Free Radicals and Antioxidants 3 (2013) 11–16

Contents lists available at SciVerse ScienceDirect

Free Radicals and Antioxidants

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

Original article

Dual effects of (+)-catechin on hemin-induced oxidative reactions: A potential pharmacological implication

Nai-Hao Lu [a,b,*], Ying-Jie He [a,c], Chao Chen [a], Rong Tian [c], Qiang Xiao [b], Yi-Yuan Peng [a,c,*]

[a] Key Laboratory of Functional Small Organic Molecule, Ministry of Education and College of Life Science, Jiangxi Normal University, Nanchang, Jiangxi 330022, China
[b] Jiangxi Key Laboratory of Functional Organic Molecules, Jiangxi Science and Technology Normal University, Nanchang 330013, China
[c] Key Laboratory of Green Chemistry, Jiangxi Province and College of Chemistry and Chemical Engineering, Jiangxi Normal University, Nanchang, Jiangxi, 330022, China

ARTICLE INFO

Article history:
Received 13 March 2013
Accepted 18 April 2013
Available online 24 April 2013

Keywords:
Hemin
(+)-Catechin
Oxidative reactions
Anti-oxidant
Pro-oxidant

ABSTRACT

Introduction: Although the anti- and pro-oxidant properties were the well-known phenomenons for flavonoids, the influence of flavonoids on free heme-dependent redox reactions was not definitely elucidated.
Methods: By spectrophotometry and Western blotting, the influence of (+)-catechin (a polyphenol identified in red wine, tea, and cocoa) on hemin-induced oxidative reactions was investigated in this study.
Results: (+)-Catechin was found to act as an efficient reducing agent for the reduction of ferryl heme to ferric state, demonstrating novel anti-oxidant pathway. However, this antioxidant had the ability to trigger hemin oxidation through producing additional hydrogen peroxide (H_2O_2). On the other hand, the effects of (+)-catechin on hemin–H_2O_2–induced protein (or methylene blue) oxidation depended on the concentrations of H_2O_2 and antioxidant. (+)-Catechin exerted significant anti-oxidant ability when lower concentration of H_2O_2 was used. However, in the presence of higher concentration of H_2O_2, (+)-catechin at low concentrations could significantly aggravate oxidative reactions and exhibit protective effects at high concentrations.
Conclusions: The anti- and pro-oxidant effects of catechin on heme-dependent redox reactions at different concentration, may provide new insights into the dietary intake and therapeutic implications of catechins with free heme and heme proteins.

1. Introduction

As the prosthetic group of numerous heme proteins, heme carries out physiological functions as a transporter for oxygen and electrons.[1] However, excess free heme have deleterious effects on DNA, polyunsaturated fatty acids, and proteins by generating reactive radicals. Evidence to support the important role of free heme in oxidative stress and a variety of diseases is widely present.[1–4] The pathologies of many diseases are linked to the interaction between the heme groups and peroxides to initiate oxidative reactions, including the generation of highly reactive ferryl heme (Fe^{4+}).[1,3,4]

Due to the deleterious effects of oxidation on biological molecules,[5,6] flavonoids have been widely used as the classic antioxidants to ameliorate oxidative damage *in vitro* and *in vivo*. Flavonoids are polyphenolic compounds which are present in plants, vegetables and fruits. Due to their anti-oxidant activities, including free radical-scavenging, lipid peroxidation-inhibiting and metal-chelating, they exert a wide range of beneficial effects on human health.[7,8]

As one of the phenolic compounds present in red wine, tea, and cocoa, (+)-catechin is a major contributor to the high anti-oxidant capacity.[7–10] Therefore, the therapeutic use and dietary intake of catechins can be associated with significant health benefits.[7,8,11] Several studies have attributed the anti-oxidant properties of tea extracts to the presence of catechins. However, catechin could react with dissolved oxygen in aqueous solution and generate hydrogen peroxide (H_2O_2),[12] which might lead to the accelerated oxidation of heme proteins.[10,13] Although the anti- and pro-oxidant properties were the well-known phenomenons for flavonoids,[7,8,10,13,14] the

* Corresponding authors. Key Laboratory of Functional Small Organic Molecule, Ministry of Education and College of Life Science, Jiangxi Normal University, 99 Ziyang Road, Nanchang, Jiangxi 330022, China. Tel./fax: +86 791 8120396.
E-mail addresses: naihaolu@gmail.com (N.-H. Lu), yiyuanpeng@yahoo.com (Y.-Y. Peng).

