Original Paper

Zinc-Boron Complex-Based Dietary Supplements for Longevity and Healthy Life

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ABSTRACT: The nutritional role of zinc (Zn) and boron (B) in the plant-animal-human food chain is highly topical worldwide research. Our data indicated that Zn-B complex (ZBC)-based dietary supplements can be used as stable non-toxic formulations, with high intestinal absorption rate, inducing alpha2-macroglobulin (A2M) expression for longevity and healthy life. ZBC is metabolized by hydrolysis, mainly at the absorption site (intestinal level), and most of it is excreted in the urine. Within seven hours from the administration in mice, almost the entire amount of orally absorbed ZBC is eliminated in a metabolized form. The highest amount of A2M protein in mouse liver was determined by immunoabsorbance assay in the chronic experiment (1000mg/kg of ZBC), followed by the subchronic experiment (at the same dose of ZBC), and by the acute experiment (5000mg/kg of ZBC).

KEYWORDS: *A2M protein, dietary supplement, healthy life, longevity, zinc-boron complex.*

Introduction

Zinc (Zn) represents an important trace element for cellular functions, being involved in antioxidant response, in brain glutaminergic transmission, and in conferring biological activity to enzymes.

Various health issues, such as growth retardation, neuronal and sensory dysfunctions, hypogonadism, and immunodeficiency are caused by abnormal Zn homeostasis.

Brain functions decline associated with aging is caused also by Zn dyshomeostasis.

One of the problems may be an altered homeostasis of metallothioneins and other Zn-binding proteins, such as alpha$_2$-macroglobulin (A2M), which are both high affinity Zn-binding proteins within cells and blood, respectively [1-3].

About 10% of all proteins may bind with Zn, a fact revealed by human genome bioinformatics research [4].

Also, in elderly, innate immune response, thymic endocrine activity, and the survival of old mice were restored and increased by Zn nutritional supplements [5-7].

A2M is an abundant plasma protein and multi-potent protease inhibitor, which can bind and inactivate proteases of all four classes [8,9].

A2M is also involved in the regulation of other important functions of the human body [10].

Moreover, A2M binds some hormones and regulates their activity.

A2M damage decline of its concentration provokes accumulation of proteinase in biological fluids and may cause immune inflammation.

An age-dependent decline in the A2M level may have detrimental effects on human health.

Moreover, low levels of Zn in the blood of elderly people are correlated with inflammation and decline in A2M expression [11].

Zn and boric acid (BA) biological interaction was demonstrated by the zinc borate (ZB) low acute toxicity in rats, with a median lethal dose (LD_{50}) value >10g/kg body weight (b.w.) as against of 3.3g/kg b.w. (LD_{50}) for disodium tetraborate pentahydrate (DTBP).

Boron (B) equivalent amounts were established for ZB and DTBP [12].

There were no toxic effects in the rat testes using 1000mg ZB/kg b.w./day (50mg B/kg b.w. equivalent dose) in a 28-day toxicity study

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applying repeated dose by oral gavage [13], although the lowest level of the noticed adverse effect was 26mg B/kg b.w.

In humans, to reduce the toxic effects, background amounts of Zn may interact with B.

Human soft tissues contain Zn levels over two times greater than in analogue tissues of the experimental animals [14-16].

In addition, Zn provides protection against testicular and developmental toxicity of cobalt and cadmium [17,18].

The toxicity of Zn-B complex (ZBC) was tested *in vitro*, on cell cultures, using L929 cell line, according to the European standard procedures and techniques [19].

L929 cell line was affected by ZBC both morphologically and in terms of viability, above the minimum toxic concentration of 0.5mM [20,21].

In our previous research, compared to other pharmacologically active zinc salts (chloride, sulfate, oxide, orotate), ZBC belongs to the class of substances with very low toxicity (Category V, practically non-toxic).

From the histopathological point of view, in the case of acute (24 hours), subchronic (28 days) and chronic (90 days) experiments, normal aspects were highlighted for all mice internal organs [20].

The aim of our paper was to demonstrate that ZBC-based nutritional supplements can be used as stable non-toxic formulations, with high intestinal absorption rate, inducing A2M expression for longevity and healthy life.

