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Copper chlorophyllin: A food colorant with bioactive properties?

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ABSTRACT

Copper chlorophyllin (Cu-Chl) is a chlorophyll-derived food colorant, commercially available as a complex mixture of different chlorin molecules. Several studies have reported great variability in the composition of the mixture as well as the presence of porphyrins and uncoppered chlorin derivatives. The intake of chlorophyllin and its relationship with possible health benefits have been also the subject of several studies and the results have drawn attention to the possibility that this compound may serve not just as a food colorant, but also as a potential bioactive product with antimutagenic, anticarcinogenic and antioxidant activities; however, its mechanisms of action are not yet well understood. Attaining knowledge of its mechanisms of absorption, bioavailability and consequent bioactivity is essential for the exploration of its potential health-related applications. The purpose of this paper is to present an overview of bioactivities concerning Cu-Chl and provide a discussion on the latest scientific research findings on its human health benefits.

1. Introduction

Copper chlorophyllin (Cu-Chl) is a semi-synthetic derivative of the natural green pigment chlorophyll. It displays some technological advantages over chlorophyll, such as greater hydrophilicity and tinctorial power and higher stability towards acid and light. Although both chlorophyll and copper chlorophyllin are allowed as food colorants in Europe (EC, 1994) and Brazil (ANVS, 2008); the latter is permitted only in one type of citrus drink in the United States (FDA, 2002).

The consumption of chlorophyll has long been associated with potential health benefits, and nowadays one can still find advertisements of many chlorophyll-containing products, mostly dietary supplements and juices. These advertisements claim that the chemical similarity between chlorophyll and the prosthetic group (heme) of hemoglobin may be responsible for protecting the human body from several diseases. Although chlorophyll and its derivatives were suggested to display hematopoietic activity in the 30s (Hughes & Latner, 1936; Patek, 1936), the later elucidation of the biosynthetic pathways of both chlorophyll and hemoglobin ruled out this hypothesis. The first steps of this biosynthesis are similar in plants, animals and fungi; however, they occur in different intracellular compartments and are catalyzed by enzymes that have the same specificities but differ in structure (amino acid sequence). In the common part of the pathway, 5-aminolevulinic acid generates an intermediate metabolite called protoporphyrinogen IX. The subsequent steps are specific to the biological role that each molecule (chlorophyll or heme group) will play (Tanaka & Tanaka, 2007).

Not long ago, Cu-Chl was used for accelerating wound healing, minimizing body odor in geriatric or ileostomized patients, in treatment of calcium oxalate renal calculi and anemia, among many other applications (as reviewed by Kephart, 1955). In most cases, its efficacy was not scientifically confirmed and its importance faded away.

Research findings on Cu-Chl bioactivities became more accessible to the scientific community in the 80s when several studies were published in international indexed journals. The potential antioxidant activity of commercial preparations of copper chlorophyllin (Sato, Fujimoto, Sakai, Kimura, & Murata, 1979) has encouraged their consumption to prevent chronic degenerative diseases. Possible antimutagenic and chemopreventive effects of Cu-Chl in human body have been discussed (Egner, Muñoz, & Kensler, 2003; Ferruzzi & Blakeslee, 2007; Kumar, Shankar, & Sainis, 2004). Other biological activities, including protection against the deleterious effects of oxidative stress or radiation on cells and modulation of the activity of enzymes involved in the detoxification process of xenobiotics, have been investigated.

Research on confirming the bioactivities of Cu-Chl has also been strongly motivated by the fact that it was widely used not long ago without reported adverse effects. For instance, Harrison, Levin, and Trabin (1954) reported neither any kind of pathology or toxicity related to copper nor any photosensitization effects in a study of rats fed a diet supplemented with Cu-Chl. However, more recent studies have shown tumor-enhancing and genotoxic effects of the complex (Chernomorsky, Rancourt, Virdi, Segelman, & Poretz, 1997; Romert, Curvall, & Jenssen, 1992; Sarkar, Sharma, & Talukder, 1994).

Other factors must also be discussed to ensure a solid analysis of its health benefits. For example, chemical composition in commercial

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preparations of Cu-Chl varies widely (Chernomorsky, Rancourt, Sahai, & Poretz, 1997; Ferruzzi, Failla, & Schwartz, 2002; Inoue et al., 1994; Mortensen & Geppel, 2007; Sato et al., 1986). Differences in experimental protocols should also be considered, namely variations among models (*in vitro* or *in vivo*), the source and concentrations of Cu-Chl, and the type of carcinogen/mutagen employed.

In order to better understand the health effects of Cu-Chl, whether positive or negative, we mainly reviewed studies published in the last 20 years. In this paper we present an overview of bioactivities most commonly assigned to Cu-Chl, providing a discussion on its biological effects and mechanisms of action, taking into account variations in the chemical composition of commercial grade preparations.

2. Synthesis of Cu-Chl and its chemical structures

The main raw material for preparation of Cu-Chl is natural chlorophyll, a macrocyclic molecule that consists of four pyrrole rings bonded by methylene bridges and coordinated to a magnesium atom. Chlorophyll also has a fifth ring (cyclopentanone) and one long hydrophobic side chain derived from phytol ($C_{20}H_{39}OH$), which imparts high hydrophobicity to this molecule. At least six different chlorophyll molecules are known: chlorophylls *a*, *b* (both are found in higher plants), *c*, *d*, *e* (found in algae) and bacteriochlorophylls (found in photosynthetic bacteria) (Hendry, 2000). They differ in their functional groups; for example, chlorophyll *a* has a methylene group, whereas chlorophyll *b* has an aldehyde group.

The main source of chlorophyll for the synthesis of Cu-Chl is dehydrated alfalfa, which is a widely available plant and can provide great amounts of chlorophyll in an economically viable manner. In general, carotenoids, xanthophylls, and sterols also present in alfalfa leaves, are extracted along with chlorophyll.

Copper chlorophyllin is formed by the saponification of chlorophyll molecules in an alkaline medium containing methanolic sodium hydroxide, leading to isocyclic ring opening and phytyl group removal (Humphrey, 1980). Magnesium is removed and replaced with a copper atom using copper sulfate in an acid medium, which gives chlorophyllin the desired chemical stability. Besides copper, divalent cations such as iron and zinc can be used. Copper, however, remains the most commonly used.

The chemical reactions involved in the synthesis of Cu-Chl also result in the formation of a complex mixture of various chlorin-based compounds, including the most commonly found copper chlorins e_4 (derived from chlorophyll *a*) and e_6 (derived from chlorophyll *b*) (Ferruzzi et al., 2002; Inoue et al., 1994; Sato et al., 1986). Other molecules, such as copper pheophorbide a and uncoppered chlorins, may also be present (Chernomorsky, Rancourt, Sahai, et al., 1997; Mortensen & Geppel, 2007). Some of these molecules and their chemical structures are depicted in Fig. 1.

Separation and identification of the components of Cu-Chl have been performed by means of several techniques, such as thin layer chromatography (TLC), mass spectrometry (MS) and infrared spectroscopy (IR) (Sato et al., 1986), high-performance liquid chromatography (HPLC) (Chernomorsky, Rancourt, Sahai, et al., 1997; Egner et al., 2000, 2001; Ferruzzi et al., 2002; Inoue et al., 1994), as well as the coupling of HPLC and mass spectrometry (HPLC–MS) to provide more accurate results (Mortensen & Geppel, 2007). The use of visible spectrophotometry is limited because the main components of Cu-Chl absorb light within a narrow wavelength range (Inoue et al., 1994).

Besides chlorins, porphyrins have been detected in some commercial Cu-Chl preparations, which reveals its qualitatively and quantitatively rich composition and its versatile nature in terms of biological activities (Chernomorsky, Rancourt, Virdi, et al., 1997). The lack of a suitable method for the characterization and quantification of the components in commercial Cu-Chl limits the development of accurate quality parameters for these preparations.

3. Biological effects and mechanisms of action

3.1. Antimutagenic and chemopreventive activities

There are some mutagenic/carcinogenic compounds of high interest in food science, including the polycyclic aromatic hydrocarbon dibenzo(*a*,*l*)pyrene (DBP), the heterocyclic amines 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) and 2-amino-3-methylimidazol-[4,5, *f*]quinoline (IQ), and the mycotoxin aflatoxin B_1 (AFB_1), which are often found in processed or *in natura* foods (Marques, Valente, & Rosa, 2009). Strains of *Salmonella typhimurium* are employed in the Ames Test (Ames, McCann, & Yamasaki, 1975; Maron & Ames, 1983), one of the most widely used *in vitro* assays for studies on antimutagenic activities.

A study conducted in the early 80s with *S. typhimurium* strain TA100 showed that an increase in the concentration of chlorophyll (extracted from different vegetables) resulted in increased inhibition of the mutagenic activities of 3-methylcholanthrene (3MC) and benzo[*a*]pyrene (BP). Also, Cu-Chl and natural chlorophyll exhibited similar inhibitory activity at equivalent levels (Lai, Butler, & Matney, 1980). Later, also in studies with *S. typhimurium*, Ong, Whong, Stewart, and Brockman (1989) compared the antimutagenic activities of chlorophyllin, retinol, β-carotene and vitamins C and E against five mutagenic complex mixtures containing BP and other polycyclic aromatic hydrocarbons, including diesel emission particles, fried beef and airborne particles. Chlorophyllin yielded the highest reduction in mutagenic activity, followed by retinol, when compared at the same concentration. The antimutagenic activity of Cu-Chl has been reported to be dose-dependent (Chernomorsky, Rancourt, Sahai, et al., 1997).

Whereas the mutagenic activity of BP is strongly inhibited by pure copper chlorin e_6 and ferric chlorin e_6, Arimoto, Kan-yama, Rai, and Hayatsu (1995) found that the mutagenicity of benzo[*a*]pyrene-4,5-epoxide (BP(4,5)E), a metabolite of BP, was more strongly reduced by commercial grade Cu-Chl in a study of the antimutagenic effects of chlorophyllin on various BP metabolites in the *S. typhimurium* assay. They also observed that the rate of degradation of the metabolite benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE) was efficiently accelerated by copper chlorin e_6. These findings suggest that Cu-Chl is able to inhibit the mutagenic activity of BP through a mechanism in which copper chlorin derivatives accelerate the degradation of BPDE (Tachino et al., 1994).

The demonstration that chlorophyllin might exhibit anticancer activity *in vivo* was first reported in studies conducted on rainbow trout (*Oncorhynchus mykiss*) by Breinholt, Hendricks, Pereira, Arbogast, and Bailey (1995). In this work, the authors observed a potential role of chlorophyllin in reducing hepatocarcinogenesis in the animal, as a consequence of the inhibition of AFB_1–DNA adducts formation (also verified by Breinholt et al., 1999).

Chemopreventive effects of Cu-Chl were investigated in Chinese volunteers at a high risk of developing hepatocellular carcinoma because of the intake of aflatoxin-contaminated foods (Egner et al., 2000, 2001, 2003). When they were given tablets containing 100 mg of commercial Cu-Chl (mainly composed of copper chlorins e_6 and e_4) three times daily, a 50–55% reduction in the urinary excretion of aflatoxin-N^7-guanine, a biomarker of DNA adducts, was observed after a four-month-period, compared to that for the control group (no Cu-Chl) (Egner et al., 2001, 2003). In both studies, there were no cases of toxicity or adverse effects as a result of the intake of Cu-Chl, but there were some reports of green-colored blood serum and dark-green-colored feces. Recently, also in study with human volunteers, Jubert et al. (2009) suggested that Cu-Chl may also influence the pharmacokinetics of AFB_1, thus limiting the bioavailability of this food toxin.

