



Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

The antioxidant protection of CELLFOOD® against oxidative damage in vitro

Serena Benedetti^{a,*}, Simona Catalani^a, Francesco Palma^b, Franco Canestrari^a^a University of Urbino "Carlo Bo", Department of Biomolecular Sciences, Section of Clinical Biochemistry, Via Ubaldini 7, 61029 Urbino (PU), Italy^b University of Urbino "Carlo Bo", Department of Biomolecular Sciences, Section of Biochemistry and Molecular Biology, Via Saffi 2, 61029 Urbino, Italy

ARTICLE INFO

Article history:

Received 16 April 2011

Accepted 4 June 2011

Available online xxxx

Keywords:

CELLFOOD®

Nutritional supplement

Oxidative damage

Antioxidant protection

Red blood cells

DNA

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, peroxy 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.

© 2011 Published by Elsevier Ltd.

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 formula-

Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane) hydrochloride; BAP, biological antioxidant potential; CF, CELLFOOD®; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DTNB, 5,5-dithio-bis-2-nitrobenzoic; GSH, glutathione; Hb, hemoglobin; H₂O₂, hydrogen peroxide; HOCl, hypochlorous acid; mtDNA, mitochondrial DNA; NaOCl, sodium hypochlorite; PBS, phosphate-buffered saline; RBC, red blood cells; ROS, reactive oxygen species.

* Corresponding author. Tel.: +39 0722 351477; fax: +39 0722 322370.

E-mail addresses: serena.benedetti@uniurb.it (S. Benedetti), simonacatalani@yahoo.it (S. Catalani), francesco.palma@uniurb.it (F. Palma), franco.canestrari@uniurb.it (F. Canestrari).

tion 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 makes 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), peroxy 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, peroxy 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, Italia) 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 peroxy radicals was obtained

from Trimital (Milan, Italy); 2',7'-dichlorofluorescein 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 (Visser et al., 1998). H_2O_2 and HOCl concentrations were determined spectrophotometrically at 230 nm ($\epsilon = 71 M^{-1} cm^{-1}$) and 292 nm ($\epsilon = 350 M^{-1} cm^{-1}$), 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, was 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 was 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.

209 2.6. Protection of lymphocytes against ROS formation

210 Heparinized blood samples were obtained from healthy volunteers via vena-
211 puncture after obtaining informed consent. Lymphocytes were isolated by centrifuga-
212 tion at 1500 rpm for 20 min in the presence of Lymphoprep™, washed two times
213 with PBS and after, re-suspended in the same buffer at a concentration of
214 1×10^6 cells/mL. Cells were incubated with DCFH-DA (25 μ M, final) at 37 °C for
215 30 min (Myhre et al., 2003); excess probe was then removed by centrifugation.
216 Intracellular ROS were detected in DCFH-DA-loaded lymphocytes incubated at
217 37 °C in the presence or absence of CF at different dilutions, with 100 μ M H₂O₂,
218 10 mM AAPH or 125 μ M HOCl (final concentrations). CF was added to the lympho-
219 cyte suspension 10 min prior to addition of the oxidizing agents. Samples of 5×10^4
220 cells were placed on 96-well plates; at the indicated time, the fluorescence emission
221 of the probe following oxidation by ROS was measured at 520 nm upon excitation
222 at 485 nm in a FluoStar Optima spectrofluorimeter (BMG Labtech, Offenburg,
223 Germany).

224 2.7. Data analysis

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

228 **3. Results**

229 3.1. CF antioxidant power

230 The evaluation of CF ferric reducing ability by the BAP test, as a
231 measure of its total antioxidant power, revealed that CF had a BAP
232 value equal to 65,2/F11Ter

theexperim using theet of

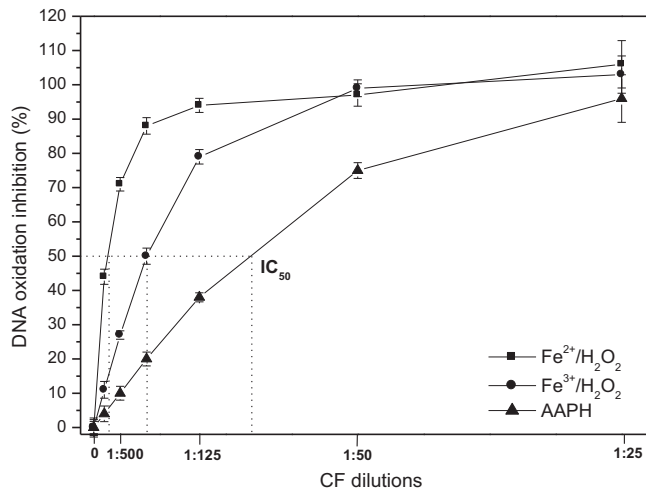


Fig. 2. The protection of DNA against H₂O₂- and AAPH-induced oxidation by CELLFOOD®. Pincio plasmid DNA (0.025 mg/mL) was incubated with Fe²⁺/H₂O₂, Fe³⁺/H₂O₂, or AAPH in the absence and presence of CF (dilution range 1:1250–1:25). Data are expressed as the mean ± SD of three experiments.

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 Fe²⁺/H₂O₂. 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 arose 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 M₁dG 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

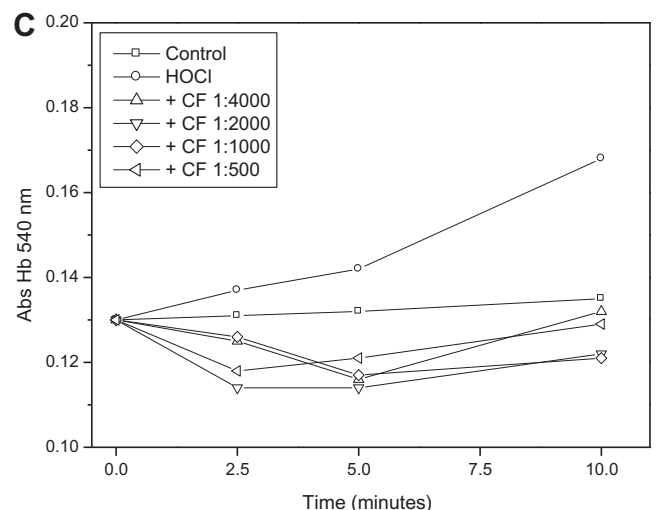
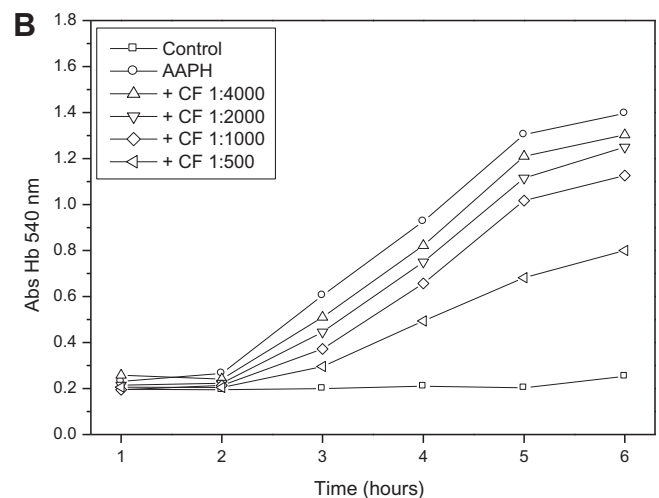
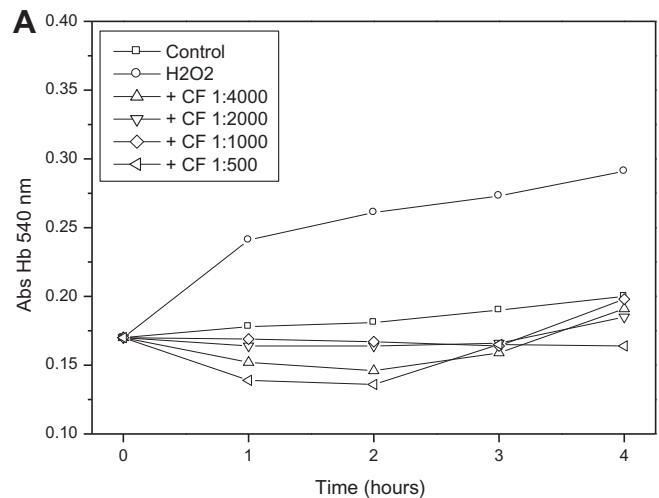


Fig. 3. The protection of erythrocytes against H₂O₂- (A), AAPH- (B) and HOCl-induced (C) hemolysis by CELLFOOD®. Erythrocytes were incubated in PBS with 100 μM H₂O₂ at 37 °C for 4 h (A), with 10 mM AAPH for 6 h (B) or with 125 μM HOCl for 10 min (C) in the absence and presence of CF (dilution range 1:4000–1:500). Data are expressed as the mean of three experiments (SD have been omitted for clarity).

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

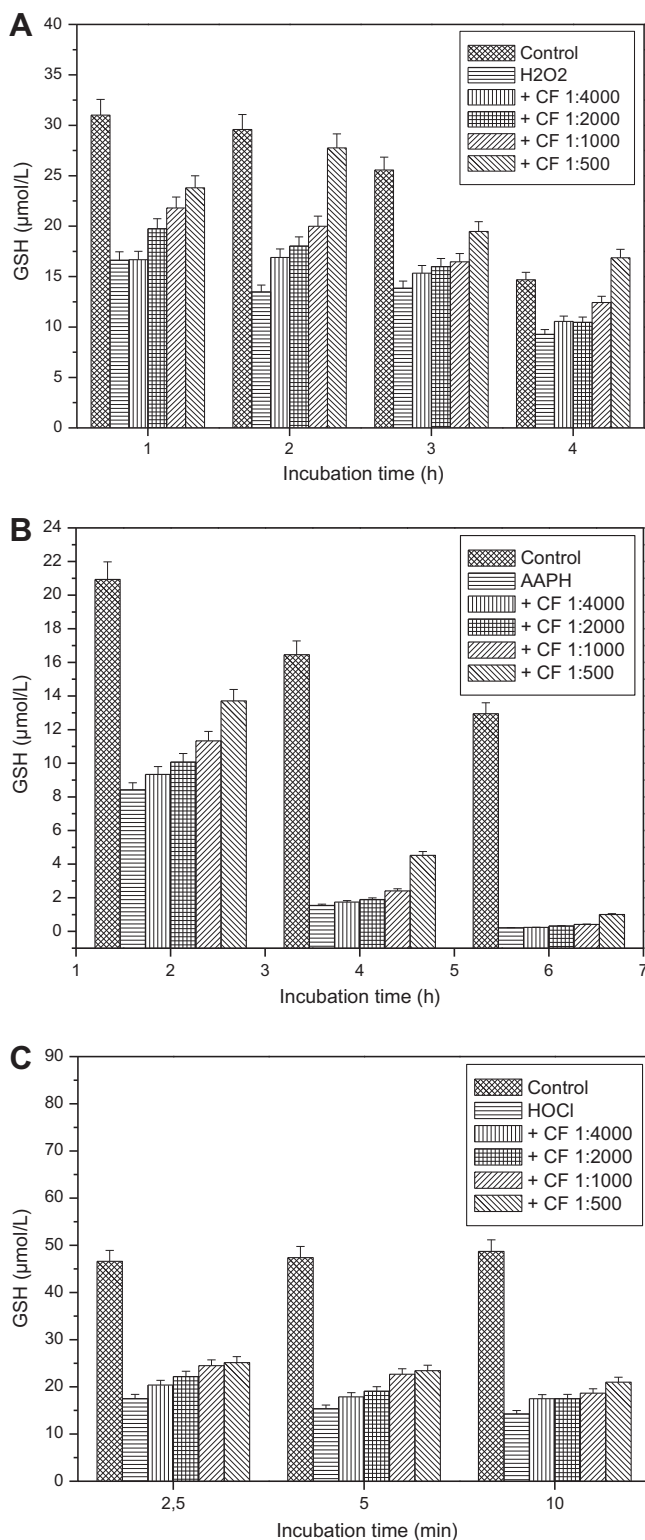


Fig. 4. The protection of erythrocytes against H₂O₂- (A), AAPH- (B) and HOCl-induced (C) GSH depletion by CELLFOOD®. Erythrocytes were incubated in PBS with 100 μM H₂O₂ at 37 °C for 4 h (A), with 10 mM AAPH for 6 h (B) or with 125 μM HOCl for 10 min (C) in the absence and presence of CF (dilution range 1:4000–1:500). Data are expressed as the mean ± SD of three experiments.

