Biometals (2008) 21:1–8
DOI 10.1007/s10534-007-9086-7

Therapeutic potentials of combined use of DMSA with calcium and ascorbic acid in the treatment of mild to moderately lead intoxicated mice

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Received: 4 August 2006 / Accepted: 18 January 2007 / Published online: 8 February 2007

Abstract The aim of this study was to explore the therapeutic efficacies of combined use of meso-2,3-dimercaptosuccinic acid (DMSA) with calcium and ascorbic acid in the treatment of mild to moderately lead-intoxicated mice. Female albino mice were exposed to lead by drinking water contaminated with 0.1% (moderate lead exposure) or 0.05% (mild lead exposure) lead acetate. After the cessation of lead exposure, mice were supplemented by gavage with saline solution, 50 mg/kg body weight (b.w) DMSA, 100 mg/kg b.w DMSA, calcium and ascorbic acid, or 50 mg/kg b.w DMSA and calcium as well as ascorbic acid, respectively. Atomic absorption spectrophotometric method was used to analyze lead levels in blood, bone, liver, kidney and brain. Activities of blood δ-aminolevulinic acid dehydratase (ALAD) were determined by colorimetric method. DMSA supplemented alone could reduce lead levels in both soft tissues and bone and reverse lead-inhibited activities of blood ALAD in mild to moderately lead-intoxicated mice. On the other hand, combined use of DMSA with calcium and ascorbic acid achieved better therapeutic efficacies in mobilizing lead in blood, liver and kidney, and reversing lead-inhibited activities of blood ALAD in moderately lead intoxicated mice than DMSA supplemented alone. Moreover, the better therapeutic efficacies were also found in mildly lead intoxicated mice in mobilizing lead in blood and bone achieved by combined use of DMSA with calcium and ascorbic acid. Combined use of DMSA with calcium and ascorbic acid seems to be the better choice in the treatment of mild to moderate lead-intoxication.

Keywords Lead intoxicated mice · Meso–2,3-dimercaptosuccinic acid (DMSA) · Calcium · Ascorbic acid · Lead body burden · δ-aminolevulinic acid dehydratase (ALAD)

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Introduction

Lead poisoning remains a common disease among children in China (Wang and Zhang 2006) and almost the other regions in the world (Canfield et al. 2003, Lidsky and Schneider 2003). Recently, Chen et al. (2005) reported a stronger relationship in children between blood lead levels (BLLs) and intelligence quotient (IQ) at 7 years of age than that between IQ at 7 years of age and the higher BLLs at 2 years of age, and the association of BLLs and IQ at 5 years of age was also stronger than that between IQ at 5 years of age and BLLs at 2 years of age. Their data suggested clearly that lead exposure would continue to be toxic to children when they reached school age, and did not support the viewpoint that all intellectual damage is done before 2 or 3 years of age. Thus, the effects of concurrent BLLs on IQ may be greater than currently believed. If higher concurrent BLLs were associated with lower IQ, it is plausible that we should attempt to keep BLLs low in children. On the other hand, a study reported by Roberts et al. (2001) showed that BLLs in children with 25–29 μg/dL required 24 months to decline to less than 10 μg/dL, although all families received education, counsel and lead hazard identification when possible. These findings underscored the need for chelating agents to reduce lead body burden more rapidly than that can be achieved through environmental efforts alone.

Despite growing concern regarding the detrimental effects on mild to moderately lead poisoned children and adults, pharmacological treatment is controversial (Dietrich et al. 2004, Peterson et al. 2004). Meso–2,3-dimercaptosuccinic acid (DMSA), is a water-soluble, sulfhydryl-containing compound which is an effective oral chelator of heavy metals, and was identified as an effective antidote to heavy metal poisoning over 50 years ago (Miller 1998). DMSA was licensed by the Food and Drug Administration in 1991, and is the first approved oral lead chelator in treatment of lead poisoning in the United States. More recent clinical use and research substantiated its efficacy and safety, and established it as the premier lead chelating agent, based on its oral administration, specificity for lead and lower urinary excretion of essential minerals compared with the other chelating agents (Nightangle 1991, Liebelt et al. 1994, Smith et al. 2000, Cremin et al. 2001, Stangle et al. 2004). However, the major disadvantage of DMSA in chelation therapy is its inability to form complexes with the intracellular lead because of its lipophobic nature. On the other hand, some adverse reactions to DMSA were also reported, including mild gastrointestinal symptoms, reversible neutropenia and transient elevation of liver enzymes when it was treated in a large dose or long term course of administration (American Academy of Pediatrics 1995).