2231-2536/$ – see front matter
http://dx.doi.org/10.1016/j.fra.2013.04.002

influence of catechin on free heme-dependent redox reactions was not definitely elucidated.

2. Materials and methods

2.1. Materials

Ferriprotoporphyrin IX chloride (hemin; which is referred to as "heme"), bovine serum albumin (BSA), catalase, (+)-catechin (Catechin), rabbit polyclonal antibody against dinitrophenol (DNP) and 2, 4-dinitrophenylhydrazine (DNPH) were purchased from Sigma (Aldrich Co.; St. Louis, MO, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and RPMI-1640 (for cell culture) were purchased from Gibco BRL (Gaithersburg, MD, USA).

2.2. Ferryl hemin reduction by (+)-catechin

Ferryl hemin was generated by addition of H_2O_2 to hemin in a 1:1 ratio at pH 7.4.[10,13] After 15 min, catalase was added to remove excess H_2O_2, and then antioxidant was added. A Hitachi U-3310 spectrophotometer was used for the optical spectra measurement of samples.

2.3. Detection of H_2O_2 in the reaction between (+)-catechin and hemin

In order to investigate the potential roles of widespread H_2O_2 in oxidation process, catalase (H_2O_2 scavenger) was used.[2,15] In the absence or presence of catalase, hemin (20 μM, final concentration, the same below) was treated with (+)-catechin (20 μM) or H_2O_2 (20 μM) at 37 °C for 20 min. The obtained reaction samples were used in optical spectra determination.

2.4. Hemin and (or) H_2O_2-induced protein (or methylene blue) oxidation in the presence of (+)-catechin

Samples of BSA in phosphate buffered saline (PBS, 0.1 M, pH 7.4) were pre-incubated (5 min at 37 °C) with flavonoid, and then treated with hemin (20 μM) and (or) H_2O_2 (0.04 or 0.5 mM) at 37 °C for 20 min. The final concentration of BSA was 0.5 mg/ml. Significant protein oxidative modifications were observed in short time incubation when high concentrations of hemin-H_2O_2 were used in many *in vitro* experiments.[10,13] These high concentrations were, therefore, chosen in our studies to conveniently compare the different effects of catechin. The obtained reaction mixtures were used in protein oxidation assays.

Due to its applicability for the simple and rapid detection of free radicals, methylene blue (MB) was selected as the substrate to further study the role of (+)-catechin in hemin−H_2O_2−induced oxidative damage.[16] In the absence or presence of (+)-catechin, samples (0.01 mg/ml MB) in PBS were incubated with hemin-H_2O_2 for 2 h. Subsequently, the concentrations of MB were determined spectrophotometrically at 665 nm.

2.5. Western blotting analysis for protein oxidation

As the widely used marker of protein oxidation, the formation of protein carbonyl groups is always accompanied with oxidative damage of protein.[5,6] For detection of protein oxidation, the carbonyl groups in proteins were first derivatized with DNPH,[6] resulting in the formation of DNP. Antibody against DNP was used for the detection of protein carbonyl groups. Western blotting analysis for protein oxidation was processed as previously described.[6,10] Alpha Imager 2200 software was used to analyze the optical density in Western blotting.

2.6. Cell culture and cell viability determination

Human hepatoma HepG2 cells were grown in RPMI-1640. Different concentrations of (+)-catechin were added to the cells for 5 min. Hemin-H_2O_2 system then was added, and the cells were incubated further at 37 °C for up to 2 h. Cell viability was quantified by using MTT assay.[14]

2.7. Statistical analysis

All data were expressed as the means ± SD of three independent experiments. Significance was assessed by using the one-way ANOVA ($P < 0.05$ as significant).

3. Results and discussion

3.1. Anti-oxidant effects of (+)-catechin on hemin oxidative damage

In addition to its ability as radical scavengers, some flavonoids had the capacity to reduce highly reactive ferryl heme (Fe^{4+}) formed in the reaction of heme with H_2O_2.[10,13] The efficiency of this polyphenol compound in functioning as ferryl hemin reducing agent was examined at physiological pH. Addition of (+)-catechin to ferryl hemin led to optical changes shown in Fig. 1A. These changes were typical of the reduction of ferryl heme to ferric heme, the increase in the absorbance from 380 to 400 nm and the decrease about 420 nm assigned to the Soret bands of ferric heme and ferryl heme, respectively.[10,13,15] Therefore, this polyphenol compound was found to act as an efficient reducing agent for the reduction of ferryl heme to ferric state.

