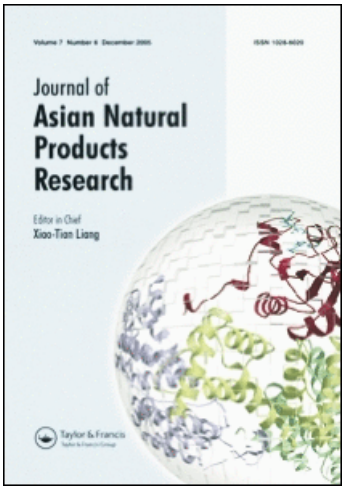

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Peroxynitrite and hemoglobin-mediated nitrative/oxidative modification of human plasma protein: effects of some flavonoids

Na Wang[a]; Dan Li[a]; Nai-Hao Lu[b]; Lian Yi[b]; Xiao-Wei Huang[ab]; Zhong-Hong Gao[b]
[a] Technology Center of China Tobacco Hubei Industrial Corporation, Wuhan, China [b] School of Chemistry and Chemical Engineering, Huazhong University of Science and Technology, Wuhan, China

Online publication date: 21 April 2010

To cite this Article Wang, Na , Li, Dan , Lu, Nai-Hao , Yi, Lian , Huang, Xiao-Wei and Gao, Zhong-Hong(2010) 'Peroxynitrite and hemoglobin-mediated nitrative/oxidative modification of human plasma protein: effects of some flavonoids', Journal of Asian Natural Products Research, 12: 4, 257 — 264
To link to this Article: DOI: 10.1080/10286021003620226
URL: http://dx.doi.org/10.1080/10286021003620226

Journal of Asian Natural Products Research
Vol. 12, No. 4, April 2010, 257–264

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Peroxynitrite and hemoglobin-mediated nitrative/oxidative modification of human plasma protein: effects of some flavonoids

Na Wang[a], Dan Li[a]*, Nai-Hao Lu[b], Lian Yi[b], Xiao-Wei Huang[ab] and Zhong-Hong Gao[b]

[a]*Technology Center of China Tobacco Hubei Industrial Corporation, Wuhan 430051, China;*
[b]*School of Chemistry and Chemical Engineering, Huazhong University of Science and Technology, Wuhan 430074, China*

(*Received 30 September 2009; final version received 13 January 2010*)

Protein tyrosine nitration is a common post-translational modification occurring under conditions of nitrative/oxidative stress in a number of diseases. The major pathways of protein tyrosine nitration *in vivo* include peroxynitrite ($ONOO^-$) and hemoglobin/NO_2^-/H_2O_2-dependent reaction. In this paper, several structural diversity flavonoids (quercetin, kaempferol, (+)-catechin, baicalein, apigenin, and naringenin) were chosen, to study their efficiencies against $ONOO^-$ or hemoglobin/$NaNO_2$/H_2O_2-mediated nitrative/oxidative damage to human plasma proteins *in vitro*. Protein nitration was efficiently inhibited by these flavonoids regardless of nitration pathways, and the inhibitory effects were consistent with their free radical scavenging activities. These flavonoids dose dependently inhibited $ONOO^-$-induced protein oxidation, while they ineffectively suppressed hemoglobin/$NaNO_2$/H_2O_2-triggered protein oxidation. These results mean that $ONOO^-$ and hemoglobin/$NaNO_2$/H_2O_2 can cause plasma protein nitrative and oxidative damage in different pathways, and those flavonoids with strong antioxidant activities may contribute their protective effect partly through inhibiting protein nitration.

Keywords: flavonoids; plasma; protein oxidation; protein tyrosine nitration

1. Introduction

Flavonoids are polyphenolic compounds present in plants, fruits, and vegetables [1,2]. They have many beneficial health effects because of their antioxidant activities, including metal chelation, free radical scavenging, and lipid peroxidation inhibition [2,3]. Evidence for the potential antioxidant activities suggests that the dietary intake and the therapeutic use of flavonoids can be associated with significant health benefits [4,5]. In France, the low incidence of heart disease and arteriosclerosis has been ascribed to a higher intake of flavonoids from red wine [4]. Many flavonoids were found to be better antioxidants than the antioxidant nutrients such as vitamin C, vitamin E, and β-carotene on a mole-for-mole basis [1].

