Biol Trace Elem Res (2007) 118:27–37
DOI 10.1007/s12011-007-0019-7

In Vitro Effects of Calcium Fructoborate on fMLP-stimulated Human Neutrophil Granulocytes

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Received: 16 October 2006 / Accepted: 20 December 2006 /
Published online: 18 May 2007

Abstract Discovery of naturally occurring boron complexes with organic compounds containing hydroxyl groups, sugars, and polysaccharides, adenosine-5-phosphate, pyridoxine, riboflavin, dehydroascorbic acid, and pyridine nucleotides led to the reassessment of the biochemical role of boron. Boron's anti-inflammatory actions were claimed but not yet demonstrated. This study investigated the effects of calcium fructoborate (CF) on the human polymorphonuclear neutrophils (PMN) that play a central role in the inflammatory response. Our results demonstrated that CF exposure induced a dose-dependent decrease in cell viability. Treatment of PMN cells, for 24 h, with 22,500 μM CF led to a decrease in cell viability by 61.1%, an inhibition of respiratory burst by 92.9% in the case of fMLP-stimulated cells, a diminution of intracellular level of superoxide anion with 59.3%, and a stimulation of superoxide dismutase activity by 72% in unstimulated PMN cells. Altogether, these results suggest the antioxidant and anti-inflammatory properties of CF.

Keywords Calcium fructoborate (CF) · Human Polymorphonuclear neutrophils (PMN) · Cytotoxicity · Apoptosis · Respiratory burst · Superoxide radicals · Superoxide dismutase (SOD)

Introduction

Boron is an essential nutrient for certain organisms, notably vascular plants and diatoms. Whereas the role of boron in the life cycle of plants is well documented, a little is known about boron homeostasis and function in animal cells. Five lines of evidence, derived in large part from animal model research, indicate that dietary boron can have beneficial

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effects on humans and animals. In amounts typically found in human and animal diets, boron improved bone health (independent of vitamin D status) by increasing bone development in frogs [1]; bone breaking strength in pigs [2], broilers [3], and growing pullets [4]; and bone calcium concentrations in chicks [5]. Secondly, boron interacts with specific steroid hormones; it increased circulating concentrations of 25-hydroxycholecalciferol in humans [6] and chicks [7] and counteracted the deleterious effects of dietary vitamin D deficiency on body growth in chicks [7] and growth plate morphology in embryonic [8] or hatched [9] chicks. In addition, boron increased the circulating concentrations of 17β-estradiol in humans [10], and together with injections of 17β-estradiol, increased trabecular bone surfaces in ovariectomized rats [11]. Thirdly, the physiologic amounts of boron apparently reduced the amount of insulin required to maintain plasma glucose in rats [12]. Fourthly, the borate or its analogues can inhibit the in vitro activities of several enzymes in the eicosanoid pathway related to inflammation and immune function [13]. Fifthly, the evidence leading to the hypothesis that boric acid may be anti-carcinogenic was derived from epidemiological screening, where the risk of prostate cancer was observed to be inversely proportional to dietary intake of boron in a dose-responsive manner [14]. Boric acid has also been reported to inhibit the growth of LNCaP prostate tumors in nude mice [15]. Studies on the prostate cancer cell lines, DU-145 and LNCaP, showed that boric acid inhibits their proliferation in a dose-dependent manner. Non-tumorigenic prostate cell lines, PWR-1E and RWPE-1, and the cancer line PC-3 were also inhibited, but required concentrations higher than observed human blood levels [16]. In a recent study on DU-145 human prostate cancer cell line, Barranco and Eckhert [17] observed that pharmacologically relevant SB treatment causes DU-145 cells to convert to highly granular, low-volume, flattened cells that have a marked reduction in their capacity to migrate, invade matrigel, and attach to synthetic substrates. Reduction in the expression of proliferation-relevant proteins, along with the upregulation of b-galactosidase activity, ultimately leads to a nonproliferating entity reminiscent of a senescent-like cell. Finally, the resulting cell accumulates intracellular acidic vesicles, while acidifying its extracellular environment.