One of the most accepted mechanisms to explain the chemopreventive and antimutagenic activity of Cu-Chl is based on the

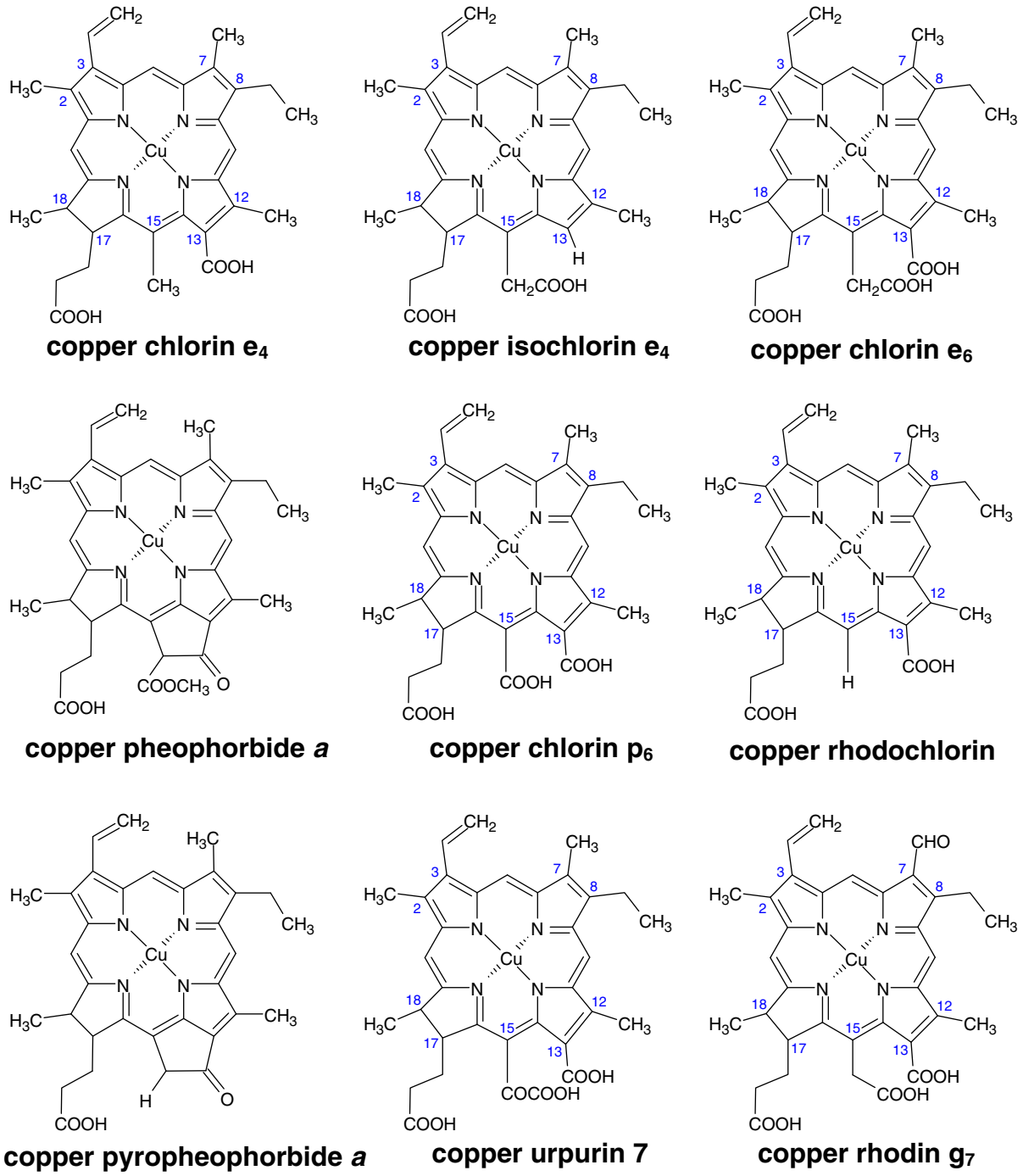

Fig. 1. Chemical structures of some chlorin-based compounds that may be found in commercial preparations of sodium copper chlorophyllin.

formation of a chlorophyllin–mutagen complex, which is thought to be maintained via interactions between the porphyrin ring of the Cu-Chl molecule and aromatic rings in mutagenic compounds (Chung, Lee, Lee, Surh, & Park, 2000). The formation of these complexes in the gastrointestinal tract may reduce the bioavailability of mutagens, reducing the possibility of DNA-adduct formation. Park, Surh, and Miller (1995) reported antitumor activity of chlorophyllin against some small aliphatic molecules and suggested a mechanism of deactivation other than that for aromatic compounds. According to them, this mechanism may involve nucleophilic sequestration or suppression of metabolic activation of the mutagenic compounds often dependent on the enzymatic activity of cytochrome P450.

In a study of chemopreventive and antimutagenic properties of Cu-Chl, Dashwood, Breinholt, and Bailey (1991) showed that the binding affinity between DNA and AFB_1 decreased in a dose dependent manner with increasing Cu-Chl concentrations, as also reviewed by Dashwood et al., 1999. The dissociation constant for chlorophyllin–AFB_1 interaction, $K_d = 1.4$ (± 0.4) μM (Breinholt, Schimerlik, Dashwood, & Bailey, 1995), is comparable to that reported by Reddy et al. (1999) for chlorophyllin–DBP interaction (with 2:1 stoichiometry, respectively), $K_d = 1.59$ (± 0.01) μM, indicating strong binding of the complex. Dashwood and Liew (1992) suggested that Cu-Chl may act as an interceptor molecule after observing that rats treated with 1% chlorophyllin two days before IQ administration

showed higher levels of the mutagen in urine and feces than animals given IQ alone.

One type of interaction that may be present in the formation of chlorophyllin–mutagen complex are the van der Waals forces, which may occur by overlapping rings (π–π interactions) of aromatic molecules, leading to stabilization of the complex (Dashwood, Yamane, & Larsen, 1996). Electrostatic interactions or hydrogen bonding may occur between functional groups of the mutagen molecule and carboxyl groups of chlorophyllin; the position and number of carboxyl groups in chlorin molecules may be relevant in this type of interaction.

However, differences in the molecular structure of carcinogens directly affect their binding affinity with chlorophyllin and, thus, their bioavailability. In a study of the chemopreventive effects of Cu-Chl, lower transport of DBP across Caco-2 cell monolayers and consequent reduced uptake were observed in comparison to those for AFB_1, indicating higher binding affinity of chlorophyllin for DBP than AFB_1 (Mata, Yu, Gray, Williams, & Rodriguez-Proteau, 2004). They also found that the absorption of PhIP was only inhibited at Cu-Chl concentrations at least 10 times as high as those able to inhibit the absorption of DBP or AFB_1. According to them, differences in stoichiometry or binding sites of mutagens may explain the results for DBP and AFB_1, but probably do not explain the results obtained for the absorption of PhIP.