Exposure of proteins to reactive oxygen species or reactive nitrogen species results in oxidative modifications of amino acid residues, altering the protein structure and function [6,7]. Among these post-translational protein modifications, the production of 3-nitrotyrosine (3-NT) has been used as a biomarker of nitric oxide-mediated oxidative stress [6]. Over the past several

*Corresponding author. Email: danli0909@163.com

ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286021003620226
http://www.informaworld.com

years, substantial evidence has been accumulated that the major pathways of protein tyrosine nitration *in vivo* include peroxynitrite ($ONOO^-$) and hemoglobin/$NaNO_2$/H_2O_2-dependent reaction in which free radicals and iron catalysis are involved [6]. The nitration of proteins modulates catalytic activity, cell signaling, and cytoskeletal organization [7]. Meanwhile, oxidative damage of protein is always accompanied by the formation of protein carbonyl groups, which has been widely used as a marker of protein oxidation [8]. Evidence for the presence of nitration and oxidation of proteins and their roles in physiology and pathology is increasing [6–9].

Due to the deleterious effect of nitration and oxidation of proteins, the utilization of flavonoids has become the usual strategy to inhibit free radical-mediated oxidative and nitrative stress. (−)-Epicatechin has been found to efficiently protect against nitration of protein tyrosine residues by peroxynitrite, but exhibits relatively ineffective protection against the oxidation simultaneously [10]. Many studies paid attention to the defense effect of antioxidants against $ONOO^-$-mediated protein nitration and oxidation, which could be usually attributed to free radical scavenging [10]. However, few studies dealt with the effect of flavonoids on hemoglobin/$NaNO_2$/H_2O_2-triggered protein nitration and oxidation.

In order to emphasize the physiological relevance, human plasma was used as the substrate, to evaluate the ability of flavonoids in preventing the formation of 3-NT and carbonyl groups (biomarkers of nitrative and oxidative stress, respectively), which were measured by Western blotting. Two well-accepted nitrating models ($ONOO^-$ and hemoglobin/$NaNO_2$/H_2O_2) were applied, and six flavonoids (quercetin (Qu), kaempferol (Ka), (+)-catechin (Ca), baicalein (Ba), apigenin (Ap), and naringenin (Na)), belonging to different subfamilies, were used as the representatives of flavonoids (Figure 1). The study will not only give the relationship between antioxidant activity and protein nitration/oxidation, but also provide a new method for the primary evaluation of flavonoids as a plasma protector.

2. Results

2.1 Effects of different flavonoids on the 3-NT and carbonyl group residue formation

As shown in Figure 2(A)–(C), the treatment of plasma with hemoglobin (25 μM)/$NaNO_2$ (1 mM)/H_2O_2 (0.5 mM) or $ONOO^-$ (0.25 mM) resulted in the significant formation of 3-NT and carbonyl groups determined by Western blotting. The six tested flavonoids (Qu, Ka, Ca, Ba, Ap, and Na), at a dose of 10 μM, which was much lower than their toxic concentration, revealed different inhibitory effects on protein nitration. The inhibition order of flavonoids on hemoglobin/$NaNO_2$/H_2O_2-induced protein nitration was Qu, Ca > Ka, Ba > Ap, Na, thus Qu and Ca exhibited the more effective protection against $ONOO^-$-triggered protein nitration (Figure 2(A)–(C)).

In contrast to their roles in protein nitration, these flavonoids were relatively ineffective in protecting plasma proteins from hemoglobin/$NaNO_2$/H_2O_2-induced oxidative damage, and protein oxidation was slightly aggravated in the presence of flavonoids (Figure 2(A),(C)). However, all tested flavonoids could significantly inhibit $ONOO^-$-triggered protein carbonyl formation (Figure 2(B),(C)).