Boron actions can be correlated with the fact that boric acid forms cyclic diesters with appropriate diols or polyols from many of the chemical substances found in the body, such as carbohydrates (sugars and polysaccharides), nucleotides (such as adenosine monophosphate and niacinamide adenine dinucleotide), and vitamins (such as ascorbic acid, pyridoxine, and riboflavin). The most stable esters are those in which boric acid is the bridge between two carbohydrate molecules, e.g., fructose–boron–fructose. Such soluble boron complexes are found naturally in phloem saps and nectars in plants. Polysaccharides containing boron in similar linkages are found in plant cell walls in the form of pectins.

Currently, two hypotheses have been advanced for the biochemical function of boron in animals, including humans. The first is that boron plays a role in cell membrane functions that influence response to hormone action, trans-membrane signaling, and trans-membrane movement of regulatory ions. Boron has been shown, in animal models, to influence the transport of extracellular calcium and the release of intracellular calcium in platelets activated by thrombin. It also influences redox actions involved in cellular membrane transport in plants. A second hypothesis is that boron acts as a metabolic regulator in several enzymatic systems. Boron may play an important role in regulating the respiratory burst, which is the reactive-oxygen-species mechanism by which white blood cells kill microorganisms. If boron does, in fact, regulate the respiratory burst, it assumes the role of a novel antioxidant, preventing some of the collateral damage that may occur when reactive oxygen species react with surrounding tissue.

In this paper, we studied the action of calcium fructoborate (CF), which has a chemical structure similar to the natural forms of B found in edible plants [18], upon studying some

functions of neutrophils in relation with its antioxidant role [19]. CF marketed by FutureCeutical Corporation (SUA) as Fruitex-B is a B-based nutritional supplement, stable, nontoxic, water soluble, known to reduce the pain and inflammation associated with osteoarthritis, improve calcium absorption and metabolism, increase steroid hormone levels of vitamin D, estrogen, and testosterone, involved in healthy prostate maintenance, and increase superoxide dismutase (SOD) levels [20, 21].

Material and Methods

Materials

CF was synthesized according to Miljkovic's patent [22]. The purity of CF was checked by nuclear magnetic resonance and high-performance liquid chromatography, and the results indicate 99.9% analytical purity. All reactants used for its synthesis; boric acid, acetone, $CaCO_3$, and fructose were acquired from the Sigma Chemicals.

Neutrophils Isolation

Fresh human neutrophil granulocytes (PMN) were obtained from healthy donors. Venous blood was drawn into heparinized syringes. The PMN cells were separated by centrifugation on Histopaque medium according to Boyum [23]. Remaining red blood cells were eliminated by hypotonic lysis. The purified neutrophils were washed and resuspended in Dulbecco's Modified Eagle's Medium (DMEM), and the cells were counted in a hemocytometer. Morphological analysis revealed that 90% of the cells were PMN. The cells were treated with CF, calcium chloride (CC), and sodium borate (SB) in the range of 450–22,500 μM for 24 h.

Phase-Contrast Microscopy

Morphological changes induced by treatment in neutrophils were examined with an inverted phase-contrast microscope Nikon Eclipse TS 100 at different stages of experiment.

Viability Test

Viability test was performed by the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT dye-reduction assay). The tetrazolium dye MTT is reduced to a colored product by the activity of NADH-dependent dehydrogenases, and this indicates the level of energy metabolism in cells. PMN cells were treated in 24-well plates and incubated with 0.5 ml/well of MTT (1 mg/ml) for 3 h at 37°C. After this time, the formazan was released from the cells with dimethyl sulfoxide. Absorbance of the supernatant was measured at 550 nm.

Observation of PMN Cells Apoptosis

The apoptotic cells were observed by annexin V/propidium iodide (PI) staining, that allows the distinction between apoptosis and necrosis. Viable cells do not bind Annexin V-FITC or PI. Early apoptotic cells with exposed phosphatidyl serine (PS) but intact cell membranes

bind Annexin V-FITC (that will appear bright apple green on the cell membrane surface) but exclude PI. Later, during the apoptotic process *in vitro*, the membrane becomes porous, and PI becomes associated with DNA in the nucleus and is visible as red fluorescence. The uptake of PI is an indication of necrosis. For apoptosis detection, a kit containing annexin V-FITC and propidium iodide (SIGMA Chemical Co) was used. Briefly, cells treated with CF in a 24-well plate were washed twice with phosphate-buffered saline (PBS) and then resuspended in 100 μL binding buffer 1× provided by the kit. In each sample, 5 μL of annexin and 10 μL PI were added, and the plate was incubated for 15 min at room temperature in the dark. After this time, the cells were monitored with the fluorescence microscope and photographed with a digital camera Nikon CoolPix 4500.

