International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

International Journal of Pharmacology 3 (1): 19-26, 2007
ISSN 1811-7775

Effects of Quercetin on DNA Damage Induced by Copper Ion

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Abstract: It is well known that DNA damage plays an important role in carcinogenesis. Quercetin, as an antioxidant and free radical-scavenger, is known to protect DNA from damage produced by Reactive Oxygen Species (ROS) such as hydroxyl free radical (·OH), Hydrogen Peroxide (H_2O_2) and superoxide ion ($O_2^{\cdot -}$). But quercetin has been reported to be carcinogenic too. Based on the contradiction in terms, we study the effect of quercetin on DNA damage in the presence of cupric ion. Present results show that quercetin has opposite effects on DNA damage induced by cupric ion depending on the concentration of cupric ion. At low concentration of cupric ion, quercetin exerted a protective role. While at higher concentration of cupric ion, quercetin promoted DNA cleavage, which was not inhibited by hydroxyl free radical scavenger. Additionally, quercetin diminished slightly the oxidation of CT DNA by cupric ion plus H_2O_2 in the 2-thiobarbituric acid-reactive substances (TBARS) assay. So, it can be concluded that oxidative stress is not the only reason for DNA damage induced by quercetin plus cupric ion. We further propose a mechanism for explaining the promoting effect that formation of quercetin copper (II) complexes binding to DNA in an intercalation mode may result in hydrolytic cleavage of DNA. The results may imply that in certain pathological situations quercetin may result in DNA damage rather than exert a protective role to bring on carcinogenesis.

Key words: DNA damage, quercetin, copper, hydrolysis, oxidation

INTRODUCTION

The presence of a variety of metals is necessary for the normal functioning of cells and the survival of organisms. However, in excess of orgamism's binding capacity metals may be highly toxic to result in diseases including cancer development. Metal is related to endogenous Reactive Oxygen Species (ROS) generation. And excess generation of ROS (i.e., hydroxyl radical) induced by metal can result in damage to many kinds of biomolecules, including DNA (Kulms *et al*., 2002), which is involved in carcinogenesis (Galaris and Evangelou, 2002; Wojciech and Kazimierz, 2002; Halliwell and Gutteridge, 1999).

Quercetin (Que, 3, 5, 7, 3', 4'-pentahydroxyflavone, Fig. 1), a bioflavonoid widely distributed in fruits and vegetables, has been reported to exert multiple biological effects as an antioxidant and free radical-scavenger (Nègre-Salvayre and Salvayre, 1992; Afanasev *et al*., 1989; Morel *et al*., 1993). It has been reported that flavonoids have a protective effect on DNA damage induced by hydroxyl radical (Russo *et al*., 2000). One of mechanisms that may explain the protective effect of the flavonoid on DNA would involve the prevention of the generation of ROS by chelating metal ions, such as copper and iron. Additionally, quercetin metal complexes have higher antioxidation than quercetin alone (Rubens de Souza and Wagner De Giovani, 2004; Armida *et al*., 2005; Zhou *et al*., 2001). Whereas, there is also considerable evidence that quercetin themselves are mutagenic and carcinogenic (Das *et al*., 1994; Duthie *et al*., 1997; Pamukcu *et al*., 1980). The mutagenicity of polyphenolic flavonoids may be due to the reactive oxygen species produced by their oxidation and redox cycling. It has been suggested that some flavonoids can exert prooxidant actions to damage DNA as a result of a combination of flavonoid and metal (Yamashita *et al*., 1998; Masataka *et al*., 1999; Pattubala *et al*., 2004).

Most studies on DNA damage induced by ROS formed by a Fenton-type reaction have used ferric or copper ion and H_2O_2 to generate oxidative stress

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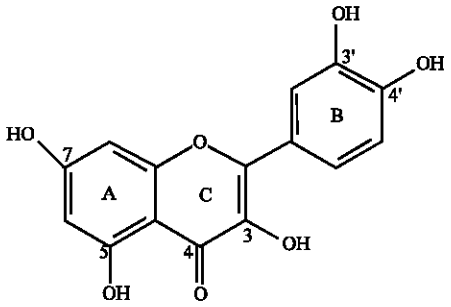