2.2 Effects of flavonoids with different concentrations on the 3-NT and carbonyl group residue formation

In the presence of flavonoids at different concentrations (0.01, 0.03, and 0.1 mM), the dose-dependent reduction of plasma protein nitration was observed in both *in vitro* models (Figure 3(A)–(C)). Flavonoid pretreatment dose dependently prevented the formation of protein carbonyl groups

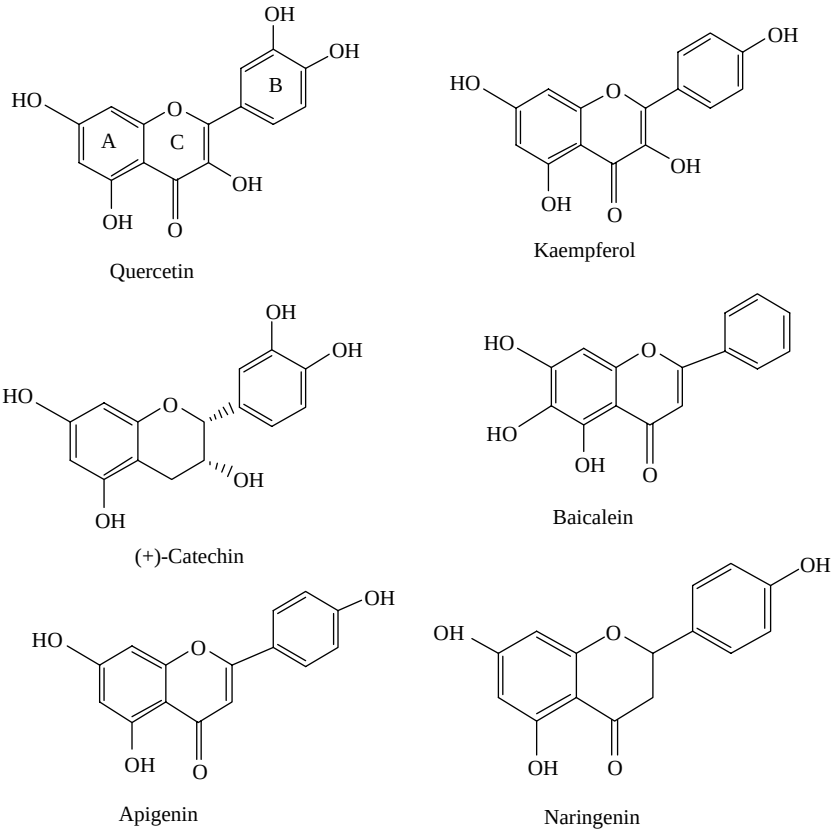

Figure 1. The chemical structures of the tested flavonoids.

induced by $ONOO^-$ (Figure 3(B),(C)), while they, to some extent, exhibited pro-oxidant effect in hemoglobin/$NaNO_2$/H_2O_2-induced protein oxidation (Figure 3(A),(C)).

2.3 Effects of flavonoids on scavenging 1,1-diphenyl-2-picrylhydrazyl radical

Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical has been applied to measure the antioxidant activities of most flavonoids. In the present study (Figure 4), the DPPH radical scavenging order of the six tested flavonoids was Qu, Ca > Ba, Ka ≫ Ap, Na.

3. Discussion

For a period of time, 3-NT was thought to be a biomarker of the existence of $ONOO^-$, and, hence, protein tyrosine nitration was the footprint of $ONOO^-$ formation in biological systems [6,7]. However, later studies demonstrated that some hemoproteins such as hemoglobin and myoglobin could catalyze $NaNO_2$/H_2O_2-dependent nitration of tyrosine to yield 3-NT [11–13]. The possible mechanism is that hemoprotein catalyzes the oxidation of nitrite to nitrogen dioxide (NO_2) which reacts with tyrosine radical ($Tyr^{\cdot}$) to form 3-NT [6]. As a hemoprotein-rich tissue, plasma is one of the major organs which are prone to oxidative damage, especially under inflammation [6,9,14].

