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Effect of Zn_7-metallothionein on oxidative stress in liver of rats with severe thermal injury[1]

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ABSTRACT

AIM: Using the model of burned animal with delayed resuscitation to study antagonistic effect of Zn_7-metallothionein (Zn_7-MT) on oxidative stress in the liver of rats suffered from severe thermal injury on skin. **METHODS:** To compare the changes in antioxidant concentrations and antioxidative enzyme activities in the liver or plasma of burned rats with or without Zn_7-MT in resuscitation fluid by biochemical assay. **RESULTS:** After injury, glutathione concentration was progressively decreased with time. At 24 h after injury, activities of glutathione reductase and glutathione peroxidase in the liver of burned rats were increased and then decreased at 48 h postburn. α-Tocopherol in plasma was reduced at 24 h and malondialdehyde in the liver was increased significantly postburn. MT and MT-1 mRNA expression in burned rats were activated. Taken together, oxidative stress in the liver of burned rats occurred. Exogenous Zn_7-MT attenuated the changes in antioxidant concentrations and antioxidative enzyme activities in the liver or plasma of burned rats. The effect of Zn_7-MT was in a concentration-dependent manner and the concentration of 10 μmol/L was the most effective. Exogenous Zn_7-MT also inhibited MT-1 mRNA overexpression and increased MT protein concentration. **CONCLUSION:** Zn_7-MT effectively antagonized oxidative stress in the liver of rats with severe thermal injury.

INTRODUCTION

Severe thermal injury in humans and animal models involves development of multiple organ failure. Horton *et al* demonstrated a dysfunction of cardiac contraction of burned rats[1]. Pintaudi *et al* reported that after moderate to extensive burning in humans there was an extensive and rapid spreading oxidative degradation of lipids[2]. The authors regarded this extensive injury as a result of systemic oxidative stress. Oxidative stress is believed to be the major causative agent to damage the organs distant from the original burn wound. Results in our previous study[3] showed an oxidative stress in the liver of rats suffered from severe thermal injury. Oxidative stress refers to the cytological consequences of a mismatch between the production of free radicals or reactive oxygen species (ROS) and the ability of the cell to defend against them. Oxidative stress can thus occur when the production of ROS increases, scavenging of free radicals or repair of oxidatively modified macromolecules decreases, or both. There have been increasing evidences for unbalance of redox or

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overproduction of ROS in patients suffered with burns or in burned animal models[4-8]. ROS could, in return, damage proteins, lipids, nucleic acids, and other biological macromolecules further impairing various tissues and organs. To support/improve the postburn systemic function, therefore, a variety of antioxidants including ascorbic acid, tocopherol, glutathione, and carotenoids have been employed to treat patients or animals with burning injury and got inspiring results[9-11].

Metallothioneins (MT)[12], a group of proteins characterized by a high thiol content and that bind bivalent metals, are thought to be a potent scavengers of ROS and protect cells from oxidative stress. Results in our previous studies evaluated the ability of MT to quench free radical signals and the signal of PBN-related oxygen free radical adducts and protect cardiac muscle from postischemic reperfusion injury[13,14]. In cultured myocardiac cells, MT also exerted the protective effect on cells from the damage by hydroxyl radicals[15].

In this work, we studied effects of Zn_7-MT on oxidative stress in the liver of burned rats by determining the changes in glutathione concentration, glutathione-related enzyme activities, α–tocopheral content, zinc level, and lipid peroxidation. Moreover, we also examined the effect of exogenous Zn_7-MT on the liver MT protein and MT-1 mRNA expression postburn.

MATERIALS AND METHODS

Chemicals According to Vasak[16], rabbit liver Zn_7-metallothionein (Zn_7-MT) was induced, separated, purified, metal-substituted and determined by our group. The molecular weight of Zn_7-MT consisting of 61 amino acid residues is about 6300. The protein contains high content of cystein (approximately 33 %). All the cysteinyl side chains are bound to Zn ions in thiolate complexes. Zn_7-MT is negatively charged at neutral pH. In lower pH condition Zn ions bound to the protein are released to yield apometallothionein.

o–Phthaldialdehyde, nicotinamide adenine dinuoleotide phosphate reduced sodium salt (NADPH), 1,1,3,3–tetramethoxypropane (malonaldehyde-*bis*-dimethylacetal), 5,5'-dithio-*bis* (2-nitrobenzoic acid) (DTNB), α-tocopherol, and hemoglobin were purchased from Sigma Chemical Co (St Louis Mo, USA). Thermus aquaticus DNA polymerase, avian myeloblastosis virus (AMV) reverse transcriptase, random primers, and guanidine thiocyanate were obtained from Promega Co. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were purchased from Serva Co. All other reagents in analytical grade or molecular biology grade were purchased from Beijing Beihua Fine Chemical Products Co, Ltd or Beijing No 2 Chemical Reagent Factory.