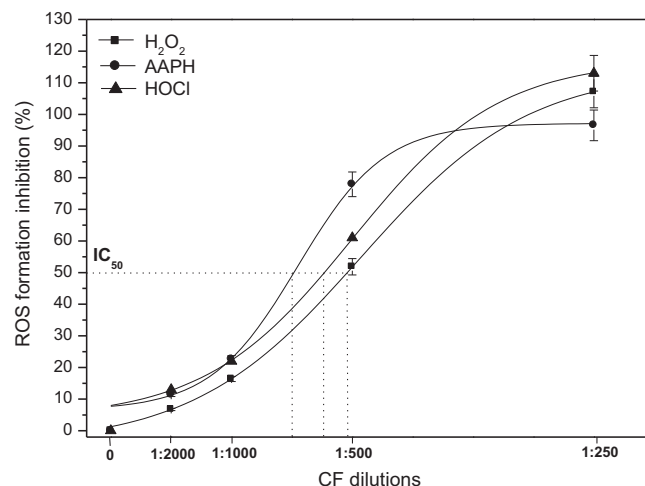


Fig. 5. The protection of lymphocytes against H₂O₂-, AAPH- and HOCl-induced ROS accumulation by CELLFOOD®. Lymphocytes were incubated in PBS at 37 °C for 10 min with 100 μM H₂O₂, 10 mM AAPH or 125 μM HOCl in the absence and presence of CF (dilution range 1:2000–1:250). Data are expressed as the mean ± SD of three experiments.

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 H₂O₂ 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

tion 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

have great relevance in contrasting the toxic effects of ROS produced by cellular metabolism (such as peroxy 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 H₂O₂, 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 suggest 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.

Acknowledgment

We thank Dr. Francesca Carducci for advice on the English language.

References

- Anderson, D., Yu, T.W., Phillips, B.J., Schmezer, P., 1994. The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in the COMET assay. *Mutat. Res.* 307, 261–271.
- Balaban, R.S., Nemoto, S., Finkel, T., 2005. Mitochondria oxidants, and aging. *Cell* 120, 483–495.
- Benedetti, S., Benvenuti, F., Pagliarini, S., Francogli, S., Scoglio, S., Canestrari, F., 2004. Antioxidant properties of a novel phycocyanin extract from the blue-green alga *Aphanizomenon flos-aquae*. *Life Sci.* 75, 2353–2362.
- Berger, M.M., 2005. Can oxidative damage be treated nutritionally? *Clin. Nutr.* 24, 172–183.
- Benzie, I.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Anal. Biochem.* 239, 70–76.
- Bohr, V.A., 2002. Repair of oxidative DNA damage in nuclear and mitochondrial DNA and some changes with aging in mammalian cells. *Free Radic. Biol. Med.* 32, 804–812.
- Cohen, R.A., Tong, X., 2010. Vascular oxidative stress: the common link in hypertensive and diabetic vascular disease. *J. Cardiovasc. Pharmacol.* 55, 308–316.
- Cordero, M.D., de Miguel, M., Carmona-López, I., Bonal, P., Campa, F., Moreno-Fernández, A.M., 2010. Oxidative stress and mitochondrial dysfunction in fibromyalgia. *Neuro Endocrinol. Lett.* 31, 169–173.
- Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D., Milzani, A., 2006. Biomarkers of oxidative damage in human disease. *Clin. Chem.* 52, 601–623.
- Dizdaroglu, M., Rao, G., Halliwell, B., Gajewski, E., 1991. Damage to the DNA bases in mammalian chromatin by hydrogen peroxide in the presence of ferric and cupric ions. *Arch. Biochem. Biophys.* 285, 317–324.
- Dyer, D.S., 2000. CELLFOOD®. Vital cellular nutrition for the new millennium, Feedback Books Inc.

