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Original

Mineral and/or milk supplementation of fruit beverages helps in the prevention of H_2O_2-induced oxidative stress in Caco-2 cells

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Abstract

Introduction: Fruit beverages are commonly supplemented with milk, vitamins and/or minerals in order to improve their healthy effects by providing some bioactive components that can act additively or synergistically against oxidative stress.

Aims: To test whether iron, zinc, and milk added to fruit beverages do not affect the cytoprotective effect against oxidative damage to Caco-2 cells through GSH-related enzymes induction and cell cycle progression preservation, in comparison with non-supplemented fruit beverage.

Methods: Caco-2 cells were incubated 24 h with the bioaccessible fraction (BF) of eight fruit beverages with/without iron and/or zinc, and/or milk, and then challenged with H_2O_2 (5 mmol L^{-1} -2 h). Mitochondrial enzyme activities (MTT test), GSH-Rd and GSH-Px enzyme activities, cell cycle progression and caspase-3 activity were measured.

Results and discussion: Fruit beverages prevented the deleterious effect of H_2O_2 on cell viability, with almost all samples reaching control basal levels. Only independent iron or zinc supplementation with/without milk exerted positive effects upon GSH-Rd activity. Both minerals with milk, afforded improved preservation of GSH-Px activity. All samples prevented the decrease in the G1 phase of cell cycle induced by H_2O_2, except iron supplemented samples with/without milk, but none of them avoided the increase in sub-G1 phase. However, this fact was not associated to caspase-3 activity, with a probable positive effect of zinc upon this parameter.

Conclusion: Mineral and/or milk supplementation of fruit beverages helps in the prevention of oxidative stress in Caco-2 cells based on cell viability maintenance, GSH-related enzymes activation, cell cycle distribution preservation and inhibition of caspase-3 activation.

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Key words: *Caco-2 cells. Fruit beverages. Minerals. Milk. Oxidative stress.*

LA ADICIÓN DE MINERALES Y/O LECHE A BEBIDAS A BASE DE ZUMO DE FRUTAS AYUDA EN LA PREVENCIÓN DEL ESTRÉS OXIDATIVO INDUCIDO POR H_2O_2 EN CELULAS CACO-2

Resumen

Introducción: En la actualidad las bebidas a base de zumo de frutas llevan adicionadas leche, vitaminas y/o minerales con objeto de mejorar sus efectos beneficiosos para la salud mediante el aporte de numerosos compuestos bioactivos que pueden actuar de forma aditiva o sinérgica frente al estrés oxidativo.

Objetivos: Evaluar si la adición de hierro, cinc y leche a las bebidas a base de zumo de frutas no afecta al efecto citoprotector frente al daño oxidativo en células Caco-2 a través de la inducción de enzimas del ciclo del GSH y la preservación de la progresión del ciclo celular, en comparación con la bebida a base de zumo de frutas no suplementada.

Métodos: Las células Caco-2 se incubaron 24 h con las fracciones bioaccesibles (FB) de ocho bebidas a base de zumo de frutas con/sin hierro y/o cinc y/o leche, y se sometieron a estrés oxidativo con H_2O_2 (5 mmol L^{-1}-2 h). Se determinó la actividad enzimática mitocondrial (test MTT), la actividad de las enzimas GSH-Rd y GSH-Px, la progresión del ciclo celular y la actividad de la enzima caspasa-3.

Resultados y discusión: Las bebidas a base de zumo de frutas previnieron del efecto perjudicial del H_2O_2 sobre la viabilidad celular, con casi todas las muestras alcanzando los niveles basales del control. Sólo la adición independiente de hierro o cinc con/sin leche ejerció efectos positivos sobre la actividad de la enzima GSH-Rd. Por otra parte, ambos minerales con leche proporcionaron una mejor preservación en la actividad de la enzima GSH-Px. Todas las muestras previnieron el descenso en la fase G1 del ciclo celular inducido por el H_2O_2, excepto la muestra adicionada de hierro, pero ninguna de ellas evitó el incremento en la fase subG1 del ciclo celular. Sin embargo, este hecho no estuvo asociado con la actividad de la enzima caspasa-3, con un probable efecto positivo del cinc sobre este parámetro.