As a reducing agent for ferryl species, (+)-catechin could exert anti-oxidant effect on heme oxidative damage. Fig. 1B showed significant increase in hemin oxidation in the presence of H_2O_2. The addition of antioxidant greatly slowed down the oxidation process. Therefore, (+)-catechin exhibited significant protective activity in ameliorating heme-related oxidative damage through the reduction of ferryl heme to native heme.

3.2. Pro-oxidant effect of (+)-catechin on hemin oxidative damage

Moreover, the influence of (+)-catechin on normal heme redox transition was investigated. Fig. 2A showed the spectra changes of hemin after the addition of (+)-catechin. After 20 min of incubation with flavonoid, the spectra of hemin shifted to resemble that of oxidized heme.[10,13,15,17,18] Therefore, (+)-catechin exhibited pro-oxidant ability in hemin oxidation.

In the presence of anti-oxidant enzyme (Fig. 2A and B), catalase could efficiently inhibit catechin-triggered hemin oxidation as indicated by the absorbance increase close to 400 nm. Based on these results, it was demonstrated that H_2O_2 was the important intermediate on the pro-oxidant mechanism of catechin. A possible explanation for the pro-oxidant effect of catechin on hemin oxidation is that itself can be the source of H_2O_2 which may lead to the accelerated oxidation of heme. Moreover, catechin would bind to both intact and degraded forms of heme proteins through its metal chelating effect,[12,19] which was convenient for catechin to react with dissolved oxygen in aqueous solution, and generates H_2O_2. Also, metal-chelating ability would make the spectral changes of hemin after the addition of (+)-catechin different from that after the treatment of H_2O_2 (Fig. 2).

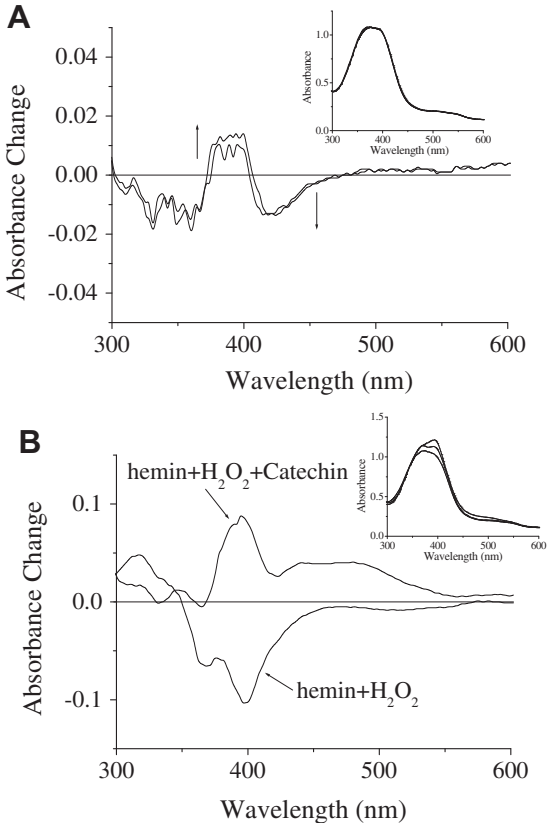

Fig. 1. (A) Spectral changes of ferryl hemin after addition of (+)-catechin. Ferryl hemin was generated by addition of H_2O_2 to hemin in a 1:1 ratio at pH 7.4. After 15 min, catalase was added (10 U) and then (+)-catechin (20 μM, final concentration). The final hemin concentration was 20 μM. Difference spectra derived from (Inset) in which initial ferryl hemin spectrum has been set to zero. Inset: absorbance spectra taken approximately every 1 min after the addition of (+)-catechin. (B) Spectral changes of hemin after addition of H_2O_2 in the presence of (+)-catechin. Hemin (20 μM) was incubated with H_2O_2 (20 μM)-catechin (20 μM) at 37 °C for 20 min. Difference spectra derived from (Insert) in which hemin spectrum has been set to zero. Inset: absorbance spectra taken approximately 20 min after the addition of (+)-catechin.

