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The antioxidant protection of CELLFOOD® against oxidative damage *in vitro*

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ABSTRACT

CELLFOOD® (CF) is an innovative nutritional supplement containing 78 ionic/colloidal trace elements and minerals combined with 34 enzymes and 17 amino acids, all suspended in a solution of deuterium sulfate. The aim of this study was to investigate, for the first time, the antioxidant properties of CF *in vitro* in different model systems.

Three pathophysiologically relevant oxidants were chosen to evaluate CF protection against oxidative stress: hydrogen peroxide, peroxyl radicals and hypochlorous acid. Both biomolecules (GSH and plasmid DNA) and circulating cells (erythrocytes and lymphocytes) were used as targets of oxidation.

CF protected, in a dose-dependent manner, both GSH and DNA from oxidation by preserving reduced GSH thiol groups and supercoiled DNA integrity, respectively. At the same time, CF protected erythrocytes from oxidative damage by reducing cell lysis and GSH intracellular depletion after exposure to the oxidant agents. In lymphocytes, CF reduced the intracellular oxidative stress induced by the three oxidants in a dose-dependent manner.

The overall *in vitro* protection of biomolecules and cells against free radical attacks suggests that CF might be a valuable coadjuvant in the prevention and treatment of various physiological and pathological conditions related to oxidative stress, from aging to atherosclerosis, from neurodegeneration to cancer.

1. Introduction

Increased oxidative stress generally describes a condition in which cellular antioxidant defenses are inadequate to completely inactivate the free radicals generated by an excessive production of reactive oxygen species (ROS), loss of antioxidant defenses, or both (Halliwell and Gutteridge, 1999). A major consequence of oxidative stress is damage to nucleic acids, lipids, and proteins, which can severely compromise cell health and viability, ultimately leading to cell death and the development of disease (Dalle-Donne et al., 2006).

Among macromolecules, mitochondrial DNA (mtDNA) is highly exposed to ROS produced by electron leakage during oxidative phosphorylation (Yakes and Van, 1997). Moreover, it is thought to be more susceptible to oxidative damage than nuclear DNA (nDNA) due to the lack of histones (Dizdaroglu et al., 1991; Ljungman and Hanawalt, 1992) and the vulnerability/insufficiency of the mitochondrial repair pathways (Bohr, 2002; Graziewicz et al., 2002). As a consequence, oxidative damage to mtDNA may lead to a loss of membrane potential, reduced ATP synthesis and cell death (Van Houten et al., 2006). An increasing amount of evidence suggests that oxidative stress and oxidative damage to mtDNA are linked to either the primary or secondary pathophysiologic mechanisms of multiple acute and chronic human diseases, such as atherosclerosis, neurodegeneration and cancer (Wallace, 1999; Cohen and Tong, 2010; Galasko and Montine, 2010; Ziech et al., 2010).

In recent years, the traditional therapeutic approach to these diseases has increasingly opened up to the contribution of antioxidant supplements, especially those from natural sources which have a higher bioavailability and therefore higher protective efficacy than synthetic antioxidants (Berger, 2005; Fusco et al., 2007; Herrera et al., 2009). Focusing our attention on natural and bioavailable sources of antioxidants, we took into consideration a nutritional supplement, namely CELLFOOD® (CF) (Nu Science Corporation, CA, USA), a highly-concentrated proprietary

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride; BAP, biological antioxidant potential; CF, CELLFOOD®; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DTNB, 5,5-dithio-bis-2-nitrobenzoic; GSH, glutathione; Hb, hemoglobin; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; mtDNA, mitochondrial DNA; NaOCl, sodium hypochlorite; PBS, phosphate-buffered saline; RBC, red blood cells; ROS, reactive oxygen species.

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formulation containing 78 ionic/colloidal trace elements and minerals combined with 34 enzymes and 17 amino acids, all suspended in a solution of deuterium sulfate (Dyer, 2000).

Preliminary evidence make CF potentially interesting as a natural supplement for the antioxidant protection against oxidative stress-related damage. First, the efficacy of CF has been evidenced in the treatment of fibromyalgia (Nieddu et al., 2007), a chronic pain syndrome without an effective cure (Smith and Barkin, 2010). Among the different hypotheses for its ethiopathophysiology, oxidative stress, generated by mitochondrial dysfunction, is one of the possibilities (Pieczenik and Neustadt, 2007; Cordero et al., 2010), suggesting that supplementation with antioxidants might be important in modulating the effects of ROS in the fibromyalgic syndrome. Accordingly, it has been demonstrated that the oral supplementation of CF for a period of six months significantly improves fibromyalgia symptoms and health-related quality of life of fibromyalgic patients with respect to placebo, thus sustaining the role of CF as a valuable source of antioxidants (Nieddu et al., 2007).

Second, the efficacy of CF has been evidenced in professional athletes (Milić and Djordjević, 2009). It is well known that during intense exercise, bodily oxygen consumption is greatly increased and more ROS are produced *in vivo* due to increased cellular metabolism and to white blood cell activation (Santos-Silva et al., 2001). If not readily neutralized, free radicals alter the permeability and functionality of muscle cell membranes thus causing a decline in performance and a slower recovery; at the same time, an anemic state may occur due to erythrocyte oxidative hemolysis (Robinson et al., 2006). Interestingly, it has been demonstrated that CF supplementation has a positive effect on the adaptation/training process and sport performance in professional cyclists (Milić and Djordjević, 2009), possibly through mechanisms involving an antioxidant protection against exercise-related oxidative damages.

With the aim of filling the existing gap in literature on the mechanisms underlying the protective role of this nutritional supplement, in the present study we investigate the antioxidant properties of CF *in vitro* by evaluating its protection against three pathophysiologically relevant oxidants such as hydrogen peroxide (H_2O_2), peroxyl radicals ($ROO^{\cdot}$) and hypochlorous acid (HOCl). It is well known that, at physiological levels (1–10 μM extracellular), H_2O_2 enhances cell proliferation and has a signaling role; on the contrary, at higher concentrations, H_2O_2 induces oxidative stress, DNA oxidation and damage, and consequent mutagenesis and apoptosis (Song et al., 2007). Similarly, peroxyl radicals are important intermediates contributing significantly to free radical mediated oxidative stress, having the ability to induce chain reactions and lipoperoxidation, causing structural changes and making the membrane lose its integrity (Kannan and Jain, 2000). Finally, HOCl is a highly reactive biological oxidant playing an important role in both bacterial cell killing and inflammatory tissue injury by neutrophils (Winterbourn, 2002). Excessive production of HOCl (up to 200 μM in pathological conditions) has toxic effects; in fact, HOCl is able to penetrate cell membranes and to react with a wide range of target molecules (lipids, proteins and DNA). Reduced glutathione is one of the preferred biological substrates of HOCl (Winterbourn and Brennan, 1997).

With this in mind, the protective effect of CF against oxidative damage was investigated in different model systems by choosing both biomolecules (glutathione and DNA) and circulating cells (erythrocytes and lymphocytes) as targets of oxidation.

2. Material and methods

2.1. Reagents

CELLFOOD® (liquid) was kindly provided by Eurodream (La Spezia, Italy) and stored at room temperature (CF is stable for several years in these conditions); Lymphoprep™ was purchased from Fresenius Kabi (Oslo, Norway); 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) as generator of peroxyl radicals was obtained from Trimital (Milan, Italy); 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 5,5-dithio-bis-2-nitrobenzoic (DTNB), glutathione (GSH) and sodium hypochlorite (NaOCl) from Sigma–Aldrich (Milan, Italy).

2.2. CELLFOOD® antioxidant power

The antioxidant power of CF was measured by the BAP (Biological Antioxidant Potential) test (Diacron International, Grosseto, Italy). The method is based on the ability of a colored solution, containing ferric ions (Fe^{3+}) adequately bound to a specific chromogenic substrate, to discolor when its Fe^{3+} ions are reduced to ferrous ions (Fe^{2+}) following the addition of a reducing system (Benzie and Strain, 1996). The intensity of decoloration is assessed photometrically at 505 nm; intra- and inter-assay coefficients of variation are less than 5.5%.

2.3. Protection of thiol groups against oxidation

As previously described (Solarska et al., 2010), GSH solution (250 μM, final) in phosphate-buffered saline (PBS, pH 7.4) was mixed in the presence or absence of CF at different dilutions, with oxidants namely 100 μM H_2O_2, 10 mM AAPH or 125 μM HOCl (final concentrations) to make up a volume of 500 μL.

HOCl was added as a solution of NaOCl in PBS; in fact at pH 7.4, this solution contains a mixture of HOCl and OCl^- in approximately 1:1 ratio and is subsequently referred to as HOCl (Vissers et al., 1998). H_2O_2 and HOCl concentrations were determined spectrophotometrically at 230 nm ($\varepsilon = 71\ M^{-1}\ cm^{-1}$) and 292 nm ($\varepsilon = 350\ M^{-1}\ cm^{-1}$), respectively (Maehly and Chance, 1954; Morris, 1966).

After a 15 min incubation at room temperature (HOCl) or a 1 h incubation at 37 °C (H_2O_2, AAPH), the samples were diluted with 1 mL of 0.1 M phosphate buffer, pH 8.0, and mixed with 10 μL of 10 mM DTNB. The absorbance of thionitrobenzoic acid was measured at 412 nm.