Conclusión: La adición de minerales y/o leche a las bebidas a base de zumo de frutas ayuda en la prevención de estrés oxidativo en células Caco-2 mediante el mantenimiento de la viabilidad celular, activación de enzimas del ciclo del GSH, preservación de la progresión del ciclo celular e inhibición en la activación de la enzima caspasa-3.

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Palabras clave: *Células Caco-2. Bebidas a base de frutas. Minerales. Leche. Estrés oxidativo.*

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Abbreviations

BF: Bioaccessible fraction.
GSH: Glutathione.
GSH-Rd: Glutathione reductase.
ROS: Reactive oxygen species.
$\Delta\psi_m$: Mitochondrial membrane potential.
SOD: Superoxide dismutase.
MTT: 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide.
CPPs: Caseinophosphopeptides.
GSH-Px: Glutathione peroxidase.

Introduction

The growing interest in functional foods has stimulated intensive research aimed at understanding their health benefits. At present, fruit beverages are commonly supplemented with milk, vitamins and/or minerals such as iron and zinc in order to improve their healthy effects by providing some of the so-called bioactive components.[1,2] Fruits[3] and citrus juices[4] contain many antioxidant compounds that can act additively or synergistically[5] against oxidative stress. In addition to their positive effect upon micronutrient bioavailability, a potential antioxidant role for isolated caseinophosphopeptides (CPPs) provided by milk, and formed during gastrointestinal digestion, has been suggested.[6,7]

Oxidative stress impairs the intracellular redox status, which is known to play a critical role in cell function and regulate cell proliferation.[8] It has been reported that human intestinal (Caco-2) cells metabolize reactive oxygen species (ROS) through the glutathione (GSH) cycle, and that intracellular GSH depletion reflects oxidative damage.[9] Physiologically, the functionality of the GSH cycle is conditioned by the mitochondrial production of reducing equivalents, and GSH precursors or intracellular GSH concentration has been found to affect cell proliferation.[10] Oxidative stress results in an imbalance between ROS accumulation and antioxidant defense systems in cells, thereby affecting mitochondrial integrity.[11,12] ROS accumulation affects the cell cycle checkpoints and control systems that regulate cell proliferation.[13,14]

Caco-2 cells have been successfully used to examine the effects of different foods, or food extracts, against oxidative stress, including phenolic apple juice extract,[15] plants (sage, rosemary and oregano)[16] and anthocyanin blackberry extracts,[17] or carotenoids[18] and flavonoids.[19] These studies have not considered aspects such as the potential instability and structural changes of antioxidants during digestion and/or interaction with other food components in the gut. Accordingly, the antioxidant capacity inherent to foods or their individual components may be overestimated.

Several *in vitro* procedures simulating the human gastrointestinal digestion process have been developed to evaluate the stability and bioaccessibility of carotenoids in commonly consumed herbs,[20] and antioxidant compounds (such as polyphenols) in raspberry[21] and in fruit beverages.[11,22-24] The antioxidant effect of bioaccessible fractions of fruit beverages, with/without skimmed milk and/or mineral supplements, against H_2O_2-induced oxidative damage to fully differentiated Caco-2 cells[11] has been described. Although the bioaccessible fraction of fruit beverages did not prevent intracellular ROS accumulation, a more preserved mitochondrial membrane potential ($\Delta\Psi m$) and thus mitochondrial enzyme activity, was observed.[11] To the best of our knowledge, little information is available on the H_2O_2-mediated effects and potential cytoprotective action of fruit beverages supplemented with minerals and/or milk upon the cell cycle progression of fully differentiated Caco-2 cells. In this respect, as far as we are aware, only Laparra et al.[7] have reported the positive effect of isolated CPPs and bioaccessible fraction of fruit beverages with/without milk upon the H_2O_2-mediated decrease in the G1 phase cell population; in addition, these samples preserved GSH-reductase activity - a sensitive biomarker of H_2O_2-induced oxidative stress.

