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Antioxidant effect derived from bioaccessible fractions of fruit beverages against H_2O_2-induced oxidative stress in Caco-2 cells

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Abstract

This work evaluates the effect of bioaccessible fractions from fruit beverages against oxidative stress (OS) in Caco-2 cells. A fruit beverage (grape + orange + apricot) (with/without milk and/or iron/zinc) was subjected to *in vitro* gastrointestinal digestion, and bioaccessible fractions were incubated with Caco-2 cell cultures. Following preincubation, OS was induced with 5 mM H_2O_2. Intracellular reactive oxygen species (ROS), mitochondrial potential ($\Delta\psi_m$), mitochondrial metabolism (MTT test), intracellular reduced glutathione (GSH) and superoxide dismutase activity (SOD) were measured. The data evidenced viable cultures with increased mitochondrial metabolism and GSH-Rd activities, without alteration in SOD activity. Accordingly, more preserved mitochondrial integrity was also evidenced, allowing the action of antioxidant systems in preincubated cultures. Based on these data, we can conclude that a cytoprotective effect is derived from bioaccessible fractions of fruit beverages, though this effect failed to prevent intracellular ROS accumulation in Caco-2 cell cultures exposed to 5 mM H_2O_2.
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Keywords: Oxidative stress; Caco-2; Fruit beverages; Milk; Minerals

1. Introduction

Fruit beverages are often commercially supplemented with milk, vitamins and/or minerals to improve their nutritional value and to provide bioactive food components with beneficial and healthy effects. It is widely known that fruits (Serafini, Bellocco, Wolk, & Ekström, 2002), and citrus juices (Vinson et al., 2002) possess many components, such as ascorbic acid, carotenoids, α-tocopherol and a wide range of polyphenols and flavonoids, with antioxidant potential. It has been postulated that dietary antioxidants can scavenge reactive oxygen species (ROS) in the body, lowering oxidative stress involved in tissue damage, accelerated aging and chronic degenerative diseases, such as cancer and cardiovascular disorders (Boyle & Langman, 2000; Ferro-Luzzi & Branca, 1995; Serafini et al., 2002; Vinson et al., 2002). Therefore, the consumption of fruit beverages, that are often supplemented with milk as source of functional bioactive peptides and fortified with minerals, could be helpful in complying with the dietary intake recommendations related to oxidative stress. Based on data for daily intakes in Spain (Mercasa, 2006), the annual mean *per capita* consumption of fruit beverages was estimated to be 18.4 l, of which 1.4, 3.9 and 4.7 l corresponded to grape, orange and apricot juices, respectively.

Most recent research on antioxidants, has evaluated the potential total antioxidant capacity (TAC) of pure components or food extracts and dietary supplements in aqueous solutions (Ou, Hampsch-Woodill, & Prior, 2001; Prior et al., 2003; Re et al., 1999). Although, the measured TAC values show possible interactions among different antioxidants and contribute relevant information to better understand the potential protective effects of food components (Manna et al., 2002), these studies lack a biological system with functions that can be impaired by oxidative stress. Furthermore, the protective effects of natural occurring

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antioxidants against oxidative stress using Caco-2 cells (a human colon cancer cell line) have been well documented in several studies. Most of these studies have monitored the antioxidant effects of carotenoids (Bestwick & Milne, 2000), flavonoids (Yokomizo & Moriwaki, 2006), and phenolic apple juice extract (Schaefer, Baum, Eisenbrand, & Janzowski, 2006) upon the accumulation of ROS. These investigations have shown that standard solutions of β-carotene fail to inhibit the ROS accumulation (Bestwick & Milne, 2000), and that phenolic compounds can also exert prooxidant activities (Schaefer et al., 2006; Yokomizo & Moriwaki, 2006). From a metabolic point of view, carotenoids exhibit a cytoprotective effect, increasing antioxidant enzyme activities (Bestwick & Milne, 2000), and both fresh and packaged orange juice increase mitochondrial enzyme activities (Ekmekcioglu, Strauss-Blasche, Leibetseder, & Marktl, 1999). Using only standard solutions, the antioxidant activities of some minerals, such as zinc (Zn), have been described (Zödl et al., 2003). However, the data found in the literature with respect to the pro- (Nuñez, Tapia, Toyokuni, & Okada, 2001) and/or anti-oxidant activity of Fe (Zödl et al., 2004) upon Caco-2 cells are less conclusive and appear contradictory.

From a physiological perspective, food (after consumption) is subjected to a gastrointestinal digestion process before reaching the proximal intestine, which is considered to be the main site of nutrient and mineral absorption (Ganong, 1990). It is also notable that, during the gastrointestinal digestive process, antioxidant and other functional components could be metabolized or not released from foods, thus affecting the native antioxidant potential of each of these components.

The aim of this study was to evaluate the cytoprotective effect against H_2O_2-induced oxidative stress in Caco-2 cells, derived from the bioaccessible fraction of fruit beverages with/without milk and minerals, after being subjected to a simulated gastrointestinal digestion procedure. In this sense, the present study could improve knowledge of the cytoprotective effects of fruit beverages, based on an intestinal epithelial model which more precisely could simulates the *in vivo* situation.

2. Materials and methods

2.1. 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, FbMFe, FbMZn, FbMFeZn. The compositions of the aforementioned samples are shown in Table 1.

Table 1
Compositions of the fruit beverages assayed

Component (g/100 g)	Sample	
	Fb	FbM
Osmosis water	58.7	57.7
Apricot puree	24.5	24.5
Grape concentrate	7.2	7.2
Orange concentrate	4.2	4.2
Sugar	5.1	5.1
Skimmed milk powder	–	1.0
Classic pectin	0.4	0.4
Vitamin C (L-ascorbic acid)	0.054	0.054

Fb, fruit beverages with or without mineral supplementation; Fe (sulphate), 3 mg Fe/100 ml fruit juice and/or Zn (sulphate), 1.6 mg Zn/100 ml fruit juice; FbM, fruit juices with milk (11% v/v) and supplemented or not with mineral.

2.2. In vitro digestion

To simulate the human gastrointestinal digestive process, samples of fruit juices (80 g) were subjected to an *in vitro* procedure, as previously described (Perales, Barbera, Lagarda, & Farre, 2005). 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 100 °C to inhibit sample proteases, and were then cooled by immersion in an ice bath. Twenty-gramme aliquots of the inactivated digests were transferred to polypropylene centrifuge tubes and centrifuged at 3890*g* for 60 min at 4 °C to separate the soluble fraction (bioaccessible fraction), which was pooled.

2.3. 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 (Laparra, Vélez, Montoro, Barberá, & Farré, 2005).

For the assays, Caco-2 cells were seeded onto 24-well plates (Costar Corp., USA), at a density of 5×10^4 cells cm^{-2}, with 1 ml of MEM (Minimum Essential Medium, MEM; Gibco BRL Life Technologies, Scotland), and culture media was changed every two days. Fifteen to eighteen days after initial seeding, the culture medium was aspirated from wells, and cell monolayers were washed with PBS warmed to 37 °C. Cell cultures were preincubated (37 °C/5% CO_2/95% relative humidity) for 24 h with bioaccessible fractions of digested samples, added in 1:1 proportion (v/v) with culture media to preserve cell viability. Afterwards, culture medium was removed and the cells were washed with PBS at 37 °C; the induction of oxidative stress was carried out by exposure to a 5 mM H_2O_2 solution in MEM for 1 h (37 °C/5% CO_2/95% relative humidity).

2.4. Intracellular accumulation of ROS and mitochondrial membrane potential changes

The intracellular accumulation of ROS in Caco-2 cells was evaluated using a 2 mM dihydrorhodamine (DHR,

Sigma) solution prepared in dimethylsulfoxide, which is oxidized to fluorescent rhodamine. Afterwards, 1 ml of PBS containing DHR (final concentration 5 μM) was added, followed by incubation for 20 min (37 °C/5% CO_2/95% relative humidity). After eliminating the supernatants, cells were washed with PBS and resuspended in a trypsin–EDTA solution (2.5 g l^{-1} of trypsin, 0.2 g l^{-1} of EDTA).

Mitochondrial membrane potential ($\Delta\psi_m$) changes were evaluated using DHR and propidium iodide (PI, Sigma) double labelling (Cai et al., 2000). Briefly, cultures were washed with PBS and incubated with 1 ml of PBS containing DHR, prepared as described above, for 30 min in the dark (37 °C/5% CO_2/95% relative humidity). Afterwards, the media were aspirated and 0.5 ml of a PI staining solution [1 mg ml^{-1} of trisodium citrate, 0.05 mg ml^{-1} of PI, and 1 mg ml^{-1} of RNase A (Sigma, P4875)] was added with an additional incubation time of 15 min under the aforementioned conditions.

Fluorescent intensities of DHR ($\lambda_{ex} = 488$ nm and $\lambda_{em} = 525$ nm) and PI ($\lambda_{exc} = 488$ nm and $\lambda_{em} = 620$ nm) were determined by flow cytometry (Coulter, EPICS XL-MCL, Miami, USA). $\Delta\psi_m$ changes were estimated by the relative amount of PI vs. DHR double negative cell populations. Control cells were used throughout each assay. At least 10,000 cells per sample were analyzed.

2.5. Mitochondrial enzyme activity

The mitochondrial activity of Caco-2 cell was evaluated by using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide) assay (Laparra et al., 2005). This colorimetric method is based on the reduction of the tetrazolium ring of MTT by mitochondrial dehydrogenases (Ekmekcioglu et al., 1999), 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 through each assay.

2.6. Intracellular GSH content and SOD activity

Cell homogenates were obtained by treating cultures with 0.2 ml of 20 mM Tris buffer solution containing 0.1% Triton (v/v). Control cells were used throughout each assay.

The GSH content was determined, measuring the formation of a fluorescent complex of *o*-phthalaldehyde (OPA) with reduced glutathione (GSH) (Hissin & Hilf, 1976). Briefly, aliquots (10 μl) of cell homogenate were incubated at room temperature with 10 μl of buffered formaldehyde (37–40% formaldehyde: 0.1 M Na_2HPO_4 in 1:4 proportion, v/v; pH 8), 170 μl of 0.1 M Na_2HPO_4–5 mM EDTA buffer, and 10 μl of the OPA solution (1 mg ml^{-1} in absolute methanol). After mixing and incubation at room temperature for 30 min, the fluorescence intensity was measured at $\lambda_{excitation}$ 355 nm and $\lambda_{emission}$ 460 nm, using a Multilabel Plate Counter VICTOR^3 1420 (Perkin–Elmer, Turku, Finland). The concentrations of GSH were standardized by determination of total proteins (Lowry, Rosebrough, Farr, & Randall, 1951).

For the determination of SOD activity, slight modifications in the volumes of reactants of a commercial kit (RANSOD SD125, Randox, Antrim, UK) were made in order to use 96-multiwell plates. Superoxide radicals were generated using a xanthine/xanthine oxidase system. Absorbance was determined at 505 nm using a Multilabel Plate Counter VICTOR^3 1420 (Perkin–Elmer). Enzyme activities were calculated as SOD units ml^{-1} of cell homogenate.

2.7. Statistical analysis

One-factor analysis of variance (ANOVA) and the Tukey test (Box, Hunter, & Hunter, 1978) were applied to determine differences in cytoprotective effects between treated and control cultures. A significance level of $p < 0.05$ was adopted for all comparisons. Statgraphics Plus version 5.0 (Rockville, Maryland, USA) was used for the statistical analysis.

3. Results and discussion

3.1. Intracellular accumulation of ROS and mitochondrial membrane potential changes

The concentration of H_2O_2 used in this study was selected from the broad range (10 μM–10 mM) previously reported by other authors to study induced oxidative stress in Caco-2 cells (Bestwick & Milne, 2000; Wijeratne, Cuppett, & Schlegel, 2005), thus allowing us to monitor the deleterious effects of H_2O_2 under the experimental conditions used in this work. In pilot studies, three concentrations of H_2O_2 (0.5, 5 and 10 mM) were used, showing that the lowest concentrations assayed (0.5 mM H_2O_2) caused neither alteration of mitochondrial potential nor deleterious effects on cell metabolism. Besides, 10 mM H_2O_2 caused a marked alteration of mitochondrial potential and cell viability during the incubation times used.

To gain insights to modification in the redox cell state, we investigated the accumulation of ROS within cells by monitoring the oxidation of DHR, which accumulates mainly within the mitochondria, as a result of its lipophilic cation nature (Haugland, 1996), where it is oxidized in the presence of peroxidases (Gomes, Fernandes, & Lima, 2005). In a previous study, monitoring the effect of kaempferol on ROS levels (Bestwick, Milne, Pirie, & Duthie, 2005), it has been suggested that DHR can be used to evaluate mitochondrial membrane potential changes, constituting a useful tool for obtaining information about the time course of the alterations in mitochondrial integrity (*see below*). Fig. 1 shows the intracellular accumulation of ROS in Caco-2 cultures pre-incubated with bioaccessible

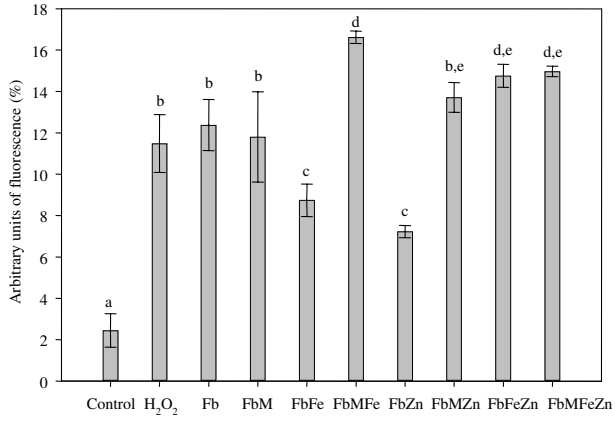

Fig. 1. Intracellular reactive oxygen species (ROS) production in Caco-2 cell cultures preincubated for 24 h with bioaccessible fractions and exposed to 5 mM H_2O_2. Results are expressed as means ± SD ($n = 4$). Different letters on the bars indicate statistically significant differences ($p < 0.05$).

fractions of selected samples, after being exposed to 5 mM H_2O_2.

After the H_2O_2-induced oxidative stimulation of cell cultures, highly increased accumulations of intracellular ROS with respect to the controls were observed in all cases. Although, fruit juices are rich in vitamins such as ascorbic acid, and in other compounds, such as carotenoids and polyphenols, for which an important antioxidant capacity has been evidenced (García-Alonso, Ros, & Periago, 2006; García-Alonso, Ros, Vidal-Guevara, & Periago, 2006; Serafini et al., 2002), these results showed that the bioaccessible fractions of the analyzed samples failed to prevent intracellular ROS accumulation. Comparing ROS levels, cultures pre-incubated with samples Fb and FbM did not show statistically significant differences ($p > 0.05$). This could suggest that the contribution of supplemental milk (sample FbM) or casein-derived phosphopeptides (CPPs), produced during *in vitro* digestion, exerted no additional effect in terms of the prevention of ROS accumulation. It is also interesting to note that the radical-scavenging and antioxidant capacities of CPPs have only been evaluated in aqueous standard solutions, and without the participation of any biological system (Kitts, 2005).

Both the FbFe and FbZn, mineral supplemented samples, produced the lowest ROS accumulation in H_2O_2-stimulated Caco-2 cultures (Fig. 1). However, the addition of milk to the latter samples (FbMFe and FbMZn) induced 2-fold higher ROS levels. These data suggest that free minerals (Fe and Zn) could exert an antioxidant effect upon the cells, and in presence of casein-derived phosphopeptides that act as a mineral carriers (Kitts, 2005), these minerals would lose their potential as antioxidants (*as discussed below*). On the other hand, a different pattern in ROS accumulation was observed in those cultures exposed to samples treated with both minerals jointly, compared with independent addition, supplemented or not with milk. Preincubation of cultures with bioaccessible fractions of FbFeZn and FbMFeZn showed no statistically significant differences ($p > 0.05$) in terms of the intracellular accumulation of ROS.

The data found in the literature in relation to the antioxidant effects of food components in preventing ROS accumulation are inconclusive. Bestwick and Milne (2000) showed that pre-incubation (24 h) of Caco-2 cells with β-carotene (0.1–50 μM) standard solutions in culture media failed to inhibit intracellular ROS accumulation. However, previous authors (Yokomizo & Moriwaki, 2006) have reported that preincubation of Caco-2 cultures with quercetin, kaempferol and luteolin significantly decreased intracellular ROS accumulation, while preincubation with apigenin and others do not. These authors (Yokomizo & Moriwaki, 2006) stressed differences in the ability of these compounds when incorporate in cells as a requisite for reducing ROS. Besides, it is recognized that phenolic compounds can also exert prooxidant activities under *in vitro* conditions, resulting in the production of H_2O_2 (Yokomizo & Moriwaki, 2006; Schaefer et al., 2006; Lee, Hur, Lee, & Lee, 2005). This latter fact could, at least in part, explain the inability of the bioaccessible fraction of fruit beverages to prevent ROS accumulation.

It has been widely reported that H_2O_2 is highly deleterious to cells, and that its accumulation causes the oxidation of cellular targets such as DNA, several proteins, and lipids, leading to mutagenesis and cell death (Hampton & Orrenius, 1997; Tada-Oikawa, Oikawa, Kawanishi, Yamada, & Kawanishi, 1999; Kowaltowski, Vercesi, Rhee, & Netto, 2000). An earlier stage in H_2O_2-induced cytotoxicity is associated with its diffusion into the mitochondrial matrix, with subsequent loss of integrity and finally cell death mediated by mitochondrial disturbances (Mronga, Stahnke, Goldbaum, & Richter-Landsberg, 2004). The evaluation of mitochondrial membrane potential ($\Delta\psi_m$) changes in Caco-2 cells preincubated with bioaccessible fractions of samples after exposure to 5 mM H_2O_2 is depicted in Fig. 2.

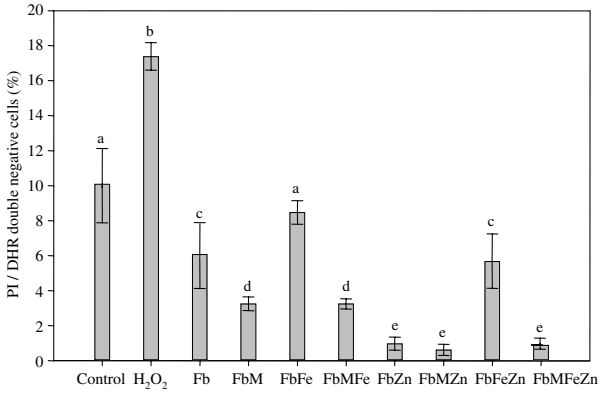

Fig. 2. Changes in mitochondrial membrane potential ($\Delta\psi_m$) in Caco-2 cell cultures preincubated for 24 h with bioaccessible fractions and exposed to 5 mM H_2O_2. Results are expressed as means ± SD ($n = 4$). Different letters on the bars indicate statistically significant differences ($p < 0.05$).

On evaluating $\Delta\psi_m$, it must be taken into account that staining with PI can identify subpopulations of cells with altered membrane permeability and associated loss of membrane integrity. A reduction in $\Delta\psi_m$ leads to the release of DHR from mitochondria and a decrease in fluorescence intensity. Alterations in $\Delta\psi_m$ can be concluded from the increased proportion of PI vs. DHR double negative cells (PI/DHR, %) with respect to the controls (Cai et al., 2000).

After 1 h of exposure to H_2O_2, a pronounced decrease in $\Delta\psi_m$ was observed only in those cultures not pre-incubated with the samples, evidencing the toxic effect upon mitochondrial integrity of the H_2O_2 (5 mM) concentration assayed. In cultures pre-incubated with bioaccessible fraction of analyzed samples, lower PI/DHR values (%) were detected, suggesting more preserved mitochondrial integrity. It must be stressed that cultures exposed to these samples supplemented with milk, and treated or not with minerals (FbM vs Fb, FbMFe vs FbFe, FbMFeZn vs FbFeZn) (Fig. 2), showed lower PI/DHR (%). This may suggest a possible key role for casein phosphopeptides, produced during *in vitro* digestion (Miquel et al., 2005), and acting as mineral carriers with the modulation of cell response. On the other hand, lower PI/DHR values (%) were detected in those cultures preincubated with samples containing Zn than in those treated with Fe (FbZn vs. FbFe, FbMZn vs. FbMFe). An antioxidant effect of Zn, resulting in the induced synthesis of metallothioneins and antioxidant enzymes, such as glutathione peroxidase, which protects protein sulfhydryl groups, allowing the activity of cytosolic SOD, has been reported (Zödl et al., 2003). Although, the addition of both minerals, Fe and Zn, either independently or jointly, exerted no deleterious effect upon mitochondrial integrity, a different behaviour was observed. The data related to Fe, and previously reported in the literature, revealed both pro- and antioxidant activities. Although their prooxidant activity is generally accepted, a previous study (El Hajji, Nkhili, Tomao, & Dangles, 2006) evidenced that Fe–quercetin complexes seem less prone to auto-oxidation than does free quercetin. In addition, when there is over-production of H_2O_2, or in sites devoid of catalase, the presence of free Fe is able to transform H_2O_2 into hydroxyl radicals (reviewed in Cremonesi, Acebron, Raja, & Simpson, 2002). This particular characteristic of Fe in the presence of certain antioxidant food compounds and high H_2O_2 levels, could at least partly explain the lower ROS levels detected (Fig. 1) with respect to other samples, and resulting in the cytoprotective effect observed in Caco-2 cells.

Taken together, the results obtained suggest that the bioaccessible fraction of the samples could exert a cytoprotective effect, preserving mitochondrial integrity. In addition, mitochondrial respiration produces a physiological amount of oxygen radicals, which suggests a possible correlation between oxidative stress and mitochondrial integrity. Mitochondria play a central role in several cellular metabolic processes, and may lose their ability to phosphorylate ADP by decreasing their membrane potential generated during oxidative phosphorylation (Stryer, 1988), thus adversely affecting the adaptive response of the cellular antioxidant defence systems.