Respiratory Burst Assay

ROS were quantified by measuring luminol-dependent chemiluminescence at 37°C using a Tecan reader at approximately one measurement per well every 25 s, in 30 min. Neutrophils (2×10^4 cells) subjected to CF treatment for 24 h were washed with DMEM and then were suspended with 25 μM luminol, 4 μg/ml cytochalasin B, and DMEM for a final volume of 200 μl per well. The cells were stimulated with 1 μM formylmethionyl-leucyl-phenylalanine (fMLP), and ROS generation was measured by integrating photon counts for 30 min after agonist addition. The solvent control, dimethyl sulfoxide (DMSO), did not stimulate ROS production.

Superoxide Dismutase Assay

The assay of SOD activity was carried out by the method described by Paoletti and Mocali [24] that consists of purely chemical reaction sequence, which generates superoxide from molecular oxygen in the presence of EDTA, manganese (II) chloride, and mercaptoethanol. NAD(P)H oxidation (pursued by the diminution of absorbance at 340 nm) is linked to the availability of superoxide anions in the medium. Values of ΔA340 recorded for samples with SOD activity will progressively decrease depending on the amount of enzyme in the assay mixture. The percent change in enzyme activity was calculated with assay data from treated and control cells.

The human neutrophils were treated for 24 h with CF, washed with PBS, resuspended in PBS, and lysed by sonication. Aliquots appropriate volume of total cell extract were used for assay of SOD activity. The assay volume (213 μl) contained 160 μl 100 mM triethanolamine–diethanolamine–HCl buffer (pH 7.4), 8 μl of 7.5 mM NADPH, 5 μl of 100 mM EDTA–50 mM $MnCl_2$ solution (pH 7.0), 20 μl of the cell extract and was kept at room temperature for 5 min to stabilize. Then, 20 μl of 10 mM mercaptoethanol was added and mixed well. Reagent blanks, which contained all assay components except cell extract glucose oxidase were used to calibrate the spectrophotometer. The decrease in absorbance at 340 nm was monitored for 20 min over a 5-min interval at 25°C in a Beckman DU 7500 spectrophotometer. All determinations were expressed as specific SOD activity in units per milligram (U/mg) protein. The assay of protein concentration was carried out by the Bradford method [25].

SOD Activity Analysis Using Native Gel Analysis

After 24 h of treatment, the cells were rinsed twice with ice-cold PBS and then sonicated on ice. Equal protein, as determined by the Bradford assay, was loaded into each lane. Proteins

were size separated in 12% polyacrylamide gels with 5% stacking gels, and SOD activity was visualized by the nitroblue tetrazolium method [26].

Changes of Intracellular Superoxide Anion Level

Any change of the intracellular superoxide anion level was determined by the ability of superoxide anion to reduce the nitroblue tetrazolium (NBT) salt to intracellular black–blue deposits. Briefly, human neutrophils were incubated with different concentrations of CF and 0.3 mg/ml NBT. After 2 h, the culture medium was removed, and cells were washed with PBS and lysed so that the reduced NBT would dissolve in the lysis buffer, and optical absorbance was measured at 520 nm using a microplate reader [27].

Statistical Methods

At least three experiments were performed in triplicate for each experimental condition. Data are expressed as the mean±SEM. Statistical differences were analyzed using Student's *t* test or analysis of variance when suitable.

