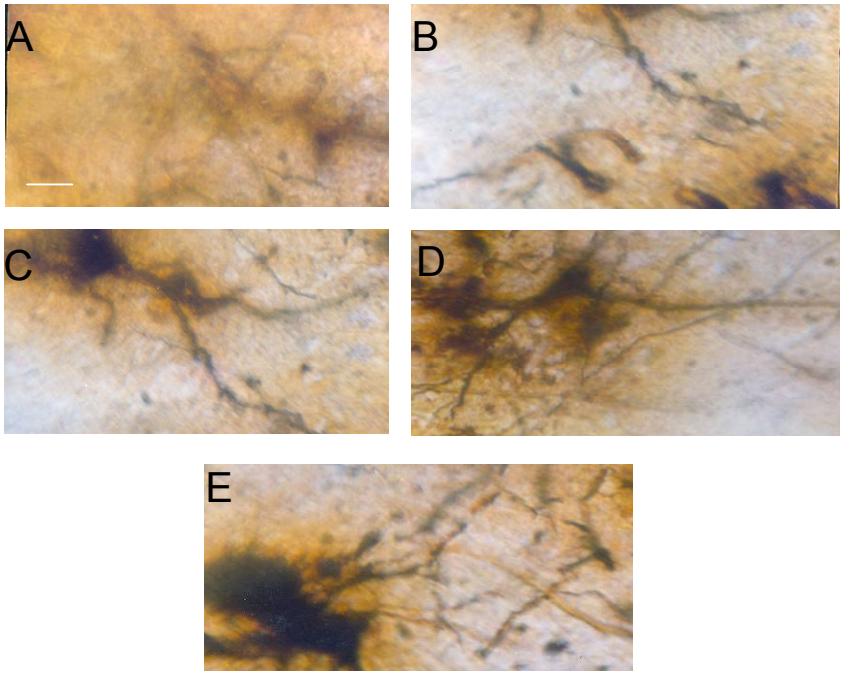

Figure 4*: Immunohistochemical staining to MT the hippocampal sections (X 40) where A: Control group; B: Control group treated with 6 mg/kg $ZnCl_2$ (iv); C: Rats exposed to ischemia for 1 h; D: Rats exposed to ischemia for 1 h plus 8 h reperfusion; E: Rats exposed to ischemia for 1 h then 8 h reperfusion and treated with 6 mg/kg $ZnCl_2$ (iv) during the first 5 min of 8 h reperfusion. Bar scale: 50 um*

Extent of lipid peroxidation

Figure (5) explains that the extent of LPO in rat hippocampal tissue in the form of MDA (nmol/mg protein) does not differ in the control group (39.50 ± 1.40) from control group injected with a single dose (6 mg / kg, iv) of $ZnCl_2$ (43.00 ± 2.10). Exposure to 60 min brain ischemia resulted in a marked increase in hippocampal MDA level (56.70 ± 2.80) when compared to control and control Zn^{+2}-treated non-ischemic rats. In the group of rats exposed to ischemia for 1 h plus 8 h reperfusion showed significantly increased hippocampal MDA level (87.40 ± 2.90) compared to all tested groups. Injection of ischemic rats subjected to 8 hr reperfusion with a single dose (6 mg/kg, iv) of $ZnCl_2$ during the first 5 min after the onset of reperfusion resulted in a marked reduction in MDA level (64.00 ± 2.10) which approximately simulate ischemic non-reperfused rats but still at a higher level than the other groups.