A new trend in chelation therapy with DMSA has emerged recently, which was to use combined therapy instead of therapy with DMSA alone (Kalia and Flora 2005). Vitamins, essential metals or amino acids, when supplemented with DMSA have been found to be beneficial in mobilizing lead in the body and providing recovery in a number of altered biochemical variables. Calcium is one of the well-studied factors used in the treatment of lead intoxication. Because calcium-binding proteins have a high affinity for lead, it was assumed competition for calcium-binding sites might underlie the mechanism of calcium and lead interactions (Fullmer 1991; Han et al. 2000). Silbergeld et al. (1988) reported that calcium intake was negatively correlated with the BLLs. Ascorbic acid is another factor which has been well studied in the treatment of lead poisoning. Beneficial effects of ascorbic acid in the treatment of lead poisoning could be attributed to its ability to scavenge free radicals and form complexes with lead, thereby, curtailing lead-induced oxidative stress and decreasing intestinal absorption as well as increasing renal lead clearance (Dalley et al. 1990; Houston and Johnson 2000; Gurer and Ercal 2000; Kleszczewska 2001; Upasani et al. 2001; Hsu and Guo 2002; Shalana et al. 2005). Although the beneficial effects of DMSA, calcium or ascorbic acid in the treatment of lead poisoning have been well-testified, the therapeutic potentials of calcium and ascorbic acid in conjunction with DMSA in chelating therapy of lead poisoning has not been extensively studied, except for a few reports on combined use of DMSA with calcium

or ascorbic acid alone (Varnai et al. 2003, 2004; Flora et al. 2003). Consequently, the objective in present study was to explore the therapeutic potentials of DMSA used with calcium and ascorbic acid in the treatment of mild to moderately lead intoxicated mice.

Materials and methods

Animals

Total 98 female albino mice, weighing 15.0 ± 1.0 g, were used, which were purchased from the animal laboratory of China Medical University. Animal room was kept at a temperature of 20 ± 2°C with a 12 h light/dark cycle and a relative humidity of 50–60%. Free access to food and water was allowed at all the time. The mice were housed five per cage in the sterilized plastic cages with wood shaving bedding. Institutional Animal Care and Use Committees in China Medical University approved our experimental protocol.

Procedures

Therapeutic potentials of DMSA used with calcium and ascorbic acid were evaluated in 2 animal models with mild to moderate lead exposure, in which the lower-dosing regimen produced lead intoxicated mice with BLLs around 20 μg/dL and the higher-dosing regimen produced BLLs around 40 μg/dL. In the first experiment (moderate lead exposure), 48 mice were divided randomly into two groups, which were lead exposed group (40 mice) and control (eight mice). Mice in lead exposed group drank distilled water contaminated with 0.1% lead acetate for consecutive 4 weeks, and in control drank distilled water. After cessation of lead exposure, mice in the lead exposed group were subdivided into 5 groups with eight mice in each group. Mice in different groups were treated with saline solution (untreated control), 50 mg/kg body weight (b.w) DMSA (DMSA alone), 100 mg/kg b.w DMSA (DMSA high), 400 mg/kg b.w calcium carbonate and 100 mg/kg b.w ascorbic acid (Ca + Vc) or 50 mg/kg b.w DMSA and 400 mg/kg b.w calcium carbonate as well as 100 mg/kg b.w ascorbic acid (DMSA + Ca + Vc), respectively. Dosing regimens of DMSA were determined based on the previous reports (Stangle et al. 2004, Kostial et al. 1999, Varnai et al. 2004). Calcium carbonate, ascorbic acid or DMSA was dissolved into redistilled water to its desired concentration, then mixed and given by gavage to mice every other day for four consecutive weeks. Mice in control were treated with saline solution by gavage. All solutions were prepared immediately prior to application. 24 h after the last supplementation, mice were anaesthetized and rapidly dissected. Blood was collected by cardiac puncture in heparinized tubes. Liver, kidney and brain as well as left thighbone were removed immediately.