Objectives

Taking these facts into account, and based on previous findings in which the total antioxidant capacity of these beverages was not reduced by either gastrointestinal digestion or mineral and/or milk supplementation,[23] the present study continues previous work[11] and reflects novel data with the aim of determining if dietary factors such as iron, zinc, and milk added to fruit beverages do not adversely affect the cytoprotective effect of these beverages against H_2O_2-induced oxidative damage to Caco-2 cells through GSH-related enzymes induction (reductase and peroxidase), cell cycle progression preservation, and avoiding cell death apoptosis related processes (rise in subG1 cell cycle phase and activation of caspase-3) in comparison to non supplemented fruit beverage.

Materials and methods

Samples

A fruit beverage (Fb) (grape + orange + apricot) with/without iron (Fe) and/or zinc (Zn), and with/without skimmed milk (M), was used in this work, with the following references: Fb, FbFe, FbZn, FbFeZn, FbM, FbFeM, FbZnM and FbFeZnM. The compositions of the aforementioned samples are shown in table I.

In vitro digestion

To simulate the human gastrointestinal digestive process, samples of fruit juices (80 g) were subjected to

Table I
Composition of the fruit beverages assayed

Component (g kg⁻¹)	Sample	
	Fb	FbM
Osmosis water	5.87	5.77
Apricot puree	2.45	2.45
Grape concentrate	0.72	0.72
Orange concentrate	0.42	0.42
Sugar	0.51	0.51
Skimmed milk powder	–	0.104
Classic pectin	0.04	0.04
Vitamin C (L-ascorbic acid)	0.0054	0.0054

Fb = Fruit beverages with/without mineral supplementation, Fe (sulphate, 0.0003 g Fe kg⁻¹ fruit beverage) and/or Zn (sulphate, 0.00016 g Zn kg⁻¹ fruit beverage).
FbM = Fruit beverages with skimmed milk (0.104 g kg⁻¹ fruit beverage) with/without mineral supplementation.

an *in vitro* procedure as previously described.[11] After gastric (pepsin-pH 2) and intestinal (pancreatin and bile extract-pH 6.5) steps, and prior to the assays with Caco-2 cells, the digests were heated for 4 min. at 100ºC to inhibit sample proteases, and were then quickly immersed in an ice bath. Twenty-gram aliquots of the inactivated digests were transferred to polypropylene centrifuge tubes and centrifuged at 3,890 g for 60 min. at 4ºC to separate the soluble fractions, which were pooled. Glucose (5 mmol L⁻¹ final concentration) and HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) (50 mmol L⁻¹ final concentration) were added to the soluble fraction, and finally water was added to adjust the osmolarity to 310 ± 10 mOsm kg⁻¹ (freezing point osmometer, Osmomat 030). The soluble fraction obtained is here referred to as the bioaccessible (BF) fraction, and represents the maximum soluble fraction of food-derived compounds in the simulated gastrointestinal media that would be available for absorption.

Caco-2 cell culture

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC 86010202, Salisbury, UK). Cultures were maintained and grown as previously described.[25] For the assays, Caco-2 cells were seeded onto 24-well plates (Costar Corp., USA) at a density of 5 x 10⁴ cells cm⁻² with 1 mL of minimum essential medium (MEM), and the culture medium was replaced every two days. Fifteen to 18 days after initial seeding, the culture medium was aspirated, and the cell monolayers were washed twice with PBS warmed to 37ºC. The cells were then incubated for 24 h with the BF of fruit beverages diluted in MEM (1:1 v/v). Posteriorly, culture medium was removed and the cells were washed twice with PBS at 37ºC. For the induction of oxidative stress, cell cultures were exposed to a 5 mmol L⁻¹ H₂O₂ solution in MEM for 2 h. Afterwards, the cultures were washed twice with PBS (37ºC) and used to monitor the biological parameters as described below.