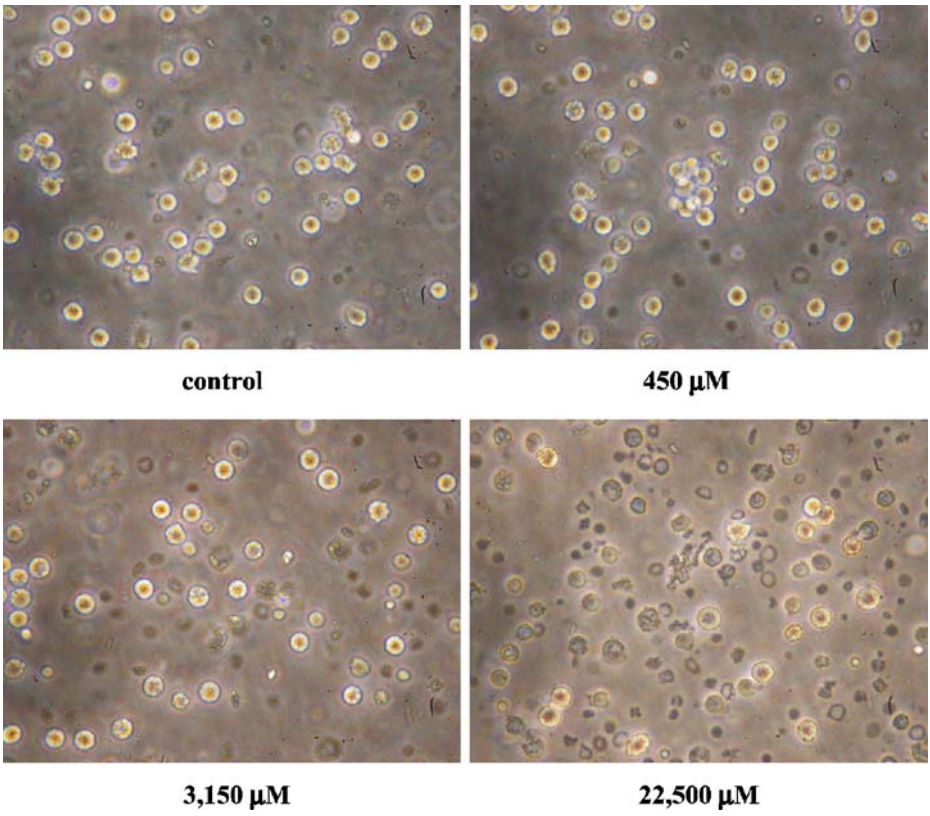

Fig. 1 Morphological appearance in phase-contrast light microscopy of PMN cells control and treated for 24 h with different concentrations of calcium fructoborate (×100)

Table 1 Cytotoxicity of Exposure of PMN Cells (24 h, 37°C) to Different Concentrations of Calcium Fructoborate, Sodium Borate, and Calcium Chloride

Treatment	Absorbance at 550 nm		
μM	Calcium fructoborate	Sodium borate	Calcium chloride
0	0.375±0.003	0.416±0.004	0.385±0.004
450	0.364±0	0.411±0	0.377±0.003
1,350	0.341±0.001	0.413±0.008	0.392±0.007
2,250	0.304±0.001	0.404±0.004	0.389±0.007
3,150	0.230±0	0.409±0.004	0.371±0.002
22,500	0.146±0	0.399±0.005	0.335±0.004

Results

The treatment of the human polymorphonuclear neutrophils with different CF doses was monitored by phase-contrast microscopy (Fig. 1). Exposure of PMN cells to CF has as a