- Foti, P., Erba, D., Riso, P., Spadafranca, A., Crisculi, F., Testolin, G., 2005. Comparison between daidzein and genistein antioxidant activity in primary and cancer lymphocytes. *Arch. Biochem. Biophys.* 433, 421–427.
- Fusco, D., Colloca, G., Lo Monaco, M.R., Cesari, M., 2007. Effects of antioxidant supplementation on the aging process. *Clin. Interv. Aging* 2, 377–387.
- Galasko, D., Montine, T.J., 2010. Biomarkers of oxidative damage and inflammation in Alzheimer's disease. *Biomark. Med.* 4, 27–36.
- Graziewicz, M.A., Day, B.J., Copeland, W.C., 2002. The mitochondrial DNA polymerase as a target of oxidative damage. *Nucleic Acids Res.* 30, 2817–2824.
- Grignani, F., Kinsella, T., Mencarelli, A., Valtieri, M., Riganelli, D., Grignani, F., Lanfrancone, L., Peschle, C., Nolan, G.P., Pelicci, P.G., 1998. High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. *Cancer Res.* 58, 14–19.
- Gungor, N., Knaepen, A.M., Munnia, A., Peluso, M., Haenen, G.R., Chiu, R.K., Godschalk, R.W., van Schooten, F.J., 2010. Genotoxic effects of neutrophils and hypochlorous acid. *Mutagenesis* 25, 149–154.
- Halliwell, B., Gutteridge, J.M.C., 1999. Free radicals in biology and medicine. Oxford University Press, New York.
- Herrera, E., Jiménez, R., Aruoma, O.I., Hercberg, S., Sánchez-García, I., Fraga, C., 2009. Aspects of antioxidant foods and supplements in health and disease. *Nutr. Rev.* 67, S140–S144.
- Hseu, Y.C., Chang, W.H., Chen, C.S., Liao, J.W., Huang, C.J., Lu, F.J., Chia, Y.C., Hsu, H.K., Wu, J.J., Yang, H.L., 2008. Antioxidant activities of Toona Sinensis leaves extracts using different antioxidant models. *Food Chem. Toxicol.* 46, 105–114.
- Kannan, K., Jain, S.K., 2000. Oxidative stress and apoptosis. *Pathol. Physiol.* 7, 153–163.
- Knight, J.A., 2000. Review: free radicals antioxidants, and the immune system. *Ann. Clin. Lab. Sci.* 30, 145–158.
- Lenaz, G., 1998. Role of mitochondria in oxidative stress and ageing. *Biochim. Biophys. Acta* 1366, 53–67.
- Ljungman, M., Hanawalt, P.C., 1992. Efficient protection against oxidative DNA damage in chromatin. *Mol. Carcinog.* 5, 264–269.
- Maehly, A.C., Chance, B., 1954. The assay of catalases and peroxidases. *Methods Biochem. Anal.* 1, 357–424.
- Magalhães, A.S., Silva, B.M., Pereira, J.A., Andrade, P.B., Valentão, P., Carvalho, M., 2009. Protective effect of quince (*Cydonia oblonga* Miller) fruit against oxidative hemolysis of human erythrocytes. *Food Chem. Toxicol.* 47, 1372–1377.
- Martarelli, D., Pompei, P., 2009. Oxidative stress and antioxidant changes during a 24-hours mountain bike endurance exercise in master athletes. *J. Sports Med. Phys. Fitness* 49, 122–127.
- Milić, R., Djordjević, S., 2009. Cycling performance and Cellfood. In: Loland, S., Bø, K., Fasting, K., Hallén, J., Ommundsen, Y., Roberts, G., Tsolakidis, E. (Eds.), *Book of Abstracts of the 14th Annual Congress of the European College of Sport Science*. Gamlebyen Grafiske A.S., Oslo, p. 230.
- Morris, J.C., 1966. The acid ionization constant of HClO from 5 to 35. *J. Phys. Chem.* 70, 3798–3805.
- Myhre, O., Andersen, J.M., Aarnes, H., Fonnum, F., 2003. Evaluation of the probes 2',7'-dichlorofluorescein diacetate luminol and lucigenin as indicators of reactive species formation. *Biochem. Pharmacol.* 65, 1575–1582.
- Nieddu, M.E., Menza, L., Baldi, F., Frediani, B., Marcolongo, R., 2007. Efficacy of Cellfood's therapy (deutrosulfazyme) in fibromyalgia. *Reumatismo* 59, 316–321.
- Ohshima, H., Tatemichi, M., Sawa, T., 2003. Chemical basis of inflammation-induced carcinogenesis. *Arch. Biochem. Biophys.* 417, 3–11.
- Pastore, A., Federici, G., Bertini, E., Piemonte, F., 2003. Analysis of glutathione: implication in redox and detoxification. *Clin. Chim. Acta* 333, 19–39.
- Piecznik, S.R., Neustadt, J., 2007. Mitochondrial dysfunction and molecular pathways of disease. *Exp. Mol. Pathol.* 83, 84–92.
- Robinson, Y., Cristancho, E., Böning, D., 2006. Intravascular hemolysis and mean red blood cell age in athletes. *Med. Sci. Sports Exerc.* 38, 480–483.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular cloning. A laboratory manual*. Cold Spring Harbor, New York.
- Santos-Silva, A., Rebelo, M.I., Castro, E.M., Belo, L., Guerra, A., Rego, C., Quintanilha, A., 2001. Leukocyte activation erythrocyte damage lipid profile and oxidative stress imposed by high competition physical exercise in adolescents. *Clin. Chim. Acta* 306, 119–126.
- Smith, H.S., Barkin, R.L., 2010. Fibromyalgia syndrome: a discussion of the syndrome and pharmacotherapy. *Am. J. Ther.* 17, 418–439.
- Solarska, K., Lewińska, A., Karowicz-Bilińska, A., Bartosz, G., 2010. The antioxidant properties of carnitine in vitro. *Cell. Mol. Biol. Lett.* 15, 90–97.
- Song, Y., Driessens, N., Costa, M., De Deken, X., Detours, V., Corvilain, B., Maenhaut, C., Miot, F., Van Sande, J., Many, M.C., Dumont, J.E., 2007. Roles of hydrogen peroxide in thyroid physiology and disease. *J. Clin. Endocrinol. Metab.* 92, 3764–3773.
- Taylor, R.W., Turnbull, D.M., 2005. Mitochondrial DNA mutations in human disease. *Nat. Rev. Genet.* 6, 389–402.
- Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell. Biol.* 39, 44–84.
- Van Houten, B., Woshner, V., Santos, J.H., 2006. Role of mitochondrial DNA in toxic responses to oxidative stress. *DNA Repair* 5, 145–152.
- Vissers, M.C., Stern, A., Kuypers, F., van den Berg, J., Winterbourn, C.C., 1994. Membrane changes associated with lysis of red blood cells by hypochlorous acid. *Free Radic. Biol. Med.* 16, 703–712.