Fig. 1: Molecular structure of quercetin

(Galaris and Evangelou, 2002; Wojciech and Kazimierz, 2002). Is oxidative stress the only reason for the influence of quercetin plus cupric ion on DNA damage? Do copper (II) complexes with quercetin exert a hydrolysis role on double-strand DNA like other copper complexes (Pattubala *et al.*, 2004)? Moreover, little is known about the influence of concentration of metal ions dependence on the protective effect that quercetin exert against DNA damage. In the present study, we describe the effect of quercetin on DNA strand breakage induced by different concentrations of cupric ion. We present evidence that quercetin exerts opposite effects on DNA strand breakage induced by cupric ion, protecting DNA from damage at low cupric ion concentrations and promoting DNA cleavage at higher concentrations of cupric ion. Moreover, the oxidative and hydrolytic mechanisms of DNA damage by quercetin copper (II) complexes were investigated. Present results imply that in certain pathological situations quercetin may result in DNA damage, bringing on carcinogenesis in normal cell and antitumor effect in tumor cell.

MATERIALS AND METHODS

Chemicals: All chemicals and reagents were purchased from commercial sources and were used without further purification. The plasmid pBR322 DNA was purchased from TaKaRa Biotechnology Co. Ltd., Dalian (China). Calf thymus DNA (CT DNA) and ethidium bromide were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Quecetin was from Superman Plant and Chemicals Co. Ltd., Chengdu (China). Copper (II) chloride dehydrate ($CuCl_2 \cdot 2H_2O$) was from Guangfu Fine Chemicals, Tianjing (China). 2-thiobarbituric acid (TBA) was from Sinopharm Chemical Reagent Co. Ltd., Shanghai (China). Agarose (molecular biology grade) was from Oxoid Limited Basingstoke, Hampshire (England). Stock quercetin solution was prepared at a concentration 10 mM in DMF solution (50%, v/v). The Tris-HCl buffer solution was prepared with triple distilled water.

DNA damage: DNA damage was measured by the conversion of supercoiled pBR322 plasmid DNA to nicked circular and linear DNA forms. Supercoiled pBR322 plasmid DNA (0.25 μg/reaction) in Tris-HCl buffer (50 mM) with 18 mM NaCl (pH = 7.2) was treated with the indicated amount of copper salts, H_2O_2 and quercetin followed by dilution with the Tris-HCl buffer to a total volume of 10 μL. The samples were incubated for 1 h at 37°C. After stopping the reaction by addition of 1/10 volume of the loading buffer (0.25% bromophenol blue, 40% sucrose, 0.25% xylene cyanole and 200 mM EDTA), the samples were loaded on 1% neutral agarose gel containing 40 mM Tris/acetate and 1 mM EDTA pH 8.0 (TAE buffer) and were subjected to electrophoresis in a horizontal slab gel apparatus and 1×TAE buffer, which was performed at 75 V for 1.5 h. The gel was stained with a solution of 0.5 μg mL^{-1} ethidium bromide for 30 min, followed by destained in water. Agarose gel electrophoresis of plasmid DNA was visualized by photographing the fluorescence of intercalated ethidium bromide under a UV illuminator. The cleavage efficiency was measured by determining the ability of the metal ions to convert the supercoiled DNA (SC) to Nicked Circular form (NC) and Linear form (L). After electrophoresis, the proportion of DNA in each fraction was estimated quantitatively from the intensities of the bands using Glyko BandScan software.

To study mechanism of the DNA cleavage reaction performed by cupric ion plus quercetin, inhibition reaction were carried out by adding different scavengers of reactive oxygen intermediates such as dimethyl sulfoxide (DMSO) (0.4 M), glycerol (0.4 M) and mannitol (0.2 M) prior to the addition of quercetin and cupric ion. Samples were treated as described above.

Thiobarbituric acid-reactive substances (TBARS) assay: Each sample containing 0.5 mM CT DNA, in 50 mM Tris-HCl buffer (pH = 7.2), 100 μM cupric ions, quercetin (0, 25, 50, 100 and 200 μM), 100 μM H_2O_2 was incubated at 37°C for 24 h in a total volume of 0.5 mL. After incubation, samples were treated with 0.5 mL of 1% (w/v) solution of 2-thiobarbituric acid in 50 mM NaOH and 0.5 mL of glacialacetic acid and were incubated at 100°C for 30 min. After cooling, absorbance at 532 nm was measured. Blanks contained all components except the metal ion.