Animals Young male Wistar rats (obtained from the Institute of Beijing Biological Products, Grade II, 0005063) were approximately 280 *g*. These animals were maintained on an unrestricted standard diet with free access to tap water for at least 1 week before starting experiments. Injury model was prepared at the Animal Experimental Center in the Union Medical University.

Experimental protocol Rats were randomly divided into 3 groups: the injury group, the normal control group and the protection group. In the injury group under brief ether anesthesia, the dorsum of the rat was shaved, exposed to 100 ° C water bath for 10 s which resulted in skin thermal injury to the dorsum covering a 30 % total body surface area with full-thickness third degree[17] (confirmed by pathological examination). At 6, 12, 24, and 36 h after injury respectively, saline (30 mL/kg) was injected intraperitoneally as delayed fluid resuscitation. After the first time of resuscitation, burned animals could be allowed free access to water. The treatments to rats in normal controls were identical to injury group except that the dorsum did not exposed to 100 ° C water. All the treatments were the same in the protection group except that Zn_7-MT was added in the resuscitation fluid. Actually, in the first part of this work (determinations of GSH and GSH-related enzyme activities) the protection group was divided into three subgroups; I, II and III corresponding to the dosage of Zn_7-MT 1, 5, and 10 μmol/L, respectively.

At 24 h and 48 h postburn, rats were anesthetized by ip injection with 20 % ethylcarbamate in saline (5 mL/kg). The blood was taken and the plasma was collected for the measurement of α-tocopherol content. Then, the liver was removed as quickly as possible. All of the preparations were stored at -70 ° C until use for analysis. For determination of zinc at 12, 24, and 48 h postburn, animal was anesthetized, serum was separated, the liver was collected and stored at -70 ° C. At 12, 24, and 48 h postburn approximately 250 mg liver removed was flash frozen in liquid nitrogen and then stored at -70 ° C until total RNA isolation.

Determination of glutathione, glutathione-related enzyme activities and lipid peroxide GSH was assayed by the methods of Hissin *et al*[18]. GSH level was determined from the standard curve and ex-

pressed as micromolar per gram tissue (μmol· g^{-1} tissue). GSH-Px activity was measured by the method[19] using H_2O_2 and GSH as substrates. An enzyme unit of activity was defined as the amount of enzyme catalyzing the conversion of 1 mmol of GSH per gram protein per minute at 37 ° C (mmol· min^{-1}· g^{-1}protein). GSH-Re activity was determined according to Inger *et al* [20] by monitoring the decrease in absorbance at 340 nm due to oxidation of NADPH at 37 ° C. The specific activity is expressed as micromolar per gram protein per minute (mmol· min^{-1}· g^{-1} protein). Lipid peroxidation in terms of malonaldehyde (MDA) was assessed by the method[21]. MDA level was determined from the standard curve and expressed as nmol· g^{-1} tissue.

Measurement of a**-tocopherol** High-performance liquid chromatography method of α-tocopherol determination was adapted[22]. The hexane extract from 0.5 mL of plasma was prepared by mixing the sample in the presence of 1 mL ethanol, 4 mL hexane, and 0.5 mL distilled water. α-Tocopherol acetate (50 mg per sample) was added as an internal standard. The hexane phase was removed, and 100 mL of the extract was injected on the column of the liquid chromatograph. α-Tocopherol was detected by its native fluorescence (excitation at 290 nm; emission at 340 nm).

Measurement of zinc level[23] Serum was diluted with 4 mL of 5 % (v/v) glycerol. Liver tissue was homogenized in 5 volume of cold water. Homogenate was lyophilized and then all the samples were digested with an acid mixture (HNO_3/$HClO_4$, 4/1, v/v). Zinc analysis was carried out with the atomic absorption spectrophotometer (WFX-1C).

Measurement of MT expression Metallothionein (MT) concentration was determined by ^{109}Cd-heme affinity assay[24]. High affinity of MT for cadmium coupled with a good stability at high temperature is the basis for the determination. Liver tissue was homogenized in 4 volumes of cold sucrose 0.25 mol/L with pH 7.4 and homogenate was centrifuged at 10 000×*g* for 20 min. The supernatant was used for determination of total MT protein. Carried-free ^{109}Cd was added in the heat-denatured supernatant. The mixture was incubated at room temperature for 10 min. Then hemoglobin solution was added to remove the excess cadmium. After heating, cooling, and centrifuging the supernatant was transferred to a γ-counting tube. The amount of radioactivity in the supernatant fraction was determined by direct γ-counting. Result was expressed as ^{109}Cd-bound protein per 1×10^6 cells.