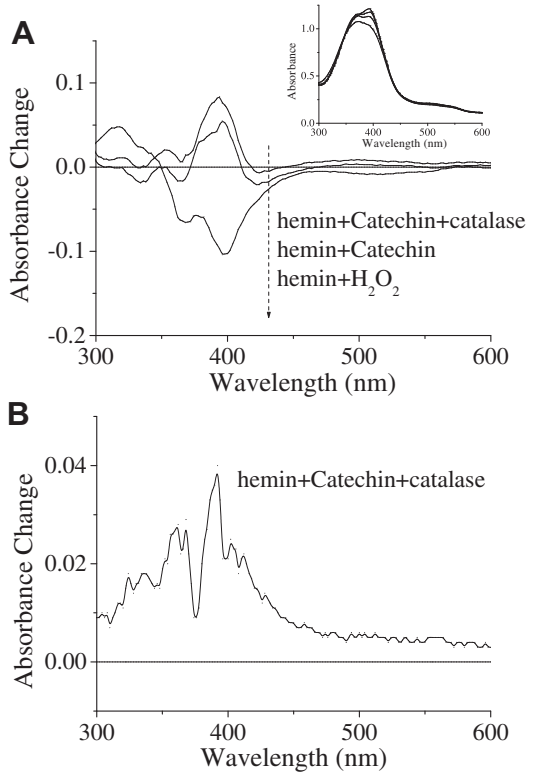

Fig. 2. Spectral changes of hemin after addition of (+)-catechin (or H_2O_2) in the presence of catalase. In the presence of catalase (10 U), hemin (20 μM) was incubated with (+)-catechin (or H_2O_2, 20 μM) at 37 °C for 20 min. (A) Difference spectra derived from (Inset) in which hemin spectrum has been set to zero. (B) Difference spectra derived from (Inset) in which spectrum of catechin treated-hemin has been set to zero. Insert: absorbance spectra taken approximately 20 min after the addition of (+)-catechin.

3.3. Effects of (+)-catechin on hemin-induced protein oxidative damage

As the classic antioxidants, flavonoids have been widely shown to ameliorate protein oxidative damage *in vitro* and *in vivo*.[7,8] As shown in Fig. 3A and B, the presence of (+)-catechin exhibited insignificant effect on hemin-triggered BSA carbonyl formation, while it significantly inhibited H_2O_2-induced BSA oxidation. The unobvious effect on hemin-induced protein oxidative damage was probably due to the ability of this antioxidant to effectively trigger additional reactive species formation (Fig. 2), which would markedly promoted protein oxidation, and consequently the classic antioxidant couldn't completely scavenge the massive reactive species.

3.4. Anti- and pro-oxidant effects of (+)-catechin on hemin–H_2O_2–induced oxidative reactions

The effect of (+)-catechin on hemin–H_2O_2–triggered oxidative reactions was then investigated. The significant formation of protein carbonyl groups was observed after the treatment of BSA with hemin-H_2O_2 system (Fig. 4A and B). (+)-Catechin pretreatment at lower concentrations (0.05, 0.1 mM) exhibited pro-oxidant effects on BSA oxidation, while the anti-oxidant effect was observed at 0.5 mM (Fig. 4).

Besides the important influence of catechin concentrations, the diverse effects of catechin on hemin–H_2O_2–induced protein oxidation also depended on the concentrations of H_2O_2. When the ratio between hemin and H_2O_2 was increased to 0.5, (+)-catechin could effectively inhibit protein oxidation in a dose-dependent manner (Fig. 5A and B). Moreover, this antioxidant may exhibit similar abilities in organic molecules oxidation. Methylene blue (MB) was also widely used as a substrate for peroxidase activity determination.[16] (+)-Catechin efficiently inhibited MB oxidative degradation when lower concentration of H_2O_2 was used (Fig. 6A). When the H_2O_2 concentration used was in large molar excess of hemin, (+)-catechin could significantly aggravate MB oxidation at low concentrations and exhibit protective effects at high concentrations (Fig. 6B).