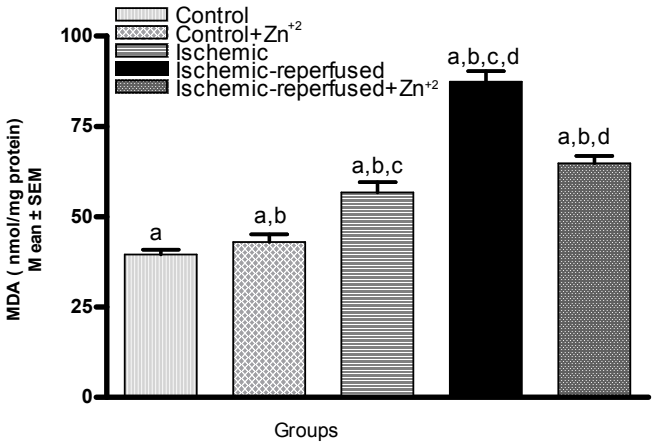

Figure 5: *MDA level (nmol/mg protein) in the hippocampus of rats (control, control-$ZnCl_2$-treated, ischemic, ischemic-reperfused and ischemic-reperfused-$ZnCl_2$ treated groups). Data are presented as mean ± SEM (n=10) and analyzed using Graph Pad Instat software by one way ANOVA followed by Tuckey Kramer post ANOVA test. groups having the same symbol (a, b, c, d) are significantly different from each other at P <0.05.*

DISCUSSION

Involvement of oxidative stress in ischemic reperfusion injury is well established.[5] Free radicals are highly reactive molecules that initiate radical chain reactions and damage cellular macromolecules, including proteins, DNA, and lipids, ultimately leading to cell death.[2,9] Elevated iron with its catalytic role in oxidative injury was observed in a region-specific manner during ischemia in both cortical and subcortical brain regions, most prominently in the hippocampus, cerebellum, midbrain, and cortical gray matter.[17]In the present investigation, hippocampal neurons, that show elevated iron content during ischemia,[17] with increased vulnerability to ischemia/reperfusion,[17,31] were chosen to be a model of iron-catalyzed cerebral ischemia/reperfusion injury *in vivo*. Our data revealed that exposure of rats to brain ischemia resulted in marked increases in the levels of hippocampal iron and MDA as well as the percentage of apoptotic cell death in comparison with either the control group or the control group intravenously injected with $ZnCl_2$. Reperfusion of ischemic rats for 8 h significantly magnified levels of hippocampal iron and MDA along with increased apoptotic percentage. These data are supported by the recent studies that apoptotic cell death occurs *in vivo* in cerebral ischemia/reperfusion models.[8,9,3] Iron-catalyzed oxygen radical production has long been hypothesized to play a prominent role in brain injury during ischemia/reperfusion.[17] It was found that treatment with iron chelators such as deferoxamine has been associated with reduced lipid peroxidation, improved post-ischemic vasoreactivity, cerebral perfusion and ATP recovery.[21,22,23] The increase of hippocampal iron along with lipid peroxidation and apoptosis in present study may be due to release of iron during ischemia/reperfusion which play a crucial role in free radical production with subsequent neuronal cell injury.[35] Also, previous studies reported that free radical generation during ischemia/reperfusion lead to lipid peroxidation-mediated chain reactions[3,36] and cytotoxicity from lipid peroxidation products e.g. 4-hydroxynonenal which may stimulate apoptosis.[37,38] The present data are in harmony with the previous study that the brain of ischemic/reperfused rats showed marked increases in lipid peroxides and

apoptosis.[2] The authors explained that these effects were due to deoxygenating–iron activated $^{-}$OH radical production. In addition, it has been concluded that neuronal apoptosis in ischemia models may be due to $^{-}$OH radicals that may be generated from H_2O_2 through an iron-activated pathway.[39] Our findings also supported by the previous study that oxidative stress is involved in cell death after cerebral ischemia due to reactivity of oxygen radicals that can injure neurons and other brain cells directly with increased evidence of redox signaling such as mitochondrial cytochrome c release, DNA repair enzymes, and transcriptor NF-κB, which might lead to neuronal apoptosis.[35]