The interaction between Cu-Chl and three DNA-intercalating compounds, acridine orange, quinacrine mustard and doxorubicin, may be proportional to the concentration of Cu-Chl in aqueous solutions as suggested in spectrophotometric analyses (Pietrzak, Wieczorek, Stachelska, & Darzynkiewicz, 2003). A posterior study noted that the amount of the complex chlorophyllin-intercalator increased as the amount of the complex DNA-intercalator decreased (Pietrzak, Wieczorek, Wieczorek, & Darzynkiewicz, 2006). Pietrzak, Halicka, Wieczorek, Wieczorek, and Darzynkiewicz (2008) also observed a strong association of chlorophyllin with the mutagen 2-methoxy-6-chloro-9-(3-(2-chloroethyl)aminopropylamino) acridine (ICR-191), in the order of $10^5\ M^{-1}$, which is stronger than the association of DNA with ICR-191. Cu-Chl seems to be a more effective interceptor molecule than polyphenols as well. Catechins, found in green tea and black tea, bind with aromatic mutagens at a constant of 10^2–$10^3\ M^{-1}$ (Hernaez, Xu, & Dashwood, 1997). Thus, once intercepted by Cu-Chl, the mutagen probably becomes unable to interact with DNA.

The dose of carcinogens also seems to play an important role in the formation of DNA adducts within a certain range of Cu-Chl concentrations. Pratt et al. (2007) used concentrations of DBP and commercial Cu-Chl in the range of 0–371.5 ppm and 0–6000 ppm, respectively, in a carcinogenesis study and observed that the presence of Cu-Chl led to a reduction in the incidence of tumors in rainbow trout (47–75% reduction in stomach and 49–83% in liver), as well as in the formation of DNA adducts at various concentrations of DBP. When they used another brand of Cu-Chl, the reduction in incidence of tumors and DNA adducts proved to be related to the total concentration of copper chlorins present in the preparation. However, at high concentrations of DBP, only the maximum concentration of Cu-Chl was effective in reducing tumor incidence. According to them, in order to avoid either underestimating or overestimating the true chemopreventive potential of Cu-Chl, the carcinogen dose must be chosen in accordance with that to which the human body is usually exposed.

The potential role of Cu-Chl in preventing tumors seems not to be restricted to pre-absorptive effects. As the theory of the interceptor molecule is debated, other studies suggest a mechanism of action based on enzymatic activity modulation. In this case, the bioactivity of Cu-Chl may be related to post-absorptive effects. Yun, Jeong, Jhoun, and Guengerich (1995) compared the inhibition constant ($K_I = 4.1\ \mu M$) of cytochrome P450 enzymes in the presence of chlorophyllin and the constants previously reported for the complex chlorophyllin-IQ, and suggested that non-specific inhibition of these enzymes may be the primary mechanism behind the chemopreventive effects of chlorophyllin. A comparison of the concentration of copper chlorin e_4 ethylester, detected in human blood serum (about 3 μM), to the inhibition constant of cytochrome P450 enzymes in the presence of chlorophyllin may indicate strong chemopreventive activity of copper chlorin-derivatives (Egner et al., 2000).

Cu-Chl has been shown to exhibit antimutagenic and chemopreventive activity in living organisms ranging from the simplest (*S. typhimurium*) to the most complex (humans). Its bioactive efficacy appears to be dependent on its dose and on the chemical structure of the toxic compound under study. The composition of the Cu-Chl also seems to play an important role in its biological effects. Higher biological activity of pure chlorin compounds, such as copper chlorin e_6, compared to that of commercial grade Cu-Chl, has been reported, suggesting that the presence of a complex mixture of chlorin derivatives may qualitatively and quantitatively influence the bioactivity of commercial grade Cu-Chl.

Moreover, the presence of coppered and uncoppered chlorins in commercial grade preparations of Cu-Chl, the chemical structure of mutagens as well as their binding affinities probably drive the type of interactions that sustain the chlorophyllin–mutagen complex and influence its stability and the formation of DNA–mutagen complexes. Also, the presence of a metal ion in the chlorophyllin molecule probably does not play a key role in the formation of complexes with mutagens, according to Arimoto, Fukuoka, Itome, Nakano, Rai, & Hayatsu (1993).

A very high dose of DBP (500 ppm) led to the death of more than a half of the trout in a carcinogenesis study by Reddy et al. (1999), pointing out the need for careful choice of experimental conditions. Otherwise, the results will not reflect the actual exposure to the mutagen/carcinogen in humans and the true potential activity of chlorophyllin.

3.2. Anticlastogenic activity and modulation of gene expression

Several studies have demonstrated the potential anticlastogenic activity of Cu-Chl, according to which, it may play a protective role against chromosomal damage induced by gamma rays and also against genotoxic effects induced by clastogenic chemicals, such as cyclophosphamide (CP), N-nitroso-N-ethylurea (ENU) and urethane (ERU) (Abraham, Sarma, & Kesavan, 1994). Olvera, Arceo, and Zimmering (2000) have reported similar results using four monofunctional alkylating agents (diethylnitrosamine (DEN), methylnitrosourea (MNU), methyl methanesulphonate (MMS) and also ENU) as clastogens. Although they found that only genotoxicity induced by ENU was inhibited in a Cu-Chl dose-dependent fashion, Abraham et al. (1994) did not observe such inhibiting behavior except for CP.

Chlorophyllin also appears to protect DNA against damaging agents. Bez, Jordao, Vincentini, and Mantovani (2001) observed that Cu-Chl led to a great reduction in the genotoxic effects induced by MMS, and that it was as efficient as chlorophylls *a* and *b*. In addition, Negraes, Jordão, Vicentini, and Mantovani (2004a, 2004b) reported a non-dose dependent anticlastogenic effect of Cu-Chl against ethyl methane sulfonate (EMS)-induced DNA damage and a reduced frequency of chromosomal aberrations in different cell cycle phases after the simultaneous administration of chlorophyllin and EMS. Therefore, Cu-Chl can also exhibit a time-dependent effect.

Gene expression may also be modulated by Cu-Chl. Okai, Higashi-Okai, Yano, and Otani (1996) investigated the inhibitory activity of several concentrations of chlorophyllin against 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indol (Trp-P-1) and mitomycin C-induced gene expression of *S. typhimurium* and noted a chlorophyllin dose-dependent effect: the higher the concentration, the greater the effect. The presence of this pigment has also led to the reduction of adverse effects on BP-

induced gene expression in normal human mammary epithelial cell (NHMEC) (John, Keshava, Richardson, Weston, & Nath, 2008). However, gene expression response varied among treatments (BP or BP + Cu-Chl) and cell strains (different donors).

Certain postabsorptive effects of Cu-Chl, such as its possible modulating action on cytochrome P450 enzymes, may explain these findings. Abraham et al. (1994) suggested that Cu-Chl absorbed in the gastrointestinal tract may inhibit the activation process of clastogens by modulating the activity of enzymes, such as cytochrome P450. In studies using NHMECs, Keshava et al. (2009) observed that treatment with Cu-Chl reduced the expression of several genes, including some that encode cytochrome P450 enzymes. Other studies also suggest that chlorophyllin may bind to RNA (Marty, Ouameur, Neault, & Tajmir-Riahi, 2004), which results in inhibition of translation, or intercalate with DNA (Tajmir-Riahi, Neault, & Diamantoglou, 2004) by binding with cytochrome P450 genes. These findings indicate the existence of direct and indirect mechanisms of action of Cu-Chl.

In order to fully understand the suggested postabsorptive effects of Cu-Chl, it is essential to consider the chemical composition of commercial preparations of Cu-Chl, because its great variability (mainly composed of different chlorins) certainly influences the absorption kinetics in cells. Further investigations are needed to find out if these effects are due to synergism among chlorins, or due to the activity of only one of them. Some scientists have suggested the use of highly pure Cu-Chl to unfold the true nature of these effects.

3.3. Copper chlorophyllin as an antioxidant

Cu-Chl has been reported to display antioxidant activity against oxidative stress or radiation-generated reactive oxygen species. This potential bioactivity may in turn reduce the risk of several chronic diseases. On the account of the fact that Cu-Chl may be at least partially absorbed into the bloodstream, investigations of its postabsorptive effects have been encouraged.

Cell membrane protective effects have been extensively investigated, mainly because of the influence of lipid peroxidation and loss of membrane–protein interactions and consequent cellular damage. Although the human body itself has mechanisms to control biomolecule oxidation processes, such as lipid peroxidation in membranes as a result of cellular respiration, some genetic disorders are known to impair the deactivation of free radicals, which induces lipid peroxidation and its undesirable effects. The antioxidant properties of some molecules, including Cu-Chl, may be related to the mechanism of their antimutagenic action (Ong, Whong, Stewart, & Brockman, 1986).

Cu-Chl was reported to display antioxidant activity against rat liver mitochondrial and microsomal lipid peroxidation induced in enzymatic (in the presence of NADPH-generating system) and non-enzymatic (in the presence of ascorbate) systems (Sato, Imai, Kimura, & Murata, 1984). Sato et al. (1986) also investigated the antioxidant activity of this pigment against Fe^{2+} and ascorbate-induced lipid peroxidation in rat liver homogenate. They observed that two of the Cu-Chl fractions isolated by thin layer chromatography (TLC) completely inhibited thiobarbituric acid reactive substances (TBARS) formation. These two fractions were identified as copper isochlorin e_4 and copper chlorin e_6, both of which displayed antioxidant activity about eight times as high as that of the commercial Cu-Chl used in the study. The presence of these two copper chlorins accounted for about 92% of the antioxidant capacity of Cu-Chl.

Cu-Chl also appears to play a positive role in protecting DNA against gamma radiation-induced damage. Kumar, Chaubey, Devasagayam, Priyadarsini, and Chauhan (1999) observed that Cu-Chl inhibited radiation-induced DNA damage in a concentration-dependent manner (90% protection at a Cu-Chl concentration of 500 μM) and more efficiently than other endogenous antioxidants, probably by scavenging peroxyl and hydroxyl radicals generated during radiation. The chlorophyllin also seems to be effective against reactive oxygen species generated during γ radiation and methylene blue photosensitization (Boloor, Kamat, & Devasagayam, 2000), and also may act as antioxidant in Fenton reaction and as a scavenger for 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radicals (Kumar, Devasagayam, Bhushan, & Verma, 2001).

Kamat, Boloor, and Devasagayam (2000) investigated the antioxidant activity of Cu-Chl against oxidative damage induced by γ-radiation, photosensitization, ascorbate-Fe^{2+}, NADPH-ADP-Fe^{3+} and 2,2′-azobis(2-propionimidinedihydrochloride) (AAPH) in *in vitro* and *ex vivo* experiments. They reported attenuation of radiation-induced damage in mitochondria from rat liver even at a low concentration of Cu-Chl (10 μM). Furthermore, the protective effects of chlorophyllin against the formation of lipid hydroperoxides (LOOH) and deactivation of superoxide dimutase enzyme (SOD), induced by γ-radiation, was greater than those reported for other antioxidants, such as ascorbate, glutathione, mannitol and tert-butanol (at equimolar concentrations — 50 μM).

When Cu-Chl was compared to six chlorophyll derivatives (chlorophylls *a* and *b*, pheophytins *a* and *b*, pheophorbides *a* and *b*), the highest antioxidant activity was found in the chlorophyllin system, probably because of the presence of a metal ion (copper) in the porphyrin ring (Lanfer-Marquez, Barros, & Sinnecker, 2005). On the contrary, Kumar et al. (2004) reported an increase in ROS levels and cell apoptosis, mainly after exposure to radiation or AAPH, 48 h after the administration of Cu-Chl, which indicates a pro-oxidant activity of the pigment, possibly due to the presence of a chlorophyllin metabolite in the system.

It was also demonstrated that chlorophyllin can induce the response of phase 2 enzymes, which play a role in protecting cells against reactive electrophiles and oxidizing compounds; one of these enzymes is NAD(P)H:quinone oxidoreductase 1 (NQO1). In a study of the ability of chlorophyll and its derivatives to induce this enzyme, copper chlorin e_4 ethyl ester proved to be more potent inducers than chlorophylls, which may be explained by the fact that this derivative is more efficiently absorbed by cells than the other chlorin-type compounds present in Cu-Chl (Fahey et al., 2005). The observations made in this study regarding the postabsorptive effects of copper chlorin e_4 ethyl ester may corroborate the results obtained by Egner et al. (2000, 2001, 2003) which concern its *in vivo* absorption..