3.5. Anti- and pro-oxidant properties of catechins in hemin–H_2O_2–induced oxidative damage

Moreover, other catechins including (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC), were used to study their efficiencies on hemin–H_2O_2–induced MB oxidation. Similar to the dual effect of (+)-catechin, the other catechins significantly inhibited MB oxidative degradation when lower concentration of H_2O_2 was used and enhanced MB oxidation at high concentration of H_2O_2 (Fig. 7A and B). Structure-activity study has indicated that the galloyl moiety at the 3-position

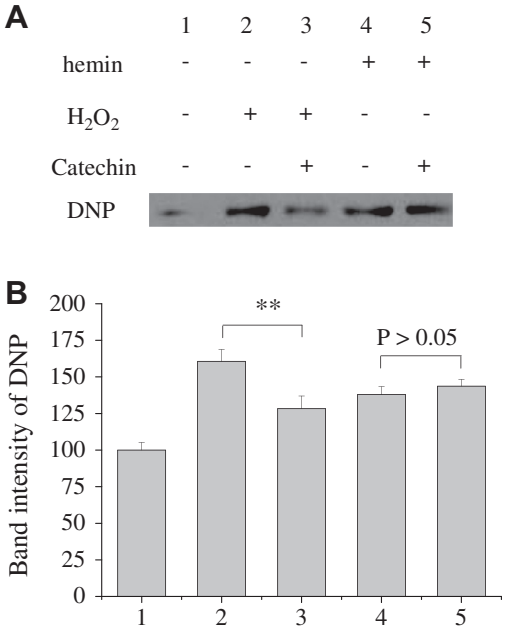

Fig. 3. Effect of (+)-catechin on hemin (or H_2O_2)-induced BSA oxidation. (A) Detection of protein carbonyl groups by Western blotting with antibody against DNP. BSA (0.5 mg/ml) was treated with hemin (20 μM) or H_2O_2 (0.5 mM), and in the presence or absence of (+)-catechin (20 μM), Blank represented the untreated BSA. (B) The corresponding densitometry analysis of protein bands (values are mean ± S.D. of three independent determinations, $^{**}P < 0.01$, $P > 0.05$ compared to respective Control group (lane 2 and 4)).

of flavan-3-ol skeleton and the ortho-trihydroxyl group in the B-ring are the most important structural features for free radical scavenging ability.[9,20] Thus, ECG and EGCG (a gallate ester moiety at the 3-position of catechins) have the higher anti-oxidant activity.

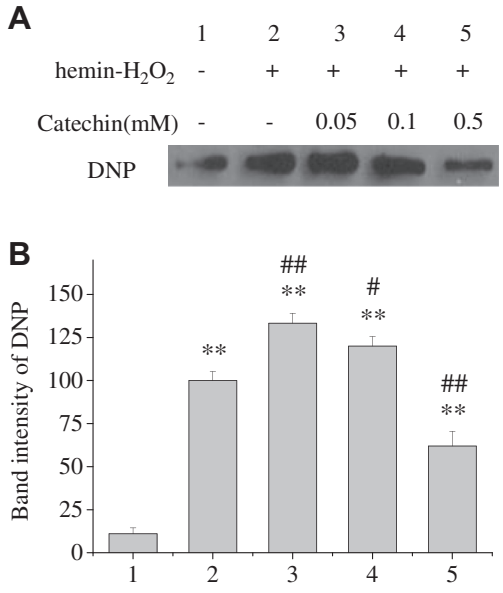

Fig. 4. Effect of (+)-catechin on hemin-H_2O_2 (0.5 mM)-induced BSA oxidation. (A) Detection of protein carbonyl groups by Western blotting with antibody against DNP. BSA (0.5 mg/ml) was treated with hemin (20 μM)-H_2O_2 (0.5 mM) (Control), and in the presence of different concentrations of (+)-catechin, Blank represented the untreated BSA. (B) The corresponding densitometry analysis of protein bands (values are mean ± S.D. of three independent determinations, $^{**}P < 0.01$ compared to Blank group (lane 1), $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ compared to Control group (lane 2)).