Evaluation of mitochondrial enzyme function

The mitochondrial functionality of the Caco-2 cells was evaluated by using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide) assay, as previously reported.[11] This colorimetric method is based on reduction of the tetrazolium ring of MTT by mitochondrial dehydrogenases,[26] yielding a blue formazan product which can be measured spectrophotometrically; the amount of formazan produced is proportional to the number of viable cells. The conversion to insoluble formazan was measured at 570 nm with background subtraction at 690 nm. Control cells were used in each assay.

Measurement of GSH-reductase (GSH-Rd) and GSH-peroxidase (GSH-Px) activities

GSH cycle enzyme activities were measured as previously described for GSH-Rd[27] and GSH-Px.[28] These methods monitor the decomposition of NADPH at 340 nm. Briefly, to determine GSH-Rd, an aliquot (50 μL) of the cell homogenate was mixed with 140 μL of 100 mmol L⁻¹ phosphate buffer containing 5 mmol L⁻¹ EDTA. Then, 15 μL of a 10 mmol L⁻¹ NADPH solution and 100 μL of a 20 mmol L⁻¹ GSSG solution were added to the cell homogenate. The decrease in absorbance (λ, 340 nm) was recorded every minute for 10 minutes using a Multilabel Plate Counter VICTOR³ 1420 (Perkin Elmer, Turku, Finland). GSH-Px activity was measured in an aliquot (50 μL) of the cell homogenate mixed with 150 μL of 100 mmol L⁻¹ phosphate buffer containing 1 mmol L⁻¹ EDTA. Then, 25 μL of 2.4 U GSH-Rd activity mL⁻¹ and 25 μL of 10 mmol L⁻¹ GSH solution in 100 mmol L⁻¹ phosphate buffer were added, and the mixture was incubated (37ºC - 5 min). Afterwards, 15 μL of a 10 mmol L⁻¹ NADPH solution was added, and the decrease in absorbance (λ, 340 nm) was recorded. Changes in the rate of absorbance were converted into units of GSH-Rd and GSH-Px using a molar extinction coefficient of 6.22 x 10³ M⁻¹ cm⁻¹, and the results were expressed as a percentage of the control. One unit of activity was defined as the oxidation of 1 μmoL of NADPH per minute.

Cell cycle analysis

Cell cycle analysis was performed by propidium iodide (PI) staining of DNA content in exposed cultures.[29] Briefly, cells were washed with PBS and resuspended in 1 mL of lysis buffer [1 mg ml⁻¹ of trisodium citrate, 1 μl ml⁻¹ of sodium dodecyl sulfate (0.5% w/v), 0.05 mg ml⁻¹ PI, and 1 mg ml⁻¹ of RNase A (Sigma, P4875)]. After
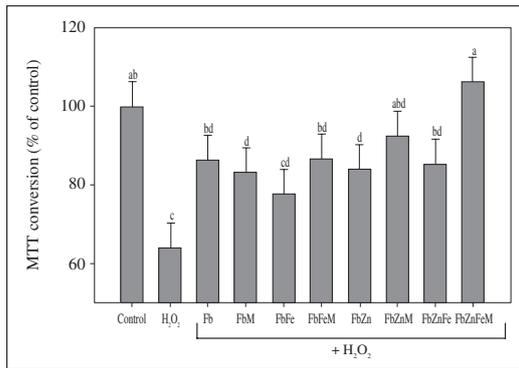
Fig. 1.—Mitochondria enzyme activities in cell cultures, preincubated (24 h) or not with fruit beverages, exposed to 5 mmol L^{-1} H_2O_2 (2 h). Fruit beverage (Fb) (grape + orange + apricot) with/without iron (Fe) and/or zinc (Zn) and with/without skimmed milk (M). Results are expressed as mean ± standard deviation (SD) (n = 4). Different letter on the bars indicate significant statistically (p < 0.05) differences analysed by one-way ANOVA followed by Fischer's LSD post hoc test.