Materials and Methods

Animals and procedures

The Law No. 43 (Romanian Parliament; April 11, 2014), the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (December 2, 2005) and the Directive No. 86/609 (European Council; November 24, 1986) on the protection of animals used for scientific objectives were considered for establishment of the experimental protocol.

The study has been approved by the University of Medicine and Pharmacy of Craiova, Romania (Ethics Committee Approval No. 51/20 April 2018).

For the entire duration of experiments, the mice were housed in standard conditions (temperature of 22±2ºC, humidity of 55±10%, light-dark cycle of 12 hours, water, and food *ad libitum*) in a separate area of the Animal Care Unit of the University of Medicine and Pharmacy of Craiova.

Pharmacokinetics of ZBC-based dietary formulation in mice

The pharmacokinetic experiment was performed on a batch of 10 BALB/c mice, eight weeks old, with an average weight of 25g, the number of females and males being equally distributed (n=5), which were orally administered (per os-*p.o.*), by gavage, in 0.2mL of distilled water, a dose of 250mg/kg b.w. ZBC (on average, 6.25 mg ZBC/0.2mL of aqueous solution was *p.o.* administered to each mouse).

The evolution of the animals was monitored throughout the experiment, at well-established time intervals [22,23].

Blood samples were collected by puncture at the tip of the tail, with a microsyringe, in a volume of 10µL from each mouse, at different time intervals: 15 minutes (1/4 hour), 30 minutes (1/2 hour), 60 minutes (1 hour), 120 minutes (2 hours), 240 minutes (4 hours), 420 minutes (7 hours), 600 minutes (10 hours), 720 minutes (12 hours), 960 minutes (16 hours) and 1440 minutes (24 hours).

The samples were preserved at -80ºC, in microtubes with anticoagulant (Ethylenediaminetetraacetic acid potassium salt) [22,23].

Urine samples were collected from a group of four mice (two males and two females), at 240 minutes (4 hours) and at 420 minutes (7 hours) from *p.o.* administration of ZBC, by bladder puncture, with a micro-syringe, 10µL for each mouse, after sacrificing the animals by euthanasia under general anesthesia with an injectable mixture of Ketamine/Xylazine (administered intraperitoneally).

Urine samples were stored at -80ºC, in Eppendorf tubes [22,23].

Blood and urine sample analysis was performed by liquid chromatography (LC)/mass spectrometry (MS), within 24 hours of the collection and appropriate processing of the samples.

Serum was obtained by blood samples centrifugation at 2000rpm for 10 minutes, and further processing in two phases: protein precipitation in methanol and then in acetonitrile.

The processing of urine samples was also carried out by the above-mentioned method of protein precipitation in two phases (in methanol and, respectively, in acetonitrile) [24,25].

The validation of the LC/MS analysis was performed based on the UHPLC/Q Exactive

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Orbitrap MS [26] and on the high-performance thin-layer chromatography (HPTLC)-ultraviolet (UV) densitometry techniques set up by our research team [20,27].

Analysis of A2M protein expression in the mouse liver

For A2M protein analysis, the experiment was performed on a batch of 10 BALB/c mice, eight weeks old, with an average weight of 25g, the number of females and males being equally distributed (n=5), which were *p.o.* administered, by gavage, in 0.2mL of distilled water, different doses of ZBC, at various time intervals, as follows: (*i*) for the acute experiment (at 24 hours), five different doses of 5mg/kg b.w., 50mg/kg b.w., 300mg/kg b.w., 2000mg/kg b.w., and 5000mg/kg b.w.; (*ii*) for the subchronic experiment (lasting 28 days), three different doses of 50mg/kg b.w., 300mg/kg b.w., and 1000mg/kg b.w.; (*iii*) for the chronic experiment (lasting 90 days), also three different doses of 50mg/kg b.w., 300mg/kg b.w., and 1000mg/kg b.w.

The control mice were monitored and maintained in the same conditions, and they received: for the acute experiment-distilled water, *p.o.*, by oral gavage; for the subchronic experiment and for the chronic one-only the drinking water normally consumed.

The evolution of the animals was monitored throughout the experiment, at well-established time intervals [23,28].

Depending on the duration of the experiments, the animals were sacrificed at intervals of 24 hours (acute), 28 days (subchronic) and 90 days (chronic), respectively, by euthanasia under general anesthesia with an injectable mixture of Ketamine/Xylazine (administered intraperitoneally), for the purpose of liver harvesting, for A2M protein analysis.

