Original Paper

In Vitro Cytotoxicity of Zinc Fructoborate, a Novel Zinc-Boron Active Natural Complex

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ABSTRACT: In recent years, the role of zinc in biological systems has been a subject of intense research. Zinc is required for multiple metabolic processes as a structural, regulatory, or catalytic ion. The objective of this study, was to assess the toxicity profile of a newly synthesized zinc-boron molecule on cultured cells. Zinc fructoborate, at different levels of concentration, was tested for its impact on the Vero kidney cell line (ATCC® CCL-81™) using the MTT assay. The compound exhibited a low cytotoxic effect on the cell line. Thus, our study demonstrates that the zinc fructoborate could become a promising dietary supplement molecule.

KEYWORDS: *zinc fructoborate, in vitro, cytotoxicity, MTT assay, ageing*

Introduction

Zinc (Zn) is an essential trace element that is widely required in the cellular functions.

It is involved in the glutaminergic transmission (signaling molecule) in the brain, in the antioxidant response and is a cofactor of many enzymes and transcription factors.

Abnormal Zn homeostasis causes a variety of health issues including growth retardation, immunodeficiency, hypogonadism, and neuronal and sensory dysfunctions.

Animal experiments have shown that, Zn deficiency may occur in aging which is associated with a decline in brain function.

Zn homeostasis is regulated through Zn transporters and permeable channels [1-3], emphasizing the physiological relevance of Zn to life.

A human genome bioinformatics study revealed that approximately 10% of all proteins may bind with Zn [4].

Physiological Zn supplementation in elderly restores the thymic endocrine activity and innate immune response (NK cell cytotoxicity) and increases the survival rate in old mice. Therefore, Zn supplementation is useful to achieve health longevity because the Zn-binding proteins may regain their original protective task against oxidative damage with a beneficial impact on the immune response [5-7].

The interaction of Zn and boric acid was characterized by the low acute toxicity of zinc borate (ZB) with an LD_{50} value higher than 10g/kg body weight (b.w.) in rats [8] compared to the disodium tetraborate pentahydrate with an LD_{50} (median lethal dose) value of 3.3g/kg b.w. (ZB and disodium tetraborate pentahydrate have equivalent boron concentration).

There was no evidence of toxic effects upon administering 1000mg ZB/kg b.w./day in a 28-day repeated dose oral gavage toxicity study, which corresponds to the equivalent dose of 50mg Boron (B)/kg b.w. [9].

The lowest-observed-adverse effect level (LOAEL) for testicular result is 26mg B/kg b.w. Background levels of Zn in humans may interact with boron to reduce the hazard of toxic effects. Zn levels in soft tissue in humans are two times higher than in comparative tissues of laboratory animals [10-12].

Zn has been also shown to protect against testicular toxicity of cobalt and cadmium [13,14].

Consequently, all these research data indicate that supplementation with stable natural active

zinc-boron complex-based nutritional formulations may delay aging.

Therefore, we tested the *in vitro* cytotoxicity of the new synthesized zinc-boron complex, the zinc fructoborate.

Material and Methods

Cell lines

In order to carry out our studies on the *in vitro* effect of zinc fructoborate (ZnFB), the Vero (ATCC® CCL-81™) epithelial cell line of monkey kidney was chosen. This cell line is recommended by ISO 10993-5: 2009 to test the cytotoxicity of certain compounds or medical devices.

The Vero cells were grown in EMEM medium (Eagle's Minimal Essential Medium) supplemented with 10% fetal bovine serum (FBS) and incubated in a humidified 5% CO_2 atmosphere at 37°C.

The cells were kept in culture until they reached an 80% confluence, after which the adherent cells were detached, by treatment with trypsin-ethylenediaminetetraacetic acid (EDTA) and planted at a density of 10^4 cells/cm^2 in 96-well plates.

After 24 hours of monolayer growth, the cells were incubated for another 24 hours in medium with sterile filtered zinc fructoborate and then subjected to analysis of cell density, morphology and cell viability.

For the treatment with ZnFB the following concentrations were used: 0.01, 0.015, 0.1, 0.5, 1.0, 1.5 and 3mM. As negative control cells were cultured under similar condition without any additives. A positive cytotoxicity control represented treatment of cells with 5% dimethyl sulfoxide (DMSO) [14,15].

Test substance

The substance that was used in the assay was the zinc fructoborate. The test agent was obtained by synthesis in our laboratory, according to Hunter's patent [16].

MTT assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test is commonly used for cytotoxicity, viability and cell proliferation studies. Cell treatment with MTT allows the evaluation of oxidative metabolism and cellular response to external factors that may have a positive or negative effect on the survival of the cell culture.

This quantitative colorimetric method is based on the reduction of the MTT yellow colored compound into the dark blue formazan.

