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# Antioxidant and micronutrient-rich milk formula reduces lead poisoning and related oxidative damage in lead-exposed mice

Yu Zhang [a], Qingqing Li [b], Xiaojie Liu [c], Hui Zhu [c], Aihua Song [b], Jingjing Jiao [b,*]

[a] Department of Food Science and Nutrition, College of Biosystems Engineering and Food Science, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058, China
[b] Department of Nutrition and Food Hygiene, School of Public Health, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058, China
[c] Analysis Center for Scientific Committee, Wahaha Group Co. Ltd., 10th Street, Hangzhou 310018, China

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ABSTRACT

Lead poisoning is a global environmental disease that induces lifelong adverse health effects. The effect of a milk formula consisting of antioxidant of bamboo leaves (AOB), vitamin C (Vc), calcium lactate (CaLac), ferrous sulfate ($FeSO_4$) and zinc sulfate ($ZnSO_4$) on the reduction of lead and lead-induced oxidative damage in lead-exposed mice was studied. The lead-reducing effect of milk formula was investigated via a 7-week toxicokinetics study and a tissue distribution level examination. The ameliorating effect of milk formula on lead-induced oxidative damage was investigated. Results demonstrated current milk formula could effectively reduce blood lead levels (BLLs) and lead distribution levels of liver, kidneys, thighbones and brain in mice based on metal ion-mediated antagonism and chelation mechanisms. This milk formula could not only protect lead-susceptible tissues against lead poisoning, but also maintain normal absorption and distribution of essential elements *in vivo*. Meanwhile, current milk formula could prevent the reduction of δ-aminolevulinic acid dehydratase (δ-ALAD) activity and enhancement of free erythrocyte protoporphyrins (FEP) levels in blood erythrocytes of mice. Also, this formula could indirectly protect blood cell membranes against lead-induced lipid peroxidation. We conclude that current optimized milk formula effectively reduces lead poisoning and lead-induced *in vivo* oxidative damage in lead-exposed mice.



## 1. Introduction

Lead exposure in environmental and occupational public continues to be a serious health problem. It is well-known that lead exposure is dangerous for humans because high blood lead levels (BLLs) could lead to neural pathogenesis and intellectual retardation. BLL, even those below 10 μg/dL, are inversely associated with children's IQ scores at 3 and 5 years of age (Bellinger et al., 1992; Bellinger and Needleman, 2003). Also, lead can remain in the bones for several decades. In adults about 90% of the total amount of lead in the body is contained in bones and teeth, while approximately 75% of lead in children's bodies is stored in their bones (Canfield et al., 2003).

The traditional medical treatment available for lead poisoning therapy is chelation, which can save lives in persons with very high BLLs. Current commonly used chelating chemicals include: (i) $CaNa_2EDTA$, which has been used for treatment of lead poisoning over the past 38 years; and (ii) *meso*-2,3-dimercaptosuccinic acid (DMSA), a new and promising chelating chemical which is classified as an investigational new drug by the US FDA (Chisolm, 1990). However, chelating drugs are not always available in developing countries and have limited value in reducing the sequelae of lead poisoning (Rogan et al., 2001; Dietrich et al., 2004). Currently, research progress has focused on eliminating lead and formulating some practical additives to reduce BLLs in both human and animal studies. Some recent studies demonstrated that ascorbic acid (Vc) supplementation could effectively ameliorate detrimental effects of lead-induced histopathological alterations and apoptosis in rats (Kermanian et al., 2010; El-Neweshy and El-Sayed, 2011). Vc combined with thiamine (vitamin $B_1$) supplementation can exhibit a protective effect in the reproductive system and liver cells by inhibiting lead-induced excessive cell apoptosis in mice (Wang et al., 2007; Shan et al., 2009). Also, trace elements (e.g. calcium, iron, zinc and selenium) were considered as another potential agent (Kordas et al., 2007; Schrauzer, 2008; Ettinger et al., 2009). Compared to the use of all of above micronutrient additives, chelation therapy has strong but short-term reduction effects in the overall long-term management of lead exposure.

*Abbreviations:* δ-ALAD, δ-aminolevulinic acid dehydratase; AOB, antioxidant of bamboo leaves; BLLs, blood lead levels; CaLac, calcium lactate; DMSA, *meso*-2,3-dimercaptosuccinic acid; FEPs, free erythrocyte protoporphyrins; GFAAS, graphite furnace atomic absorption spectrometer; MDA, malondialdehyde; PBG, porphobilinogen; Vc, vitamin C.

* Corresponding author. Tel.: +86 571 8820 5790; fax: +86 571 8820 5791.
*E-mail address:* jingjingjiao@zju.edu.cn (J. Jiao).

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It is well known that excess lead exposure inevitably affects function and structure of blood erythrocytes and induces the occurrence of oxidative damage (Quintanar-Escorza et al., 2007). Meanwhile, lead can reduce the activity of superoxide dismutase and antioxidant related enzymes in blood erythrocytes (Rendón-Ramírez et al., 2007). Cytoplastic enzyme δ-aminolevulinic acid dehydratase (δ-ALAD), the second enzyme in the heme synthesis pathway, catalyzes the condensation of two molecules of δ-aminolevulinic acid to generate one molecule of porphobilinogen (PBG) and is highly sensitive to lead exposure (Wang et al., 2010). The protection of δ-ALAD activity plays an important role in the prevention of lead poisoning and oxidative/peroxidative damage of blood erythrocytes. However, little attention has been paid to protein damage caused by lead-induced oxidative damage, though it has been suggested that antioxidant or micronutrient treatment could prevent these toxic alterations.

Recently, we demonstrated that some dietary supplements, i.e. calcium lactate (CaLac), zinc sulfate ($ZnSO_4$), ferrous sulfate ($FeSO_4$) and Vc, and natural antioxidants [extract of wolfberry, extract of white chrysanthemum and antioxidant of bamboo leaves (AOB)] have promising capacity of reducing BLL in lead-exposed mice (Jiao et al., 2011). To get a deeper understanding of the reducing effect of supplements and antioxidants on lead poisoning and related oxidative damage, we now conducted toxicokinetics, tissue distribution and lead-induced oxidative damage studies for investigating the functionality of selected antioxidants and micronutrients in lead-exposed mice. CaLac, $ZnSO_4$, $FeSO_4$, Vc and AOB were selected as additives for the preparation of milk formula due to their promising reducing effect on lead poisoning according to our previous work (Jiao et al., 2011).

## 2. Materials and methods

### 2.1. Materials

Milk was freshly prepared each time before oral gavage by dissolving fat-free powdered milk (15 g, Wahaha Group Co., Ltd., Hangzhou, China) into water (100 mL). Finally, the calcium concentration of this prepared milk was 1.83 mg/mL, which was determined by GFAAS analysis in the present work. The stock solution of AOB (25 mg/mL) was freshly prepared by dissolving AOB concentrated solution into deionized water. AOB concentrated solution was provided by Zhejiang University Innoessen Co., Ltd. (Hangzhou, Zhejiang, China). AOB was prepared from the bamboo leaves of *Phyllostachys nigra* var. *henonis* identified by Research Institute of Subtropical Forestry of the Chinese Academy of Forestry (Hangzhou, Zhejiang, China) according to our previous study (Zhang et al., 2008). Briefly, fresh bamboo leaves were collected during the autumn season in Anji district (Zhejiang, China) and then air dried. The coarse powder of bamboo leaves was obtained after comminution and filtration (20–40 mesh) and 10 g powder was extracted for 1 h by 100 mL of 30% (v/v) ethanol aqueous solution using the hot reflux method. The filtrate was then isolated by membrane filtration to remove macro- and micro-molecular components such as polysaccharides and minerals and AOB concentrated solution was finally obtained after concentrating in vacuum. AOB including flavonoids, lactones and phenolic acids were analytically characterized and reported previously (Lu et al., 2005). Three kinds of milk formula containing different levels of antioxidants and micronutrients were freshly prepared via adding different quantities of AOB, Vc, CaLac, $FeSO_4$ and $ZnSO_4$ into above-prepared milk (100 mL) (Table 1). The addition levels of supplements in milk formula were selected according to Regulations of Codex Alimentarius Commission and China National Standards GB 2760-2007 (CAC/GL, 2008; China National Standard, 2007). Meanwhile, the stock solution of $CaNa_2EDTA$ (1.6 mg/mL) was prepared for gavage feeding in the positive control group.

### 2.2. Animals

Five-week old healthy male imprinting control region (ICR) mice (average weight: 30 g, $n = 105$) were obtained from Zhejiang University School of Medicine Laboratory Animal Center (Hangzhou, China) and used in the present work. Mice were kept in cages (20 × 15 × 15 cm, 5 mice each) with free access to basal feed and deionized water under a 12 h light/12 h dark cycle in a humidity (50 ± 10)% and temperature (20 ± 1) °C controlled room. Mice were placed in the experimental environment in advance for a 2-week adaptation period. This study was reviewed and approved by Zhejiang University School of Medicine Ethical Review Board Committee.

### 2.3. Establishment of high BLL models in mice

To establish high BLL models, mice received peritoneal injection (ip) with lead acetate solution (12.8 μg/g bw, i.e. 7 μg $Pb^{2+}$/g bw) once daily for 7 d according to previous study (Zhu et al., 2004). Mice in negative control group were administered with deionized water. Then, mice were feed-deprived overnight but allowed to drink deionized water *ad libitum*. Blood samples (0.5 mL) were collected using the retroorbital vein bleeding method. Finally after the quantification of BLLs, mice used for the establishment of high BLL models were screened and selected according to previous report (China Ministry of Health, 2002; Jiang et al., 2006). Mice with BLLs of more than 60 μg/dL were regarded as successful models. However, mice with BLLs of less than 60 μg/dL or more than 600 μg/dL were excluded.

### 2.4. Experimental groups and toxicokinetics study

The mice with eligible BLLs (~80% of original 105 mice) were then randomly divided into 6 groups ($n = 12$): (i) high BLL model control group; (ii) positive control group ($CaNa_2EDTA$); (iii) milk group (without the use of any additive); (iv) milk formula group I; (v) milk formula group II; and (vi) milk formula group III. The milk formula groups I–III referred to the experimental groups of milk with addition of different levels of AOB, Vc, CaLac, $FeSO_4$ and $ZnSO_4$ (Table 1). Mice not pretreated with lead acetate were considered as an additional negative control group ($n = 12$). For each group except negative and high BLL model control groups mice were administered with $CaNa_2EDTA$ (16 μg/g bw), milk or milk formula by gavage feeding. Mice in negative and high BLL model control groups were administered with deionized water, respectively. In the toxicokinetics study, all of mice were administered once daily for 9 weeks. Blood samples (0.5 mL) were collected using the retroorbital vein bleeding method while related BLLs were quantified at day 0, day 1 and at the end of the first, third, fifth, seventh and ninth week. Besides the gavage feeding with $CaNa_2EDTA$, milk or milk formula, mice got free access to basal feed and deionized water. To consider whether the absorption of other essential minerals (e.g. calcium, iron and zinc) was affected, blood calcium, iron and zinc levels were also determined during the whole period of toxicokinetic study. To observe the growth of lead-exposed mice in each group, the body weights of mice in all groups were recorded and compared at the time when blood samples were collected.

### 2.5. Tissue distribution

It is well known that the absorption and metabolism of lead may induce the accumulation of lead *in vivo*. To investigate the effect of milk formula on lead distribution and accumulation in tissues, mice in all groups were killed after 9-week experimental period. Blood samples (1 mL) were collected in anticoagulant tubes after feed-depriving overnight using the ophthalmectomy bleeding method. Some tissues which easily accumulate lead toxin (liver, kidneys, thighbone and brain) were separated and their lead, calcium, iron and zinc levels were quantified and compared.

### 2.6. Determination of lead, calcium, iron and zinc levels in serum and tissues

Lead, calcium, iron and zinc levels in serum and tissues were analyzed by graphite furnace atomic absorption spectrometer (GFAAS) method (Parsons and Slavin, 1993; ISO 6869:2000, 2005; ISO/TS 6733:2006, 2006). Serum samples in all experimental groups were routinely isolated from collected whole blood (500 μL). After killing of mice at the end of toxicokinetic study, liver, kidneys, thighbone and brain samples (0.5–2 g) were wet-weighed and completely homogenized. Blood or homogenized tissue samples were then processed by routing dry-ashing procedures (Szkoda and Żmudzki, 2005). After dry-ashing, samples were diluted 1:10 in an optimized phosphate matrix modifier containing 0.5% (v/v) of Triton X-100 and 0.2% (v/v) of dilute nitric acid. Then, diluted serum or homogenized tissues (12 μL) were deposited in the graphite furnace for quantitative analysis. An atomic absorption spectrometer (Perkin–Elmer 4110 ZL) equipped with graphite furnace and autosampler (Perkin–Elmer As-72) was used for the determination of lead, calcium, iron and zinc levels. The method performance of GFAAS analysis was validated via limit of detection (LOD), limit of quantification (LOQ), precision, spiked recovery and quality control (QC) proficiency test according to our previous study (Jiao et al., 2011).

### 2.7. Evaluation of lead poisoning induced oxidative damage in mice

Lead can reduce the activity of catalase and superoxide dismutase, which breakdown hydroxyl and superoxide free radicals and other reactive oxygen species in blood erythrocytes (Mousa et al., 2002). Therefore, lead poisoning could increase reactive oxygen species and thus induce an oxidative process and damage to proteins in blood erythrocytes. To demonstrate whether milk formula prepared in the present study (containing AOB and Vc as antioxidant agents) could protect δ-ALAD of blood erythrocytes in lead-exposed mice, δ-ALAD activity, FEP levels and lipid peroxidation status were determined at the end of this toxicokinetic study. Analysis of δ-ALAD activity, FEP levels and TBARS levels in blood erythrocytes were