The biological samples were stored at-80ºC, and the analysis was performed in max. 10 days after harvesting [23,29].

The amount of native and transformed A2M was determined by immunoabsorbance assay (Biomac, Leipzig), expressed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), based on the method developed by our research team [20,30].

Statistical analysis

The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) ver. 19.0 for Windows (SPSS, Chicago, IL, USA). Data were reported as mean±standard deviation (SD).

Student's *t*-test was used to determine the degree of significance.

For statistical significance, a value of $p<0.05$ was established.

Results

Pharmacokinetics of ZBC

Based on the absorption-elimination curve as a function of time, in the case of ZBC, a mono-compartmental pharmacokinetic model for extravascular administration (*p.o.*) was highlighted.

From the graphic representation of this curve, it can be seen that the function starts from 0, increases to a maximum value, after which it decreases "exponentially" towards 0.

The following pharmacokinetic parameters were determined starting from the experimental data: the maximum concentration (C_{max}) reached by ZBC in the blood, at equilibrium: 980µg/mL; the maximum time (t_{max}) at which the maximum concentration in the blood was reached: 110 minutes; the biological (plasma) half-life ($t_{1/2}$), *i.e.*, the time when the amount of the active substance in the blood was halved, after reaching the distribution equilibrium at the blood level: 200 minutes (Figure 1).

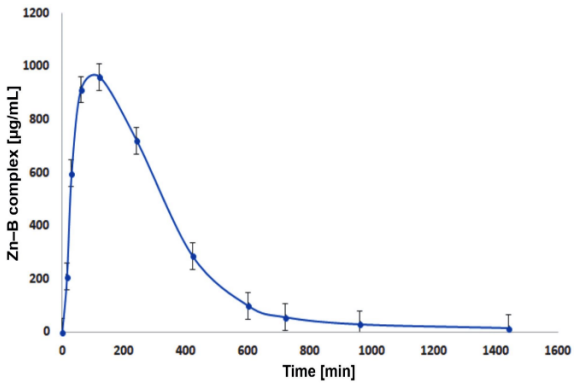

Figure 1. Absorption-elimination curve for ZBC administered p.o., in a dose of 250mg/kg, in mice. p.o.: Per os; ZBC: Zinc-boron complex.

Analysis of A2M protein expression

The highest amount of A2M protein in the mouse liver, expressed relative to GAPDH, was determined for the chronic experiment (2.6 A2M/GAPDH; 1000mg/kg b.w. of ZBC), followed by the subchronic experiment (2.1 A2M/GAPDH; the same dose of ZBC), and by the acute experiment (1.6 A2M/GAPDH; 5000mg/kg b.w. of ZBC) (Figures 2-4).

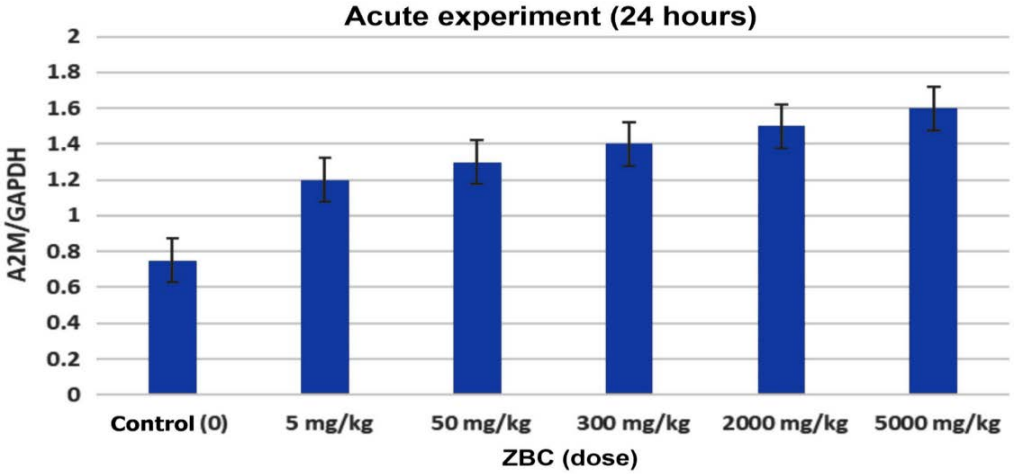

Figure 2. Determination of A2M protein levels (expressed relative to GAPDH) in mouse liver, after p.o. administration of five different doses of ZBC, in an acute experiment (n=5; mean±standard deviation, Student's t-test). A2M: α2-Macroglobulin; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; p.o.: Per os; ZBC: Zinc-boron complex.