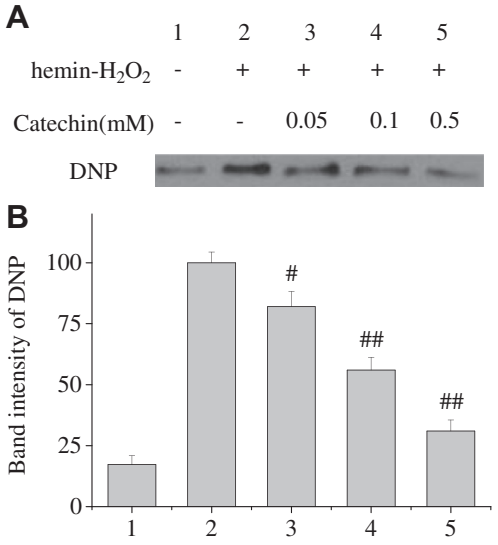

Fig. 5. Effect of (+)-catechin on hemin-H_2O_2 (0.04 mM)-induced BSA oxidation. (A) Detection of protein carbonyl groups by Western blotting with antibody against DNP. BSA (0.5 mg/ml) was treated with hemin (20 μM)-H_2O_2 (0.04 mM) (Control), and in the presence of different concentrations of (+)-catechin, Blank represented the untreated BSA. (B) The corresponding densitometry analysis of protein bands (values are mean ± S.D. of three independent determinations, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ compared to Control group (lane 2)).

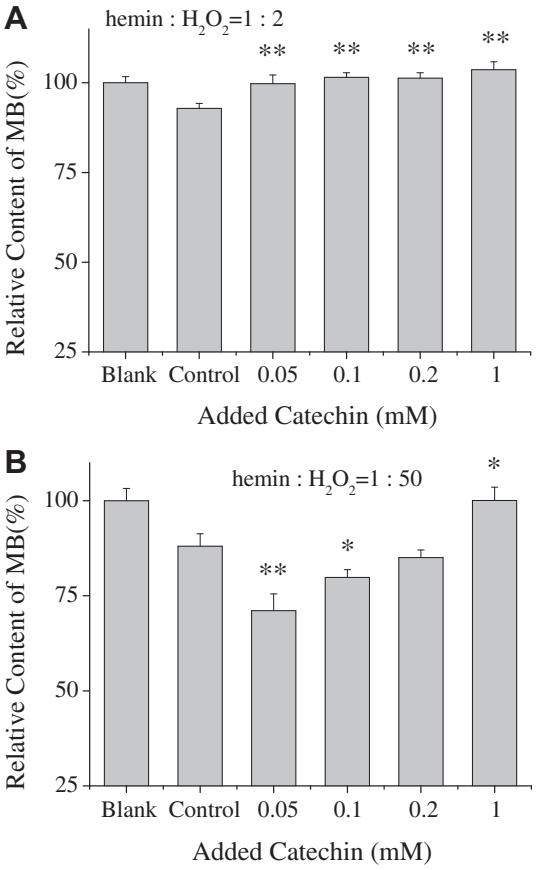

Fig. 6. Effect of (+)-catechin on hemin-H_2O_2 (different ratios)-induced MB oxidative degradation. Degradation of MB was determined by spectrophotometry. MB (0.01 mg/ml) was treated with hemin (10 μM)-H_2O_2 (Control, 0.02 mM (A), 0.5 mM (B)), and in the presence of different concentrations of (+)-catechin, Blank represented the untreated MB. (Values are mean ± S.D. of three independent determinations. The respective Blank values were set to 100%, $^{**}P < 0.01$, $^{*}P < 0.05$ compared to respective Control group).

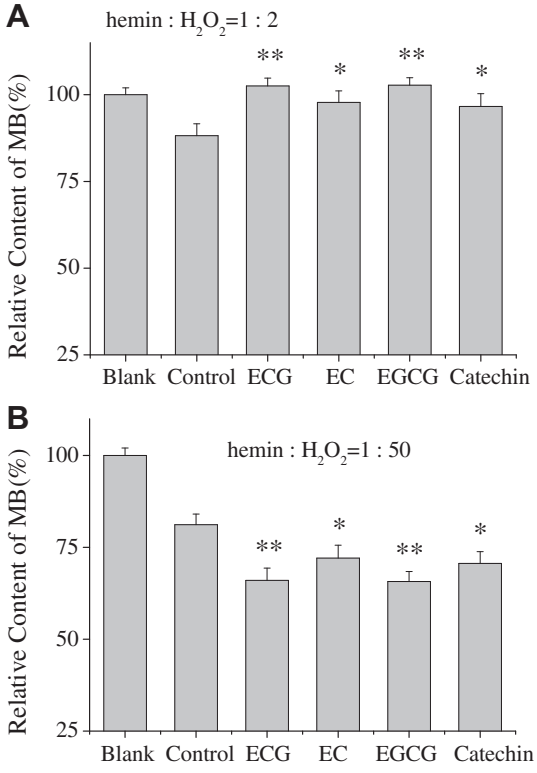

Fig. 7. Effect of (+)-catechin (or other catechins) on hemin–H_2O_2–induced MB oxidative degradation. Degradation of MB was determined by spectrophotometry. MB (0.01 mg/ml) was treated with hemin (20 μM)-H_2O_2 (Control, 0.04 mM (A), 1 mM (B)), and in the presence of different catechins (30 μM), Blank represented the untreated MB. (Values are mean ± S.D. of three independent determinations. The respective Blank values were set to 100%, $^{**}P < 0.01$, $^{*}P < 0.05$ compared to respective Control group).