It has been found that flavonoid intake was significantly associated with a decrease in various diseases because of their antioxidant activities [3–5].

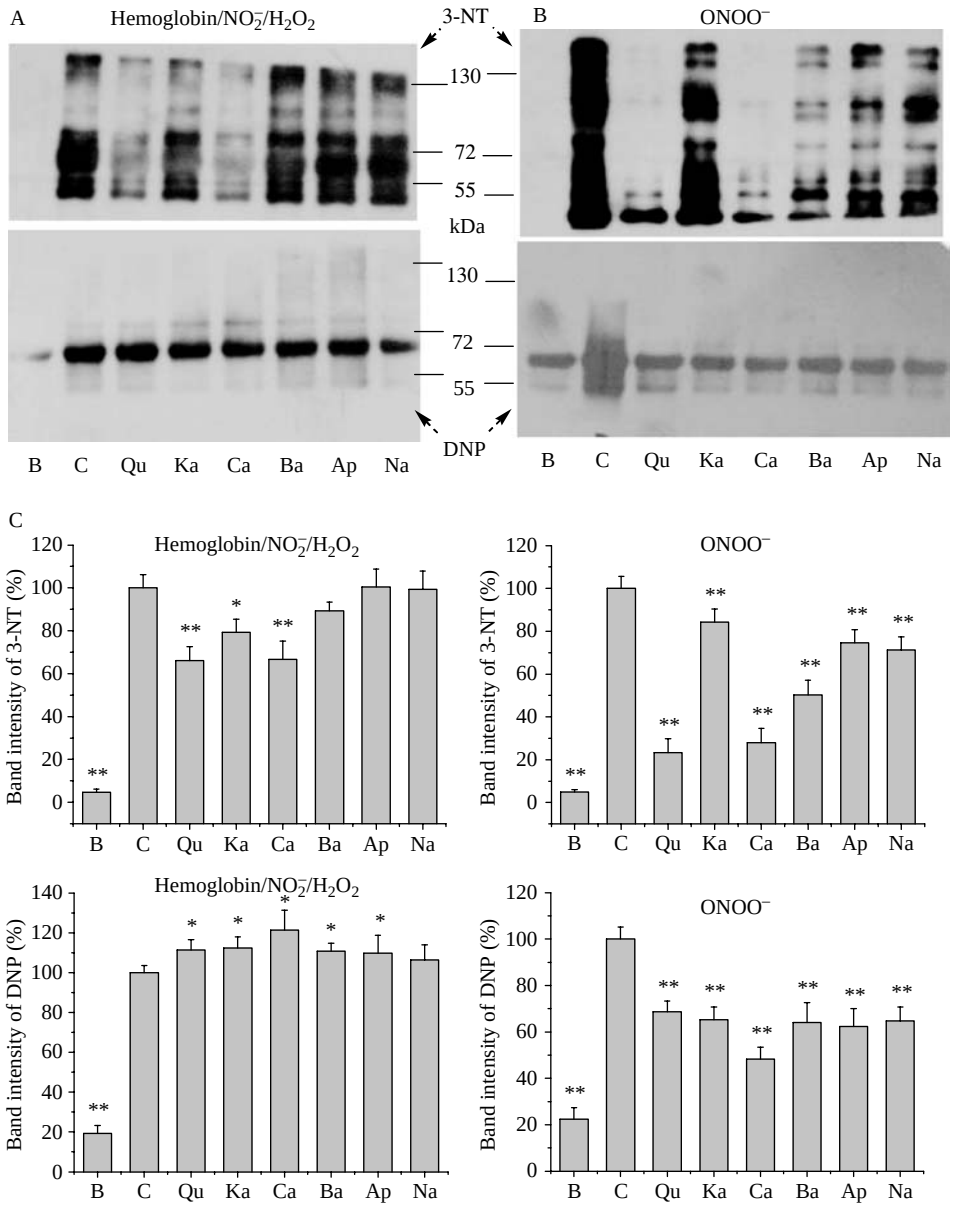

Figure 2. Effect of different flavonoids on plasma protein nitration and oxidation induced by (A) hemoglobin/NO_2^-/H_2O_2 or (B) $ONOO^-$. Detection of 3-NT and carbonyl group residues by Western blotting with antibody against 3-NT or DNP in plasma proteins. Plasma was treated with hemoglobin (25 μM)/$NaNO_2$ (1 mM)/H_2O_2 (0.5 mM) or $ONOO^-$ (0.25 mM; control), and in the presence of 0.01 mM flavonoids. Blank represented the untreated plasma. (C) The corresponding densitometry analysis of protein bands ($^{**}P < 0.01$, $^{*}P < 0.05$ vs. control group).

The protective effects of flavonoids may represent a defense against oxidative and nitrative damage [15]. It was well documented that flavonoids could protect against $ONOO^-$-mediated protein nitration and oxidation reactions [10,16], but the effect of flavonoids on hemoglobin/NO_2^-/H_2O_2-induced nitrative and oxidative

