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Delphinidin, Luteolin and Halogenated Boroxine Modulate *CAT* Gene Expression in Cultured Lymphocytes

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ABSTRACT

Luteolin and delphinidin are the flavonoids with known protective roles. They inhibit genotoxic effects induced by halogenated boroxine (HB) *in vitro*. Statistically significant decrease in the number of micronuclei and nuclear buds and decrease in proportion of abnormal cells were observed before, but mechanism of their anti-genotoxic activity is still not clear. In our experiment we aimed to quantify HB effects on the relative expression of *CAT* (*catalase*) gene and explore antioxidative effects of luteolin and delphinidin via restoration of *CAT* gene activity. Cell cultures from peripheral blood lymphocytes of five healthy donors were established and treated with single and combined treatments of HB with luteolin or delphinidin. Total RNA was isolated from harvested cells and reverse-transcribed. SYBR based Real-Time PCR amplification method was used. Relative gene expression measurements were done using normalization of ratio of target (*CAT*) and housekeeping (*GAPDH*) genes. Intergroup variance analysis was done with REST® software. Luteolin itself lead to downregulation of relative *CAT* gene expression as well as HB. But simultaneous treatment of HB and bioflavonoids lead to upregulation. Delphinidin as indenpendent treatment and as simultaneous treatment caused upregulation of relative *CAT* gene expression. Obtained results may suggest protective role of delphinidin and luteolin to oxidative-stress damage caused by HB, and also that new approaches to the treatment applications of HB should include bioflavonoids and monitoring corresponding antioxidant system. Our findings indicate that there is a quantifiable effect of luteolin and delphinidine on antioxidant genes which could be used in exact monitoring of oxidative stress related events.

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Introduction

Oxidative stress presents the imbalance in cellular oxidation-reduction (redox) reactions in favor of the oxidation, leading to oxidative damage [1]. Oxidative-stress damage may influence the structure and function of numerous biomolecules (nucleic acids, proteins, carbohydrates, polyunsaturated lipids), which results in changes in the structure and

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function of cells, tissues and organs [2]. The resulting damage may disturb ion homeostasis, signal transduction in cells, gene transcription, and thus lead to other disorders. Oxidative stress has a significant role in the etiopathogenesis of cardiovascular and infectious disorders, cancer, fibrosis and the aging process [3].

Antioxidant enzymes fulfill the major role in antioxidant defense [1]. Catalase has a key antioxidant enzymatic role in the bodies defense against oxidative stress. It catalyses degradation of the reactive oxygen species (ROS) hydrogen peroxide to water and oxygen and thereby protects cells against ROS toxic effects [4]. Catalase is encoded by the *CAT* gene. It is reported that various polymorphisms in this gene cause decrease in catalase activity and confer to various diseases [4]. The levels of catalase are strongly affected by *CAT* gene expression modulation [4].

Bioflavonoids present natural, polyphenolic compounds with numerous protective antioxidant, antimutagenic and anticarcinogenic properties in human cells. These properties of selected bioflavonoids (delphinidin and luteolin) have been addressed in several studies before. Luteolin showed antiproliferative and anticarcinogenic activity against various cancers [5-7]. It has also increased level of other antioxidative enzymes i.e. glutathione-S-transferase, glutathione reductase or superoxide dismutase [8]. Delphinidin prevented muscle atrophy and upregulated miR-23a expression, inhibited tumor transformation of mouse skin JB6 P+ cells and also showed cytotoxic effects in human osteosarcoma cells and protective effects against ROS-induced injuries in epithelial cells of human retina [9-12].

Halogenated boroxine (HB; dipotassium-trioxohydroxytetrafluorotriborate) is a compound with suggested activity in treatment of benign and malignant skin changes [13]. It has been shown to have suppressive role in division of various cell types both *in vitro* and *in vivo*, and at higher concentrations exhibit proven genotoxic effects [14-15]. It’s suggested that it might act as a pro-oxidant at higher concentrations [16]. It’s observed that it can also inhibit activity of catalase [17].