Therefore, the anti-oxidant order of these catechins on hemin-induced oxidative reactions was: ECG, EGCG > EC, Catechin (Fig. 7A), which was similar to the pro-oxidant ability of these compounds (Fig. 7B). The results also supported that the anti-oxidant effects of catechins were always accompanied by their pro-oxidant activities.

3.6. Proposed mechanism for the anti- and pro-oxidant property of catechin in hemin-induced oxidative reactions

These results in this study revealed that the diverse effects of catechin on hemin–H_2O_2–induced protein (or MB) oxidation depended on the concentrations of H_2O_2 and antioxidant (Figs. 4–7). When lower concentration of H_2O_2 and higher concentration of antioxidant were used, the anti-oxidant ability of catechin would occupy the major effect, and subsequently free radical-induced oxidation process was effectively inhibited.[10] In addition to acting as an antioxidant, (+)-catechin also showed pro-oxidant effect on hemin–H_2O_2–induced oxidative reactions at higher concentration of H_2O_2 and lower concentration of antioxidant. In contrast to the anti-oxidant activities, flavonoids also have the abilities to act as pro-oxidants under certain conditions.[20,21] Usually, these pro-oxidant activities involve interactions of polyphenols with transition metal ions. Meanwhile, oxidation of polyphenols produces potentially cytotoxic species, including $O_2^{\cdot-}$, H_2O_2 and a complex mixture of quinones and semiquinones.[17,20,21]

The abnormal effect of catechin on promoting oxidative reactions was also probably related to its ability to interfere in heme-dependent redox reactions. In the presence of high concentration of H_2O_2, free iron would be significantly released from heme proteins or free heme,[1,2,18] which has the potential to promote oxidative damage via classical Fenton reaction or Haber–Weiss reactions.[22] Catechins have been reported to exhibit a pro-oxidant activity through recycling redox reactions between Cu (I) and Cu (II), and generating H_2O_2.[19] Moreover, this phenolic antioxidant at low concentration efficiently promoted Fe^{3+}-H_2O_2-induced protein oxidation (Fig. 8A and B). Catechin, therefore, could reduce Fe^{3+} to Fe^{2+} and produce reactive species and lead to extensive oxidative damage. On the other hand, catechin acted as a reducing substrate for ferryl hemin, thereby recycling it to ferric hemin. The ferric hemin would again participate in remaining H_2O_2-dependent redox reaction and subsequently promote peroxidase-triggered oxidative reactions.[23] Simultaneously, this classic antioxidant at low concentration couldn't completely scavenge the massive reactive species. Catechin at low concentration, therefore, exhibited pro-oxidant effect on hemin-induced oxidative reactions. The pro-oxidant ability may reveal previously unrecognized physiologic effects of tea extract (catechin) in connection with heme bioactivity.

The effect of (+)-catechin on hemin–H_2O_2–mediated cell viability was further investigated. In this study, H_2O_2 was generated in situ by glucose–glucose oxidase system.[14] After 2 h incubation, the addition of hemin-H_2O_2 system was sufficient to significantly reduce cell viability. Compared with the properties on oxidative reactions *in vitro*, catechin had different effects on heme-induced loss of HepG2 cell viability (Fig. 9). Due to a balance between the anti- and pro-oxidant properties, the presence of (+)-catechin at low concentrations had insignificant influence on cell viability. This compound would exert significant anti-oxidant ability and effectively inhibited hemin–H_2O_2–cytotoxicity only when the high concentration was used. Also, catechin did not cause significant change of hemin (or H_2O_2)-mediated cell viability (data not shown). Therefore, the anti- and pro-oxidant effects on heme-dependent oxidative reactions should be taken into account