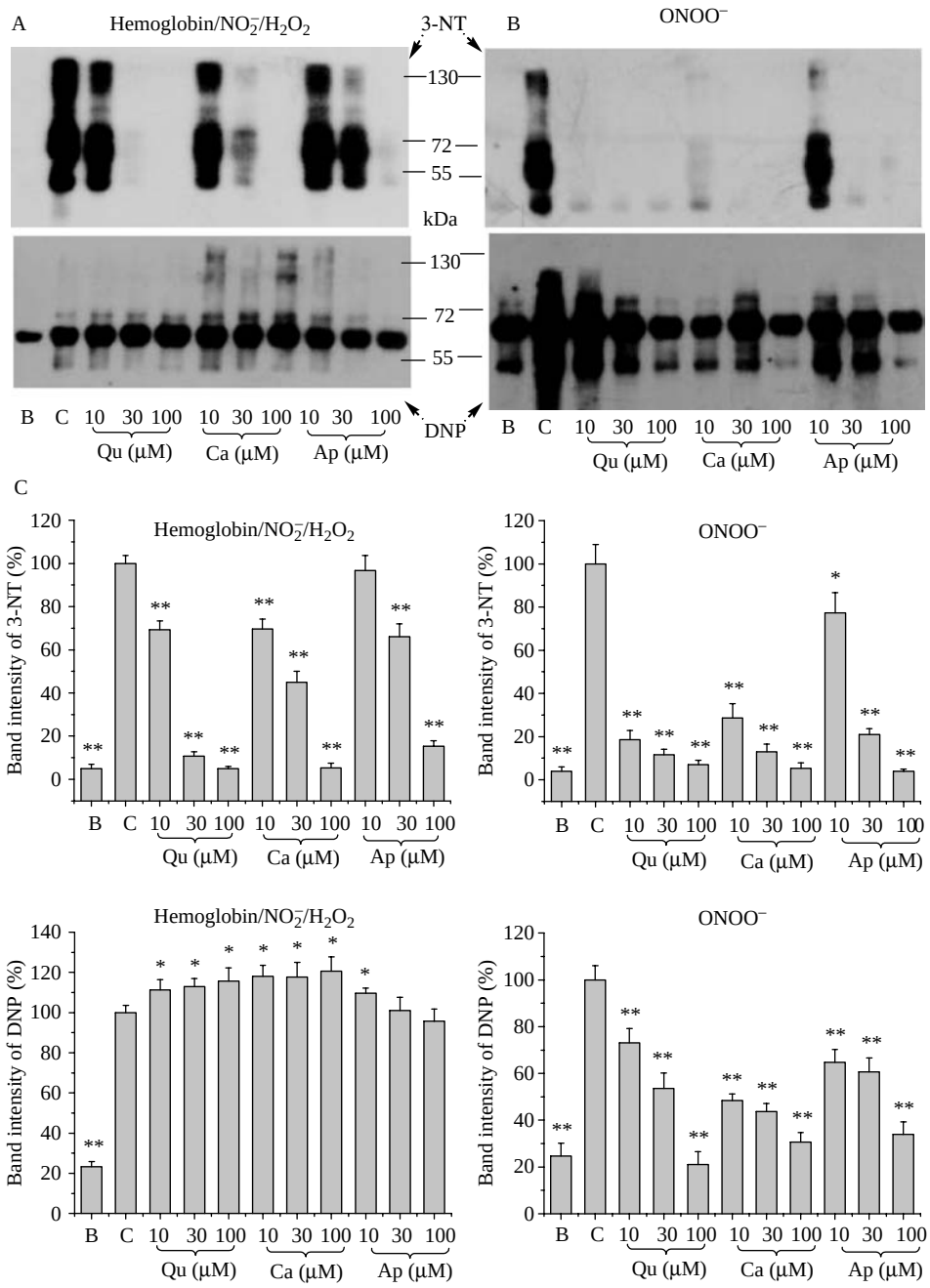

Figure 3. Effect of flavonoids with different concentrations on plasma protein nitration and oxidation induced by (A) hemoglobin/NO_2^-/H_2O_2 or (B) $ONOO^-$. Detection of 3-NT and carbonyl group residues by Western blotting with antibody against 3-NT or DNP in plasma proteins. Plasma was treated with hemoglobin (25 μM)/$NaNO_2$ (1 mM)/H_2O_2 (0.5 mM) or $ONOO^-$ (0.25 mM; control), and in the presence of 0.01, 0.03, and 0.1 mM flavonoids. Blank represented the untreated plasma. (C) The corresponding densitometry analysis of protein bands ($^{**}P < 0.01$, $^{*}P < 0.05$ vs. control group).

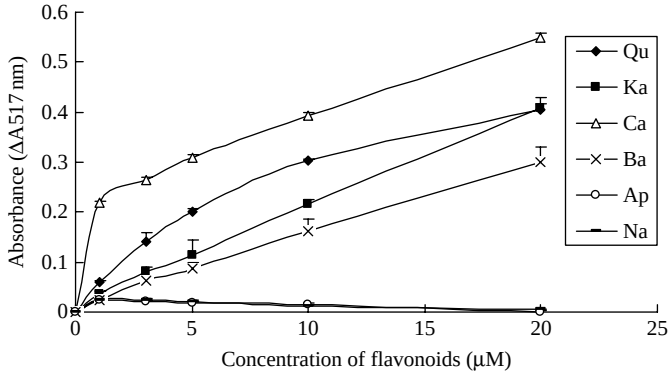

Figure 4. Effects of flavonoids on scavenging DPPH radical. After adding different concentrations of flavonoids to 2.5 ml diluted DPPH-ethanol solution, the absorbance reading was taken exactly 10 min after initial mixing. Values are mean ± SD of three determinations.

injury was relatively unknown. So, we set out to elucidate the anti-nitration and antioxidation activities of flavonoids in the two well-accepted nitrating models. Regardless of the nitration pathways, protein tyrosine nitration was efficiently inhibited by these antioxidants when the concentration was high enough. The decrease in 3-NT formation in proteins may be due to the metal-chelating and competitive reaction of tyrosyl radical with flavonoids [10,16]. Those flavonoids with higher hydrogen donation abilities, or to be more clear, with higher free radical scavenging abilities, could be more efficient on inhibiting 3-NT formation. The abilities of flavonoids to scavenge radicals are influenced by the presence of a functional group in their structure, mainly the B-ring catechol, the 3-hydroxyl group, and 2,3-double bond conjugated with the 4-oxo function [2,3]. In the present experiment, the inhibitive abilities of flavonoids on protein nitration were similar to their DPPH radical scavenging abilities. Among the six tested flavonoids, Qu and Ca were the most effective. This order is consistent with the conclusion that the ortho-hydroxyl structure in the B ring of flavonoids would show more potent effect on free radical scavenging and inhibiting protein nitration.