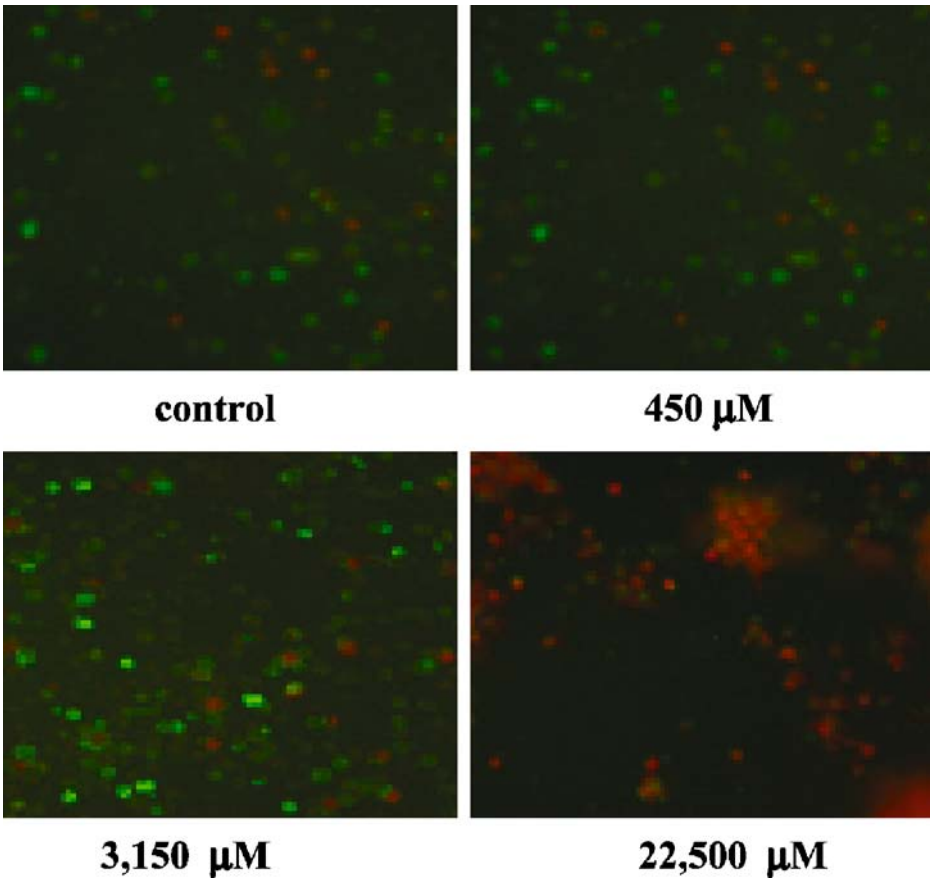

Fig. 2 Fluorescence micrographs showing the PMN cells with positive Annexin V and PI signals after treatment with different concentrations of calcium fructoborate (×100)

result the dose-dependent morphological changes of cells. Light microscopy analysis indicated that the cell viability decreased with the increase of the CF concentration. The most obvious cytotoxic effects were seen at the maximum concentration used in the study; 22,500 μM. No differences were seen between control, CC- and SB-treated neutrophils.

To explain these effects, changes in viability/cytotoxicity of PMN cells treated for 24 h with different concentrations of CC, SB, and CF were evaluated by MTT assay (Table 1). We found that CF exerted the highest cytotoxic effects, in a dose-dependent manner, more evident at doses of 3,150 and 22,500 μM CF. As a result of the PMN cells treatment with 3,150 and 22,500 μM CF for 24 h, we noticed a decrease in the cell viability by 38.7 and 61.1%, respectively, compared with control, which was considered to be 100%. The minimal effect was at SB treatment. The treatment of PMN cells, for 24 h with 22,500 μM SB and CC has, as a result, a decrease in their viability by 4.1 and 13%, respectively.

To study the mechanism of action of CF, we investigated the relationship between PMN cells apoptosis and cytotoxic effects of this compound. Staining with FITC-conjugated annexin V and propidium iodide (PI) can identify subpopulations of cells with membrane changes and the associated loss of membrane integrity. Figure 2 shows the results of analysis by fluorescence microscopy of the PMN cells treated with CF and stained with annexin V-FITC and PI.

We have observed the presence of some apoptotic cells in control experiment after 24 h of culture. The treatment of PMN cells with 3,150 μM CF induced shrinkage in size and loss of cell membrane integrity, a classical feature of necrotic or late apoptotic cells. These