UV-visible spectra measurement of copper chelation and DNA-binding: The modifications of the absorption spectra of 100 μM quercetin solution when combined with 50 μM cupric ions, in 50 mM Tris-HCl buffer (pH = 7.2) at 37°C, was spectrophotometrically analyzed with UV-visible spectrophotometer 756MC. All the spectra

were run against blanks containing the buffer and the cupric ion. The cupric chelation was evaluated from the change in absorbance and/or spectral shift. The sample containing 100 μM quercetin, 50 μM cupric chloride in 50 mM Tris-HCl buffer (pH = 7.2) was kept at 37°C and the spectral titration measurement was carried out by adding increasing concentrations of CT DNA (25, 50, 100, 150 and 200 μM).

RESULTS

DNA damage induced by quercetin plus cupric ion: The undamaged double-stranded plasmid pBR322 exists in a compact Supercoiled Conformation (SC). Upon formation of strand breaks, supercoiled form of DNA is disrupted into a Nicked Circular form (NC) and Linear form (L). If one strand is cleaved, the supercoiled form will relax to produce a nicked circular form. If both strands are cleaved, a linear form will be produced. When the plasmid DNA is subjected to electrophoresis, relatively fast migration is observed for supercoiled form. Nicked circular form migrates slowly and linear form migrates between SC and NC (Masataka *et al*., 1999). Hence DNA strand breaks were quantified by measuring the transformation of the supercoiled form into nicked circular and linear forms.

DNA strand breaks were dependant on cupric ion concentration in the presence of H_2O_2 and the absence of quercetin. The increase in copper ion promoted formation of double strand breaks (Fig. 2). Quercetin alone induced a little or no DNA damage in the absence of cupric ion. However quercetin had opposite effects on the DNA damage induced by cupric ion plus H_2O_2. At $[Cu^{2+}]$ = 25 μM, quercetin decreased the formation of single strand breaks. However, at cupric ion concentrations equal or higher than 50 μM quercetin markedly increased the rate of strand breaks and even at 100 μM copper ion plasmid DNA was completely degraded as indicated by the disappearance of the main bands of the DNA in the gel and by the presence of a smear in the corresponding electrophoretic lane.

In the presence of quercetin, cupric ion plus H_2O_2 caused the cleavage of SC DNA into NC DNA and L DNA. However, the increasing amount of quercetin did not markedly increase formation of double strand breaks (Fig. 3). In the presence of H_2O_2, DNA cleavage induced by cupric ion plus quercetin was more efficient than that induced by cupric ion, as seen from the formation of linear DNA. At same concentration of cupric ion, higher concentration of quercetin slightly increased formation of double strand breaks in absence of H_2O_2 (Lane 7 and 8 in Fig. 3).

Effects of scavengers on DNA damage induced by quercetin plus cupric ion: Figure 4 shows the effects of scavengers on DNA damage induced by quercetin plus cupric ion. Typical hydroxyl free radical (·OH) scavengers such as DMSO, glycerol and mannitol, showed no or little inhibitory effect on DNA damage induced by quercetin plus copper ion in the presence of H_2O_2. There was a similar result in the absence of H_2O_2. DMSO slightly inhibited the DNA damage in the absence of H_2O_2.

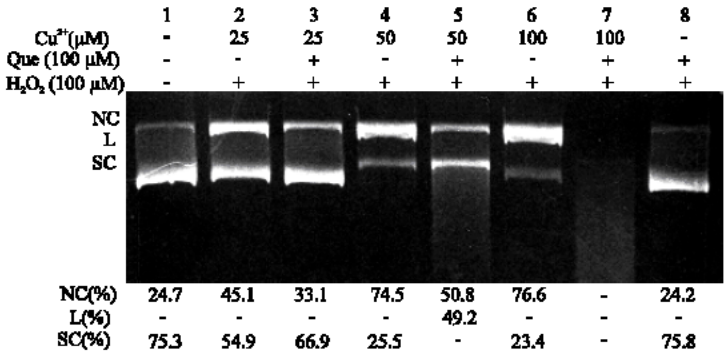

Fig. 2: Effect of quercetin on DNA damage induced by hydrogen peroxide and the increasing concentrations of cupric ions. The plasmid pBR 322 DNA was incubated for 1 h with the indicated additions in the absence and in the presence of 100 μM quercetin. After electrophoretic separation of pBR 322 DNA molecules in 1% agarose gel, this was stained with ethidium bromide and agarose gel electrophoresis was visualized by photographing the fluorescence under a UV illuminator and the bands were quantified using the Glyko BandScan software. The percentage of SC, OC and L form of pBR 322 DNA was calculated. Lane 1, DNA control. Abbreviations: NC, Nicked Circular; L, Linear; SC, Supercoiled; Que, Quercetin