incubation overnight at 4ºC, the released nuclei were resuspended by agitation with a Pasteur pipette, and the fluorescence was analyzed by flow cytometry (Coulter, EPICS XL-MCL, USA) at l_{exc} = 536 nm and l_{em} = 617 nm. Control cells were used in each assay.

Caspase-3 activity

Caspase-3 colorimetric determination (CASP-3C kit, Sigma) was based on hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA, Sigma) by caspase-3, resulting in release of the p-nitroaniline (pNA) moiety. The concentration of the pNA released from the substrate was calculated from the absorbance values at 405 nm and from a calibration curve prepared with defined pNA solutions. Results were expressed as a percentage of active caspase-3-positive cells in control cultures.

Statistical analyses

Results are presented as means ± SD (n = 4). One-way analysis of variance and Fischer's LSD *post hoc* test were applied. A significance level of p < 0.05 was adopted for all comparisons. Statgraphics Plus version 5.1 (Rockville, Maryland, USA) was used for the statistical analysis.

Results

Mitochondrial enzyme activities

The incubation period with BF (1:1, v/v in MEM) of fruit beverages was not overtly toxic to the cell cul-

tures, as concluded from the MTT conversion values (74.9-132.5%) determined in the cell cultures prior to the induction of oxidative stress. The effects of mineral and/or milk supplementation of fruit beverages against H_2O_2-mediated oxidative stress in Caco-2 cultures are shown in figure 1. Direct H_2O_2 exposure of cell cultures caused a sharp (p < 0.05) decrease in MTT conversion values, demonstrating the deleterious effect of H_2O_2-induced oxidative stress upon cell cultures. On the other hand, cell cultures incubated with the BF of fruit beverages exhibited more preserved mitochondrial enzyme function after exposure to H_2O_2 with almost all samples reaching control basal levels. As regards mineral supplementation, neither Fe nor Zn exerted a negative effect upon MTT conversion. Milk supplementation had a clear positive effect compared with its counterpart, when both minerals were present together.

GSH cycle enzyme activities: GSH-Rd and GSH-Px

The incubation of cell cultures with the bioaccessible fraction of fruit beverages not subjected to oxidative stress did not impair either GSH-Rd or -Px activity, as concluded from the values ranging between 94.4-265.4% of the control values. The effect of mineral and milk supplementation upon GSH cycle enzyme activities after H_2O_2-exposure is shown in figure 2. Direct H_2O_2 exposure of cell cultures primarily affected GSH-Px activity (p < 0.05), but surprisingly not GSH-Rd activity. In general, pre-treatment of cells with BF prior to oxidative stress provoked an induction of both enzymes (with the exception of Fb and FbZnFe for GSH-Px) what could be expected as a preparation of the cell against a potential oxidative injury. Mineral supplementation exerted a positive effect upon both GSH-Rd
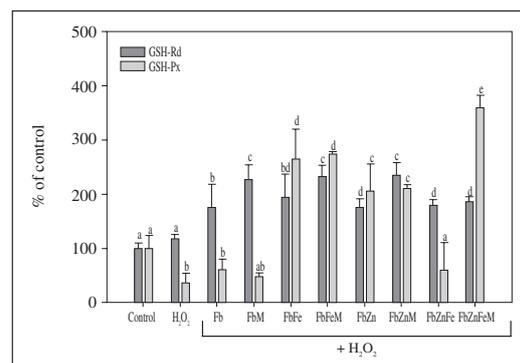

Fig. 2.—GSH reductase (GSH-Rd) and GSH-peroxidase (GSH-Px) enzyme activities in Caco-2 cultures, preincubated (24 h) or not with fruit beverages, exposed to 5 mmol L^{-1} H_2O_2 (2 h). Fruit beverage (Fb) (grape + orange + apricot) with/without iron (Fe) and/or zinc (Zn) and with/without skimmed milk (M). Results are expressed as mean ± standard deviation (SD) (n = 4). Different letter on the bars indicate significant statistically (p < 0.05) differences analysed by one-way ANOVA followed by Fischer's LSD post hoc test.

