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A synergistic effect of Cu^{2+} and norbixin on DNA damage

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ABSTRACT

Annatto and its derivatives are members of carotenoids with long-chain conjugated polyenes, which are widely used as food additives and antioxidant. However, carotenoids can also act as pro-oxidant under certain circumstances. To explore the biochemical behavior of annatto and its derivatives, the DNA damage effects by norbixin to the copper(II) ions mediated DNA damage was evaluated herein. It has been found that norbixin was capable of promoting copper(II) caused DNA damage on supercoiled plasmid DNA, whereas the long-chain saturated system such as lauric acid did not. These DNA damage showed strong dependency on both the concentration of norbixin and the interaction time. For the mechanism of damage promotion by norbixin, the long-chain conjugated polyenes unit may participate in the reductive reaction of copper(II) ion to generate free radical, and gave stronger DNA damage through the interactions between DNA and the radicalized carotenoids. The control experiments showed that the redox cycle between Cu(I) and Cu(II), hydroxyl radical, singlet oxygen and hydrogen peroxide may play essential roles in the cleavage reaction.

1. Introduction

Under certain conditions, carotenoids such as β-carotene, lutein, and lycopene can act as antioxidants in lipid phases by trapping, scavenging and deactivating free radicals, including peroxyl radicals, or physically quenching singlet oxygen (Sies et al., 1992) both *in vitro* and *in vivo* (Bast et al., 1998). In the presence of a sufficiently high concentration of other antioxidants, carotenoids may behave as antioxidants, even though they present a pro-oxidant character in the absence of other additives (Palozza, 1998). For example, mixtures of norbixin with ascorbic acid, ascorbyl palmitate and δ- or α-tocopherols enhanced the antioxidant effect beyond that of the phenolic antioxidants in both oils and emulsions, and synergistic effects were observed with the tocopherols and ascorbic acid in the emulsion system (Kiokias and Gordon, 2003). However, there are some contradictory results obtained in several studies that after several years of supplementation of placebo, 50 mg α-tocopherol alone, 20 mg β-carotene alone, both α-tocopherol and β-carotene, or a combination of 30 mg of β-carotene and 25,000 IU of retinal (vitamin A) per day, the dietary supplementation had no benefit and may have had an adverse effect on the incidence of lung cancer and on the risk of death from lung cancer, cardiovascular disease, and any cause in smokers (Omenn et al., 1996; Heinonen et al., 1994).

The yellow-red pigment obtained from annatto seeds is a mixture of carotenoids such as bixin, norbixin, β-carotene, cryptoxanthin, lutein, zeaxanthin and methylbixin (Alves de Lima et al., 2003). The diverse carotenoids have been reported to protect against chemically-induced transformation of cultured cells from oxidized damage (Pung et al., 1993) and prevent or modulate the genetic damage caused by some known mutagens/carcinogens (Mure and Rossman, 2001). Bixin, a carotenoid with two carboxylic acid groups, one of which is esterified, is major oil-soluble pigment present in annatto extract. It is reported that bixin (the main colorant of annatto), one of the most effective biological singlet molecular-oxygen quenchers and scavenger of O_2^-, peroxynitrite and ·OH (Dimascio et al., 1990; Zhao et al., 1998), can strongly inhibit the auto-oxidation of rapeseed oil triglycerides, though other carotenoids, such as lutein or lycopene, had no antioxidant effect (Haila et al., 1996). Norbixin (Scheme 1), a water-soluble annatto, which is derived from bixin by hydrolysis of the ester group, is also found to retard oxidative deterioration with both of oils and oil-in-water emulsions, and it showed particularly effective in the emulsion. In the research of DNA cleavage induced by reactive oxygen species (ROS), particularly by hydroxyl radicals, Kovary and co-workers observe both pro- and antioxidant effects of norbixin on Sn^{2+} or Fe^{2+}-mediated plasmid DNA damage (depending upon the Sn^{2+}:norbixin ratio), in the presence/absence of H_2O_2 (Kovary et al., 2001). They have shown that norbixin can protect plasmid DNA against oxidative damage *in vitro* but can also increase the extent of oxidative damage of genomic DNA when murine fibroblasts submitted to H_2O_2-induced oxidative stress.

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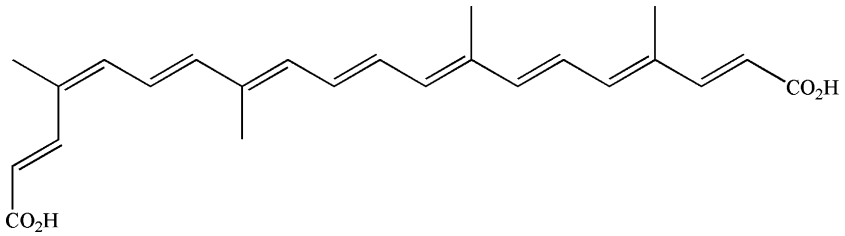

Scheme 1. Chemical structures of norbixin.

To explore the role of carotenoids in cancer prevention and their antioxidant properties, in the light of the pioneering work by Kovary, the present study was designed to systematically evaluate the possible toxicological or protective potential of annatto color powder against induced DNA damage in the presence of various metal ions, particularly copper ion. It has been found herein that norbixin was capable of promoting copper(II) ions caused damage on supercoiled plasmid DNA, whereas the long-chain saturated system such as lauric acid did not. These DNA damage showed strong dependency on the concentration of those carotenoids together with the reaction time, with effective dosage as low as 5 μM. The results reported here bear implications to the DNA damage caused by the synergism between metal ions and carotenoids.

2. Materials and methods

2.1. Materials

pBR322 plasmid DNA (4361 base pairs), prepared from DH5α cells, was purified by standard procedures (Sambrook et al., 1989). DNA concentration was determined by measuring the absorbance at 260 nm and assumed that one unit of absorbance corresponds to 50 mg/ml double-stranded DNA. Metal salts including cupric chloride, zinc chloride, manganese sulfate, cobalt sulfate, nickel sulfate, AR reagents, were dissolved in sterile Millipore water and passed through sterile 0.22 μm filters to remove any impurities. Dimethyl sulfoxide (DMSO) was high purity reagent (>99.99%). Lauric acid, AR reagents, was dissolved in DMSO to give a stock solution. Neocuproine and several active oxygen species scavengers were commercially available and were used without further purification. The liquid chromatography-mass spectra (LC-MS) were analyzed on a LCQ Advantage (Finnigan, USA). The UV–visible spectra were recorded on a CARY 100 Bio (Varian, USA). Analyses of the carotenoids were performed on an Agilent 1100 HPLC system (Agilent, USA), equipped with a LC-8 column. The mobile phase consisted of acetonitrile + 0.1% CF_3COOH in water at a ratio of 50:50 (v/v). The NMR data were collected on AVANCE 600 (Brucker, Switzerland). Purification of norbixin from annatto seeds and its conversion to norbixin by saponification have been described elsewhere (Kovary et al., 2001). The purity of norbixin was higher than 99% based on HPLC analysis. Norbixin in 10 mM Tris buffer (pH 8.0) was identified by spectrophotometry (by maximum absorption at 453 and 482 nm). ^1H NMR for norbixin (400 MHz, DMSO): 7.80 (d, 1H, J = 15.6 Hz), 7.25 (d, 1H, J = 15.6 Hz), 6.43–6.81 (m, 10 H, 10 × CH), 5.87 (d, 1H, J = 15.6 Hz), 5.84 (d, 1H, J = 15.6 Hz), 1.92, 1.93, 1.97, 1.98 (4s, 12H, 4 × CH_3). LC-MS analysis of the norbixin sample afforded an m/z ratio of 380.3 (cal. m/e 380.2). The dried and crystallized norbixin were dissolved in DMSO and kept dark at –20 °C, until further use.

2.2. Plasmid DNA cleavage

The plasmid DNA cleavage reactions consisted of cleavage agents and 1 μg pBR322 DNA in 50 μL of 10 mM Tris buffer at pH 8.0. Cu^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} were the sources of the metal ions used in the cleavage reactions. All DNA cleavage reactions were performed under normal atmospheric conditions at 37 °C for 12 h incubator. All control experiments were carried out in the similar conditions expected that some molecules were chosen to replace reactants. After the reaction, the samples were precipitated by ethanol, redissolved in a dye solution (0.04% bromophenol blue, 0.04% xylene cyanol FF, and 5% glycerol) and frozen at –20 °C prior to running the gel. All gels were run on 1% agarose slab gels at 3–5 voltage/cm in 0.5× TBE buffer (45 mM Tris–Borate acid and 1 mM EDTA). Gels were stained with ethidium bromide and pictures taken with a digital camera. The resulting single- and double-stranded breaks (relaxed and linear forms, respectively) as well as the residual supercoiled form were subsequently estimated by using the software – program NIH Image J. The amount of supercoiled DNA was multiplied by a factor of 1.22 to account for reduced ethidium bromide intercalation into supercoiled DNA. Each cleavage reaction was repeated for 3–5 times, and the data were the average of the parallel experiments.

2.3. Statistical analysis

All gel data are expressed as mean ± SEM (standard error of the mean) and were analyzed statistically by one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test using GraphPad InStat program (GraphPad Software, Inc.). Differences were considered statistically significant when $P < 0.01$.