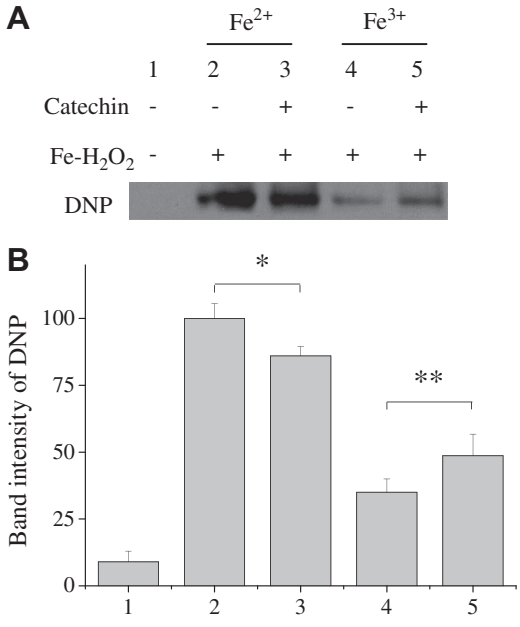

Fig. 8. Effect of (+)-catechin on Fe^{3+} (Fe^{2+})–H_2O_2–induced BSA oxidation. (A) Detection of protein carbonyl groups by Western blotting with antibody against DNP. BSA (0.5 mg/ml) was treated with free iron (20 μM)-H_2O_2 (0.3 mM) (Control), and in the presence of (+)-catechin (20 μM), Blank represented the untreated BSA. (B) The corresponding densitometry analysis of protein bands (values are mean ± S.D. of three independent determinations, $^{**}P < 0.01$, $^{*}P < 0.05$ compared to respective Control group (lane 2 and 4)).

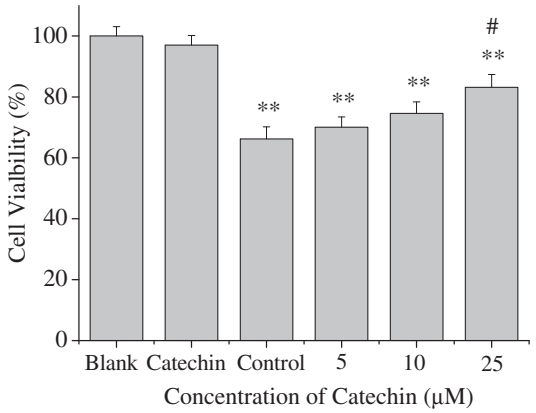

Fig. 9. Effect of (+)-catechin on hemin–H_2O_2–mediated loss of cellular viability. Different concentrations of (+)-catechin were added to HepG2 cells, and incubated with hemin (10 μM)-H_2O_2 (10 mU glucose oxidase/glucose) for 2 h. Blank represented the untreated cells, CAT represented (+)-catechin (5 μM)-treated cells, Control represented hemin–H_2O_2–treated cells. Cellular viability was measured by using MTT assay (values are mean ± S.D. of three independent determinations, $^{**}P < 0.01$ compared to Blank group; $^{\#}P < 0.05$ compared to Control group).

when this classic flavonoid was used to protect proteins against oxidative stress *in vivo* and *in vitro*.

4. Conclusions

In addition to its abilities as a free radical scavenger and reducing agent, catechin also had the ability to trigger additional reactive species formation. Moreover, the diverse effects of catechin on hemin–H_2O_2–induced oxidative reactions depended on the concentrations of H_2O_2 and antioxidant. These novel results showed that catechin could possess anti- and pro-oxidant activities through interfering in heme-dependent redox reactions. The balance between anti-oxidant and pro-oxidant abilities of catechins, therefore, should be taken into account in their medical and nutritional applications.

Conflicts of interest

All authors have none to declare.

Acknowledgments

Financial support from the National Natural Science Foundation of China (Nos. 31100608, 31260216 and 21162012), the Natural Science Foundation of Jiangxi Province (Grant No. 20114BAB204013), Education Department of Jiangxi Province (No. GJJ12183), The Sponsored Program for Cultivating Youths of Outstanding Ability in Jiangxi Normal University, the Open Project Program of Key Laboratory of Functional Small Organic Molecule, Ministry of Education, Jiangxi Normal University (No. KLFS-KF-201224), China Postdoctoral Science Foundation (No. 2013M531557) and the Graduates' Innovation Fund of Jiangxi Province (No. YC2012-S054).

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