In the second experiment (mild lead exposure), mice in the lead exposed group drank distilled water contaminated with 0.05% lead acetate for 4 consecutive weeks. Totally, five groups were divided with 10 mice in each group, which were untreated control, DMSA alone, DMSA high, DMSA + Ca + Vc and control. The other procedures were the same as those in the first experiment. Blood and left thighbone were taken in the second experiment.

All samples were kept in –80°C freezer until analysis.

Reagents and laboratory wares

All reagents used in present study were analytical grade, and acids were of specific grade for pollutant metal analysis. All glasses and plastic wares were washed with detergent and acid, and then rinsed with redistilled water to be free of metal leaching. Water used in this study was redistilled. δ-aminolevulinic acid (δ-ALA) was purchased from Sigma Chemical Co., St. Louis, MO. DMSA, ascorbic acid and calcium carbonate were purchased from the Chemicals Company in Shanghai, P. R. China.

Analysis procedures

Lead levels

About 100 mg of brain, kidney, liver and bone were wet digested by heating with 1 ml of nitric

acid and 0.5 ml of perchloric acid. Blood was digested by 0.1% nitric acid. Atomic absorption spectrophotometry-Graphite Furnace (Varian spectra-AA 40P, USA) was used following a standardized analytical method (Jin et al. 2000) with an accuracy of ± 5 μg/L. Instrumental parameters used for sample analysis were: drying for 65 s between 85 and 120°C, charring for 30 s between 300 and 480°C, atomization for 3 s at 1850°C, cleaning for 4 s at 2,700°C. Photometry was performed at a wavelength of 283.3 nm, used a lead hollow cathode lamp with a current supply of 7.5 mA, taken advantage of Zeeman background correction. Duplicate determinations were carried out for each sample and the average was taken as a measure. Quality control was performed by determination of the reference samples from the Centers for Disease Control (CDC) in the United States as participation in the CDC Proficiency Testing Program. The test results were in good agreement with the reference values.

ALAD activity

Determination was by the method of Berlin and Schaller (1974). Briefly, 0.02 ml of whole blood was added into 0.48 ml redistilled-water. The reaction was started by adding substrate (δ-ALA) into the hemolysate and incubated for 60 min at 38°C. The reaction product (porphobilinogen) was determined using modified Ehrlich's reagents and measured at 555 nm. Enzyme activity was evaluated by the amount of porphobilinogen formed at 38°C. One unit of ALAD activity was defined as increase of absorbance per 0.1 by 1 ml of blood. Results were reported in U/g hemoglobin.

Concentrations of hemoglobin in blood were measured by cyanmethemoglobin method using Drabkin's reagent.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). SPSS for Windows, version 11.5, (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The significance of difference was evaluated by one-way analysis of variance (ANOVA) followed by Post Hoc tests of Student–Newman–Keuls test (SNK) to compare means among different groups. The statistical significance was defined as $P < 0.05$.