3. Results

3.1. Cooperative damage effect produced by Cu(II) and norbixin on plasmid DNA

Plasmid pBR322 was incubated with norbixin, without and with 50 μM Cu^{2+}, and the resulting DNA damage was estimated by gel electrophoresis. In the absence of Cu^{2+}, norbixin didn't display damage effect to plasmid DNA (Fig. 2A, lane 4). However, in the presence of Cu^{2+}, the adding of norbixin markedly enhanced the DNA damage by Cu^{2+} (Fig. 1A). The percentage of the relaxed form of plasmid DNA was much higher than that of Cu^{2+} itself (Fig. 1A, lane 2). As the concentration of norbixin ranged from 1 to 1000 μM, the damage degree of norbixin to Cu^{2+} exhibited clearly increasing trend (Fig. 1A, lanes 3–10). The concentration of norbixin that visibly enhanced DNA damage by Cu^{2+} was as low as 5 μM (Fig. 1A, lane 3). These results implied that a cooperative effect has been produced when norbixin was added to the Cu^{2+}-induced damage system. As shown in Fig. 1C and D, the DNA damage by Cu^{2+} in the existence of norbixin enhanced as the reaction time elongated. In the cooperative system of Cu(II) ions and norbixin, the intact form I DNA was firstly cleavage to nicked form. As this damaged form II accumulated to a certain level, the double-linear backbone of DNA broke, and then DNA completely became linear (Fig. 1D).

3.2. Control experiments

In order to explore the mechanism of cooperative effects on DNA damage by annatto and Cu^{2+}, a series of control experiments were set up. Firstly, a long-chain saturated alkyl acid–lauric acid was chosen to replace norbixin. At the same reaction condition, in the absence or presence of 50 μM Cu^{2+}, no obvious DNA damage promotion was observed while adding to the system with 50 μM lauric acid (Fig. 2A, lanes 7 and 8). However, in such concentration significant DNA damage has been generated by associated action of copper(II) ion and norbixin (Fig. 2A, lane 5). So did the concentration of lauric acid up to 500 μM (data not shown). This points out that the DNA damage promotion by norbixin and Cu^{2+}-induced system were specific, probably due to the conjugated alkene-containing molecular structure of norbixin. On the other hand, some transition metals bivalent cations in the same group, e.g., Zn^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+} and Ni^{2+} were used to instead of Cu^{2+}. In the presence of norbixin with the same concentration, 50 μM Zn^{2+} displayed few variety of the percentage of supercoiled DNA (Fig. 2A, lanes 9 and 10). The other three replaced metal cations also exhibited similar results to that by Zn^{2+} (Fig. 2A, lanes 11–18). We postulate that the less ability of carrying on redox process under our

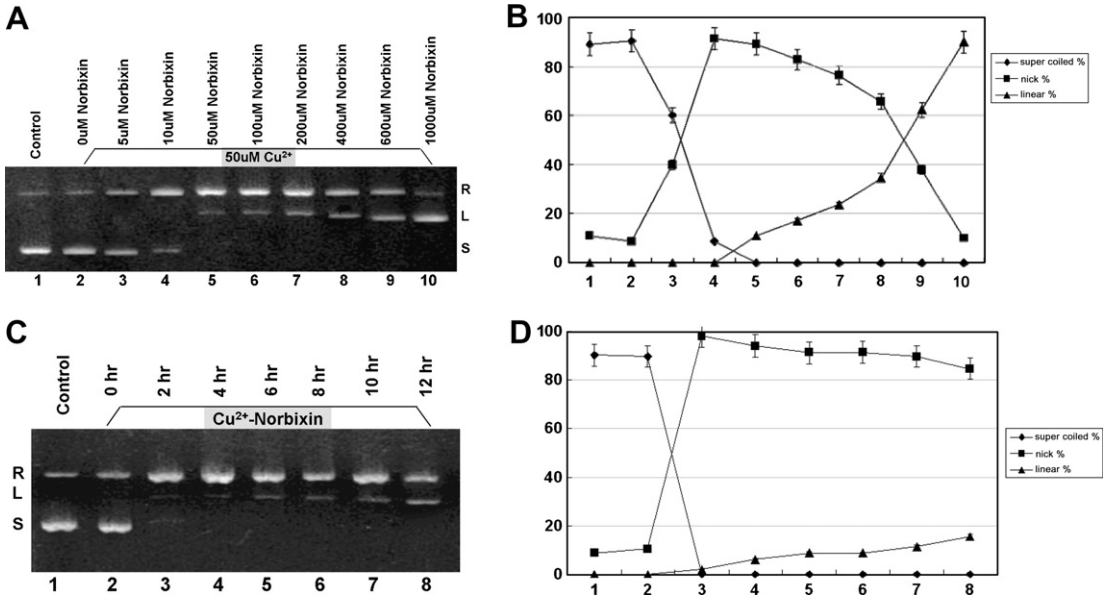

Fig. 1. Cooperative damage effect produced by copper(II) ion and norbixin on plasmid DNA. (A) 1 μg of plasmid DNA (pBR322) was treated with norbixin in the presence of 50 μM copper(II). Different concentrations of norbixin (1–1000 μM) were tested, and after incubated at 37 °C for 12 h, the reaction solution were precipitated by ethanol and submitted to gel electrophoresis. (C) 1 μg of plasmid DNA (pBR322) was treated with norbixin (50 μM) and Cu(II) (50 μM) at 37 °C with different time from 0–12 h. These results were evaluated by ethidium bromide staining and UV transillumination. Representative electrophoretograms (A and C) are shown to illustrate each type of oxidative reaction. R, relaxed form; S, supercoiled form; L, linear form. The corresponding percentage of various form of plasmid DNA in each reaction was calculated by gel densitometric analysis (B and D).

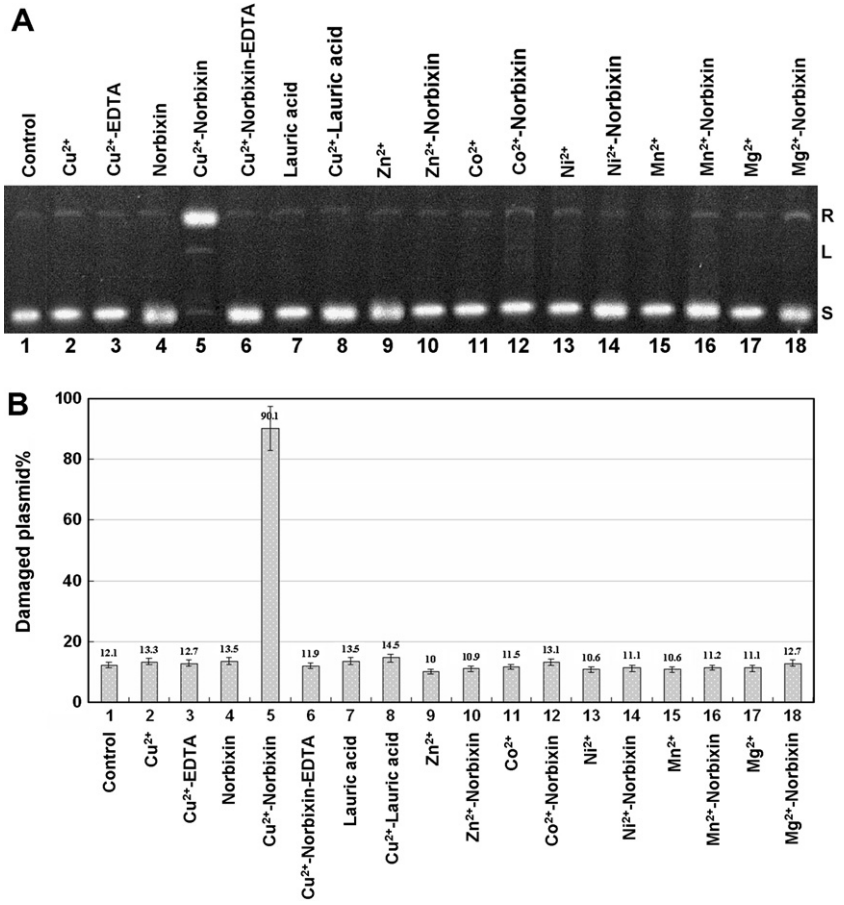

Fig. 2. Contrastive experiments. The DNA damage reaction conditions and data analysis are the same as described in Fig. 1A. However, lauric acid was used instead of norbixin, and other transition metal ions such as Zn2+, Co2+, Ni2+, Mn2+ and Mg2+were used instead of Cu2+. The electrophoretograms (A) and gel densitometric analysis (B) were illustrated.

experimental conditions by these metal ions may contribute the observed result here. The combined effect of EDTA and Cu^{2+} was also examined. When 100 μM EDTA was added to either Cu^{2+} (Cu^{2+}/EDTA = 1:2) or Cu^{2+}/norbixin (Cu^{2+}/norbixin/EDTA = 1:1:2) cleavage reaction system, no significant DNA damage was observed (lanes 3 and 6 in Fig. 2A). This was an expected result, as the chelation of Cu^{2+} by EDTA would make Cu^{2+} unavailable for the participation in the DNA relaxation reaction.

3.3. Effects of scavengers and chelators in the damage of Cu^{2+}/norbixin

To further determine the essential roles in Cu^{2+}/norbixin induced DNA cleavage, neocuproine and several free radical scavengers were added to the reaction mixture. As neocuproine has already been confirmed to bound Cu^{+} specifically and not hydrolyze DNA (Singh et al., 2001), our result showed that Cu^{+} is essential for this cleavage reaction (Fig. 3A, lane 3). Neocuproine

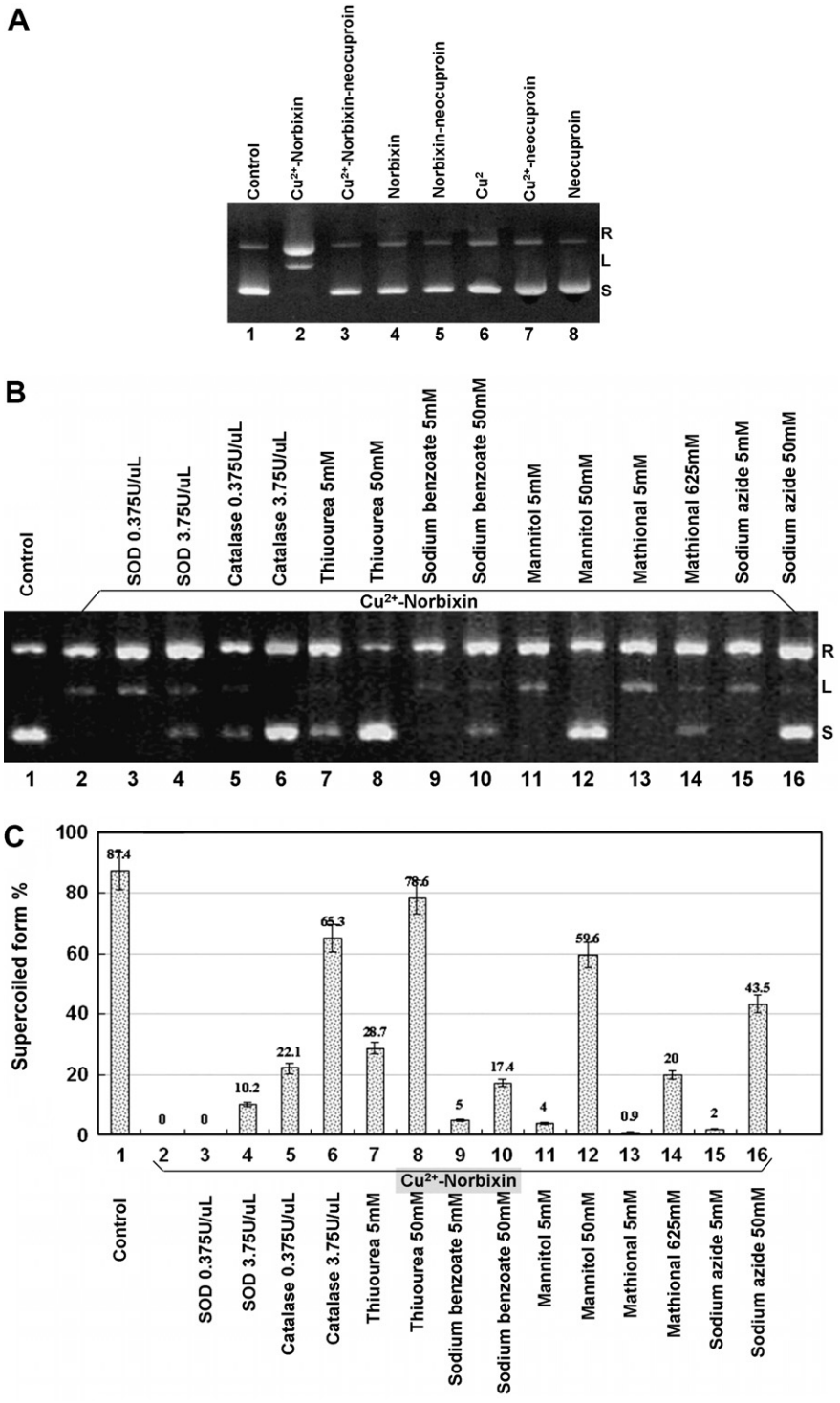

Fig. 3. Effects of scavengers and chelators in the damage of Cu(II)/norbixin. The DNA damage reaction conditions and data analysis are the same as described in Fig. 1A. The electrophoretograms (A and B) and gel densitometric analysis (C) were illustrated.