Although various studies have demonstrated the antioxidant potential of Cu-Chl under different oxidative stress conditions, many of them have solely attributed its activity to the presence of a specific copper chlorin (Fahey et al., 2005; Sato et al., 1986). Therefore, authors are concerned about the use of impure commercial Cu-Chl and suggest the use of copper chlorin compounds, as pure as possible.

4. Other related bioactivities

Other bioactivities attributed to chlorophyllin, such as immunomodulatory and antiapoptotic effects, were studied by Sharma, Kumar, and Sainis (2007) using *in vivo* models. Cu-Chl has also been reported to suppress the gene expression of proteins involved in inflammatory response (Yun, Jeon, Yang, Ju, & Han, 2006).

Cu-Chl has proved to be effective in treating trimethylaminuria, a disorder characterized by the inability to metabolize trimethylamine (TMA) to trimethylamine N-oxide (TMAO) and by strong body odor, which affects the family and social life of the people afflicted with it. When Japanese volunteers were treated with Saclophyl (branded Cu-Chl), a decrease in the urinary levels of free TMA was observed, suggesting that Cu-Chl may act as an interceptor molecule forming a non-absorbable complex with TMA (Yamazaki et al., 2004).

5. Bioavailability

Dietary natural chlorophyll is poorly absorbed although the magnesium atom present in the center of the porphyrin ring can be easily

released during digestion (Baxter, 1968). However, in simulated digestion experiments, Ferruzzi, Failla, & Schwartz, 2001 noted that chlorophyll was more easily uptaken by intestinal cells in the form of Mg-free pheophytin derivatives. On the other hand, in a study using dogs as models, chlorophyll showed poor absorption results and pheophytin *a*, the major chlorophyll derivative, was found in feces (Fernandes, Gomes, & Lanfer-Marquez, 2007) The uptake and degradation of chlorophyll in humans and animals as well as its biological activities have been extensively reviewed by Lanfer-Marquez (2003).

The stability of sodium copper chlorophyllin under *in vitro* digestion conditions and its accumulation by Caco-2 human intestinal cells (enterocyte-like cells) were investigated by Ferruzzi et al. (2002). They observed a higher recovery of copper chlorin e_4, which indicates greater stability of this chlorin compared to copper chlorin e_6. The degradation of copper chlorin e_6 during digestion was suggested to be related to oxidative processes. Also, the intracellular levels of Cu-Chl (μg Cu-Ch mg^{-1} cell protein) were proportional to the levels of Cu-Chl in the culture medium (μg Cu-Chl mL^{-1} medium) and corresponded to 45 to 60% of them (Ferruzzi et al., 2002).

Unlike the Cu-Chl mixture as a whole, some of its individual components may be absorbed and be available to other tissues in human body. Copper chlorin e_4 ethyl ester, a less polar chlorin present in commercial Cu-Chl and more likely to be absorbed, was abundantly found in human blood serum (Egner et al., 2000). Thus, at least one of Cu-Chl main components, copper chlorin e_6, is probably degraded during digestion and absorption. In addition, some pigments present in commercial grade Cu-Chl, such as chlorin e_6, may have their absorption limited probably by the action of transport proteins located at the luminal membrane of intestinal epithelial cells, which prevents these pigments from producing possible phototoxic effects (Robey, Steadman, Polgar, & Bates, 2005). However, the limitation of absorption of Cu-Chl components leads to decreasing bioavailability and biological activity through post-absorptive mechanisms.

The Cu-Chl absorption and distribution to different tissues were also investigated in rats by Gomes et al. (2009), who observed the absence of copper chlorin e_6 in blood serum and tissues (liver and kidney), possibly due to its non-absorption, its degradation or its interaction with other dietary components. However, they reported the presence of copper chlorin e_4 in both serum and tissue extracts (liver and kidney). Also, lipid peroxidation in rat brain was effectively reduced after administration of Cu-Chl, confirming significant absorption and bioavailability.

Although the degradation of copper chlorin e_6 has been suggested as a possible mechanism of metabolism of Cu-Chl, the exact mechanism remains unknown. Similarly, knowledge of the mechanisms involving the transport, absorption and availability of the other chlorins remains limited. Further studies on the absorption and bioavailability of commercial Cu-Chl and its pure components are thus needed to better understand its biological activities.

6. Antagonistic effects and lack of bioactivity

The copper atom is tightly bonded in the porphyrin ring of Cu-Chl, which provides resistance to strong acids; it is not absorbed by the body but excreted in complex form (Humphrey, 1980). Previously, Harrison et al. (1954) had reported neither copper-related toxic nor photosensitizing effects of Cu-Chl. Furthermore, the LD_{50} of potassium sodium copper chlorophyllin was established as 190 μg $gram^{-1}$ body weight for mice. Similarly, Sharma et al. (2007) observed no toxic effects of Cu-Chl, administered to mice at a maximum concentration of 200 μg $gram^{-1}$ body weight, even after 6 months of treatment. However, some toxic effects of Cu-Chl have been reported in the literature.

Nelson (1992) investigated Cu-Chl for its tumor-promoting effects on 1,2-dimethylhydrazine (DMH)-induced colorectal cancer in mice. The rates of tumor incidence ranged from 23% to 47% in mice fed Cu-Chl, whereas the incidence rate for the control group was about 10%. Chernomorsky, Rancourt, Virdi, et al. (1997), using Cu-Chl from the same batch as that of Nelson (1992), observed loss of myeloma cell viability. The incubation of cells in a medium containing Cu-Chl (300 μM) resulted in 100% loss of cell viability, and the cytocidal and cytostatic effects noted proved to be dependent on Cu-Chl concentration. Also, they suggested that the possible accumulation of chlorophyllin in cell organelles along with its interference with the activity of certain enzymes may explain its cytotoxicity.