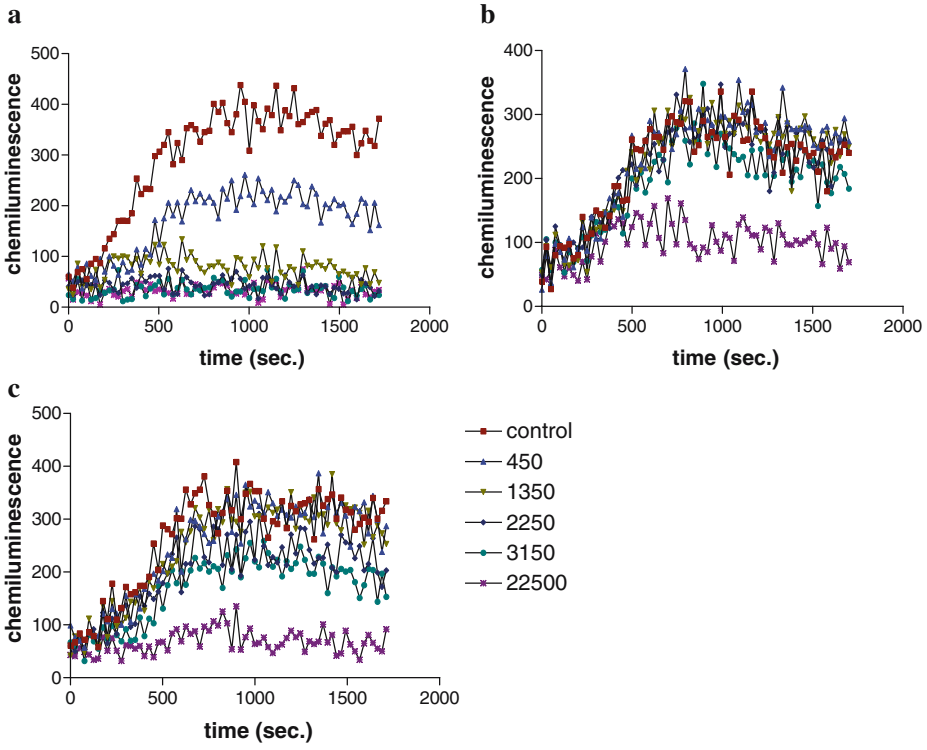

Fig. 3 The respiratory burst of fMLP-stimulated PMN cells treated with different concentrations of calcium fructoborate (A), calcium chloride (B) and sodium borate (C)

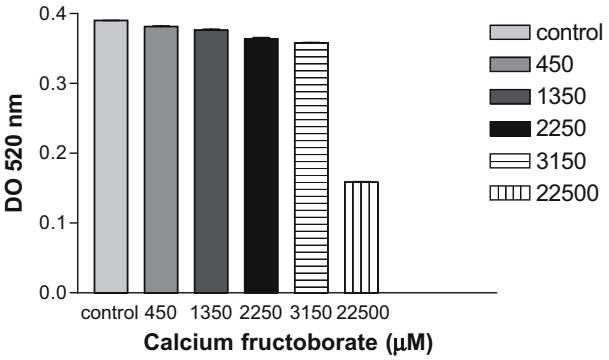

Fig. 4 Changes in the intracellular superoxide anion level as result of the treatment for 24 h of unstimulated PMN cells with different concentrations of calcium fructoborate

PMN cells in late apoptosis are both Annexin V-FITC and PI positive. As a result of treatment with maximal dose of CF (22,500 μM), we observed a massive necrosis of PMN cells, with fragmentation of the nucleus and dispersion of the PI positive material.

PMN cells play an essential role in the body's innate defense against pathogens and are one of the primary mediators of the inflammatory response. To defend the host, PMN cells use a wide range of microbicidal products, such as oxidants, microbicidal peptides, and lytic enzymes. We have studied the effects of CF, CC, and SB on fMLP-stimulated PMN's respiratory burst, evaluated by luminol-dependent chemiluminescence method (Fig. 3). The results obtained in our study suggest that the CF treatment significantly inhibits reactive oxygen species (ROS) generation in a dose-dependent manner. In rapport with control experiment, the ROS level decreased with 50, 78.5, 87.2, and 92.9% as a result of PMN cells treatment with 450, 1,350, 2,250, 3,150 and 22,500 μM CF, respectively.

The cell treatment with the same doses of SB has lower inhibitory effects on the respiratory burst. Thus, by comparison with control, the intensity of respiratory burst of fMLP-stimulated PMN cells decreased with 27.7 and 66.9%, as a result of cell treatment with 450 and 22,500 μM borate, respectively. At concentrations ranging from 450 to 3,150 μM, Ca^{2+} ions had a slight or no influence on this function of PMN cells. Nevertheless, increasing the concentration of CC up to 22,500 μM caused a decrease in respiratory burst by 53.3%.

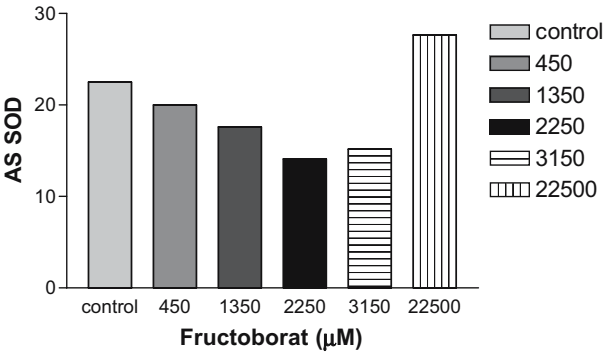

Fig. 5 Changes in the superoxide dismutase activity as result of the treatment for 24 h with different concentrations of calcium fructoborate

The next experiment has addressed the question of whether CF treatment affects the intracellular level of superoxide anion from the unstimulated PMN cells. As illustrated in Fig. 4, the level of this reactive oxygen species decreased only slightly when the cells were treated for 24 h with 450–3,150 μM CF and with 59.3% at a maximal dose taken in study.