3.2. Effects on GSH levels and SOD activity

The effects of bioaccessible fractions of samples on intracellular GSH concentration and SOD activity in Caco-2 cells are shown in Fig. 3. Only cultures pre-incubated with the bioaccessible fraction of samples showed significant differences ($p < 0.05$) in their intracellular GSH concentrations with respect to the controls. These results are expected on the basis of the data related to mitochondrial integrity recorded in this work (Fig. 2), and are in good agreement with previously reported GSH depletion (Katayama, Xu, Fan, & Mine, 2006) and antioxidant enzyme response (Wijeratne et al., 2005) in Caco-2 cultures exposed to H_2O_2. Intracellular GSH concentration was decreased to a similar extent in all cases, and significant differences ($p < 0.05$) were only seen between cultures preincubated with the bioaccessible fraction of samples FbFe and FbMFe with respect to FbZn. The GSH depletion in cell cultures clearly reflects the alteration in cell redox status, in agreement with the high ROS levels detected (Fig. 1).

The samples supplemented with milk showed no significant differences ($p > 0.05$) in intracellular GSH content with respect to the samples not containing milk. These results may suggest that CPPs produced from milk digestion made no contribution to the protective effect against H_2O_2-induced oxidative stress in Caco-2 cells. However, in a previous study (Katayama et al., 2006), the authors reported an increased GSH concentration in Caco-2 cultures preincubated for 2 h with oligophosphopeptides isolated from hen egg yolk. This was probably due to de novo GSH-induced synthesis, as suggested by the increased

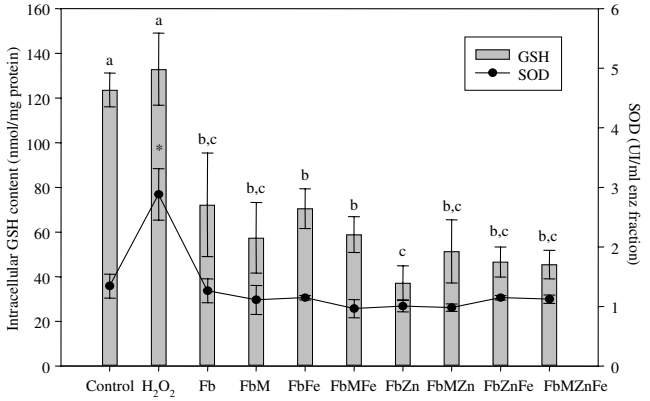

Fig. 3. Effect of 5 mM H_2O_2 on intracellular GSH content and superoxide dismutase (SOD) activity in Caco-2 cultures preincubated or not for 24 h with bioaccessible fractions. Results are expressed as means ± SD ($n = 4$). Different letters on the bars indicate statistically significant differences ($p < 0.05$) in intracellular GSH content. * Indicates statistically significant differences ($p < 0.05$) in SOD activity with respect to the controls.

glutathione reductase activity detected. These authors stressed that the cytoprotective effect of oligophosphopeptides against H_2O_2 exposure also implicate inhibition of the production of proinflammatory cytokines such as interleukin 8. The higher H_2O_2 concentration and shorter incubation time used in our study may be responsible for the differences in cell adaptation response observed in our study with respect to the results obtained by Katayama et al. (2006). In the present work, we can also speculate on the possibility of a more preserved cell metabolic adaptation response against H_2O_2-induced oxidative stress in cultures exposed to samples containing milk (see below).

When considering mineral addition of samples, neither independent nor joint addition of Fe and Zn showed differences ($p > 0.05$) in terms of intracellular GSH. Taking into account the prooxidant (Ferruzza, Scacchi, Scarino, & Sambuy, 2002; Nuñez et al., 2001) and antioxidant (Zödl et al., 2003) activities previously reported for Fe and Zn, respectively, differences in GSH concentration in the cultures should be expected. In this context, Zödl et al. (2003), using standard aqueous solutions of zinc sulphate, have shown that concentrations higher than 30 μM could promote an antioxidant effect in postconfluent Caco-2 cultures, with induction of certain antioxidant enzyme activities, such as catalase and glutathione peroxidase (GPx), contributing to increased prevention of oxidative stress to cell proteins and structures. It should be stressed that, in the present work, cell cultures were exposed to Zn concentrations higher than 100 μM when incubated with samples treated with this mineral. Besides, Nuñez et al. (2001), showed that, in Caco-2 cells, increasing concentrations (25–50 μM) of Fe for 4–6 days resulted in increased protein oxidative damage, and also in marked induction of DNA oxidation. In the same way, Ferruzza et al. (2002) observed that Fe(II) treatment (between 50 and 100 μM) produced F-actin disorganization and subsequent disruption of the cytoskeletal microtubules. It should be taken into account that, among the cellular antioxidant defence systems, GSH is one of the main nonenzymatic antioxidants involved in the adaptation to and prevention of cell oxidative stress (Baker & Baker, 1993), being implicated in the reversible thiolation/dethiolation of oxidized protein cysteinyl thiols. In addition to the effect of the minerals, it cannot be ruled out that phenolic compounds are generally recognized as antioxidants, though they can exert prooxidant activities under certain conditions, such as in the presence of transition metal ions (Lee et al., 2005). This effect could also contribute to the decreased intracellular GSH concentrations observed. To the best of our knowledge, a decreased GSH/GSSG ratio, due to the prooxidative effects of polyphenols, has been previously evidenced only in erythrocytes (Ko et al., 2006).