Besides the inhibitory effects of flavonoids on protein nitration, to further compare the different effects of flavonoids against oxidative damage, the effects of the corresponding flavonoids on protein oxidation were also studied. The effects of flavonoids on protein oxidation were different between the two models. Flavonoids dose dependently prevented the formation of protein carbonyl groups induced by $ONOO^-$, which was well explained by its reaction with peroxynitrite-derived radicals (such as $^{\cdot}OH$, $^{\cdot}NO_2$). In the case of hemoglobin/$NaNO_2$/H_2O_2-induced oxidative damage, flavonoids enhanced plasma protein carbonyl formation more or less. In contrast to their antioxidant activity, phytophenolics also have the potential to act as pro-oxidants under certain conditions [17,18]. Usually, these pro-oxidant effects involve interactions of polyphenols with transition metal ions. Oxidation of polyphenols produces $O_2^{\cdot-}$, H_2O_2, and a complex mixture of semiquinones and quinones, all of which are potentially cytotoxic. In general, the more the hydroxyl substitutions exist, the stronger the antioxidant and pro-oxidant activities are [19]. Our results indicated that the effects of flavonoids on inhibiting protein nitration

and promoting hemoglobin-induced protein oxidation were closely related to their antioxidant activities.

In this study, $ONOO^-$ and hemoglobin/$NaNO_2/H_2O_2$ were used as nitrative and oxidative agents to cause human plasma protein nitration and oxidation which were related to free radical formation. The six tested flavonoids had the ability to effectively inhibit plasma protein nitration in both nitrating models due to the free radical scavenging or chelating action, while these antioxidants decreased the formation of plasma protein carbonyl groups induced by $ONOO^-$. However, flavonoids slightly enhanced plasma protein carbonyl formation when hemoglobin/$NaNO_2/H_2O_2$ was used. These results mean that $ONOO^-$ and hemoglobin/$NaNO_2/H_2O_2$ can cause plasma protein nitration and protein oxidation in different pathways. The research of antioxidant flavonoids that prevent protein tyrosine nitration from oxidative/nitrative injury may offer a unique therapeutic option in disease prevention and health promotion, and the flavonoids may be supplemented from dietary and medicine intake.

4. Materials and methods

4.1 Reagents

Ba, Ca, methemoglobin, 2,4-dinitrophenylhydrazine (DNPH), rabbit polyclonal antibody against NT and 2,4-dinitrophenylhydrazone (DNP) were purchased from Sigma-Aldrich (St Louis, MO, USA). Qu, Ka, Ap, and Na were purchased from Shaanxi Huike Botanical Development Co., Ltd (Xi'an, China), and recrystallized in methanol; the purities of the flavonoids were more than 98% based on HPLC analysis.

4.2 Sample treatment

Human blood from a healthy volunteer was collected and centrifuged (1000*g*, 15 min) to obtain plasma. Samples of human plasma were pre-incubated (5 min at 37°C) with the tested flavonoids (at final concentrations of 0.01, 0.03, and 0.1 mM, the same below), and then treated with cold $ONOO^-$ (0.25 mM) or hemoglobin (25 μM)/$NaNO_2$ (1 mM)/H_2O_2 (0.5 mM) at 37°C for 30 min. The final concentration of plasma protein was 1.2 mg/ml. The obtained reaction mixtures were used in latter assays.

4.3 Western blotting analysis for protein nitration (3-NT) and protein oxidation

For the detection of protein oxidation, the carbonyl groups in proteins were first derivatized with DNPH, resulting in the formation of DNP. The reaction was stopped with the addition of neutralization solution, and then the DNP-derivatized proteins were subsequently separated by electrophoresis.

For the detection of protein tyrosine nitration, samples were directly mixed with loading buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to a nitrocellulose membrane, and then immunoblotted with a rabbit polyclonal antibody against 3-NT or DNP. The antibody was detected using an anti-rabbit secondary antibody conjugated with horseradish peroxidase. Chemiluminescence was used to identify specific proteins according to the ECL system.