prevents DNA from damage in the present reaction implied that the redox between Cu^{2+} and Cu^{+} may act important role in Cu^{2+}/norbixin induced DNA cleavage. In order to find out the essential role of oxygen free radicals in plasmid DNA cleavage by Cu^{2+}/norbixin, several active oxygen species scavengers were tested in the reaction mixture (Fig. 3B and C). The scavengers of hydroxyl radical – thiourea and mannitol exhibited inhibition on DNA cleavage to some extent. So did the scavenger of singlet oxygen-sodium azide. Catalase (catalyses conversion of hydrogen peroxide to water and molecular-oxygen) also showed inhibition effect. These results revealed that hydroxyl radical, singlet oxygen and hydrogen peroxide might participate in the reaction. Whereas, SOD (capable of catalyzing the reduction of superoxide anions to hydrogen peroxide) and methional (capable of scavenging both ·OH and species with weaker reactivity) did not exhibit significant inhibition effect. It should be noted that the overall effects for various free radical scavengers tested are the inhibition of the DNA damage, albeit with quite different efficacy. That might implies free radical process is for DNA damage observed. Note that the possible strong chelator (catalase, thiourea, mannitol and sodium azide in Fig. 3) for copper caused most efficient inhibition to DNA damage, this showed strong chelation effect in the inhibition of DNA damage in the process as reported in the literature (Zhu et al., 2002). At the moment, we cannot fully rule out the hydrolysis mechanism for DNA damage observed.

4. Discussion

Copper is an important structural metal ion in chromatin (Lewis and Laemmli, 1982), being present at about one copper ion per kilobase. Though the Cu(II) ion does not cleave DNA, in the presence of reducing agent and/or H_2O_2 it indeed induces significantly more DNA damage than that of the other biologically relevant transition metal ions (Drouin et al., 1996). Exposure of DNA to copper ions has been reported to result in single- and double-strand breaks, modified bases, abasic sites and DNA-protein crosslinks (Halliwell and Aruoma, 1991; Dizdarogu, 1992). For this reason, there is increased interest in the ability of copper(II) ion to participate in DNA-damaging reactions *in vivo* and *in vitro*.

There are three effects of metal ions in DNA breakage (Li et al., 2001). Firstly, they can propagate free radical chains on which continued production of free radical depends. Secondly, traces of metal ions are required for Fenton type reactions, whereby ferrous or cuprous ions can convert H_2O_2 to hydroxyl radicals. Finally, metal ions provide for site-specific production of active species. These radicals all belong to reactive oxygen species (ROS). The interaction between ROS with DNA is of considerable interest because of the potential patho-biological significance of ROS-induced DNA damage (Wallance, 1994). Oxidative damage to DNA often result in oxidized purine and pyrimidine bases and gross DNA changes such as strand breaks (Dizdarogu, 1992). Such damage may be promutagenic and has been linked to the pathogenesis of human diseases, such as cancer and aging (Guyton and Kensler, 1993). Hydroxyl radical, one of the most powerful ROS that breaks DNA, can be generated through ionizing radiation (von Sonntag, 1987) and contact of certain transition metal ion chelates with H_2O_2, especially those of copper and iron. In general, the reduced forms of these metal ions (Cu^{+}, Fe^{2+}) produce ·OH at a faster rate upon reaction with H_2O_2 than do the oxidized forms (Cu^{2+}, Fe^{3+}). However, both Cu^{2+}- and Fe^{3+}-complexes do generate hydroxyl radicals upon reaction with H_2O_2 and some kind of reducing agent (Singh et al., 2001; Hadi et al., 2002). Binding of these metals by DNA might provide centers for repeated generation of free radicals close to sites susceptible to breakage.

By using a plasmid DNA as the target molecule and Cu^{2+} ion as oxidant, we evaluated the capacity of norbixin to protect DNA against single- and double-strand breaks (Fig. 1). As visualized by gel electrophoresis, the DNA breakage promoted at submicromolar concentration of norbixin. Metal ions, such as magnesium, calcium, iron, cobalt, nickel, manganese, copper, zinc and iron are necessary element in the human body. Among them in +2 state, copper(II) ion is amongst the strongest oxidant in the solution state. In the reaction system we used, Cu^{2+} might produce free radical that damaged DNA strand if it formed complexes with carotenoids and was reduced to the reductive state. Whereas, other metal ions did not show any visible cleavage to DNA strands (Fig. 2) as they might not produce or produce not enough free radicals that break DNA, even some metal ions are in the same group on the elemental table as copper. The literature reports in H_2O_2–Fe(II)/Sn(II) system showed that norbixin can protect plasmid DNA against oxidative damage *in vitro* (Kovary et al., 2001). DNA breakage induced by Cu^{2+} and norbixin in this paper conferred a synergistic DNA oxidative damage that might be related to the redox activities of Cu(II) ions in the reaction, which might be crucial for the generation of free radicals that induce a cascade of radical reaction that might be mediated by polyene system such as carotenoids to generates DNA damage in the reaction system.

The prolonged reaction time of DNA and norbixin in the presence of Cu(II) increased the DNA damage (Fig. 1), indicating that there may be the existence of Cu(II)–Cu(I) cycles to repeat generation of free radicals. The polyenes structure of norbixin presented several conjugated π double bonds with high electron density that might facilitate the formation of metal–π complex (Haiduc and Zuckerman, 1985). Further, the copper–π complex could lead to the reduction of Cu^{2+} possibly through epoxidation requiring the involvement of a hydroxyl radical in the presence of molecular oxygen (Singh et al., 2001). In the course of the reduction of Cu^{2+} to Cu^{+}, the produced hydroxyl radical participated in the DNA cleavage process, and the amount of nicked form of plasmid DNA increased greatly. On the contrary, when the long saturated chain compounds such as lauric acid was added to the Cu^{2+} system, there were not much radicals could be induced and DNA damage promotion effect was not observed. In this case, the carotenoids such as norbixin in a Cu^{2+} system would no longer act as radical scavenger, but preferentially behaved as a kind of pro-oxidant molecules and/or as a reducing agent that promote the production of hydroxyl radical to damage DNA. As neocuproine has been added into the reaction system, no significant DNA damage was observed (Fig. 3A), which implied that Cu^{+} is essential for this cleavage reaction and the restriction of Cu(II)–Cu(I) cycle by neocuproine is essential for DNA damage in the present cooperative reaction system. The elimination of Cu(I) in the reaction leads to the prevention of DNA damage. If EDTA was added to the Cu^{2+} cleavage reaction system, the metal ions would be preferentially chelated by EDTA, no obvious DNA damage was observed (Fig. 2). The above results implied that the effectively chelated Cu would no longer participate in the redox reaction, thus few hydroxyl radicals were produced and less DNA damage was observed. When several active oxygen species scavengers such as thiourea and catalase were tested in the reaction mixture (Fig. 3B), significant inhibition effect for DNA damage implies that these radicals (hydroxyl radical, singlet oxygen and hydrogen peroxide) may participate in the reaction and the reaction process may exist oxidation cycles containing the three radicals. The DNA damage promotion by norbixin was effective at as low as 5 μM according to the gel densitometry analysis. When norbixin and Cu^{2+} were present at ratios below 1:1, a condition that favors the presence of free Cu^{2+} over norbixin molecules, an enhancement of the damage effects by Cu^{2+} on DNA had already been observed. The two carboxylic groups at the terminal of norbixin molecule gave two negative charges at weak basic environment; the carboxylic terminal might be in favor of binding with free copper ions to form the copper–π complex between the

carotenoid and the metal ions (Singh et al., 2001).The actual chemical mechanism of this synergic DNA damage effect *in vitro* and/or *in vivo* is warranted further study.

In summary, our results firstly demonstrated that the adding of norbixin enhanced DNA damage induced by copper(II) ion, whereas the long-chain saturated system such as lauric acid did not. These DNA damage showed strong dependency on both the concentration of norbixin and the interaction time. Note that the ingestion of the carotenoid reaches a certain concentration 58 μg/L (about 0.2 μM) in the plasma (Levy et al., 1997). Elucidating the biochemical role of the carotenoids during oxidative stress would be benefit for recognizing the safety of annatto extract and its nutrition.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References

Alves de Lima, R.O., Azevedo, L., Ribeiroa, L.R., Salvadori, D.M.F., 2003. Study on the mutagenicity and antimutagenicity of a natural food colour (annatto) in mouse bone marrow cells. Food Chem. Toxicol. 41, 189–192.

Bast, A., Haenen, G.R., Van den Berg, H., 1998. Antioxidant effects of carotenoids. Int. J. Vitamin Nutr. Res. 68, 399–403.

Dimascio, P., Devasagayam, T.P.A., Kaiser, S., Sies, H., 1990. Carotenoids, tocopherols and thiols as biological singlet molecular-oxygen quenchers. Biochem. Soc. Trans. 18, 1054–1056.

Dizdarogu, M., 1992. Oxidative damage to DNA in mammalian chromatin. Mutat. Res. 275, 331–342.