	Sample	sub-G_1	G_1	S	G_2/M
H_2O_2	Control	0.60 ± 0.10[a]	71.46 ± 2.18[ab]	9.32 ± 2.16[a]	14.85 ± 3.23[abc]
	H₂O₂	5.36 ± 0.56[b]	58.82 ± 0.66[c]	11.35 ± 1.02[abc]	15.71 ± 1.84[bc]
	Fb	3.40 ± 0.87[c]	65.16 ± 3.97[de]	13.18 ± 2.61[cd]	15.08 ± 2.39[abc]
	FbM	5.74 ± 1.99[b]	71.26 ± 5.46[ab]	14.36 ± 1.94[de]	12.09 ± 1.99[a]
	FbFe	8.35 ± 0.48[d]	61.00 ± 0.45[c]	10.25 ± 0.13[ab]	17.28 ± 0.90[c]
	FbFeM	4.73 ± 0.55[bc]	63.19 ± 1.85[ce]	17.53 ± 1.69[f]	16.31 ± 3.11[c]
	FbZn	8.60 ± 0.88[de]	65.63 ± 0.17[de]	14.86 ± 0.87[de]	15.26±0.89[abc]
	FbZnM	5.30 ± 0.98[b]	67.89 ± 0.25[de]	16.58 ± 1.01[ef]	12.80 ± 1.31[ab]
	FbFeZn	10.34 ± 2.05[e]	71.69 ± 2.59[ab]	13.67 ± 0.86[cd]	14.00 ± 1.79[abc]
	FbFeZnM	8.42 ± 0.17[d]	72.68 ± 3.03[a]	12.40 ± 0.37[bcd]	14.85 ± 1.48[abc]

Results are percentage of cell population in each cycle phase and are expressed as mean ± standard deviation (n = 4). [a-c]Different case letter indicate significant statistically (p < 0.05) differences in the same column analysed by one-way ANOVA followed by Fischer's LSD post hoc test.

and -Px activity, although GSH-Px in cell cultures incubated with the sample FbFeZn did not differ from the controls. On the other hand, milk supplementation exerted a significant (p < 0.05) effect upon GSH-Rd activity relative to its respective counterparts, except when both minerals were supplemented together (FbFeZn and FbFeZnM). In contrast, GSH-Px activity proved highest in cultures incubated with FbFeZnM.

Cell cycle analysis and caspase-3 activity

Oxidative stress-induced alterations in the cell cycle phase populations are shown in table II. Cell cultures directly exposed to H₂O₂ showed a marked decrease (17.6%) in the G1 phase population with respect to control cultures. The incubation of cell cultures with samples supplemented with Fe, with/without milk, did not prevent the decrease in G1 phase population, and only cell cultures incubated with samples FbM, FbFeZn and FbFeZnM exhibited a cell cycle profile closely resembling the controls. The other samples showed higher G1 phase than H₂O₂ stressed cells without reaching control levels. These observations were accompanied by corresponding increases (p < 0.05) in the S phase populations, but not in cell cultures directly exposed to H₂O₂ and FbFe sample. In all cases, the G2/M phase population was unaltered, probably because of the short H₂O₂ exposure time (2 h) involved. In addition, none of the samples avoided the increase in sub-G1 phase evoked by H₂O₂.

