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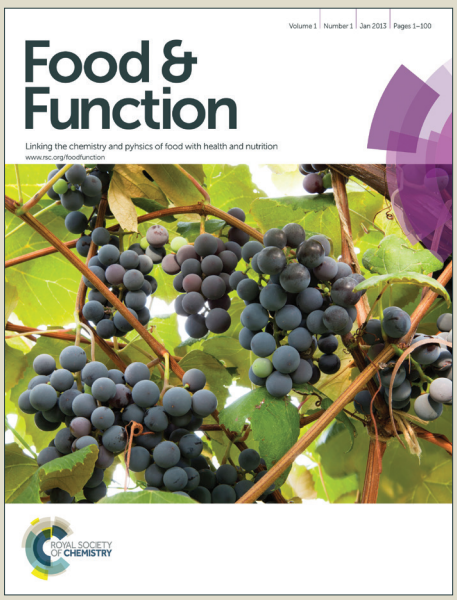

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ARTICLE

Effects of rosmarinic acid on liver and kidney antioxidant enzymes, lipid peroxidation and tissue ultrastructure in aging mice

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Rosmarinic acid (RA), a natural polyphenol, was isolated from *Rosmarinus. officinalis* L. The aim of this study was to evaluate the effects of rosmarinic acid on liver and kidney antioxidant enzymes and tissue ultrastructure in aging mice. RA was administrated at a dosage of 50, 100 or 200 mg.kg^{-1} once a day with a normal control group and an aging control group for 30 days. Liver and kidney were harvested for antioxidant enzymes activities and histological assessments. RA produced significant ($p < 0.05$ or $p < 0.01$) increases in the activity of superoxide dismutase (SOD) catalase (CAT) and glutathione peroxidase (GSH-Px) with a decrease in malondialdehyde (MDA) at 200 mg.kg^{-1} compared to the aging control. Histopathological study showed RA may induce significant structural changes in liver and kidney tissues at 200 mg.kg^{-1}. Results in this study demonstrate that RA has the potential for promoting *in vivo* antioxidant enzymes activity.

Introduction

In living organisms, the oxidative stress is generally associated with the generation of reactive oxygen species (ROS), such as O^{2-}, OH · and H_2O_2. [1] These highly reactive compounds have different molecular targets in cells, e.g., lipids or proteins, [1, 2] which cause the levels of antioxidant cellular defences systems were decreased. And this imbalance is usually accompanied with the development of various chronic diseases, for instance, carcinogenesis, coronary heart disease, and rheumatoid arthritis. [3]

Oxidative damage caused by ROS may be the major cause of the aging process. [4] To protect cells from ROS, living organisms possess antioxidant mechanisms, which are classified in enzymatic, e.g. SOD, CAT, GSH-Px; and non-enzymatic systems, e.g. vitamins, minerals and polyphenols. [5]

Antioxidant enzymes are capable of eliminating reactive oxygen species and lipid peroxidation products. During aging many physiological functions appear to change, due to decreased levels of antioxidant defences, such as SOD, CAT and GSH-Px, whereas MDA, a good indicator of lipid peroxidation, extremely increased in all cases, of which SOD and CAT are considered primary enzymes since they are involved in the direct elimination of ROS. [6] SOD is an important defence enzyme and scavenges superoxide anion form H_2O_2 and hence diminishes the toxic effects due to this radical or other free radicals derived from secondary reaction. [7] CAT is a hemoprotein, which catalyses the reduction of hydrogen peroxides [8] and known to be involved in detoxification of H_2O_2 concentrations [7]. GSH-Px catalyses the reduction of hydrogen peroxide to molecular oxygen and water, with the simultaneous conversion of reduced glutathione to oxidised glutathione. [9] Consequently, exogenous antioxidants that scavenge ROS may be beneficial in such a case.

Rosmarinic acid is a natural polyphenolic compound extracted from *Rosmarinus officinalis* L, a famous aromatic plant. Presence of phenolic -OH groups (see Fig. 1) play important roles in antioxidant activities and have been demonstrated by Rice-Evans *et al*. [10] Many experiments have reported the strong capacity of RA scavenging the free radicals, which showed that the antioxidant activity is over three times than trolox (water dissolved derivative of α-tocopherol) that rosmarinic acid can inhibit the activity of Xanhine Oxidase, and it is used to scavenge the surplus free radicals in the body. [11] Additionally, RA has preventive effects in the mouse model of ALS, [12] and other biological activities such as antiviral, antibacterial, anti-inflammatory and antifungal. [13-17] The biological activities of RA have been reviewed recently by Bulgakov *et al*.[18]

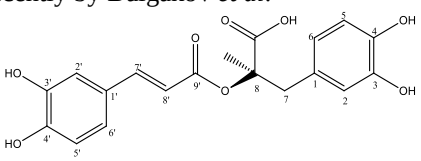

Fig. 1 Structure of rosmarinic acid.

Sometimes endogenous antioxidants are not sufficient to protect the body from damage caused by ROS induced oxidative stress. Hence, in recent years, there has been an upsurge of interest in the therapeutic potentials of natural products, as antioxidants in reducing free radical induced tissue injury. [19, 20] The antioxidation process of RA against biomolecule oxidation, such as lipid peroxidation, is very

attractive for clarifying not only its anti-oxidation mechanism of polyphenolic antioxidants, but also its relation to its various bioactivities. In the present study, the *in vivo* antioxidant activity of RA was evaluated by measuring the changes in activities of antioxidant enzymes including SOD, GSH-Px, CAT and the values of the MDA in liver and kidney in aging mice. Moreover, the ultra-microstructure of liver and kidney was assessed under transmission electron microscope (TEM).

Materials and methods

Materials and regents

Fresh leaves of *R. officinalis* L. were harvested from Fuyang rosemary base of Zhejiang HiSun Pharmaceutical Co., China in September, 2012. Rosmarinic acid standard was purchased from Sigma-Aldrich Co. (St. Louis, MO). Bovine serum albumin (BSA) protein Assay Kit, SOD Assay Kit, CAT Assay Kit, GSH-Px Assay Kit and MDA Assay Kit were purchased from Institute of Biological Engineering of Nanjing Jiancheng, Nanjing, China. All other reagents and solvents were either of HPLC or analytical grade.

Preparation of rosmarinic acid

Leaves of *R. officinalis* were shade dried and powdered. 100 g of powder was placed in a round-bottom flask with 1L of deionised water. The solution was steam-distilled for 60 min in a Clevenger-type apparatus for essential oil isolation. The mixture was centrifuged at 2000 g for 15 min at room temperature (LXJ-IIB, Shanghai, China), then the supernatant was filtered by filter paper and the filtrates were concentrated under vacuum at 60 ℃ to a volume of about 100 mL and extracted with ethyl acetate. This procedure was repeated three times, and then the combined ethyl acetate phases were concentrated in a rotary evaporator (Shanghai Qingpu Huxi Instrument Factory, Shanghai, China) at 50 ℃ and the residue were chromatographed on a silica gel column, eluting with ethyl acetate: *n*-hexane (2:1, v/v), and to give rosmarinic acid (RA) 0.46 g.

HPLC analysis

The content of RA was analysed quantitatively by HPLC on a Jasco HPLC system equipped with a UV detector (Jasco International Co. Ltd., Tokyo, Japan). Analyses were performed on a Curosil-PFP (4.6 mm×250 mm, 5 μm) reversed-phase column (Phenomenex Inc., Torrance, CA). The UV detector was set at the wavelength of 330 nm. The mobile phase was methanol: 0.5% phosphoric acid (50:50, v/v) at a flow rate of 1 $mL.min^{-1}$ and the injection volume was 10 μL.

Animal treatment

Male Kunming mice including forty healthy aging (20 months old, 47.56 ± 3.61 g) and ten young negative control mice (6 weeks old, 20 ± 2 g) were purchased from the Shanghai SLAC Laboratory Animal Co. LTD of China. Mice were kept in individual metal cages at room temperature of 25 ℃ and at 45-55% relative humidity with a 12-hour light-dark cycle. The mice were fed food and water ad libitum. They were kept in those conditions for a 7-day period of adaptation prior to start of the experiment. Aging mice were then randomly divided into 4 groups of ten animals each. The aging mice were i. g. administrated RA which extracted by ourselves (50, 100 and 200 $mg.kg^{-1}$, dispersed in 0.9% saline) once a day for 30 days, respectively. The aging control group (AC group) and normal control group (NC group) were intragastric gavage (i. g.) administered with 0.2 mL of saline each. This study was carried out with national standard "Laboratory Animal-Requirements of Environment and Housing Facilities" (GB 14925-2001), and the care of the animals and the animals experimental operation have conforming to "Beijing Administration Rule of Laboratory Animal", et al.

Tissue collection

After 24 h of 30th day treatment the animals were sacrificed by cervical dislocation and liver and kidney were harvested. The liver and kidney were cut into pieces in ice-cold saline, and then tissue homogenate (10%, w/v) were prepared and centrifuged at 2000 g for 10 min (1-15k, