ISSN : 0974 - 7435 Volume 6 Issue 8,9

Trade Science Inc.

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 6(8,9), 2012 [289-293]

The cyto- and radioprotective effects of a whey protein isolate

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Received: 29th July, 2012 ; Accepted: 30th August, 2012

ABSTRACT

Here we report an in vitro investigation concerning some protective properties of a whey protein isolate (WPI), a commercially available product obtained from milk by ion exchange and ultrafiltration. The study aim was the evaluation of WPI cellular toxicity, product influence on doxorubicin (Dox) induced toxicity in cells pretreated with WPI and on the DNA damage produced by γ-rays in human lymphocytes.

WPI practically showed no cytotoxicity within a large interval of concentrations (900-9 μg cys/ml). In associated treatments with Dox, WPI at doses of 900, 180 and 90 μg cys/ml attenuated the drug cytotoxic side-effects in normal cells (Hfl-1) in a dose linear dependent manner. γ-rays induced genotoxicity was significantly reduced by WPI, particularly by accelerating the DNA repair of the radioinduced damage in human lymphocytes, in a dose-effect relationship. Significant differences were found in the number of repaired lesions ($p < 0.01$), at 2 hrs (2.93, 2.06 and 1.98 fold lower at doses of 450, 180 and 90 μg cys/ml, respectively).

KEYWORDS

Cytotoxicity;
Doxorubicin;
Radioprotection;
Whey protein isolate.

INTRODUCTION

Reactive oxygen species (ROS), generically known as free radicals, are highly reactive molecules constantly produced in living organisms as a consequence of metabolic and other biochemical reactions as well as of external factors[1-3]. ROS are essential for life because they play major roles in vital processes (apoptosis, signal transduction, bactericidal activity of phagocytes, etc.)[3-5] and their physiological levels are controlled by endogenous enzymatic defense mechanisms[5,6].

The oxidative stress is a biochemical condition that is characterized by a disturbed balance between high levels of reactive species and the organism defense capacity. This biochemical status leads to various conditions (cancers, cardiovascular and neurological diseases) and ageing[7,8].

There is a lot of evidence that the harmful, undesirable effects of oxidative stress can be diminished or even blocked by an array of natural products from fruits

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and vegetables which, due to their antioxidant properties, act as potential free radicals scavengers[7,9].

We focused our attention on a whey protein isolate (WPI), a commercially available product, enriched in cysteine (cys), obtained from milk by ion exchange and ultrafiltration. This product contains a heterogeneous group of proteins as well as other bioactive substances like growth factors and cytokines which can play important physiological roles[10,11].

Our study concerned the *in vitro* WPI cellular toxicity, product effects on Dox induced toxicity and its influence on DNA damage produced by γ-rays in human lymphocytes.

EXPERIMENTAL

Chemicals

WPI was obtained from New Zealand Trade March., Ltd. (NZ). Fetal calf serum (FCS), glutamine, penicillin, Dulbecco's Phosphate Buffered Saline (PBS), Histopaque 1077, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and all the reagents for Comet assay were from Sigma-Aldrich (Germany). Doxorubicin was from Zhejiang Hisun Pharm. Co. (P.R.China).

Cell cultures

Human lung fibroblasts, Hfl-1, were from ECACC (European Collection of Cell Cultures) and the human ovary carcinoma, Mls, was a gift from dr.Y.Shifenbauer, Medisel Technologies, Israel.

Cluture media (from Sigma-Aldrich, Germany) were: F-12 Nutrient Mixture for Hfl-1 and DMEM for Mls. All media contained 10% FCS, 1% L-glutamine and 1% penicillin/ streptomycin.

Blood samples were collected from volunteers by venopuncture and the lymphocytes were isolated using Histopaque, according to the procedure indicated by the producer (Sigma-Aldrich).

Cellular toxicity assay

Toxicity of WPI was evaluated by MTT test, described by Mosmann (1983)[12]. Cells seeded in 96-well plates were treated, at subconfluent cell-density with 9 doses (180000 - 9 μg cys/ml)[1] of WPI. In associated treatments, three concentrations of WPI (900, 180 and 90 μg cys/ml) in PBS were given 30 minutes before cumulative doses of Dox (200 – 0.1 μM). The cells were incubated for 24, 48 and 72 h, respectively and the colorimetric measurements were done in the presence of MTT dye, at 492 nm with a plate reader (Tecan, Sunrise, Austria).

DNA damage assay (Comet assay)

Immediately after isolation, the lymphocytes were incubated in RPMI medium with 20 % FCS at 37ºC and 5% CO_2. Thirty minutes before irradiation, cells were treated with WPI (450, 180 and 90 μg cys/ml). Irradiation was carried out with 2 Gy (Theratron 1000, Canada). After irradiation, the cells were processed by alkaline comet assay in order to measure the DNA damage. The alkaline comet assay was performed according to Tice (2000)[13] protocol. Conditions used for electrophoresis of single cells embedded in agarose, were 0.83 V/cm and 300 mA for 30 minutes.

To evaluate the process of cellular repair, the irradiated cells were incubated at 37ºC and 5% CO_2, for 120 minutes and the residual DNA damage was again measured by Comet assay.

Statistical analysis

Dose response curves were calculated using GraphPad Prism software program, version 5.0 (GraphPad, San Diego, Ca., USA). Data were given as mean value ± standard error of the mean (SEM). Statistical comparison between groups was made by one-way ANOVA followed by Dunett multiple comparison test and Student's t-test, respectively; p-values under 0.05 were considered as statistically significant.

RESULTS

WPI cytotoxicity in normal and tumor cells

The WPI toxicity in normal (Hfl-1) and tumor (Mls) cells was estimated by measuring their viability in the presence of WPI, the preparate doses being spread over a large interval of concentrations (180000 - 9 μg cys/ml).

In normal fibroblasts (Hfl-1), WPI solutions practically exhibited no toxic effects (Figure 1a). Even more, at high contents of cysteine (180000 – 90000 μg cys/

ml), a stimulation of growth was found at both 24 and 48 hrs. At lower doses (900 – 9 μg cys/ml) the measurements indicated a quasi flat profile without significant differences in the cellular growth as a function of WPI doses.

Instead, at 72 hrs, an inhibitory effect occurred as a consequence of a diminished proliferation rate and/or cellular death (data not shown). The inhibitory effects vs. logarithm of doses follows a sigmoidal curve shape which allowed the calculation of IC_{50} value (1.24 μg cys/ml) i.e. the WPI concentration required to reduce cells survival by 50%.

In the Mls cells, after 24 hrs at high doses of WPI (180000 – 90000 μg cys/ml) an inhibition of cellular growth was found. At 48 hrs, for the same tumor cells, there was no evidence of toxic effects (Figure1b).

We investigated the Hfl-1 cells viability following the administration of eight doses of Dox alone (within 200 – 0.1 μM) and in pretreatment regimens (30 minutes before) with three variable doses of WPI (900, 180 and 90 μg cys/ml).

The IC_{50} values of Dox at 24 h were quantified in each of the four treatments. They were scored from the four parameters sigmoidal curves fit and are given in TABLE 1. Cytotoxic activity of Dox was decreased by WPI (higher toxic effects means lower IC_{50} values) in a dose-effect relationship. These findings demonstrate that, in associated treatments with Dox, WPI produced a linear dose dependent chemoprotective effect in Hfl-1 cells (R^2=0.996).

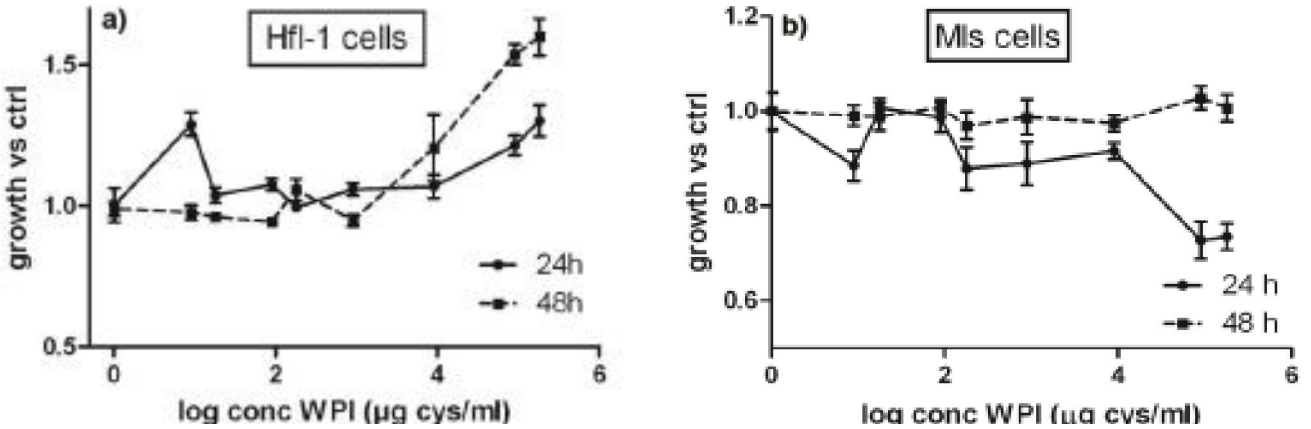

Figure 1: WPI toxicity in: a) Hfl-1 and b) Mls cells, at 24 and 48 h; the results were expressed as the mean ± SEM in triplicate measurements from 2 separate experiments (n = 6).

Therefore, WPI very low cytotoxicity recommends its potential use, within a large interval of concentrations (900 - 9 μg cys/ml), in associated treatments with other drugs at exposure times of 24 and 48 h.

The synergistic effect of WPI in associate treatments with doxorubicin

Doxorubicin is an antineoplastic agent broadly used in the treatment of a large variety of malignancies (solid and hematological). Nonetheless, the Dox clinical use is restricted because of frequent induction of dose-dependent toxic side-effects including cardio- and mielotoxicity, as a consequence of free radicals production by the cytostatic[14,15].

An immediate approach against Dox prooxidative effects on normal cells might be the combination of drug delivery with antioxidants[15,16].

Radioprotective effect of WPI on irradiated human lymphocytes

Single cell electrophoresis in agarose gel (Comet assay) is a sensitive and efficient method to detect DNA lesions induced by ionizing radiation at cellular level, the γ rays-induced genotoxicity being detected before producing cell death[17]. We examined the influence of WPI on the DNA damage and repair capacity of human blood peripheral lymphocytes irradiated with γ-

TABLE 1: IC_{50} values of Dox (μM) in associate treatments with WPI, in Hfl-1 cells, at 24 hrs (n = 3).

	Dox	Dox + WPI (μg cys/ml)		
		900	180	90
IC_{50} (μM)	13.07	25.18***	18.95**	17.65**

*****p<0.001, **p<0.01 by one-way ANOVA and Dunett multiple comparison test.**

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rays, by using Comet assay. The comets were visually assigned using an Eclipse E-100 fluorescence microscope (Nikon, Japan). At least 250 cells per slide were visually analyzed and included in one of the 5 categories (Figure 2).

If there is no DNA damage, the nucleoids shows no tail. When DNA damage occurred, the nucleoids have

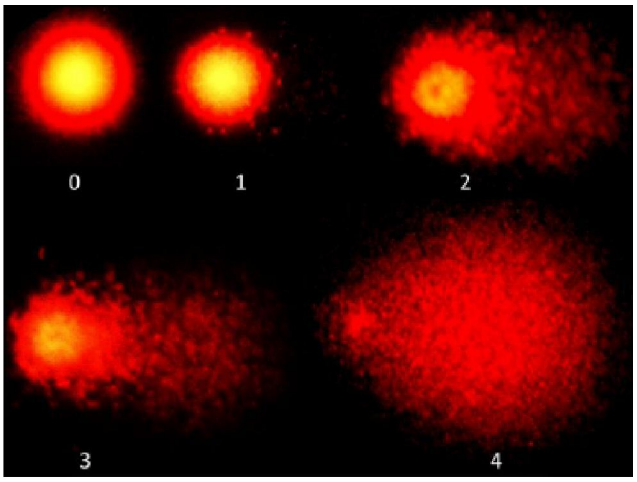

Figure 2 : Representative comet images showing different levels of DNA damge in human lymphocytes

tails like comets. The DNA damage was calculated as tail factor and the results obtained after 2 Gy irradiation with and without WPI are shown in Figure 3.

Radioinduced DNA damage was suppressed by 1.17 fold (vs. the respective control, p<0.05) when lymphocytes were treated with 450 µg cys/ml WPI. The effect attenuated progressively with WPI doses decrease (TABLE 2).