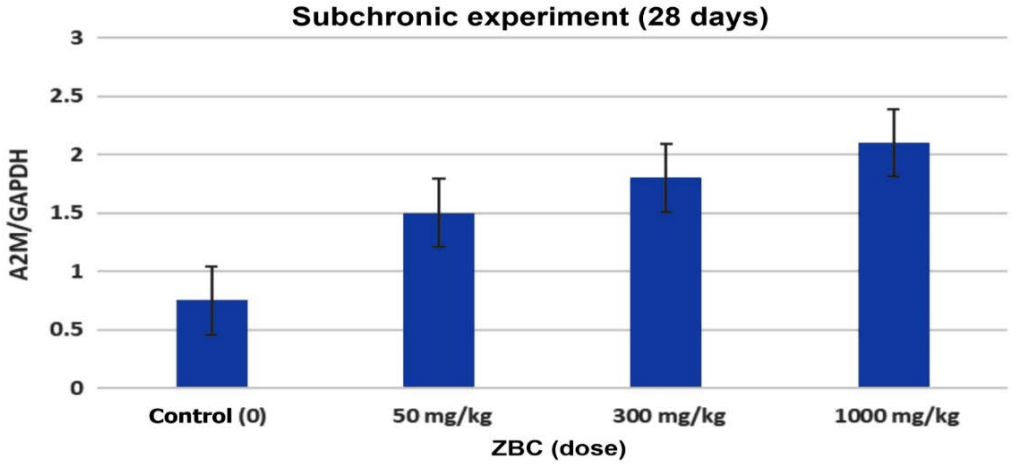

Figure 3. Determination of A2M protein levels (expressed relative to GAPDH) in mouse liver, after p.o. administration of three different doses of ZBC, in a subchronic experiment (n=5; mean±standard deviation, Student's t-test). A2M: α_2-Macroglobulin; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; p.o.: Per os; ZBC: Zinc-boron complex.

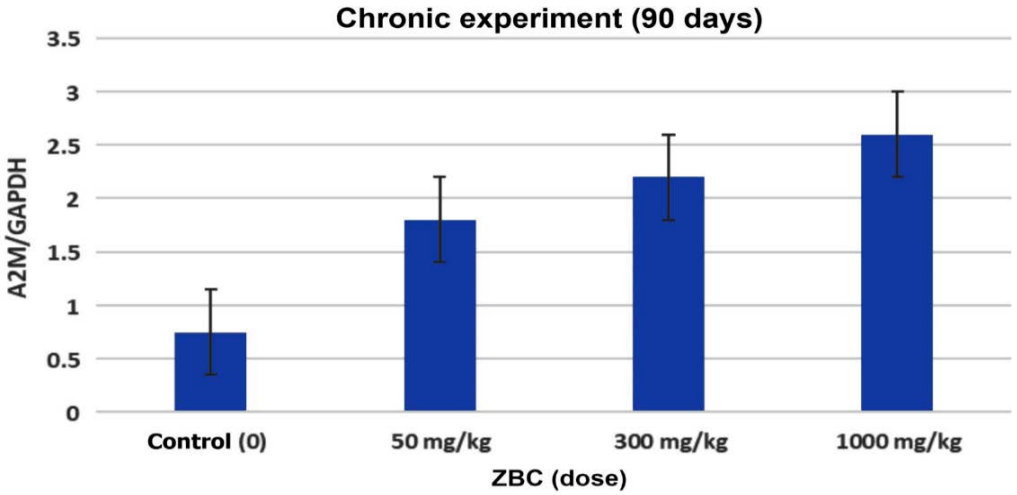

Figure 4. Determination of A2M protein levels (expressed relative to GAPDH) in mouse liver, after p.o. administration of three different doses of ZBC, in a chronic experiment (n=5; mean±standard deviation, Student's t-test). A2M: α_2-Macroglobulin; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; p.o.: Per os; ZBC: Zinc-boron complex.

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Discussion

ZBC biodegrades mainly at the absorption site, at the intestinal level and less after absorption as such, at the blood and urinary level.