- 546 Vissers, M.C., Winterbourn, C.C., 1995. Oxidation of intracellular glutathione after
547 exposure of human red blood cells to hypochlorous acid. *Biochem. J.* 307, 57–62. 559
- 548 Vissers, M.C., Carr, A.C., Chapman, A.L., 1998. Comparison of human red cell lysis by
549 hypochlorous and hypobromous acids: insights into the mechanism of lysis. 560
- 550 *Biochem. J.* 330, 131–138. 561
- 551 Wallace, D.C., 1999. Mitochondrial diseases in man and mouse. *Science* 283, 1482–
552 1488. 562
- 553 Wang, J., Sun, B., Cao, Y., Tian, Y., 2009. Protection of wheat bran feruloyl
554 oligosaccharides against free radical-induced oxidative damage in normal
555 human erythrocytes. *Food Chem. Toxicol.* 47, 1591–1599. 563
- 556 Winterbourn, C.C., Brennan, S.O., 1997. Characterization of the oxidation products
557 of the reaction between reduced glutathione and hypochlorous acid. *Biochem. J.*
558 326, 87–92. 564
- 559 Winterbourn, C.C., 2002. Biological reactivity and biomarkers of the neutrophil
560 oxidant, hypochlorous acid. *Toxicology* 181–182, 223–227. 561
- 562 Yakes, F.M., Van, H.B., 1997. Mitochondrial DNA damage is more extensive and
563 persists longer than nuclear DNA damage in human cells following oxidative
564 stress. *Proc. Natl. Acad. Sci. USA* 94, 514–519. 565
- 566 Yap, Y.W., Whiteman, M., Cheung, N.S., 2007. Chlorinative stress: an under
567 appreciated mediator of neurodegeneration? *Cell Signal.* 19, 219–228. 568
- 569 Ziech, D., Franco, R., Georgakilas, A.G., Georgakila, S., Malamou-Mitsi, V.,
570 Schoneveld, O., Pappa, A., Panayiotidis, M.I., 2010. The role of reactive oxygen
571 species and oxidative stress in environmental carcinogenesis and biomarker
572 development. *Chem. Biol. Interact.* 188, 334–339. 573

UNCORRECTED PROOF