Results

Changes of lead levels in blood and bone and activities of blood ALAD among different groups in moderately lead-exposed mice were shown in Table 1. Lead levels in blood and bone increased, while activities of blood ALAD decreased significantly after lead exposure. DMSA Supplemented alone could reduce lead levels in blood and bone significantly, and its chelating efficacies increased along with the amounts treated. On the other hand, BLLs were lower significantly in mice treated with DMSA + Ca + Vc than in mice treated with ''DMSA alone'', and the lowest in mice treated with ''DMSA high'', ''DMSA alone'' and DMSA + Ca + Vc. Activities of blood ALAD were significantly higher in mice treated with DMSA + Ca + Vc than in untreated mice, whereas the difference of activities of blood ALAD between mice treated with ''DMSA alone'' and untreated was not significant. Comparison of lead levels in bone between mice treated with DMSA + Ca + Vc and ''DMSA alone'' was not significant. Supplementation of calcium and ascorbic acid achieved no benefit in reducing lead levels in blood and bone and increasing activities of blood ALAD.

Changes of lead levels in liver, kidney and brain among different groups in moderately lead-exposed mice were shown in Table 2. Lead levels in liver, kidney and brain increased significantly after lead exposure. DMSA supplemented alone could reduce significantly lead levels in these organs. Lead levels in liver were lower significantly in mice treated with DMSA + Ca + Vc than in mice treated with ''DMSA alone''. Comparison of lead levels in kidney and brain between mice treated with DMSA + Ca + Vc and ''DMSA alone'' was not significant, however lead levels in kidney were lower significantly in mice treated with DMSA + Ca + Vc than in untreated

Table 1 Comparison of lead levels in blood and bone and activities of blood ALAD among groups in moderate lead exposure (mean ± SD)

Group	N	Blood lead levels (μg/L)	Blood ALAD activities (U/g Hb)	Bone lead levels (μg/g wet weight)
Untreated	8	423.23 ± 51.32a	53.41 ± 13.44a	373.15 ± 39.53a
DMSA alone	8	332.31 ± 51.61b	85.41 ± 27.66ab	282.24 ± 53.21b
DMSA high	8	273.74 ± 22.74c	141.85 ± 57.35c	227.39 ± 45.26c
Ca + Vc	8	401.03 ± 62.88a	94.43 ± 21.62ab	372.45 ± 30.42a
DMSA + Ca + Vc	8	241.39 ± 21.36c	119.80 ± 18.03bc	275.52 ± 32.74b
Control	8	14.56 ± 5.74d	191.18 ± 45.32d	0.47 ± 0.19d

Notes: Means with different letter are significant ($P < 0.05$)

Mice in ''Untreated group'' was treated with no agent in addition to lead, so it was positive control; mice in ''DMSA alone'' treated with 50 mg/kg b.w DMSA; ''DMSA high'' treated with 100 mg/kg b.w DMSA; ''Ca + Vc'' treated with 400 mg/kg b.w calcium carbonate and 100 mg/kg b.w ascorbic acid; ''DMSA + Ca + Vc'' treated with 50 mg/kg b.w DMSA , 400 mg/kg b.w calcium carbonate and 100 mg/kg b.w ascorbic acid. Mice in ''Control'' were not exposed to lead, it was negative control

Table 2 Comparison of lead levels in liver, kidney and brain among groups in moderate lead exposure (mean ± SD μg/g wet weight)

Group	N	Lead levels in liver	Lead levels in kidney	Lead levels in brain
Untreated	8	1.13 ± 0.26a	5.22 ± 0.72a	0.86 ± 0.12a
DMSA alone	8	0.83 ± 0.33b	4.31 ± 1.06ab	0.67 ± 0.23bc
DMSA high	8	0.73 ± 0.24bc	2.23 ± 0.70c	0.42 ± 0.17d
Ca + Vc	8	1.30 ± 0.30a	5.13 ± 0.92a	0.81 ± 0.14ab
DMSA + Ca + Vc	8	0.50 ± 0.15c	3.54 ± 0.66b	0.63 ± 0.09c
Control	8	0.09 ± 0.10d	0.32 ± 0.15d	0.03 ± 0.02e

Notes: Means with different letter are significant ($P < 0.05$)