The H₂O₂-mediated deleterious effect upon Caco-2 cultures is evidenced by the sharp increase (p < 0.05) in caspase-3 activity compared to the controls (fig. 3). The results suggest that fruit beverages likely exerted a positive effect against H₂O₂-induced caspase-3 activation, as concluded from the statistically non significant (p > 0.05) differences recorded versus the controls.

Mineral, but not milk supplementation, seemed to interfere with caspase-3 activation. The potential pro-oxidant contribution of Fe supplementation to fruit beverages did not exert any additional effect upon caspase-3 activation. In addition, the data suggested a likely positive effect of Zn supplementation, alone and/or with Fe, upon caspase-3 activation, since its presence in BF is related to a suppressing in H₂O₂-induced enhancement of caspase-3 activity.

Discussion

Oxidative stress was induced in human intestinal cell cultures (Caco-2) with a concentration of H₂O₂ chosen

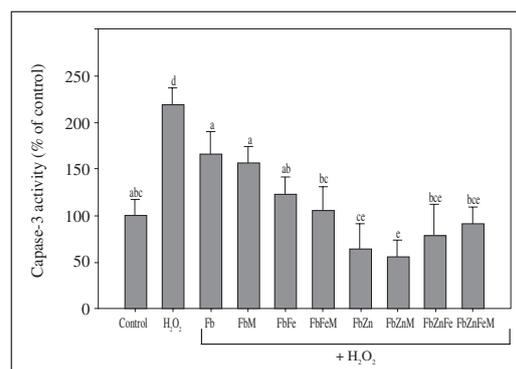*Fig. 3.—Effects of 5 mmol L⁻¹ H₂O₂ (2 h) on caspase-3 activity in Caco-2 cells preincubated (24 h) or not with bioaccessible fractions of a fruit beverage (Fb) (grape + orange + apricot) with/without iron (Fe) and/or zinc (Zn) and with/without skimmed milk (M). Results are expressed as mean ± standard deviation (SD) (n = 4). Different letter on the bars indicate significant statistically (p < 0.05) differences analysed by one-way ANOVA followed by Fischer's LSD post hoc test.*

from the broad range (10 μmol L^{-1} to 10 mmol L^{-1}) reported in the literature,[18,30] and established for these experiments in previous studies by our group.[7,11] H_2O_2-induced oxidative cytotoxicity is associated with H_2O_2 diffusion into the mitochondrial matrix, and the subsequent cytochrome c-mediated degradation of phospholipids.[12,18] Accordingly, the decreased MTT conversion values (%) evidence the deleterious effect of H_2O_2-induced oxidative stress upon cell mitochondrial enzyme activities. The better-preserved MTT conversion values in cultures incubated with fruit beverages suggest a likely positive effect of the latter against oxidative stress, maintaining cell viability.

It is accepted that intracellular GSH depletion reflects oxidative stress,[31,32] and changes in GSH cycle enzymes have been proposed as fairly sensitive biomarkers of Caco-2 cellular response to H_2O_2-induced oxidative stress.[18,30] Caco-2 cells exhibit antioxidant enzyme mechanisms - a reduced/oxidized glutathione balance (GSH/GSSG) being one of the principal systems involved in the adaptation and prevention of cell oxidative damage.[9,30] The accumulation of H_2O_2 within cells, and the subsequent impairment of internal mitochondrial membrane integrity,[11] cause uncoupling in cell metabolism and the production of reducing equivalents, which could explain the observed decrease in GSH-Px. A similar effect, although accompanied by increased GSH-Rd, was observed in cultures exposed to FbM sample. Despite the unequivocal potential benefits of Fe supplementation for nutritional status, there is controversy as to whether such supplementation may contribute to oxidative stress. In our study, mineral supplementation did not impair the protection afforded by fruit beverages against H_2O_2-induced oxidative damage. Antioxidant effects have been attributed *in vitro* to mineral solutions; Zn (0-200 μmol L^{-1}) preserved intracellular sulfhydryl groups because of the induced synthesis of metallothioneins,[33] and Fe could catalyze the decomposition of H_2O_2.[34] In this study, the positive effect of milk supplementation upon GSH-Rd activity agrees with the reported effects of purified phosphopeptides from casein in skimmed milk[7] and oligophosphopeptides from hen egg yolk[31] upon Caco-2 cells. However, in this study we cannot rule out the participation of milk components other than CPPs in the milk-mediated positive effect observed.