The MTT reduction by the mitochondrial enzymes (especially succinate dehydrogenase) is directly proportional to the number of cells viable, being an index of cellular/mitochondrial integrity.

Optical density is evaluated spectrophotometrically, resulting in a direct relationship between absorbance, dye concentration and the number of viable and metabolic active cells [17-25].

Twenty-four hours after treatment with ZnFB, the cellular monolayer was washed with a phosphate-buffered saline (PBS) followed by adding the MTT solution (1mg/mL in serum-free culture medium).

After incubating at 37°C for three hours, the formazan crystals formed in the metabolically viable cells were dissolved in DMSO.

The resulting solution was densitometrically analyzed at 584nm.

Cell density and morphology

The density and morphology of the Vero cells were observed using the contrast microscopy: an Olympus type CKX41 microscope equipped with a video camera and an image capture system with CellSens software.

Results

At first, the *in vitro* effects of ZnFB were evaluated by observing the density and cell morphology in contrast phase microscopy.

The Vero cells were grown in the presence of low concentrations of ZnFB as typical epithelial cells with a polygonal, elongated and flat morphology.

Cell density was not significantly affected by this compound at low concentrations.

Treatment of Vero cells with ZnFB for 24 hours significantly affected the cells at a concentration equal or higher than 0.05 M (Fig. 1).

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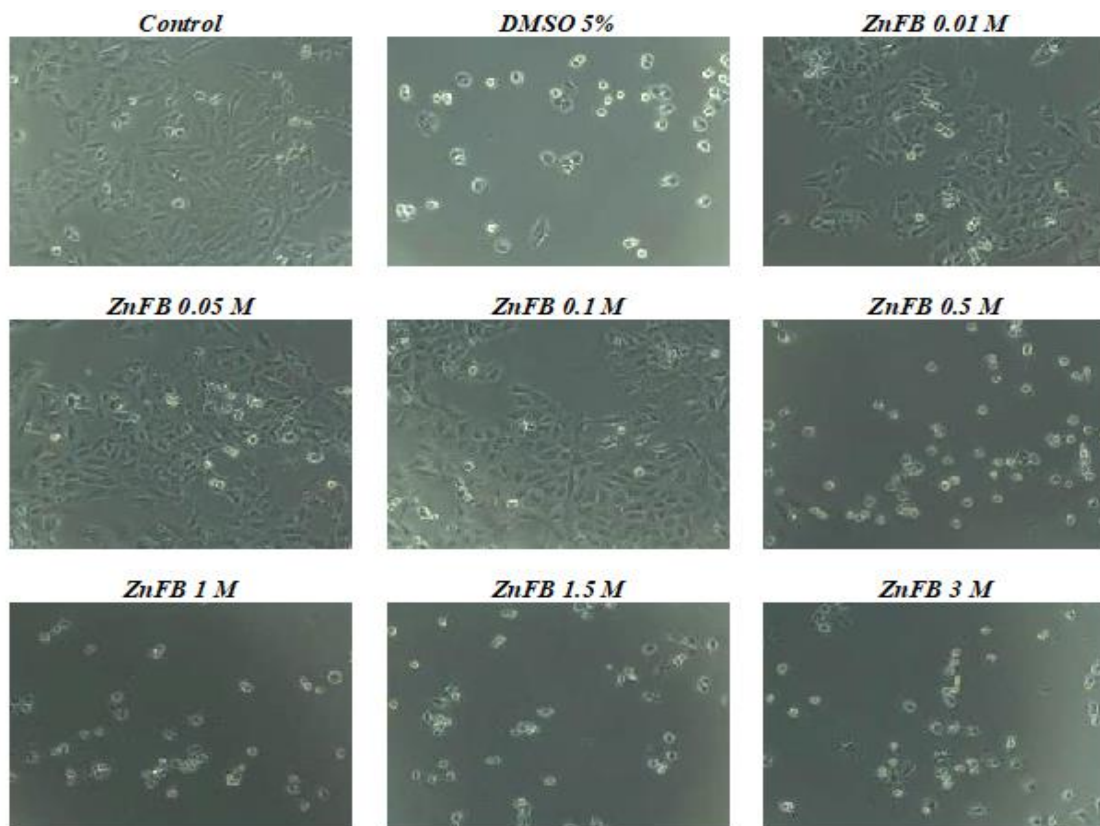

Fig.1. Effect of ZnFB on Vero cells morphology and density

Furthermore, the survival potential (viability) of cells in the presence of ZnFB was assessed by mean of the MTT assay. The results of this test confirmed the above observations with respect to cell morphology and density. ZnFB does not affect cell viability at doses ranging from 0.01 to 0.1mM. Contrarily, at concentrations between 0.5 to 3mM, cells became metabolically inactive comprising only 8% of the activity of control cells. In addition, the cell survival is much lower than in the positive control cytotoxicity (DMSO) (Fig. 2).