In treatment of HB in combination with bioflavonoids (delphinidin and luteolin), its genotoxic effects were significantly decreased. In the presence of the HB, luteolin showed more potent to decrease the number of micronuclei and nuclear buds. In similar setting

delphinidin suppressed the occurrence of aberrant cells [18]. It was also observed that the expression of *hTERT* gene was upregulated in samples treated in combine with HB and selected bioflavonoids (luteolin and delphinidin) [19]. Still the mechanism of their anti-genotoxic activity is not clear. Since HB is recognized as inhibitor of catalase activity as a major antioxidant enzyme, we designed experiment to assess antioxidative effects of luteolin and delphinidin on level of relative expression of *CAT* (*catalase*) gene.

Materials and Methods

Sample collection, cell cultures and treatments

Five healthy female volunteers donated blood samples. They were informed about the study details and they signed informed consent forms. Total volume of 400 μL heparinized (BD Vacutainer Systems, Plymouth, UK) peripheral blood was mixed with 5 mL of the PB-MAX karyotyping medium (GIBCO Invitrogen) and cultured for 72 h at 37 °C. Each culture was treated with luteolin, delphinidin and halogenated boroxine—$K_2(B_3O_3F_4OH)$ (HB) individually, and with two substances simultaneously. So, six different treatments were conducted for each blood sample.

Treatments preparation

HB, a white powder soluble in water, dimethyl sulfoxide (DMSO) and ethanol, was synthesized according to modified protocol which was described by Ryss and Slutskaya [20]. In our experiment HB was dissolved in the PB-MAX karyotyping medium (GIBCO-Invitrogen, Carlsbad, CA, USA) at a definitive concentration of 0.1 mg/mL (397.4 μM), for which has been shown to exhibit genotoxic potential. Bioflavonoids delphinidin, -delphinidin chloride (96.70%; HPLC) and luteolin (98.34%; HPLC) (Phyto-Lab GmbH & Co. KG, Germany) were also dissolved in DMSO (Panreac Quimica, Barcelona, Spain). Their final concentration added in cultures was 50 μM.

RNA isolation and reverse transcription

After the cultivation, Quick-RNA^{TM} Mini Prep Plus kit (Zymo Research) was used for isolation of total RNA from harvested cell cultures. Isolated RNA was quantified on Qubit 2.0. Fluorometer (Invitrogen, Life Technologies), using a high-sensitivity RNA assay kit

and then reverse transcription of total RNA (10 ng) was done using Proto Script First-Strand cDNA Synthesis kit (New England BioLabs).

Relative gene expression analysis

SYBR based Real-Time PCR amplification method was used to analyze relative gene expression level in treated cultures. Realtime PCR was conducted in the 7300 Real-time PCR System (Applied Biosystems) and power SYBR Green Master Mix (Applied Biosystems,USA) was used according to the manufacturer's instructions. The following cycling conditions were set: 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min following standard program for dissociation curve. For the amplification of *CAT* target and *GAPDH* housekeeping gene highly purified primers obtained from BioTeZ Berlin-Buch GmbH (Germany) were used (Table 1.).

Relative Expression Software Tool (REST®) was used for the analysis of results - normalization of ratio of target (*CAT*) and housekeeping (*GADPH*) gene and statistical analysis (Pair Wise Fixed Reallocation Randomisation Test©) were done. Based on calculated relative expression ratio REST© gives us regulation factor. For up-regulation, the regulation factor is equal to the value of expression ratio and for down-regulation, the regulation factor is presented as a reciprocal value [21].

Table 1 Primers used for amplification of target and housekeeping gene in Real Time PCR

Gene	Forward	Reverse
GAPDH	TGAAGGTCGGAGTCAACGGA	CATCGCCCCACTTGATTTTGG
CAT	GAACTGTCCCTACCGTGCTC	GAATCTCCGCACTTCTCCAG

Result and Discussion

In luteolin-treated cultures *CAT* gene expression was down regulated when compared against control and HB-treated cultures, although not statistically significant. In all other cultures, when they were compared against culture treated with luteolin individually, *CAT* gene expression was statistically significant up regulated. It was observed that *CAT* expression was significantly upregulated in delphinidin-treated cultures and cultures treated simultaneously with HB and bioflavonoid (HB+delphinidin and HB+luteolin) when compared to cultures treated with HB only. In combined treatments of HB and antioxidants,

when compared against delphinidin-only treated cultures, relative *CAT* gene expression was up-regulated but not statistically significant. Also, there was no significant difference in *CAT* gene expression level between simultaneously treated cultures but it was up regulated in cultures treated with HB and luteolin. The results of our study have not detected any significant differences of *CAT* gene expression level in any treatment, single or combined, when compared to untreated cell-cultures (control).

Table 2 Results of comparative analysis of relative *CAT* gene expression. Regulation factor-Direction of change in expression-P-value are shown in each cell, respectively**.**
(* statistically significant change observed; ↓ - down regulated expression of *CAT* gene, ↑ - up regulated expression of *CAT* gene)

Treatment	NC	HB	LU	DE	HB+DE	HB+LU
HB	7,226 (↓) 0,817					
LU	23,778 (↓) 0,336	3,092 (↓) 0.194				
DE	5,315 (↑) 0,659	38,409 (↑) 0,005*	118,764 (↑) 0,001*			
HB+LU	12,201 (↑) 0,420	88,173 (↑) 0,007*	272,636 (↑) 0,003*	2,296 (↑) 0,163	1,115 (↑) 0,832	
HB+DE	10,946 (↑) 0,498	79,1 (↑) 0,005*	244,584 (↑) 0,001*	2,059 (↑) 0,249	-	-

In comparison with control, expression of *CAT* gene in any treatment wasn't significantly different. But, in luteolin-single tratment and HB-single treatment *CAT* expression was down-regulated, while in every other treatment it was up-regulated. In higher concentrations, HB exhibits genotoxic effect [14] and might act as a pro-oxidant [16]. Also, it can inhibit catalase activity [17]. So, the results obtained in this study for HB are kind of expected, as for the luteolin it could be surprising considering that bioflavonoids as

antioxidants are directly engaged in the suppression of damage of genetic structures [22] and that antigenotoxic activity of selected bioflavonoids *in vitro* has been confirmed [18]. However, luteolin also showed genotoxic effects and inhibited proliferation of human lymphocyte culture [23]. Furthermore, in the previous study of Hadzic *et al.* [19] in combined treatment of HB and luteolin, when compared to treatment with luteolin only, *hTERT* gene was significantly upregulated. This is in a way in correlation with our results as when we compared individual luteolin treatment and simultaneous HB and luteolin treatment, in cultures treated with simultaneous treatment *CAT* gene expression was significantly up regulated and by highest observed factor in the study.

In almost every culture treated with delphinidin, simultaneously or individually, *CAT* gene expression was up regulated, except when compared against culture treated with HB and luteolin simultaneously. It is familiar from previous studies that delphinidin reduces the proportion of micronuclei *in vivo* [24], has cytostatic [25] and concentration-dependent cytotoxic effects [11]. Concerning its genotoxic effects, in some studies it showed neither aneugenic nor clastogenic activity [23], while in others it showed significant inhibitory potential to the frequencies of total defect cells in the presence of genotoxins, but also induced them in individual treatments with delphinidin [26]. In this study, delphinidin did not cause down regulation of expression of *CAT*, as was the case with luteolin. Nevertheless, the upregulation in cultures treated with simultaneous treatment of HB and delphinidin compared to those treated with delphinidin respectively as well as higher regulation factors associated with simultaneous treatment than those associated with individual treatment, may indicate that this antioxidant also shows stronger antioxidant activity in the presence of prooxidants.

Non-concordant results from our study concerning luteolin effects could be explained by the fact that in numerous studies it has been shown that some well-known antioxidants such as vitamin C, flavonoid myricetin, hormone melatonin and various carotenoids can exhibit bimodal effects, both antioxidant and prooxidant, depending on the conditions [27-30]. Hence, though delphinidin and luteolin in combined treatment with HB have showed positive effect on *CAT* gene expression, there is a need for additional studies to better

understand the precise role of these flavonoids and to elucidate whether they could be double-edge sword in certain conditions.

Conclusion

Obtained results may indicate protective role of delphinidin and luteolin in combined treatment with HB, and also that potential treatment use of HB should include bioflavonoids and monitoring corresponding antioxidant system. Still, for more precise determination of luteolin and delphinidin antioxidative effects in combined treatments, more treatments and effector genes should be explored.

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