Total RNA was prepared from liver tissue homogenate by acid guanidine thiocyanate-phenol-chloroform extraction method[25]. RNA yield, purity and integrity were monitored by recording absorbance at 260 nm (the ratio at 260/280 was between 1.7 and 2.0), and were confirmed by 1 % (w/v) agarose gel electrophoresis. Each sample of RNA (2 μg) was reverse transcribed into cDNA with AMV reverse transcriptase and cDNA was amplified by PCR techniques with *Taq* polymerase and PCR buffer. The composition of PCR primers (from 5' to 3') was as the follows: GAPDH (254 bp): GTA AAG GTC GGT GTC AAC G and GGT GAA GAC GCC AGT AGA CTC; and MT-I (186 bp): GTC ACT TCA GGC ACA GCA CG and CCC AAC TGC TCC TGC TCC AC[25]. Amplification of the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was always involved to serve as control of reaction efficacy. After an initial denaturation step of 5 min at 94 ° C, PCR amplification with 45 s at 94 ° C, 55 ° C, 72 ° C was run and terminated by a final elongation step of 7 min at 72 ° C using Gene Amp PCR system 2400 (Perkin-Elmer). Reactions were run for 20 cycles for GAPDH and 25 cycles for MT-1. The amplified products were separated by electrophoresis in a 1.5 % (w/v) agarose gel containing ethidium bromide. The DNA bands were captured under UV light and intensity of the bands was quantified by densitometric scanning. Signal intensities of the MT-1 products were normalized to those of GAPDH products as ratios to produce arbitrary units of relative abundance.

Data analysis Results are expressed as mean±SD. Group *t*- test was used to evaluate the significant difference between the normal control group and injury group, and ANOVA was used to compare the differences between the injury and protection groups. Differences were considered significant when $P\leq0.05$.

RESULTS

All the burned animals survived and the wound kept dried The decrease in GSH concentration and α-tocopherol content, the changes in GSH-related enzyme activities, the redistribution of Zn element, the increase in the liver peroxidation and the liver MT protein, and MT-1 mRNA overexpression suggested an oxidative stress in the liver of burned rats (Tab 1, 2, Fig 1, 2).

In the protection group (including subgroup I, II and III), the area and depth of thermal injury were the same as those in the injury group.

Tab 1. Ameliorative effects of Zn_7-MT on changes in glutathione level and glutathione-related enzyme activities in the liver of burned rats. *n*=8-12 rats. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* the corresponding normal control. [d]*P*>0.05, [e]*P*<0.05, [f]*P*<0.01 *vs* the corresponding injury group.

	Treatment		GSH/ mmol·g^{-1}(tissue)	GSSG/ mmol·g^{-1}(tissue)	GSH-Px/mol·min^{-1}·g^{-1}(protein)	GSH-Re/mmol·min^{-1}·g^{-1}(protein)
24 h	Control		4.3±0.6	0.54±0.07	441±10	143±10
	Injury		3.6±0.4[c]	0.67±0.08[c]	501±9[c]	173±7[b]
	Protection	I	3.8±0.3[d]	0.63±0.07[d]	498±9[d]	178±8[d]
		II	3.9±0.5[e]	0.59±0.06[e]	475±13[d]	167±8[e]
		III	4.1±0.4[e]	0.57±0.02[e]	466±19[d]	152±3[e]
48 h	Injury		3.5±0.1[c]	0.75±0.06[c]	396±6[c]	128±3[b]
	Protection	I	3.6±0.3[d]	0.74±0.09[d]	411±12[d]	128±9[d]
		II	3.9±0.7[e]	0.68±0.09[e]	431±10[e]	134±5[e]
		III	4.2±0.1[f]	0.61±0.02[f]	453±16[f]	139±3[e]

Protection group I, II and III corresponding to the dosage of Zn_7-MT 1, 5, and 10 μmol/L, respectively.