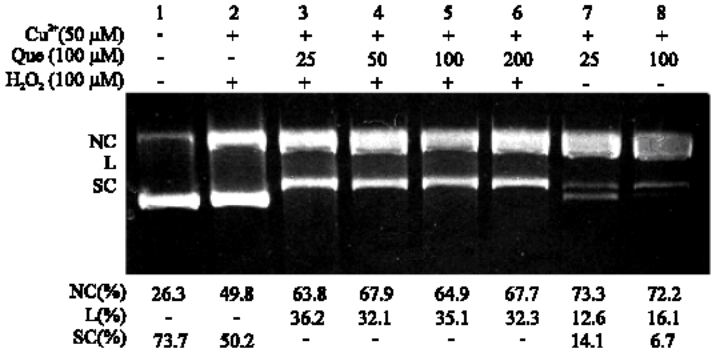

Fig. 3: Effect of the increasing concentrations of quercetin on DNA damage induced by cupric ions in the presence and the absence of hydrogen peroxide. Experiments were conducted as described in the legend of Fig. 2. Lane 1, DNA control

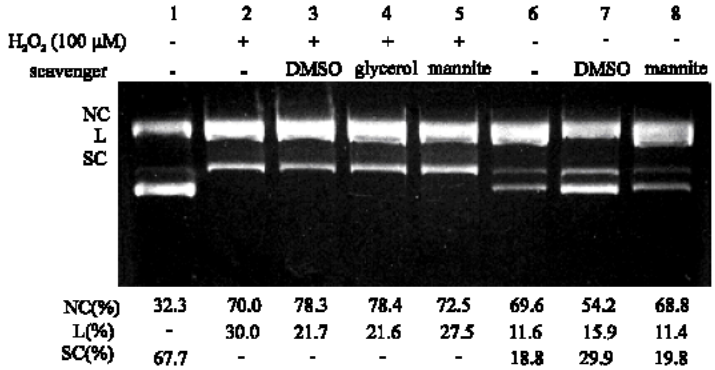

Fig. 4: Effects of scavengers on DNA damage induced by quercetin plus cupric ion. The pBR322 DNA was incubated for 1 h with scavengers, 50 μM cupric ions and 100 μM quercetin in the presence and the absence of hydrogen peroxide. Scavengers were added as follow: 0.4 M DMSO, 0.4 M glycerol, 0.2 M mannite, prior to the addition of quercetin and cupric ions. Lane 1, DNA control

The formation of 2-thiobarbituric acid reacting substances: In order to determine whether quercetin may increase the formation of ROS produced by H_2O_2 and relatively high concentration of cupric ion, the formation of 2-thiobarbituric acid reacting substances (TBARS) produced by CT DNA oxidative damage were measured. The increase in the amount of quercetin resulted in a slight decrease in the amount of TBARS (Fig. 5). So, quercetin had a protective effect on the oxidative degradation of CT DNA. However, the same concentration of quercetin promoted DNA cleavage induced by cupric ion as described above.

The formation of quercetin-Cu complexes and interaction of complexes with DNA: The quercetin spectra typically consist of two absorption maxima at 256 nm (band II related to ring B) and 376 nm (band I related to ring A).

Table 1: UV data of quercetin cupric complexes compared with quercetin

	λ_{max} (nm)		Absorbance	
Compounds	Band I	Band II	Band I	Band II
Quercetin	376	256	1.19	1.30
Quercetin copper complexes	435	254	1.02	1.35

The interaction of quercetin with cupric ion at pH 7.2 produced large bathochromic shifts (59 nm) in band I maxima which were associated with decreases in absorbance. Band II demonstrated a very small hypsochromic shift (2 nm) and a small increase in absorbance (Table 1). The data indicated that quercetin can form complexes with cupric ion.

On the addition of CT DNA, band II maxima markedly decreased in the spectra. At [DNA]/[Cu] = 4, absorbance in band II maxima decreased by 25% (Fig. 6) which indicated strong binding of the complexes to DNA.

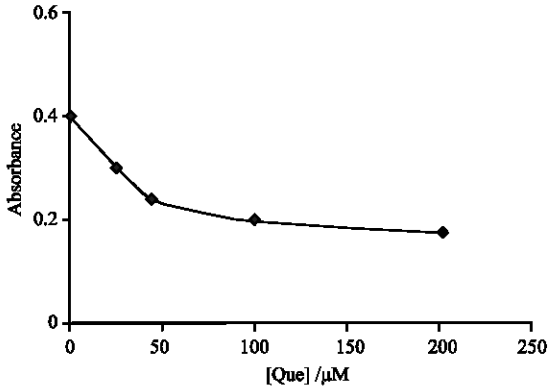

Fig. 5: Effect of quercetin on DNA oxidative damage induced by cupric ions plus H_2O_2. 0.5 mM CT DNA in 50 mM Tris-HCl buffer (pH = 7.2), containing 100 μM H_2O_2 was incubated at 37 °C for 24 h with 100 μM cupric ions in the presence of variable amounts of quercetin. TBARS formation was determined by measuring the absorbance at 532 nm. Results are the mean of triplicate determinations

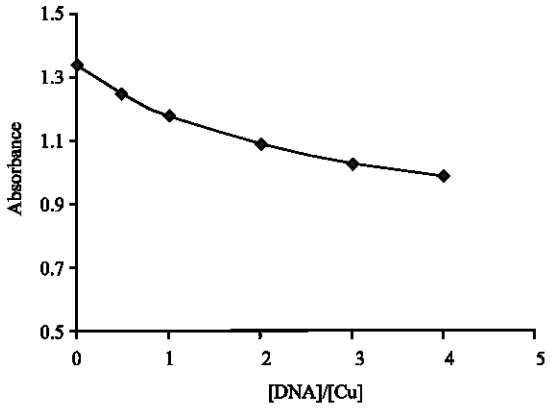

Fig. 6: Effect of increasing amounts of CT DNA on band II maxima of quercetin copper complexes. [Cu] = 50 μM, [DNA]/[Cu] = 0-4

DISCUSSION

A large part of weakly mutagenic and nonmutagenic carcinogens appear to induce carcinogenesis through metal-mediated oxidative DNA damage. Particularly, endogenous metal ions, such as copper and iron, react with superoxide anion ($O_2\cdot^-$) and hydrogen peroxide (H_2O_2) to produce highly reactive species such as hydroxyl free radical (·OH) and metal-oxygen complexes in Fenton-type reactions in biological systems, resulting in oxidative DNA damage (Galaris and Evargelou, 2002; Kennedy *et al.*, 1997; Lloyd *et al.*, 1998). *In vitro* study showed that DNA damage was induced at thymine and guanine by generating ROS in the presence of Cu (II) and H_2O_2 (Yamamoto and Kawanishi, 1989). Additionally copper can induce oxidative DNA damage by activating H_2O_2 to form reactive Cu (II)-hydroperoxo complex (Galaris and Evangelou, 2002).

Bioflavonoids protect DNA from damage induced by Reactive Oxygen Species (ROS) including ·OH, H_2O_2 and $O_2\cdot^-$ (Russo *et al.*, 2000). The protective effect of quercetin on DNA is attributed to scavenge ROS either as a free molecular or at the site of the quercetin binding to DNA. On the other hand, quercetin, as a phenolic compound, can prevent the production of ROS by complexing cations such as copper and iron that participate in hydroxyl radical formation (Alina and Juan, 2001; Naruto *et al.*, 1999).