Drouin, R., Rodrihuez, H., Gao, S.W., Gebreyes, Z., O'Conner, T.R., Holmquist, G.P., Akman, S.A., 1996. Cupric ion/ascorbate/hydrogen peroxide-induced DNA damage: DNA bound copper ion primarily induces base modifications. Free Radical Biol. Med. 21, 261–273.

Guyton, K.Z., Kensler, T.W., 1993. Oxidative mechanisms in carcinogenesis. Brit. Med. Bull. 49, 523–544.

Hadi, N., Malik, A., Azam, S., Khan, N.U., Iqbal, J., 2002. Serotonin–Cu(II)-mediated DNA cleavage: mechanism of copper binding by serotonin. Toxicol. In Vitro 16, 669–674.

Haiduc, I., Zuckerman, J., 1985. Journal of Basic Organometallic Chemistry, Walter de Gruyter, New York, NY.

Haila, K.M., Lievonen, S.M., Heinonen, M.I., 1996. Effects of lutein, lycopene, annatto, and δ-tocopherol on autoxidation of triglycerides. J. Agr. Food Chem. 44, 2096–2100.

Halliwell, B., Aruoma, O.I., 1991. DNA damage by oxygen-derived species. FEBS Lett. 281, 9–19.

Heinonen, O.P., Huttunen, J.K., Albanes, D., Haapakoski, J., Palmgren, J., 1994. The effect of vitamin E and β-carotene on the incidence of lung cancer and other cancers in male smokers. New Engl. J. Med. 330, 1029–1035.

Kiokias, S., Gordon, M.H., 2003. Antioxidant properties of annatto carotenoids. Food Chem. 83, 523–529.

Kovary, K., Louvain, T.S., Costa e Silva, M.C., Albano, F., Pires, B.B.M., Laranja, G.A.T., Lage, C.L.S., Felzenszwalb, I., 2001. Biochemical behaviour of norbixin during in vitro DNA damage induced by reactive oxygen species. Brit. J. Nutr. 85, 431–440.

Levy, L.M., Regalado, E., Navarrete, S., Watkins, R.H., 1997. Bixin and norbixin in human plasma: determination and study of the absorption of a single dose of annatto food color. Analyst 122, 977–980.

Lewis, C.D., Laemmli, U.K., 1982. Higher order metaphase chromosome structure: Evidence for metalloprotein interactions. Cell 29, 171–181.

Li, A.S.H., Bandy, B., Tsang, S.S., Davison, A.J., 2001. DNA breakage induced by 1,2,4-benzenetriol: relative contributions of oxygen-derived active species and transition metal ions. Free Radical Biol. Med. 30, 943–956.

Mure, K., Rossman, T.G., 2001. Reduction of spontaneous mutagenesis in mismatch repair- deficient and proficient cells by dietary antioxidants. Mutat. Res., 85–95.

Omenn, G.S., Goodman, G.E., Thornquist, M.D., Balmes, J., Cullen, M.R., Glass, A., Keogh, J.P., Meyskens, F.L., Valanis, B., Williams, J.H., Barnhart, S., Hammar, S., 1996. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. New Engl. J. Med. 334, 1150–1155.

Palozza, P., 1998. Pro-oxidant actions of carotenoids in biological systems. Nutr. Rev. 56, 257–265.

Pung, A., Franke, A., Zhang, L.X., Ippendorf, H., Martin, H.D., Sies, H., Bertram, J.S., 1993. A synthetic C22 carotenoid inhibits carcinogen-induced neoplastic transformation and enhances gap junctional communication. Carcinogenesis 14, 1001–1005.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, NY.

Sies, H., Stahl, W., Sundquist, A.R., 1992. Antioxidant functions of vitamins. Vitamins E and C, beta-carotene, and other carotenoids. Ann. NY Acad. Sci. 368, 7–19.

Singh, S., Asad, S.F., Ahmad, A., Khan, N.U., Hadi, S.M., 2001. Oxidative DNA damage by capsaicin and dihydrocapsaicin in the presence of Cu(II). Cancer Lett. 169, 139–146.

von Sonntag, C., 1987. The Chemical Basis of Radiation Biology. Taylor & Francis, London.

Wallance, S.S., 1994. DNA damage processed by base excision repair: biological consequences. Int. J. Radiat. Biol. 66, 579–589.

Zhu, B.Z., Antholine, W.E., Frei, B., 2002. Thiourea protects against copper-induced oxidative damage by formation of a redox-inactive thiourea-copper complex. Free Radical Biol. Med. 32, 1333–1338.

Zhao, W.E., Han, Y., Zhao, B., Hirota, S., Hou, J., Xin, W., 1998. Effect of carotenoids on the respiratory burst of rat peritoneal macrophages. Biochim. Biophys. Acta 1381, 77–88.