Several studies have reported MT as an antioxidant, free radical scavenger and metal binding acute phase protein because its synthesis is induced in response to various stress insults in different tissues.[40] Immunohistochemical staining to MT using specific antibodies in the brain tissue in our work showed increased level of staining in both ischemic and ischemic/reperfused rat brain sections (in the order of: ischemic/reperfused > ischemic) when compared to control and/or control-$ZnCl_2$ injected rats. Also, present study found that intravenous injection of $ZnCl_2$ into ischemic reperfued rats intensified the stain of MT in the hippocampus whereas same treatment to control has no effect. These data are supported by previously reported induction of MT synthesis in ischemic rat brains.[41] The increase of MT after reperfusion in comparison with ischemic non-reperfused rat brain may be a response to increased rate of free radical formation by reoxygenation of ischemic brains.[2] However, the intensified level of brain MT after injection of $ZnCl_2$ in the ischemic/reperfused animals but not in control animals may indicate a combined effect of acute phase response of MT and induction of MT synthesis by penetration of Zn into brain during reperfusion phase as a consequence of disruption of BBB permeability. 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.[42,43,44] Although the induction of MT by zinc has been reported in various tissues, the inability of Zn^{+2} to cross the BBB limits the study of these effects *in vivo*.[45,46] These facts may explain why the level of MT in the control rats injected with $ZnCl_2$ (iv) was unchanged but induced in the brain of ischemic rats after $ZnCl_2$ injection (iv) early during reperfusion. It has been reported that brain ischemia and reperfusion engage multiple independently-fatal terminal pathways involving loss BBB integrity, release of calcium and iron along with lipid peroxidation and apoptosis.[47] On the other hand, it has been reported that, in acute CNS injuries, such as cerebral ischemia, the BBB is still intact, at least in part, however reperfusion lead to its deterioration which allows entrance of iron and ferritin and their accumulation in the brain.[9,47] By this way $ZnCl_2$ can penetrate into brain during reperfusion. These foundations make it acceptable to conclude that, during ischemia and reperfusion the excessive iron–activated free radical production with subsequent peroxidation of membrane lipids allowing penetration of intravenously injected Zn^{+2} which can induce brain MT.

The acute antioxidant effects of zinc are generally manifested in the presence of a demonstrable short-term increase in levels of this metal.[10] Our data explain that intravenous injection of $ZnCl_2$ into rats exposed to brain ischemia for 1 h followed by 8 h reperfusion during the first five min resulted in significant reduction in the hippocampal levels of iron, MDA and the percentage of apoptosis compared with reperfused rats with no administration of $ZnCl_2$ but still at a higher increased values than other groups. These values are not significantly different from those estimated in the ischemic non-reperfused animals and still at higher significant levels than both control and control-$ZnCl_2$-trated groups. These effects have a strong credit as they support the previous findings when we consider the ability of Zn^{+2} to cross BBB during reperfusion[9,47] and the reported antioxidant properties of zinc.[10] The protective effect of zinc was suggested to be due to maintenance of an essential sulfhydryl group of the antioxidant enzymes secondary to

decreased reactivity.[48] Also, zinc has been shown in numerous systems to antagonize the catalytic properties of the redox-active transition metals iron and copper with respect to their abilities to promote formation of $^{\cdot}OH$ from H_2O_2 and superoxide.[10] These findings can reflect the reduced extents of lipid peroxidation and apoptosis in our study as vast majority of studies have confirmed iron-catalyzed lipid peroxidation and apoptosis in ischemic reperfusion injury.[8,9,34,47,49,50]

In the present study, induction of brain MT is considered as an additional mechanism by which Zn^{+2}can act against hippocampal injury in ischemic reperfused rats. Recent studies have hypothesized that the metallothioneins are a link between cellular zinc and the redox state of the cell.[10] It has been suggested that the resistance against oxidative stress, known to occur in MT-rich cells, may be a consequence of autophagic turnover of MT, resulting in reduced iron-catalysed intralysosomal peroxidative reactions.[26]The authors concluded that upregulation of MT by zinc resulting in reduced concentration of lysosomal redox-active iron along with less intralysosomal peroxidation and consequently diminished release of lysosomal apoptogenic factors to the cytosol. On the basis of the in vitro properties of these proteins, the functional importance of metallothionein in astrocytes could be as a free radical scavenger and metal ion chelator.[51]

In conclusion, data from this study suggest that reperfusion of ischemic rat brain resulted in deterioration of BBB permeability. This can open a therapeutic window through which Zn^{+2} penetrates into the brain and induces brain MT with subsequent protection against ischemia/reperfusion-induced neuronal cell injury. Such protection is mediated, at least in part, by inhibition of iron-catalyzed production of ROS which may provide new therapeutic targets to improve the outcome of stroke patients.

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