The data presented in the present study may suggest that the Caco-2 cell line metabolizes ROS mainly through the glutathione cycle, with the hexose monophosphate shunt as the source of reducing equivalents as previously described (Baker & Baker, 1993). In the glutathione cycle, GSH is oxidized to GSSG by glutathione peroxidase (GSH-Px), while GSSG is reduced by glutathione reductase via NADPH, which in turn is regenerated through the activity of the hexose monophosphate shunt (Stryer, 1988), for which mitochondrial integrity is required. Nevertheless, we cannot rule out that in H_2O_2-treated cultures, inhibition of the glutathione cycle could cause the accumulation of superoxide radicals in cells. In this sense, the protective effect of superoxide dismutase (SOD) upon GSH–Px activity against inactivation by superoxide radicals, by protecting cellular functions in Caco-2 cells, has been reported (Wijeratne et al., 2005). We next evaluated SOD activity in all exposed cultures (Fig. 3).

Following the preincubation of cultures with the bioaccessible fraction of selected samples, exposure to H_2O_2 caused no statistically significant differences ($p > 0.05$) in SOD activity with respect to the controls. In this context, it must be stressed that a previous study (Bestwick & Milne, 2000), evaluating the antioxidant effect against H_2O_2 oxidative stress in Caco-2 cultures preincubated with standard solutions of β-carotene for 24 h or 5 days, has shown a progressive increase in SOD and other antioxidant enzyme systems such as GSH reductase, glutathione-*S*-transferase, and catalase after exposure to 10 mM H_2O_2 (6 h). Probably, other food components present in the samples analyzed in our study could be responsible for the unaltered SOD activity.

The observed cell response to SOD activities is in good agreement with the cytoprotective effect in relation to mitochondrial integrity (Fig. 2) which, as stated before, is required to produce reducing equivalents in the GSH/GSSG cycle. Those cultures with more preserved mitochondrial integrity showed no increase in SOD activity, which may suggest that cellular functions are not negatively affected by H_2O_2 stimulation. Mineral addition did not increase SOD activity, in accordance with the results obtained by previous authors (Zödl et al., 2003; Zödl et al., 2004), who observed no effect on SOD activity in Caco-2 cells exposed to standard aqueous solutions of Zn (0–200 μM) or Fe (100–3000 μM) for 24 h.

3.3. Assays monitoring mitochondrial enzyme function

MTT conversion could provide an indirect measure of cell metabolism, since MTT reduction to formazan in viable cells takes place via reactions catalyzed by mitochondrial dehydrogenases, coupled to oxidative phosphorylation (Ekmekcioglu et al., 1999). The effects of H_2O_2-induced oxidative stress on MTT conversion in cultures preincubated with bioaccessible fractions of fruit beverages are shown in Fig. 4.

Stimulation with H_2O_2, caused a slight and statistically non-significant ($p > 0.05$) reduction (up to 20%) in MTT conversion with respect to the controls. This result agrees with previous studies in HepG2 and Caco-2 (Lee et al., 2005) cells, which reported this effect due to inhibited cell proliferation caused by the intracellular accumulation of H_2O_2. However, as can be seen in this work, the mitochon-