Xu et al. (2001) investigated the mutagenic effects of chlorophyllin in male rats after tumor initiation with DMH and IQ. Different doses of chlorophyllin (at a level as low as practicable for dietary exposure studies) were administered one week after the administration of the last dose of the mutagens. Rats treated with 0.001% chlorophyllin showed increased DMH-induced colon cancer incidence, but no significant change in IQ-induced cancer incidence. Treatment with 0.1% chlorophyllin, on the other hand, resulted in suppression of IQ-induced liver cancer, compared to the control group (only IQ). Blum et al. (2003) also observed promoting and inhibiting effects of chlorophyllin on DMH and IQ-initiated colon tumors in rats. Post-initiation treatment with 0.001% Cu-Chl led to tumor cell proliferation in the colonic crypts only in the group fed DMH. The highest concentration of Cu-Chl (1%) resulted in suppression of IQ-induced tumor, as a result of an increased rate of cell apoptosis, compared to cell proliferation.

Antagonistic effects were also observed in genotoxicity studies in the *Drosophila* wing spot test using γ radiation (Pimentel, Cruces, & Zimmering, 1999; 2000) and chromium oxide VI (CrO_3) (Cruces, Pimentel, & Zimmering, 2003) as inducers of genetic damage. Chlorophyllin genotoxic effects were reported to be time-dependent (Cruces et al., 2003). Both effects (as a tumor promoter and as an inhibitor) were again observed in another study with *Drosophila* (Cruces, Pimentel, & Zimmering, 2009). No evidence of genotoxicity was found for Cu-Chl alone, but promoting effects in the presence of γ radiation and at low Cu-Chl concentrations (0.03–4.3 mM) as well as inhibiting effects at high concentrations (mainly at 69 mM) were observed. Pretreatment with Cu-Chl followed by radiation 2–3 days later led to genotoxic damage, which was probably due to the progressive reduction of the initial concentration of Cu-Chl. These findings may confirm that mutagenesis can be induced at low concentrations of Cu-Chl in combination with a genotoxic agent. In a study using trout as models by Simonich et al. (2008), chlorophyllin co-fed with DBP produced a significant reduction in tumor incidence in liver and stomach. However, when Cu-Chl was given after initiation with DMB, tumor incidence increased by 28% in swim bladder, 3% in liver and 8% in stomach.

The absence of either positive or negative effects was also observed. Dietary chlorophyllin was investigated for protective effects against acrylamide neurotoxicity in rats and no apparent beneficial activity of Cu-Chl was observed (Woo et al., 2007). None of the mechanisms of action of Cu-Chl previously described (interceptor molecule, radical scavenging, modulation of enzymatic activity) seems able to explain this lack of activity on acrylamide.

These findings have demonstrated that both suppressing and promoting effects may be dependent on the dose of chlorophyllin and the type of mutagen employed. The concentrations of Cu-Chl used in studies are highly relevant, as already pointed out by Bez et al. (2001). They found no evidence of genotoxic effects of chlorophyllin on fibroblast cells (concentration range 0.1375–0.55 μM), suggesting that genotoxicity induced by chlorophyllin may be due to high concentrations. In order to understand the tumor-promoting effects of Cu-Chl, it is important to consider that its action may depend on the target organ for the mutagen and the different mechanisms of action involved in tumor promotion. The adverse effects of chlorophyllin may also be dependent on the type of mutagenic/toxic compound used, its bioavailability, metabolism and uptake by cells (pharmacokinetics), and even the interactions of this compound with dietary chlorophyllin, and of both with other dietary elements.

7. Final considerations and future research

This review revealed wide variation in the health-related biological activities of Cu-Chl across studies. The reasons for this variation may lie in the different models of study used in not only *in vitro* but also *in vivo* experiments and the degree of purity and composition of the commercial grade Cu-Chl preparations employed. A compositional analysis of samples of Cu-Chl preparations, performed in a study by Chernomorsky, Rancourt, Virdi, et al. (1997), indicated the presence of non-chlorophyll derivatives. *In vivo* studies of the biological activities of chlorophyllin usually make use of complex mixture of compounds (commercial Cu-Chl) in an equally complex environment (the body).

Non-chlorophyll derivatives present in these formulations may interact with some molecules in the animal organism and provide contradictory data. According to Dashwood (1997), the antimutagenic and anticlastogenic effects of commercial Cu-Chl should be interpreted considering the content of copper chlorins in the preparations. Therefore, studies on the benefits of Cu-Chl in humans using commercial grade preparations and chlorin derivatives should be carried out in parallel in order to truly elucidate whether the biological effects and mechanisms of action of Cu-Chl are related to a specific compound. Given that the use of complex mixtures such as chlorophyllins makes it difficult to interpret its health-related effects and, hence, understand its mechanisms of action, the use of pure chlorin derivatives is recommended.

A summary showing the diversity of results already published concerning the bioactivity of Cu-Chl is presented in Table 1 — only studies that used molar concentrations of Cu-Chl were selected for the purposes of comparison. Although the brand of the commercial Cu-Chl employed is usually provided in studies, its real composition and content of chlorins (coppered and uncoppered) are not often indicated. The diversity (and sometimes disparity) of results may also be attributed to the different models of study. The use of bacteria,

Table 1
Comparative results of the bioactivities of copper chlorophyllin in different studies.

Test system	Cu-Chl dose	Brand	Reference	Results
In vitro				
Supressive effect on gene expression in *S. typhimurium* TA1535/pSK 1002 induced by Trp-P-1 (10 μg mL^{-1}) and MMC (25 μg mL^{-1})	20 nM, 200 nM and 2 μM	Chl, Sigma Chemical Co.	Okai et al., 1996	Reduced β-galactosidase activity (2 μM > 200 nM > 20 nM) compared to that only in the presence of Trp-P-1 or MMC.
Antimutagenic activity using *S. typhimurim* TA100 in the presence of mutagens MNNG (5 μg/plate) and 3-MC (50 μg/plate)	0.04–3.5 μM	Four different batches of Cu-Chl-Na, Sigma Chemical Co.	Chernomorsky, Rancourt, Virdi, et al., 1997	Inhibitory activity against mutagens approximately 10-times as great as that against 3-MC and compared to MNNG.
Citotoxicity assay using mouse myeloma cell line P3X63Ag8	0–300 μM	Chl-Cu-Na Batch 17 F0307, Sigma Chemical Co.	Chernomorsky, Rancourt, Virdi, et al., 1997	Mainly cytostatic effects on tumor cells at low concentrations, but cytocidal effects at higher concentrations
Gamma radiation-induced DNA damage in plasmid pBR322 (from *E. coli*) at 1.25 Gy min^{-1}	1, 10, 50, 100 and 500 μM added prior to plasmid exposure to γ-irradiation	Cu-Chl-Na, Sigma Chemical Co.	Kumar et al., 1999	Effective protection of plasmid DNA against ionizing radiation-induced strand breaks.
Antioxidant effect against membrane damage induced by γ-rays from a ^{60}Co source (450 Gy) in rat liver mitochondria	1–100 μM	Cu-Chl-Na, Sigma Chemical Co.	Kamat et al., 2000	Dose-dependent inhibition of radiation-induced TBARS formation. At 50 μM Cu-Chl, inhibition was 95.5%.
Genotoxic and antigenotoxic effects of Chl on DNA damage induced by 400 μM MMS using Chinese hamster lung fibroplast V79 cells	0.1375, 0.275 and 0.55 μM	Cu-Chl-Na, Sigma Aldrich	Bez et al., 2001	No genotoxic effects of Cu-Chl. Reduction of 74–117% in genotoxic effects of MMS
Transport assay using 1 μM of each mutagen (DBP, AFB1 and PhIP) across Caco-2 cells	0, 1, 10 and 100 μM	Cu-Chl-Na, Sigma-Chemical Co	Mata et al., 2004	Maximized reduction in DBP transport at ratios 1:1 and 1:10 of DBP:Cu-Chl. Significant reduction of AFB1 transport only at ratio 1:100. Reduction of PhIP transport and absorption only at high ratio (1:1000).
Antioxidant activity using splenic lymphocytes exposed to 2.5 Gy γ-radiation (dose rate 7.1 Gy min^{-1}) or AAPH (1 mM)	0.1–10 μM prior to radiation exposure or addition of AAPH	Sigma Chemical Co.	Kumar et al., 2004	Reduced ROS levels even at 0.1 μM after radiation exposure and great protective effects at 10 μM. Cu-Chl at 0.1 μM did not significantly reduce ROS generation after AAPH exposure, but reduction at 1 μM and great protective effects at 10 μM were observed.
Antimutagenic effect on human promyelocytic leukemic HL-60 cells exposed to acridine mutagen ICR-191 (1.2 μM)	1 μM for 1 h	Cu-Chl-Na, Sigma Chemical Co.	Pietrzak et al., 2008	Total prevention of DNA damage induced by ICR-191 (1.2 μM) at 1 μM of Cu-Chl; suppressive effects even at 0.5 and 0.1 μM.
Down-regulation of gene expression in normal NHMECs (strain 98013) exposed to BP (4 μM)	5 μM (cells exposed for 24 h, washed and exposed to BP + Chl for 24 h)	Cu-Chl, Sigma Aldrich	Keshava et al., 2009	Cu-Chl displayed maximum inhibition of BP-induced gene expression (>85% for CYP1A1 gene and >70% for CYP1B1), and down-regulated other genes (interferon-associated, inflammatory, signal transduction and metabolism genes).
Ex vivo and *in vivo*				
Persistent effect against radiation (20 and 10 Gy) in somatic cells of *Drosophila*	Pretreatment at 48 h with Chl 5% (69 mM)	Without indication	Pimentel et al., 1999	Overall radioprotective effect
Antimutagenic activity in somatic cells of *Drosophila* exposed to four mutagens: ENU (0.5, 1 mM), MNU (0.15, 0.3, 0.5 1 mM), MMS (1 mM) and DEN (1 mM)	Pretreatment at 24 h with Chl 5% (69 mM) (except for ENU, concentrations of Chl — 1.25, 2.5 and 5%)	Without indication	Olvera et al., 2000	60% reduction in MMS-induced mutagenicity (27%–61% for MNU, 44% and 48% for ENU). Inhibitory effect also for DEN.
Protective effect against genetic damage induced by CrO_3 (2.5 and 5 mM) in somatic cells of *Drosophila*	Pretreatment with Chl 5% (69 mM)	Without indication	Cruces et al., 2003	Treatment with CrO_3 immediately or one day after Cu-Chl treatment — inhibitory effects of Cu-Chl; treatment with CrO_3 2 or 3 days after Cu-Chl treatment — genetic damage promoting effects
Effect of Chl on genetic damage induced by gamma rays (10 Gy)in somatic cells of *Drosophila*	Pretreatment at 24 h with Chl, 11 concentrations in the range 0–69 mM	Cu-Chl-Na, Sigma Chemical Co.	Cruces et al., 2009	Strong mutagenic effects at 0.03–1.1 mM, persisting at 4.3 mM. Great inhibitory effect at 69 mM, with 54% reduction in mutagenic effect.

trout, rats, mice and *Drosophila* as models can provide a rough estimate of bioactivity, but it does not represent what actually occurs in humans.

In addition to its antimutagenic, anticlastogenic, anticarcinogenic and antioxidant effects, Cu-Chl has been reported to have cancer therapeutic effects (Chimploy et al., 2009). This finding suggests interesting possibilities for new cancer treatments; therefore, further investigations are needed to confirm it and determine the most effective Cu-Chl dose ranges as well as the existence of adverse effects. Based on this finding, we speculate that Cu-Chl may have many other health-related effects.

The antioxidant activity of chlorophyllin after photosensitization also requires further investigation. Some results obtained in our laboratory indicate that when excited by a red light source, Cu-Chl may exhibit pro-oxidant activity, lower than that induced by photosensitizers with high yield of singlet oxygen generation (unpublished results). The pro-oxidant activity of chlorophyllin should also be investigated not only for its possible *in vivo* effects, but also for its negative effects on food products. For example, some compounds present in chlorophyllin-containing food products, such as lipids, may experience oxidation under certain light conditions. Finally, there is also an increasing need for identification and characterization of the compounds present in Cu-Chl commercial preparations commonly used in studies. The presence of uncoppered chlorins and porphyrins has been confirmed by using singlet oxygen ($^1O_2$) emission analysis of Cu-Chl preparations (unpublished data by our group), considering that quantum yield of $^1O_2$ generation by metalloporphyrins was found to be 0 (Redmond & Gamlin, 1999). Although this technique does not allow distinction among different types of chlorins, it at least indicates the presence of molecules that could photodamage biomolecules. There is also an urge for the development of a simplified method for the characterization of Cu-Chl preparations for lab and field use.

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