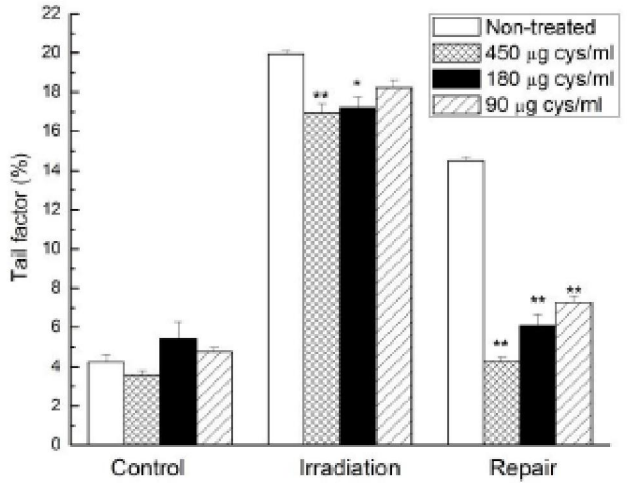

Figure 3 : Effect of WPI treatment on DNA damage in 2 Gy-irradiated human lymphocytes; the values presented as mean ± SEM, n = 2 (* p < 0.05, ** p <0.01)

TABLE 2 : Tail factor in comet assay of lymphocytes pretreated with WPI and irradiated with 2 Gy γ-rays.

WPI (µg cys/ml)	Tail factor (mean ± SEM)		
	Control	Irradiation	Repair
0 (non-treated)	4.2 ± 0.4	19.9 ± 0.2	14.5 ± 0.2
90	4.7 ± 0.2	18.2 ± 0.4	7.3 ± 0.2**
180	5.4 ± 0.8	17.2 ± 0.6*	6.1 ± 0.6**
450	3.6 ±0.2	16.9 ± 0.5**	4.2 ± 0.2**

****p<0.01 (comparison with the corresponding non-treated cells)**

***p<0.05 (comparison with the corresponding non-treated cells)**

Surprisingly larger differences vs. the corresponding irradiated cells were found in the number of residual lesions at 2 hrs. Thus, WPI significantly reduced the levels of DNA damage ($p < 0.01$) by 2.93, 2.06 and 1.98 fold, at doses of 450, 180 and 90 µg cys/ml, respectively. A linear correlation between WPI concentrations and the repair of the radiation induced damage (R^2=0.99).

DISCUSSION

The amino acid analysis of WPI indicates a complex profile of 18 amino acids with an elevated content in cysteine (2.26 g /100 g product). Cysteine is reported as being the crucial limiting amino acid in intracellular glutathione (GSH) synthesis[18]. Glutathione antioxidant system is the principal protective mechanism of the cell and experimental studies demonstrated that cysteine-rich whey protein concentrates represent an effective cysteine delivery system for GSH replenishment[19]. Thereafter, the involvement of GSH in cancer protection includes a reduction in free radical and other reactive species amounts.

Cell culture studies showed that whey protein concentrates were able to inhibit selectively cell growth in human breast[11,20,21] and prostate cancers[11]. Animal models, usually for colon and mammary tumors, nearly always show that whey protein is superior to other dietary proteins for suppression the tumor development[22]. Case reports are presented which suggest an antitumor effect of a whey protein dietary supplement in some uro-genital cancers[23].

We particularly attempted to exploit the cysteine-rich content of WPI so we combined it in pretreatments with Dox or irradiation. The concentrate very low tox-

icity is a favorable premise for this type of treatments and the product has proved to attenuate the toxic side-effects of the cytostatic drug. Previously, we demonstrated that a red grape seed extract, in association with Dox, synergistically reduced the negative cytotoxic effects of drug in normal cells in a dose-dependent manner, without weakening, even increasing, the drug antiproliferative activity on tumor cells[24], whilst a tocotrienol containing palm oil protected unselectively both normal and tumor cells[25]. On the other side, antioxidants acting as free radical scavengers might have a key role in radioprotection because radiation induced genotoxicity is mediated also through generation of free radicals which attack inclusively the DNA macromolecules in biological systems[19,26]. γ-rays induced genotoxicity was significantly reduced by WPI, particularly by accelerating the DNA repair damage induced by radiation in human lymphocytes in a manner related to dose. Hencefore, we can conclude with the hypothesis suggested in literature[9] that WPI may protect the cells indirectly by donating cysteine for GSH synthesis. Confirmation of this hypothesis requires more in depth *in vitro* experiments.

ACKNOWLEDGEMENTS

This paper has been supported by the Romanian Ministry of Education and Research, CEEX grant nr.15/2005.

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