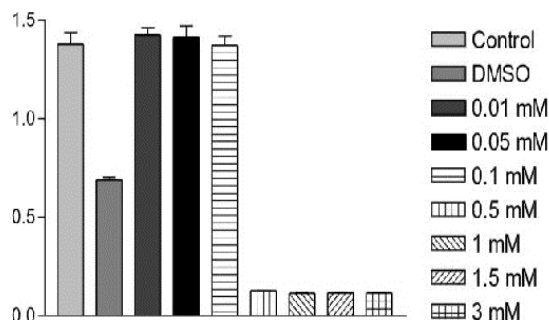

Fig. 2. Cell viability with different concentrations of ZnFB after 24 hours

Following the 24-hours exposure time, the IC_{50} of ZnFB was also determined (Table 1, Fig. 3).

Table 1. Statistics concerning IC_{50} and IC_{10} values for ZnFB

Best-fit values	
LogIC_{50}	-0.709
LogIC_{10}	-0.9053
Slope	-4.861
IC_{50}	0.1954
IC_{10}	0.1244
Standard error	
LogIC_{50}	0.09204
Slope	1.491

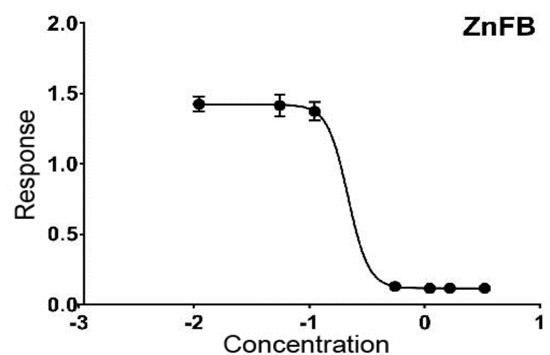

Fig. 3. IC_{50} graph (ZnFB: Zinc fructoborate)

Discussions

Zn^{2+} is a crucial metal, vital for the activity of numerous enzymes engaged in a wide range of metabolic mechanisms [26-31]; zinc has additionally regulatory [29,32] and structural functions [32]. Correlated with other trace elements, zinc is generally non-toxic *in vivo* [26,27,33].

The rather low harmfulness of zinc *in vivo* might be attributed to a mix of homeostatic mechanisms, which direct gastrointestinal assimilation and discharge of zinc [27-29,33,34], the activity of an assortment of hormonal stimuli, which control cellular metabolism [29], fast redistribution of Zn in the body [30] and cellular adaptive mechanisms [34,35].

There have been a few studies that report the inhibitory as well as toxic effects of zinc *in vitro* in different types of cells, for instance, HeLa cells [36,37], McCoy and human prostate cells [36,38], lymphoid cells [39], fibroblasts [40,41], Cloudman S91 and B16 melanoma cells [42,43]. Most of these reports and the present investigation propose that Zn^{2+} *in vitro* becomes cytotoxic over 0.5-1mM and surpasses the toxicity of many other metal ions [37,40].

The evidence indicates that Zn uptake by the cells might be essential for the expression of cytotoxicity.

The noticeable cytotoxic activity of zinc is fairly enigmatic.

A feasible method of activity could be the dislocation of different metals from vital intracellular compartments.

Zinc is known to compete with calcium, iron, copper, lead and cadmium for similar binding sites [27].

The susceptibility of cells *in vitro* to the harmful impacts of zinc might be reliant both on the rate of zinc uptake and the limitation of the defensive components.

It was found that Zn^{2+} salts reveal toxic effects toward PC12 cells in the following diminishing order: zinc citrate, zinc sulphate >zinc orotate, zinc acetate, zinc chloride, zinc gluconate >zinc histidinate. This suggests that, regarding neuronal cells, uptake of various Zn^{2+} salts greatly influence toxicity.

The IC_{50} determined for the ZnFB was 0.195±0.005mM. ZnFB affects the viability and morphology of the cultured cells only at a concentration equal or higher than 0.5mM. Comparing to the other studies, ZnFB showed a relatively low toxicity, comparable to that of zinc histidinate (IC_{50} 0.3mM).

Conclusions

Zinc is required for multiple metabolic processes as a structural, regulatory, or catalytic ion. Our study shows that ZnFB, a novel zinc-boron active natural complex, has a low cytotoxic effect on the Vero kidney cells. The level of toxicity is lower than that of the mostly used supplement, zinc orotate. Altogether, the ZnFB could be successfully used as a new source of Zn^{2+} supplement.

Conflict of interest

The authors declare that there is no conflict of interests.

Acknowledgements

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