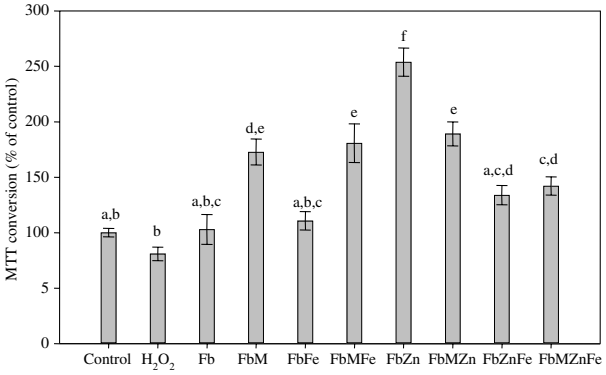

Fig. 4. MTT conversion in Caco-2 cell cultures preincubated for 24 h with bioaccessible fractions and exposed to 5 mM H_2O_2. Results are expressed as means ± SD ($n = 4$). [a–f] Different letters indicate statistically significant differences ($p < 0.05$).

drial enzyme activities of Caco-2 cells remain unaltered in those cultures preincubated with bioaccessible fractions of the fruit beverage with either milk and mineral addition. In the same way, Ekmekcioglu et al. (1999) have evidenced no negative effects on MTT conversion in Caco-2 cultures exposed to both fresh or packaged orange juice (among cola, green and black tea, and energy drinks). These latter authors only reported higher MTT conversion rates in cultures incubated with fresh orange juice, which they speculate could be attributed to food components, such as ascorbic acid, that stimulate mitochondrial metabolism by increasing succinate cytochrome c reductase (Ekmekcioglu et al., 1999). In the same way, increased MTT conversion rates have been reported in HepG2 cells preincubated with extracts of a phenolic-rich (0–30 μM) juice in relation to t-BOOH and H_2O_2-induced oxidative damage (García-Alonso et al., 2006; García-Alonso, Ros, Vidal-Guevara, et al., 2006).

Although those cultures preincubated with bioaccessible fractions of samples treated with milk and both Fe and/or Zn showed higher MTT conversion percentages than the controls, the highest percentages were observed in those exposed to samples treated only with zinc (FbZn). The increased MTT conversion detected after exposure to all of these samples accords with the more preserved mitochondrial integrity observed in the present work (Fig. 2). In addition, the results suggest that these samples could exert a certain cytoprotective effect, or at least as indicated previously (Ekmekcioglu et al., 1999) a potential modulating effect upon mitochondrial metabolic enzymes which could contribute to the increased adaptive response observed against the oxidative stress caused. In this context, consideration is also required of the antioxidant effect reported for Zn (Zödl et al., 2003) and discussed above in the present work, and which could be implicated in the observed effect.

On the other hand, preincubation with samples treated only with Fe caused no alteration in MTT conversion with respect to the controls (Fig. 4). This observation coincides with the findings of Zödl et al. (2004) in postconfluent Caco-2 cells exposed to standard solutions of Fe at concentrations of 100–3000 μM. However, in the present work, those cultures exposed to the bioaccessible fraction of fruit beverage treated with Fe also showed higher MTT conversion percentages when treated with milk, FbMFe vs. FbFe, an effect opposite to that observed for Zn. When both minerals were added jointly, the MTT conversion-increasing effects of Zn and milk were abolished (FbFeZn and FbMFeZn), reaching values similar to the controls. However, as stated above, a more preserved $\Delta\psi_m$ (Fig. 2) in those cultures exposed to FbMFeZn was detected. This could suggest a possible implication of milk in the mod ulating effect of metabolic enzymes derived from the presence of CPPs, as previously suggested (Ekmekcioglu et al., 1999).

In summary, the data presented in our work evidence viable cultures with a more preserved mitochondrial integrity and metabolic response, suggesting cell adaptation to the oxidative stress caused. In addition, it has been seen that the maintenance of mitochondrial integrity is essential for metabolizing intracellular ROS accumulation, and for preserving the activity of the antioxidant enzyme systems. It is also possible to postulate a cytoprotective effect derived from bioaccessible fractions of fruit beverages, because all preincubated cultures showed lower PI/DHR (%), though it must be stressed that all assayed samples failed to prevent intracellular ROS accumulation in Caco-2 cultures exposed to 5 mM H_2O_2. However, as might be expected, this observation was not correlated with the presence of any of the minerals used for supplementation. Although the approach used in the present work could contribute to a better understanding of the effects derived from the consumption of fruit beverages, we should pay close attention to the *in vitro* data which could not be completely extrapolable to the *in vivo* situation. Our results might suggest that fruit beverages used in our study exhibit a similar antioxidant effect. Furthermore, the mineral supplementation could be useful for surmounting mineral deficiency in sensitive population groups and improve nutritional value by the milk addition. However, further studies and complementary data within human trials are needed to confirm this asumption.

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