Considerable scientific evidence indicates that redox signaling mechanisms function in cell regulation and growth control[35,36] - GSH participating in DNA synthesis and cellular resistance to apoptosis.[37] It has been reported that peroxides cause alteration of the G1 checkpoint in cycle progression,[13] and specifically H_2O_2 causes the targeted oxidation of cellular molecules such as DNA, proteins, and lipids - leading to mutagenesis and cell death.[38] These findings could explain the decreased G1 cell proportion and the observed increase in S phase population, which may reflect the tissue response. This hypothesis is supported by the increased GSH-Rd/Px activities observed, and is in agreement with the up-regulation of c-glutamylcysteine synthetase gene previously reported.[38]

In cell cycle analysis, the sub-G1 population is commonly regarded, though not exclusively, as representing hypo-diploid cells and could be considered as an indicator of apoptotic cell death;[17] however, H_2O_2-mediated DNA strand rupture cannot be ruled out when concentrations higher than 1 mmol L^{-1} are used.[39] In this study of the relationship between the increased sub-G1 cell population and the potential participation of apoptotic cell death we determined caspase-3 activity, since it is one of the major executing enzymes in programmed cell death. At a first glance, the results suggest likely DNA strand rupture under the experimental conditions used, since no relationship between the sub-G1 phase populations and caspase-3 activity was found. This could be concordant with the increased sub-G1 peak in HepG2 cells challenged with bisphenanthroline-coumarin-6,7-dioxacetatocopper (II) complex, attributed to peripheral chromatin condensation and large-scale DNA fragmentation.[40] The similar ($p > 0.05$) caspase-3 activity observed in cell cultures incubated with mineral supplemented samples coincides with previous reports.[33,41,42] It has been indicated that metallothioneins, because of their nuclear localization, responding to Zn and Zn-Fe treatments, may play a role in preventing DNA damage and apoptosis.[43] Furthermore, Zn enhanced the Bcl-2/Bax ratio and reduced caspase-3 activity in Caco-2 cells treated with a H_2O_2-generating system.[42] Regarding milk supplementation, Phelan et al.[44] have also recently reported the absence of genoprotective effects of casein hydrolysates against H_2O_2-induced DNA damage in Caco-2 cells. However, H_2O_2 exposure of cell cultures incubated with isolated CPPs from skimmed milk did not result in significantly increased sub-G1 phase population values[7].

Conclusion

The results obtained in the present study suggest that fruit beverages exert positive effects against H_2O_2-induced oxidative stress in Caco-2 cell cultures. In addition, these effects are improved by mineral- and/or milk-supplementation. This conclusion is based on the observation of better-preserved GSH cycle enzyme activities, cell cycle distribution preservation and the fact that caspase-3 activity was not induced in cultures incubated with fruit beverages and challenged with H_2O_2-induced oxidative stress. However, fruit beverages failed to prevent the increase in sub-G1 phase population, which we hypothesize is primarily due to H_2O_2-induced DNA fragmentation, but only affects as much as 10% of cell population in agreement with cell viability maintenance. To summarize, mineral- and milk-supplementation together

could help in nutritional strategies designed to comply with the dietary intake recommendations of antioxidants and minerals. However, it is important to point out that *in vitro* studies do not completely reflect the *in vivo* situation; accordingly, animal and human trials would be needed to confirm the beneficial effects of these fruit beverages.

Acknowledgements

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