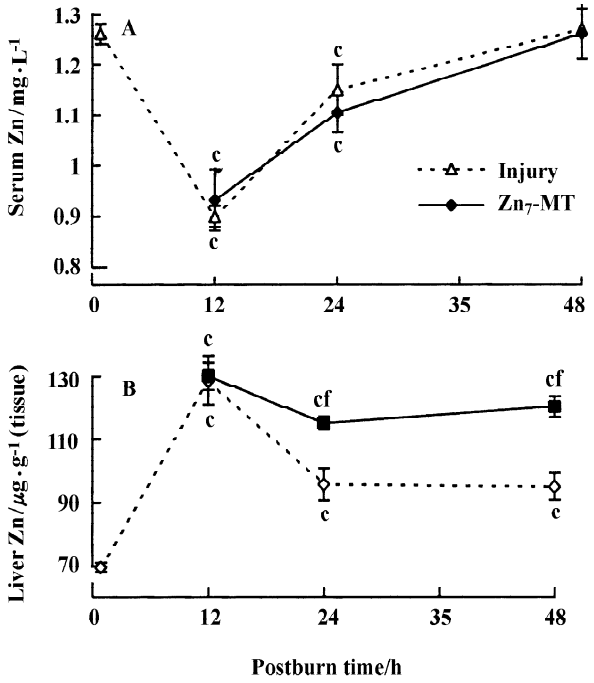

Fig 1. Ameliorative effects of Zn_7-MT (10 mmol/L) on Zn concentrations in the serum (A) and liver (B) at different time points. *n*=5. Mean±SD. [c]*P*<0.01 *vs* the normal control. [f]*P*<0.01 *vs* the corresponding injury group.

Zn_7-MT antagonized oxidative stress in the liver of burned rats (Tab 1). The antagonizing effects of Zn_7-MT on the changes in GSH level and GSH-related enzyme activities were in a concentration-dependent manner. Of the three concentrations (1, 5, and 10 μmol/L) of Zn_7-MT, 10 μmol/L was the most effective. Zn_7-MT 10 μmol/L effectively attenuated the changes in the levels of both GSH and GSSG at 24 h and 48 h postburn (P<0.05, P<0.01). At 24 h postburn the activity of GSH-Px in the Zn_7-MT 10 μmol/L group was lower but the difference was not significant (P>0.05), whereas GSH-Re activity was significantly lower (P<0.05) in comparison with that in injury group. However, at 48 h after injury, both activities of GSH-Px (P<0.01) and GSH-Re (P<0.05) in the protection group were higher than those in the injury group.

Zn_7-MT 10 μmol/L also attenuated the decrease in plasma α-tocopherol concentration of burned rats (P<0.05) in contrast to injury group (Tab 2).

In comparison with injury group Zn_7-MT 10 μmol/L

Tab 2. Ameliorative effects of Zn_7-MT (10 mmol/L) on the levels of plasma a-tocopherol, liver MT and MDA in the burned rats. *n*=8-9 rats. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* the corresponding normal control. [e]*P*<0.05 *vs* the corresponding injury group.

	Treatmet	α-Tocopherol/ μmol·L^{-1}	MDA/ nmol·g^{-1} tissue	MT/ nmol·g^{-1} protein
24 h	Control	14.7±1.9	53±7	35±3
	Injury	9.4±1.3[b]	300±4[c]	171±24[c]
	Zn_7-MT	12.7±0.4[e]	264±8[e]	208±21[e]
48 h	Injury		320±14[c]	235±30[c]
	Zn_7-MT		267±16[e]	321±32[e]