To further characterize the antioxidant action of CF on human unstimulated PMN cells, we evaluated the effects of CF on superoxide dismutase (SOD) activity, an enzyme that protects oxygen-metabolizing cells against harmful effects of superoxide free radicals. CF in concentrations ranging from 450 to 2,250 μM induced a relatively modest inhibition of the SOD activity. Treatment of PMN cells, for 24 h, with 22,500 μM CF led to an increase of this scavenger activity by 72% in accord with the low intracellular level of superoxide anion (Fig. 5).

Discussion and Conclusions

PMN cells constitute 60% of the circulating leukocytes and represent the first line of defence against microbes [28]. These cells are terminally differentiated end cells and are produced in the bone marrow from myeloid stem cells. PMN cells survive for a short time (8–20 h) in the blood and undergo apoptosis, presenting morphological changes such as diminution in cell volume, nuclear condensation, and cytoplasmic vacuolation [29]. It is noteworthy that the half-life of the PMN cells increases severalfold once they enter infected or inflamed tissues [30]. Several inflammatory cytokines including granulocyte/macrophage colony-stimulating factor, IL-2, TNF-α, and IL-15 have been reported to prolong PMN cells survival [31]. The resolution of inflammation is associated with PMN clearance, which occurs upon apoptosis of PMN cells *in situ* and their subsequent engulfment by tissue macrophages.

Our *in vitro* studies show that CF has profound effects on human PMN cells related to its concentration. Thus, the treatment with CF of fMLP-stimulated PMN, for 24 h, resulted in a decrease of respiratory burst, in a dose-dependent manner. The decrease of ROS level was evident even when non-cytotoxic doses were used (for example, by 50% at 450 μM CF), which underlies the fact that CF is a superoxide anion scavenger and may have anti-inflammatory effects. These data must be interpreted considering the previous observations that neutrophils stimulation with fMLP results in an increase of superoxide anion generation and apoptosis level [32]. It is possible that CF inhibits activation of the enzymatic complex NADPH-oxidase that directly reduces molecular oxygen to generate superoxide anion, which is later converted into other reactive intermediates. It is worth mentioning that this action is due to CF and not to calcium ions or borate residue from its composition). Actually, Granfeldt et al. [33] showed that an increase in $[Ca^{2+}]_i$ mediated through binding of fMLF to its receptor is part of a signaling cascade that activates the plasma membrane-localized oxidase. At this moment, it is difficult to propose a mechanism by which CF inhibits NADPH-oxidase function because this membrane-bound enzyme complex is comprised of both integral membrane and cytosolic proteins, and it is subjected to a complex control. A better understanding of the mechanisms used by the CF to inhibit the NADPH oxydase will allow to develop a new therapeutic approach to deal with the phenomenon.

Hunt [34] proposed an essential role of boron as a regulator of respiratory burst by suppression of serine proteases released by inflammation-activated white blood cells, inhibition of leukotriene synthesis, reduction of reactive oxygen species generated during the neutrophil's respiratory burst, and suppression of T-cell activity and antibody concentrations.

Investigation of CF effects on the intracellular level of superoxide anions in unstimulated PMN cells strengthens the idea of the antioxidant activity exerted by CF, previously proposed by us [19], according to which CF is a scavenger for superoxide anions.

The superoxide dismutase (SOD) protect the PMN contents against oxidizing activity by destroying superoxide anions (O_2^-); SOD reduces both the oxidative stress and the activation of mediators of inflammatory response [35]. In our study, we pointed out a low level of superoxide anions at the cytotoxic dose of CF as a result of an increase in SOD activity. Low superoxide dismutase activity in *PMN cells* may explain the small discrepancies between the data regarding the superoxide anions and SOD activity. These data suggest that PMN cells apoptosis and CF-induced cytotoxicity is not mediated by the superoxide release.

Additional studies are required to determine the exact mechanism of antioxidant action of CF which may allow a better understanding of the mechanism of this nutritional supplement.

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