4.4 Antioxidant activity assay on flavonoids

The free radical scavenging capacity of the compound tested was determined by DPPH [20]. After adding different concentrations of flavonoids to 2.5 ml DPPH solution (90 μM in ethanol), the total volume was adjusted to 3 ml and mixed thoroughly, and the absorbance was recorded at 517 nm exactly at 10 min.

The decrease in the solution absorbance, due to proton donating activity by flavonoids, was measured as the antioxidant activity of the tested compound.

4.5 Statistical analysis

All data were expressed as the mean ± SD of three independent experiments. Significance was assessed by using Student's *t*-test ($P < 0.05$ as significant).

References

[1] C.A. Rice-Evans, N.J. Miller, G.P. Bolwell, P.M. Bramley, and J.B. Pridham, *Free Rad. Res.* **22**, 375 (1995).
[2] B.H. Havsteen, *Pharmacol. Ther.* **96**, 67 (2002).
[3] K.E. Heim, A.R. Tagliaferro, and D.J. Bobilya, *J. Nutr. Biochem.* **13**, 572 (2002).
[4] M.B. Katan, *Am. J. Clin. Nutr.* **65**, 1542 (1997).
[5] J.W. Erdman, Jr., D. Balentine, L. Arab, G. Beecher, J.T. Dwyer, J. Folts, J. Harnly, P. Hollman, C.L. Keen, G. Mazza, M. Messina, A. Scalbert, J. Vita, G. Williamson, and J. Burrowes, *J. Nutr.* **137**, 718s (2007).
[6] G. Peluffo and R. Radi, *Cardiovasc. Res.* **75**, 291 (2007).
[7] F.J. Schopfer, P.R.S. Baker, and B.A. Freeman, *Trends Biochem. Sci.* **28**, 646 (2003).
[8] I. Dalle-Donne, R. Rossi, D. Giustarini, A. Milzani, and R. Colombo, *Clin. Chim. Acta* **329**, 23 (2003).
[9] A. Ceriello, F. Mercuri, L. Quagliaro, R. Assaloni, E. Motz, L. Tonutti, and C. Taboga, *Diabetologia* **44**, 834 (2001).
[10] P. Schroeder, L.O. Klotz, D.P. Buchczyk, C.D. Sadik, T. Schewe, and H. Sies, *Biochem. Biophys. Res. Commun.* **285**, 782 (2001).
[11] A. Grzelak, A. Balcerczyk, A. Mateja, and G. Bartosz, *Biochim. Biophys. Acta* **1528**, 97 (2001).
[12] K. Kilinc, A. Kilinc, R.E. Wolf, and M.B. Grisham, *Biochem. Biophys. Res. Commun.* **285**, 273 (2001).
[13] N. Lu, Y. Zhang, and Z. Gao, *Toxicol. In vitro* **23**, 627 (2009).
[14] E. Gussakovsky, O. Jilkina, Y. Yang, and V. Kupriyanov, *Anal. Biochem.* **382**, 107 (2008).
[15] M. Bixby, L. Spieler, T. Menini, and A. Gugliucci, *Life Sci.* **77**, 345 (2005).
[16] M. Sadeghipour, R. Terreux, and J. Phipps, *Toxicol. In Vitro* **19**, 155 (2005).
[17] B. Halliwell, *Arch. Biochem. Biophys.* **476**, 107 (2008).
[18] L. Mira, M.T. Fernandez, M. Santos, R. Rocha, M.H. Florencio, and K.R. Jennings, *Free Rad. Res.* **36**, 1199 (2002).
[19] G. Cao, E. Sofic, and R.L. Prior, *Free Rad. Biol. Med.* **22**, 749 (1997).
[20] F. Nanjo, K. Goto, R. Seto, M. Suzuki, M. Sakai, and Y. Hara, *Free Rad. Biol. Med.* **21**, 895 (1996).