Present results clearly demonstrate that quercetin has opposite effects on the DNA damage induced by cupric ion in the presence of H_2O_2. At low concentration of cupric ion quercetin protects DNA from oxidative damage as free radical-scavenger, which agrees well with the results found by others (Osako *et al.*, 2005). While at high concentration of copper ion quercetin can promote DNA cleavage and even causing the disappearance of the supercoiled, nicked circular and linear forms of the pBR322 DNA. Typical ·OH scavengers show no or little inhibitory effects on DNA damage. DNA oxidative damage induced by only ROS produces a little TBARS. Quercetin can decrease the amount of TBARS from DNA oxidative damage, while quercetin promotes DNA cleavage in electrophoresis assay. Present data provide evidences that ROS involved in DNA oxidative damage may be copper (II)-hydroperoxo species (Cu (II)OOH) (Komiyama *et al.*, 1999) other than hydroxyl free radical (·OH) and that oxidative role of ROS is not a matter of primary importance in DNA cleavage. These results could be explained by assuming that DNA damage induced by quercetin plus cupric ion could be the result of the combination of two mechanisms, i.e., oxidative damage and hydrolysis. As shown in Fig. 7, two possible mechanisms of DNA damage induced by quercetin plus cupric ion are related to the autoxidation and chelation of quercetin.

There are two metal-complexing sites within a quercetin molecule containing hydroxyl groups at 3, 5, 7, 3' and 4' positions (Fig. 1). The metal ions bound exclusively to 3', 4'-dihydroxy groups and to 3-OH and 4-oxo groups (Rubens de Souza and Wagner De Giovani, 2004). Because the 3-hydroxy group has a more acidic proton, the 3-OH and 4-oxo groups are the first sites involved in the complexation process. However, 3'-OH and 4'-OH in quercetin have the higher antioxidant

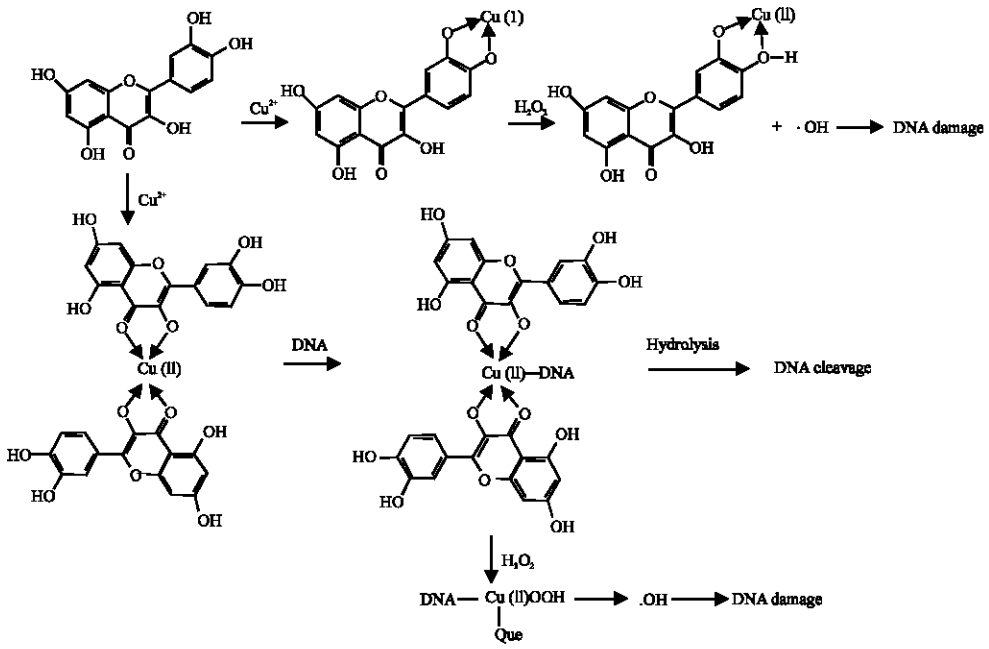

Fig. 7: Possible mechanisms of DNA damage induced by quercetin copper complexes

activities than 3-OH. So Cu (II) bound to 3', 4'-dihydroxy groups are reduced to Cu (I) effectively. Hydrogen peroxide can react with yielding copper(I) to obtain ·OH in Fenton-type reaction, as described following by:

$$CuL^{+} + H_2O_2 = CuL^{2+} + OH^{-} + \cdot OH \text{ (L = quercetin)}$$

In fact, copper ion is primarily coordinated with 3-OH and 4-oxo groups of quercetin to form a planar complex molecule, as shown in Fig. 7. On the addition of CT DNA, the complex shows a decrease in molar absorptivity of the $\pi \rightarrow \pi^*$ absorption band, which indicates strong binding of the complexes to DNA. These changes are as a result of a complex bound to DNA through intercalation involving insertion of planar quercetin in between the base pairs of DNA. In addition, there are electrostatic interaction between cupric ion and phosphate diester backbone and hydrogen bonding interaction between coordinated -NH- and two carboxylate oxygens with the functional groups positioned in the edge of the DNA bases, which promotes strong DNA-binding affinities with quercetin copper complexes to form DNA-Cu (II) - quercetin complexes. So, cupric ion, with strong Lewis acid property, coordinated with oxygen in phosphate diester backbone, could promote a nucleophilic attack of a water oxygen to the phosphorus to give a five co-ordinate phosphate intermediate (Komiyama *et al.*, 1999; Selvakumar *et al.*, 2006), resulting in hydrolytic cleavage of DNA. Moreover our results indicate that H_2O_2 can promote DNA cleavage induced by quercetin copper complexes. In the presence of H_2O_2 a ternary complex possibly formed by quercetin copper complexes and DNA could facilitate the generation of the ROS (i.e., Cu (II)OOH and ·OH as shown in Fig. 7) in the proximity of DNA (Pedreño *et al.*, 2005), which then immediately would attack DNA and produce more chain breaks on DNA than in the case of ROS produced in the bulk. So in the presence of H_2O_2 DNA damage induced by quercetin and copper ion could be ascribed to a combination of oxidation and hydrolysis involved in DNA-binding with quercetin copper complexes. The latter plays a primary role in DNA cleavage induced by quercetin copper complexes. Additionally that at low concentration of cupric ion excessive quercetin protect DNA from damage is explained as follows. Quercetin can intercalate strongly into DNA base pairs at preferred sites (Ahmed and Ramesh, 1994). When excessive quercetin intercalate into DNA, there remains no sites that quercetin-Cu complexes bind, inhibiting DNA damage. Further research is necessary to confirm these mechanisms.

The results presented here could have some physio-pathological significance because it is widely accepted that *in vivo* both quercetin and metal ions may interact with DNA. A large part of weakly mutagenic and nonmutagenic carcinogens appear to induce carcinogenesis through metal-mediated oxidative DNA damage. Particularly, endogenous metal ions, such as copper and iron, play an important role in ROS generation

from various carcinogens, which can bring on DNA damage (Galaris and Evangelou, 2002; Kawanishi *et al.*, 2002). Because the essential metals, copper and iron, are under strict physiological control during uptake, transport and metabolism, they were not considered as agents of oxidative DNA damage *in vivo*. Levels of free iron or copper ions in the normal cell are thought to be very low, so the average dietary intake of quercetin would diminish their potential oxidative damage to DNA *in vivo* in normal circumstances. However, there is an emerging view that this control may not be sufficient in certain pathological situations, such as inflammation, intoxication or intense oxidative metabolism (Wojciech and Kazimierz, 2002). Hence intracellular levels of free iron or copper may increase. In these cases normal dietary intake of quercetin could enhance cellular DNA strand breakage, especially in the mitochondria, where the active aerobic metabolism and the absence of histones may render mtDNA more susceptible to the oxidative attack (Pedreño *et al.*, 2005; Hussain *et al.*, 2000). So, quercetin can show both mutagenicity and carcinogenicity (Das *et al.*, 1994; Duthie *et al.*, 1997; Pamukcu *et al.*, 1980), which is related to DNA damage in the presence of copper ions in agreement with our results.

From the above discussion, the conclusion can be reached that in normal cellular conditions quercetin may protect DNA from the damaging attack induced by ROS, but quercetin could promote DNA strand breakage in certain pathological situations resulting in carcinogenicity. Although we suggest that hydrolysis play an important role in DNA damage induced by quercetin copper complexes, the material mechanisms of these opposite effects of quercetin on DNA damage observed in vitro remain to be further determined. Additionally it has been reported that quercetin copper complexes have a much higher antitumor activity than quercetin (Zhou *et al.*, 2001). It is possible that quercetin copper complexes have a similar antitumor mechanism to Bleomycin through breaking DNA. However insufficient evidences are available on this possibility. Work now is in progress with the aim of establishing a relation between antitumor activity and DNA cleavage induced by quercetin copper complexes. Considerable more work, hopefully, will be done in this area.

ACKNOWLEDGMENTS

This work was financially supported by the Science and Technology Project of Education Commission of Chongqing (Grant No. KJ051503). We are grateful to the Chongqing Education College Science Research Project for support of this research.

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