Mice in ''Untreated group'' was treated with no agent in addition to lead, so it was positive control; mice in ''DMSA alone'' treated with 50 mg/kg b.w DMSA; ''DMSA high'' treated with 100 mg/kg b.w DMSA; ''Ca + Vc'' treated with 400 mg/kg b.w calcium carbonate and 100 mg/kg b.w ascorbic acid; ''DMSA + Ca + Vc'' treated with 50 mg/kg b.w DMSA , 400 mg/kg b.w calcium carbonate and 100 mg/kg b.w ascorbic acid. Mice in ''Control'' were not exposed to lead, it was negative control

mice, whereas they were not significantly different between mice treated with ''DMSA alone'' and untreated. Supplementation of calcium and ascorbic acid failed to show any benefits in decreasing lead levels in liver, kidney and brain.

Changes of lead levels in blood and bone and activities of blood ALAD in mildly lead- exposed mice were shown in Table 3. Lead levels in blood and bone increased, while blood ALAD activities decreased significantly after lead exposure. DMSA Supplemented alone could also reduce lead levels in blood and bone, and ameliorate activities of blood ALAD. BLLs in mice treated with DMSA + Ca + Vc were the lowest in mice treated with ''DMSA high'', ''DMSA alone'' and DMSA + Ca + Vc as shown in moderately lead exposed mice, although differences among them were not significant. On the other hand, comparison of blood ALAD activities between mice treated with DMSA + Ca + Vc and ''DMSA alone'' was not significant, however the difference between mice treated with ''DMSA high'' and ''DMSA alone'' was significant, whereas it was not significant between mice treated with ''DMSA high'' and DMSA + Ca + Vc. Lead levels in bone were lower significantly in mice treated with DMSA + Ca + Vc than in mice treated with ''DMSA alone''.

Discussion

Our study provided an experimental evidence for the beneficial role of combined use of DMSA

Table 3 Comparison of lead levels in blood and bone and activities of blood ALAD among groups in mild lead exposure (mean ± SD)

Group	*N*	Blood lead levels (μg/L)	Blood ALAD activities (U/g Hb)	Bone lead levels (μg/g wet weight)
Untreated	10	213.37 ± 41.12a	95.76 ± 46.64a	197.59 ± 44.36a
DMSA alone	10	165.71 ± 36.82b	94.44 ± 49.46a	176.81 ± 38.10ab
DMSA high	10	143.73 ± 33.51b	156.47 ± 53.84bc	161.98 ± 28.47bc
DMSA + Ca + Vc	10	137.72 ± 19.37b	118.72 ± 30.82ab	135.48 ± 21.96c
Control	10	13.35 ± 5.11c	193.10 ± 34.00c	0.88 ± 0.32d

Notes: Means with different letter are significant ($P < 0.05$)

Mice in ''Untreated group'' was treated with no agent in addition to lead, so it was positive control; mice in ''DMSA alone'' treated with 50 mg/kg b.w DMSA; ''DMSA high'' treated with 100 mg/kg b.w DMSA; ''Ca + Vc'' treated with 400 mg/kg b.w calcium carbonate and 100 mg/kg b.w ascorbic acid; ''DMSA + Ca + Vc'' treated with 50 mg/kg b.w DMSA , 400 mg/kg b.w calcium carbonate and 100 mg/kg b.w ascorbic acid. Mice in ''Control'' were not exposed to lead, it was negative control

with calcium and ascorbic acid in the treatment of mild to moderately lead-intoxicated mice. To our knowledge, this study was the first report to explore therapeutic efficacies of DMSA in conjunction with calcium and ascorbic acid in the treatment of mild to moderate lead-intoxication. Our findings disclosed that pronounced therapeutic efficacies could be achieved when DMSA supplemented with calcium and ascorbic acid in the treatment of mild to moderately lead-intoxicated mice. On the other hand, our data suggested higher doses of DMSA should be used in order to achieve the pronounced therapeutic efficacies in the treatment of mild lead-intoxication when DMSA applied alone. As mice were treated after cessation of lead exposure, the chelating efficacies to lead reported in this study were assumed to be achieved after the level of intestinal absorption. Supplementation of calcium and ascorbic acid failed to achieve any benefits in reducing lead levels in the body and ameliorating activities of blood ALAD, which was in agreement with the viewpoints proposed by the other researchers that the chelating effects of calcium and ascorbic acid were mainly achieved in the gastrointestinal tract.