2.4. Protection of DNA against oxidative damage

To evaluate the protective effect of CF against DNA oxidation, a cell-free system composed of plasmid DNA, which resembles the structure of mtDNA, was used. The plasmid PINCO (Grignani et al., 1998) and pUC18 were propagated according to the standard molecular techniques (Sambrook et al., 1989).

The plasmid DNA was purified using the QIAGEN Plasmid Midi Kit. The purified plasmid was spectrophotometrically assayed and diluted in TE (10 mM Tris–HCl, pH 8.0 and 1 mM Na_2EDTA) to a concentration of 1 mg/mL. The pUC18 was linearized with *Eco* RI overnight at 37 °C, then was purified using QIAGEN Plasmid Mini Kit and diluted in TE to a concentration of 0.5 mg/mL.

After purification, 10 μL of PINCO plasmid DNA (0.025 mg/mL) in PBS, containing linearized pUC18 (0.01 mg/mL) as internal standard, were mixed with 10 μL of PBS or 10 μL of different CF dilutions in PBS (range 1: 1250–1:25, final) and incubated 10 min at room temperature. After incubation, 1 μL of 100 mM AAPH (5 mM final concentration), freshly made, was placed along the wall of the tube. The tube was vortexed and incubated at 37 °C per 10 min. Fifteen microliters of the reaction were mixed with 5 μL of 5× Loading Buffer and analyzed in a 0.8% agarose Tris Acetate EDTA (TAE) gel and electrophoresed for 45 min at 75 V. The gel was stained with GelRed Nucleic Acid Gel Stain (Biotium, USA) and visualized by UV light in a Gel Doc 200 (Bio-Rad). Quantification was carried out by densitometric analysis using Quantity One Software 4.01 (Bio-Rad). Oxidative damage was assessed as a decrease in the level of the supercoiled form of plasmidic DNA.

The same procedure was used for the other ROS generating systems, in which AAPH was replaced with either 1 μL of 20 mM H_2O_2 + 2 mM ferric citrate (Fe^{3+}, 100 μM; H_2O_2, 1 mM, final concentrations) or 1 μL of 60 μM $FeSO_4$ + 112 μM of Na_2EDTA, pH 8.0 + 2 mM H_2O_2 (Fe^{2+}, 3 μM; EDTA, 5.6 μM; H_2O_2, 100 μM, final concentrations) or 1 μL of HOCl 4 mM (200 μM final concentration).

2.5. Protection of erythrocytes against oxidant-induced hemolysis and glutathione depletion

Heparinized blood samples were obtained from healthy volunteers via venapuncture after obtaining informed consent. As previously reported (Benedetti et al., 2004), red blood cells (RBC) were isolated by centrifugation at 2500 rpm for 10 min, washed two times with PBS and finally re-suspended using the same buffer to a hematocrit level of 5%. The RBC suspension was then incubated at 37 °C in the presence or absence of CF at different dilutions, with 100 μM H_2O_2, 10 mM AAPH or 125 μM HOCl (final concentrations). CF was added to the RBC suspension 10 min prior to addition of the oxidizing agents. The erythrocyte suspension incubated with PBS served as control. At the indicated time, an aliquot of reaction mixture (1 mL) was removed and centrifuged. RBC hemolysis was evaluated by measuring the content of hemoglobin (Hb) in supernatants at 540 nm, while GSH levels were determined at 412 nm by titration with DTNB after the addition of distilled water to the RBC pellet in order to lyse the cells.

2.6. Protection of lymphocytes against ROS formation

Heparinized blood samples were obtained from healthy volunteers via venapuncture after obtaining informed consent. Lymphocytes were isolated by centrifugation at 1500 rpm for 20 min in the presence of Lymphoprep™, washed two times with PBS and after, re-suspended in the same buffer at a concentration of 1×10^6 cells/mL. Cells were incubated with DCFH-DA (25 μM, final) at 37 °C for 30 min (Myhre et al., 2003); excess probe was then removed by centrifugation. Intracellular ROS were detected in DCFH-DA-loaded lymphocytes incubated at 37 °C in the presence or absence of CF at different dilutions, with 100 μM H_2O_2, 10 mM AAPH or 125 μM HOCl (final concentrations). CF was added to the lymphocyte suspension 10 min prior to addition of the oxidizing agents. Samples of 5×10^4 cells were placed on 96-well plates; at the indicated time, the fluorescence emission of the probe following oxidation by ROS was measured at 520 nm upon excitation at 485 nm in a FluoStar Optima spectrofluorimeter (BMG Labtech, Offenburg, Germany).