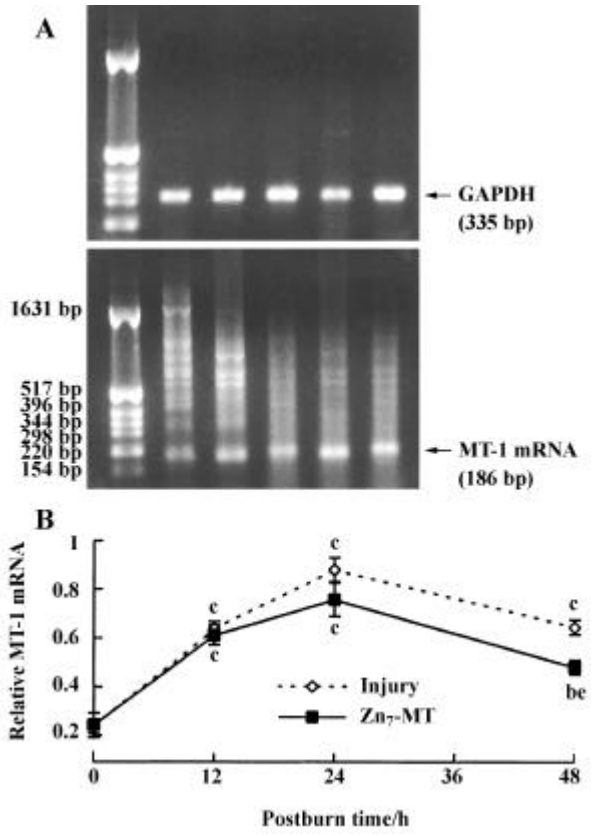

Fig 2. Ameliorative effects of Zn_7-MT (10 mmol/L) on liver MT-1 mRNA overexpression of burned rats at different time points. RT-PCR products were electrophoresed on 1.5 % agarose gels containing ethidium bromide (Fig 2A). Relative MT-1 mRNA abundance is expressed as relative MT-1 mRNA/GAPDH mRNA signal intensity (Fig 2B). *n*=(3-4) dishes of 4 independent experiments. Mean±SD. ^{b}*P*<0.05, ^{c}*P*<0.01 *vs* the normal control. ^{e}*P*<0.05 *vs* the corresponding injury group.

effectively inhibited the increase in MDA level both at 24 h (P<0.01) and 48 h (P<0.05) (Tab 2).

After administration of Zn_7-MT 10 μmol/L, Zn concentration in serum of burned rats did not show significant change at the time points tested. However, the concentrations of Zn (P<0.01) and MT protein (P< 0.05) in the liver was increased both at 24 h and at 48 h compared with the injury group (Fig 1, Tab 2).

Zn_7-MT 10 μmol/L given postburn partially inhibited MT-1 mRNA overexpression at 48 h (P<0.05) compared with the injury group (Fig 2).

DISCUSSION

In the present study, the liver GSH depletion, GSSG and MDA accumulation, reduction of α-tocopherol in plasma, and changes in GSH-related enzymes suggested an oxidative stress in the liver of rats with severe thermal injury.

GSH-related enzymes are essential components of antioxidative system. GSH, as the substrate of GSH-Px, protects against oxidative stress by spontaneous reaction with oxidants. GSH-Re catalyses the conversion of GSSG to GSH. Several studies associated an increase in antioxidant activities with a state of oxidative stress in cells[27]. Results in the present study showed increase in GSH-Px and GSH-Re activities at 24 h postburn, an increased expression and activity of antioxidant enzymes could give an effective defense against ROS. However, at 48 h the decline in both GSH-Px and GSH-Re activities may be due to modification of the sulfhydryl groups in these two enzymes by ROS or by MDA[28,29].

Usually, during oxidative stress conditions the element Zn is redistributed[30]. After severe thermal injury, Zn accumulation in the liver suggested that Zn-demand enzymes were actively synthesized to support the systemic function postburn.

Two features of metallothionein[12] make this protein attracting. One is its unusual amino acid composition – one-third of its 61 residues are cysteines and the other is its high metal content – 7 g atoms of metal for every mole protein. Compared with GSH, thionein moiety in MT would be 20 times in the number of sulfhydryl group, so MT has a high reactivity and no formation of S-S bonds. Sulfhydryl groups confer a high degree of reactivity for free radicals and electrophiles. Therefore, MT can serve as a sacrificial target for attack of ROS and thus protect cells. Moreover, MT is an acute phase protein, whose concentration could be changed substantially in response to oxidative stress[3,31,32]. In the present work, severe thermal injury resulted in decrease in the level of natural antioxidants, however, MT overexpression would be a compensative mechanism of natural defense system.

Obviously, results in this study showed that Zn_7-MT (10 μmol/L) in resuscitation fluid effectively antagonized oxidative stress in the liver of burned rats. Zn_7-MT represents that in one MT molecule there are 7 zinc ions as the metal ligand coordinated with sulfhydryl groups. The affinity of Zn for the binding sites is less than that of Cd, Cu, and Ag[12], suggesting that zinc release from MT is easier. Being a nutrient, zinc is the element at active sites in over 300 enzymes with remarkably diverse function. Therefore, exogenous Zn_7-MT given postburn increased the concentrations of both MT protein and Zn element in the liver. The thionein in

Zn_7-MT regulated the redox state in cells and the Zn released from this protein could supply Zn-demand enzyme to regulate gene transcription, cell proliferation, differentiation and other Zn-dependent cellular processes and to enhance the repair potency after severe thermal injury. Last, it should be mentioned that in protection group MT-1 mRNA overexpression was partially inhibited and it was most likely due to significant inhibition of oxidative stress in the liver by exogenous Zn_7-MT.

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