As a chelator, DMSA can form water-soluble complexes with lead in the body, and increase its urinary excretion. DMSA was reported to deplete lead effectively in soft tissue and assumed not to redistribute it to brain. Our findings were consistent with those viewpoints. Because of the hydrophilic and lipophobic properties, DMSA can not cross the cell membrane and form complexes directly with intracellular lead. The most likely explanation for depletion of intracellular lead is that DMSA can form complexes with lead in serum and deplete it, then increase the lead concentration gradient between serum and cells, which would favor efflux of intracellular lead. Thereby, the higher amount of DMSA given, the larger concentration gradients could be achieved, and more efflux of lead, therefore, the better chelating efficacies to lead could be achieved.

Varnai et al. (2004) reported that calcium supplemented during lead exposure could reduce tissue lead, but had no effect when applied after lead exposure. Combined use of calcium with DMSA during lead exposure caused a greater reduction in tissue lead than either DMSA or calcium treated alone. However, when administered after lead exposure, they had no advantage over DMSA applied alone. A significant increase in tissue lead was observed in lead exposed rats consuming low levels of calcium and attributed to less competition with lead for calcium-binding sites in gastrointestinal tract (Markowitz et al. 2004). Interaction between lead and calcium can occur at several sites in the body, including cellular mechanisms that regulate ion transports across cell membrane, competition for these sites might hamper lead from entering into the cells (Pounds 1991).

Dawson et al. (1999) reported supplementation of 1000 mg ascorbic acid daily could result in a significant decrease of BLLs, however, urinary lead excretion showed little change, suggested its

protective effects were due to the inhibition of intestinal absorption of lead. On the other hand, it was reported that treatment with ascorbic acid could result in amelioration of oxidative stress without a significant decline in tissue lead burden (Patra et al. 2001; Simon and Hudes 1999). Studies reported by Flora et al. (2003) and Varnai et al. (2003) showed that when ascorbic acid was given along with DMSA, it was capable of reversing lead-inhibited activities of blood ALAD compared to DMSA given alone, however, combined use of ascorbic acid and DMSA during lead exposure was substantially less effective than DMSA treatment alone, and did not affect DMSA chelating efficacy when administered after lead exposure.

Our findings suggested supplementation of DMSA with calcium and ascorbic acid after lead exposure could achieve pronounced therapeutic efficacies in depleting tissue lead and ameliorating activities of blood ALAD compared to DMSA given alone. A study reported by Kalra et al. (2002) showed a pronounced chelating efficacy when DMSA given along with calcium, ascorbic acid, iron and zinc in treatment of children with symptomatic lead poisoning. Although we have no immediate explanation for the results observed in present study, there is a possibility that as an antioxidant, ascorbic acid can scavenge reactive oxygen species and protect the thiols of DMSA from oxidation, thereby benefit the chelating status of DMSA. Moreover, ascorbic acid can form complexes with intracellular lead and may benefit its efflux. On the other hand, calcium can compete with lead for its extra- and intra- cellular binding sites, which could benefit DMSA and ascorbic acid to form complexes with lead in both extra- and intra- cellular sites. Therefore, it is reasonable to speculate that it is the combined beneficial effects of calcium and ascorbic acid on chelating efficacies of DMSA, provided the pronounced therapeutic efficacies in lead poisoning.

According to the results in present study, supplementation of DMSA along with calcium and ascorbic acid seems to be the better therapeutic choice in the treatment of mild to moderate lead-intoxication. However, much more data are needed before reaching such conclusions, since the results have been obtained only in limited animal models.

Acknowledgments This study was funded by the Liaoning Scientific foundation, China. The project number is 20032063 and 2001102049.

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