2.7. Data analysis

All the presented results are mean values ± standard deviations (SD) from three independent experiments. Graphs were obtained using Origin 6.0 (Microcal Software, Inc., Northampton, MA, USA).

3. Results

3.1. CF antioxidant power

The evaluation of CF ferric reducing ability by the BAP test, as a measure of its total antioxidant power, revealed that CF had a BAP value equal to 65,205 ± 1676 μM.

3.2. CF protection against GSH oxidation

In the experiments using GSH as the target of oxidation, we found that H_2O_2, AAPH and HOCl caused a strong oxidation of the GSH thiol group thus leading to a significant reduction in GSH concentration in the reaction mixture (approximately a reduction of 70% with respect to control was observed, data not shown). When oxidation occurred in the presence of CF at different dilutions (range 1:5000–1:50), we observed that CF inhibited the oxidation of GSH induced by the three oxidants in a dose-dependent manner (Fig. 1). The best protection against thiol group oxidation was observed when H_2O_2 was used as the oxidant (IC_{50} at CF dilution equal to 1:385) rather than AAPH (IC_{50} at 1:140) or HOCl (IC_{50} at 1:130).

3.3. CF protection against DNA oxidation

The protective effect of CF versus DNA oxidation was investigated in a cell-free system. Plasmid DNA that was not oxidized (control) contained 48 ± 2% of supercoiled (CCC) and 50 ± 2% of circular (OC) forms; while plasmid DNA treated with the different ROS generating systems showed a depletion to approximately 33% of the CCC form (data not shown). When oxidation induced by Fe^{2+}/H_2O_2, Fe^{3+}/H_2O_2 and AAPH occurred in the presence of CF at different dilutions (range 1:1250–1:25), a dose-dependent inhibition of DNA oxidation was observed (Fig. 2). The best DNA protection was evidenced when Fe^{2+}/H_2O_2 was used as the oxidant (IC_{50} at CF dilution equal to 1:909) rather than Fe^{3+}/H_2O_2 (IC_{50} at 1:250) or AAPH (IC_{50} at 1:84). A different trend was found by using HOCl as the oxidant agent; in fact, at the highest CF dilution tested (1:1250), a 100% protection toward DNA damage was already evidenced (not shown).

3.4. CF protection against RBC oxidation

In the experiments using RBC as the target of oxidation, we found that RBC oxidation by H_2O_2, AAPH and HOCl caused a time-dependent cell lysis leading to Hb release and absorbance increment at 540 nm (Fig. 3A–C). On the contrary, RBC incubated in PBS (controls) were stable and little hemolysis was observed during the different incubation periods. When AAPH-mediated oxidation took place in the presence of CF at different dilutions (range 1:4000–1:500), a dose-dependent reduction of RBC hemolysis was evidenced (Fig. 3B). When H_2O_2- and HOCl-induced oxidation took place in the presence of CF at different dilutions (range 1:4000–1:500), RBC hemolysis was not evidenced, indeed cell lysis was even lower than controls (Fig. 3A and C).

Following oxidation by H_2O_2, AAPH and HOCl, a time-dependent intracellular GSH depletion was also observed in RBC incubated at 37 °C (Fig. 4A–C), while little consumption of GSH was evidenced in controls during the different incubation periods. When RBC oxidation occurred in the presence of CF at different dilutions (range 1:4000–1:500), a dose-dependent inhibition of GSH consumption was observed (Fig. 4A–C).

3.5. CF protection against lymphocyte oxidation

Little fluorescence emission was evidenced in DCFH-DA-loaded lymphocytes incubated with PBS (controls); on the contrary, the addition of H_2O_2, AAPH and HOCl to lymphocytes caused a strong fluorescence emission indicating a rapid accumulation of intracellular ROS (approximately 5 times higher than control, data not shown). When oxidation took place in the presence of CF at different dilutions (range 1:2000–1:250), a dose-dependent reduction of cellular oxidative stress was evidenced, with an inhibition of ROS formation equal to 100% for the highest CF concentration tested (Fig. 5). The best protection of CF against ROS accumulation was observed when AAPH was used as the oxidant (IC_{50} at CF dilution equal to 1:647) rather than HOCl (IC_{50} at 1:500) or H_2O_2 (IC_{50} at 1:483).

4. Discussion

In this study, the *in vitro* antioxidant properties of CELLFOOD® were investigated for the first time. The first analysis conducted on CF (liquid) involved an evaluation of its ferric reducing ability as a measure of its total antioxidant power. As a result, we found that CF had a BAP value of approximately 65,000 μM; this means a very high antioxidant capacity considering that human plasma from healthy subjects normally has a BAP value ranging from

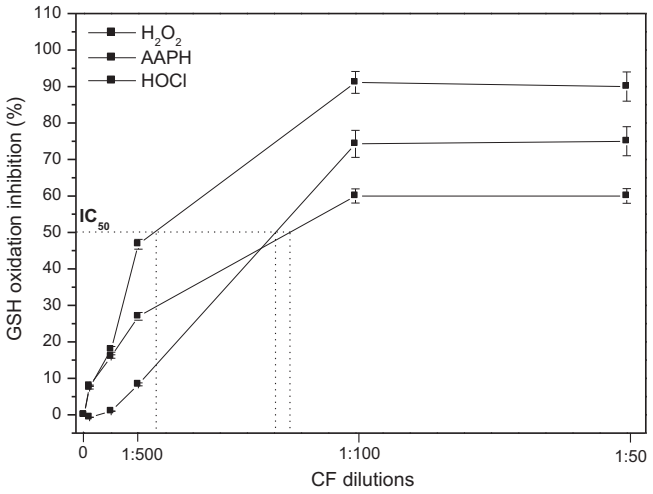

Fig. 1. The protection of GSH against H_2O_2-, AAPH-, and HOCl-induced thiol group oxidation by CELLFOOD®. GSH (250 μM) was incubated with 100 μM H_2O_2, 10 mM AAPH or 125 μM HOCl (final) in the absence and presence of CF (dilution range 1:5000–1:50). Data are expressed as the mean ± SD of three experiments.

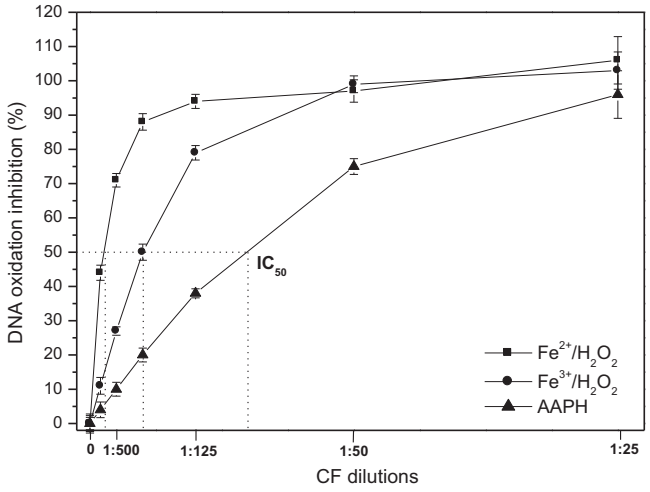

Fig. 2. The protection of DNA against H_2O_2- and AAPH-induced oxidation by CELLFOOD®. Pinco plasmid DNA (0.025 mg/mL) was incubated with Fe^{2+}/H_2O_2, Fe^{3+}/H_2O_2, or AAPH in the absence and presence of CF (dilution range 1:1250–1:25). Data are expressed as the mean ± SD of three experiments.

2200 to 4000 μM (Martarelli and Pompei, 2009). This power is obviously not absolute but relative to the tested substrate (i.e. ferric ions). However, considering that these ions are naturally occurring components, we believed that the BAP test provided a reliable measure of CF antioxidant power against the attack of free radicals in physiological conditions.

With this in mind, we decided to investigate CF antioxidant protection against three physiologically relevant oxidizing agents, namely hydrogen peroxide, peroxyl radicals and hypochlorous acid, starting with GSH as a target molecule of oxidation. As a result, we observed that CF inhibited the oxidation of GSH induced by the three oxidants in a dose-dependent manner, thus maintaining GSH thiol groups in their reduced state. GSH is the most abundant non-enzymatic antioxidant present in mammalian cells and plays a very important role in maintaining cellular redox homeostasis (Pastore et al., 2003). During oxidative stress, the cellular pool of GSH is depleted, consequently, exogenously introduced radical scavengers, such as CF, may protect GSH levels in cells by preventing them from being consumed in reactions with free radicals (Valko et al., 2007).

We also evaluated the protective effect of CF against free radical-induced DNA damage using a cell-free system composed of plasmid DNA which resembles the structure of mtDNA. Our experiments indicate a high protection of CF towards DNA oxidation, especially when induced by the radical generating system Fe^{2+}/H_2O_2. These radical species (hydroxyl radicals) are mainly produced in mitochondria (Lenaz, 1998) and it has been demonstrated that the oxidative damage to mtDNA is implicated in physiological senescence and age-related disorders such as sporadic neurodegenerative disorders, type II diabetes, cancer and cardiac diseases (Balaban et al., 2005; Taylor and Turnbull, 2005). Our results suggest that CF supplementation might play a role in mitochondria viability and the long term consumption of CF may be helpful in diseases related to mitochondrial dysfunction. Accordingly, the employment of CF in fibromyalgic patients demonstrated an improvement in fibromyalgia symptoms and health-related quality of life (Nieddu et al., 2007).

Another aspect that aroses from the DNA protection assay was the behavior of CF in relation to HOCl-mediated oxidation. HOCl induces different types of DNA damage (single-strand breakage, 8-OHdG and M_1dG formation) (Gungor et al., 2010) and our data revealed that CF offered a high level of protection against DNA single-strand breakage. It is well known that HOCl produced by

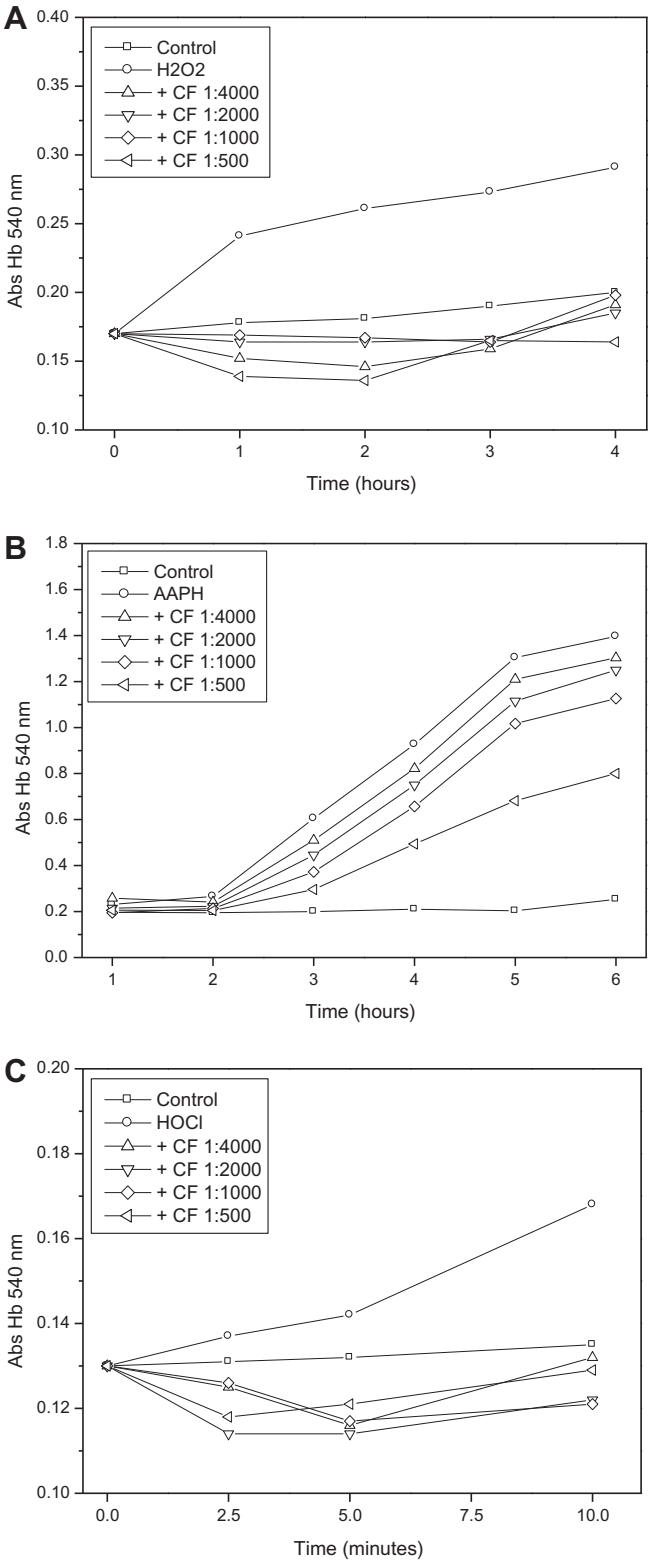

Fig. 3. The protection of erythrocytes against H_2O_2- (A), AAPH- (B) and HOCl-induced (C) hemolysis by CELLFOOD®. Erythrocytes were incubated in PBS with 100 μM H_2O_2 at 37 °C for 4 h (A), with 10 mM AAPH for 6 h (B) or with 125 μM HOCl for 10 min (C) in the absence and presence of CF (dilution range 1:4000–1:500). Data are expressed as the mean of three experiments (SD have been omitted for clarity).

activated neutrophils can initiate chemical mutagenesis and carcinogenesis by producing oxidative damage to the genome in the inflammatory environment (Ohshima et al., 2003). Indeed,

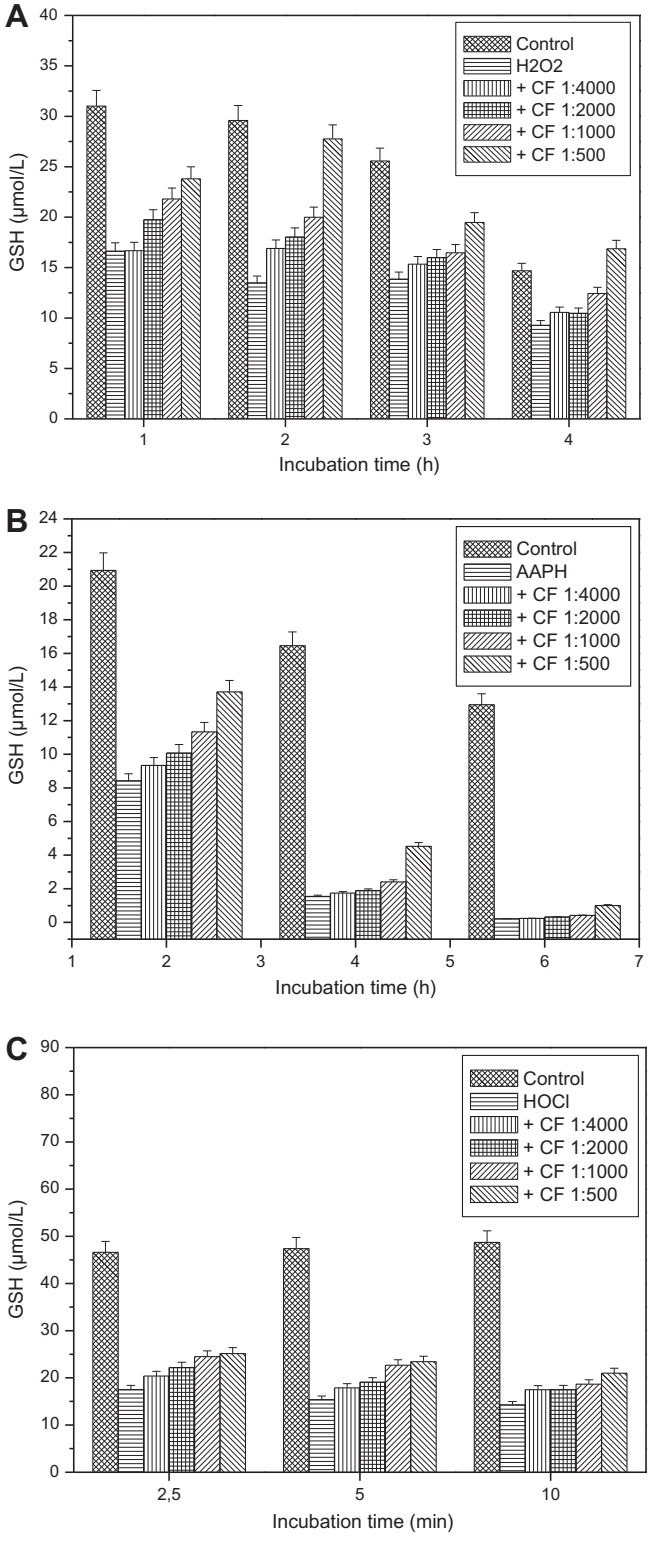

Fig. 4. The protection of erythrocytes against H_2O_2- (A), AAPH- (B) and HOCl-induced (C) GSH depletion by CELLFOOD®. Erythrocytes were incubated in PBS with 100 μM H_2O_2 at 37 °C for 4 h (A), with 10 mM AAPH for 6 h (B) or with 125 μM HOCl for 10 min (C) in the absence and presence of CF (dilution range 1:4000–1:500). Data are expressed as the mean ± SD of three experiments.

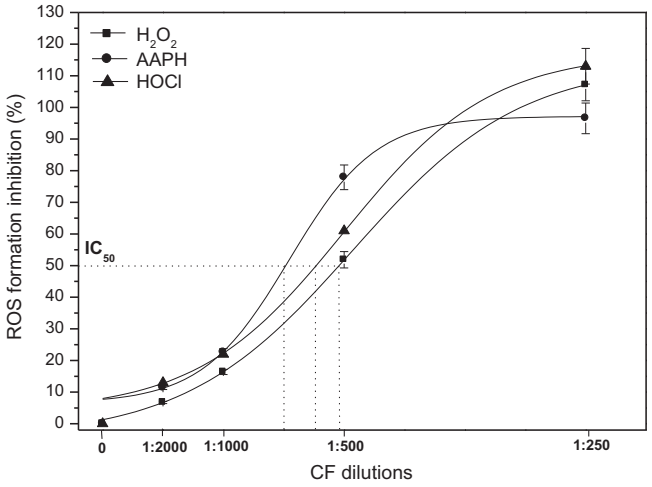

Fig. 5. The protection of lymphocytes against H_2O_2-, AAPH- and HOCl-induced ROS accumulation by CELLFOOD®. Lymphocytes were incubated in PBS at 37 °C for 10 min with 100 μM H_2O_2, 10 mM AAPH or 125 μM HOCl in the absence and presence of CF (dilution range 1:2000–1:250). Data are expressed as the mean ± SD of three experiments.

activation of neutrophils during lung inflammation should be considered a significant factor in genotoxicity towards the epithelial cells (Gungor et al., 2010). Chlorinative stress is also related to neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis, disorders in which the role of inflammation is subject to increased scrutiny (Yap et al., 2007). The findings of the present study reveal that CF exhibits genome protection activity exploitable in preventive medicine in chronic and degenerative pathologies in which inflammatory states are known to play an etiological role.

Being particularly susceptible to oxidative damage, erythrocytes were also chosen as a model system for investigating CF antioxidant properties. RBC have a high cellular concentration of Hb and are directly exposed to molecular oxygen. At the same time, they have a high polyunsaturated fatty acid content in their membranes making them highly susceptible to peroxyl radical mediated oxidation that leads to leakage of Hb and a decrease in intracellular GSH levels (Hseu et al., 2008; Magalhães et al., 2009; Wang et al., 2009). In our experiments, we found that CF effectively protected RBC from Hb release and GSH depletion induced by H_2O_2 and AAPH, thus furnishing protection against oxidative hemolysis and preserving the endogenous antioxidant defense system.

In addition, CF protection was also observed in the case of RBC oxidation by HOCl, which represents a good model system for investigating neutrophil-mediated cell injury (Winterbourn and Brennan, 1997; Vissers et al., 1998; Winterbourn, 2002). Exposure of RBC to HOCl results in the loss of intracellular GSH that precedes oxidation of membrane thiols and the formation of chloramines (Vissers and Winterbourn, 1995). The well-known end result of RBC exposure to HOCl is cell hemolysis (Vissers et al., 1994); indeed, HOCl treatment causes an immediate change in the RBC membrane structure that affects membrane deformability and permeability. In our experiments, we found that CF promptly reduced RBC lysis and GSH depletion induced by HOCl.

The overall protection of RBC by CF against oxidation might explain some of the positive effects of CF supplementation on the training process and sport performance in professional athletes (Milić and Djordjević, 2009). The rise in cellular metabolism and in Hb turnover during intense exercise may favor ROS production within the RBC and activation of leukocytes leading to the release of neutrophil activation products (HOCl) (Santos-Silva et al., 2001). Considering that most ROS are able to diffuse through the RBC membrane and that the RBC has limited repair mechanisms, the oldest RBC may not overcome the oxidative stress developed within the cell; the youngest RBC may develop and/or accumulate oxidative lesions. As a final result, an anemic state may occur (Robinson et al., 2006). In this context, CF supplementation might

have great relevance in contrasting the toxic effects of ROS produced by cellular metabolism (such as peroxyl radicals) or by neutrophil activation (such as HOCl), thus preserving RBC from hemolysis and thus athletes from anemia.

Finally, we used lymphocytes as cellular targets of oxidation. Lymphocytes, being involved in the immune response, are normally subjected *in vivo* to oxidative stress (Knight, 2000). Moreover, since lymphocytes are immediately exposed to variations in antioxidants in the blood due to modifications in dietary habits, they represent a reliable cellular line to study the effect of dietary antioxidants on cell protection (Anderson et al., 1994; Foti et al., 2005). In our experiments, we tested CF protection against free radical formation in DCFH-DA-loaded lymphocytes subjected to H_2O_2, AAPH and HOCl oxidation. The fluorogenic compound DCFH-DA has been utilized extensively as a marker for oxidative stress, in fact, it emits fluorescence when it is oxidized to DCF, thus measuring the formation of reactive species in cells (Myhre et al., 2003). Even in this case, we observed that CF significantly reduced the intracellular ROS formation induced by the three oxidants in a dose-dependent manner.

5. Conclusions

In the present study we have clearly evidenced that CELLFOOD® is a nutritional supplement that furnishes effective antioxidant protection against pathophysiologically relevant oxidant agents. The *in vitro* protection of cells and biomolecules against free radical attacks suggests that CF might be a valuable coadjuvant in the prevention and treatment of various physiological and pathological conditions related to oxidative stress, from aging to sport anemia, from fibromyalgia to neurodegeneration and cancer.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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