ZBC is metabolized by hydrolysis, mostly into fructose, boric acid (BA) and Zn^{2+} ions.

In urine, fructose-B complex is partially degraded (approx. 10%) to BA and fructose.

In mice, ZBC excretion is mainly carried out in the urine.

In the first 240 minutes from the *p.o.* administration was removed approx. 50% of ZBC in metabolized form.

Within seven hours of *p.o.* administration, almost the entire amount of ZBC absorbed *p.o.* (approx. 90%) was eliminated in a metabolized form (fructose-B complex, BA and fructose).

BA is well absorbed *p.o.* from the gastrointestinal tract.

At least 90% of the absorbed amount, after oral administration, is eliminated as such at the renal level, in maximum 96 hours.

The $t_{1/2}$, depending on the ingested dose, has values between 5-10 hours, sometimes even up to 21 hours, in the case of serious poisoning.

The blood normal level of BA is between 0 and 7.2μg/mL. In most cases of BA ingestion, the plasma amount 10 times higher than the maximum allowed (70μg/mL) is asymptomatic.

Moreover, it has been established that the plasma levels of BA can reach a maximum of 340μg/mL without toxic manifestations.

Renal excretion of BA is achieved according to first-order pharmacokinetics, with a $t_{1/2}$ of approx. 24 hours.

B is not metabolized by humans or rodents [23,31,32].

Our results are consistent with those obtained at subchronic (90-day study) and genetic safety evaluation of calcium fructoborate (CaFB) in male and female rats.

No adverse effects and no histopathological changes were recorded when CaFB was orally administered in increasing doses (386, 775 and 1161mg/kg b.w./day for male rats; 392, 785 and 1171mg/kg b.w./day for female rats).

In addition, CaFB did not show a genotoxic (Chinese hamster V79 cells) or mutagenic (bacterial assay) effects [23].

Practically, acute ingestion of BA has minimal toxic effects for most patients: *e.g.*, diarrhea, vomiting, abdominal cholic, headache, lethargy [31].

Recently, our research team developed monoclonal antibodies useful for the detection of different forms of A2M in human plasma.

Our studies have shown a significant decrease in A2M levels, especially in the age range between 20-50 years, when many chronic diseases start.

Furthermore, we found that the human A2M protein is a strong inhibitor of tumor growth.

Also, preliminary experiments demonstrated that Zn supplementation led to the stimulation of A2M protein synthesis in the liver [20,21,30,33,34].

Obtained by purification from human plasma, A2M protein can be converted into A2M* (its active form) by thiol ester fragmentation purified, thus retaining its native structure [8,35-37].

This induces a major conformational change, as indicated by pore-gradient polyacrylamide gel electrophoresis (PAGE) analysis, by binding to immobilized low-density lipoprotein receptor-related protein 1 (LRP1) and thereby inhibiting the response to its receptor-associated protein (LRPAP1).

The absence of lipopolysaccharides (LPS) in the A2M* was highlighted by *in vitro* and *in vivo* inconsiderable stimulatory effects on leukocytes; the MS technique cannot be used for the detection of A2M impurities [38-41].

A2M protein has an important role in the hemostasis regulation by inhibition of tissue plasminogen activator (tPA), factor Xa, thrombin, plasmin, activated protein C, urokinase.

Depending on the patient's age, A2M biosynthesis is increased in some diseases with inflammatory substrate (*e.g.*, sepsis, acute kidney injuries, diabetes), being influenced by the proinflammatory cytokines [38].

The most important pharmacodynamic experiments aimed at the influence of A2M protein on longevity and healthy life were performed on naked mole-rats, a long-lived species with a peculiar resistance to cancer.

Compared to mice and humans, higher amounts of messenger ribonucleic acid (mRNA) of A2M protein in the liver of naked mole-rat, revealed by transcriptome analysis, contribute to the longevity, anti-aging effects and cancer resistance [34,41].

Conclusions

The biological link between Zn and B adjuvant therapy and a defined target at cellular and tissue level may open the door for testing active ZBC-based dietary supplements in different diseases and in aged persons.

Our data indicated that ZBC-based dietary supplements can be used as stable non-toxic formulations, with high intestinal absorption rate, inducing A2M expression for longevity and healthy life.

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Conflict of interests

None to declare.

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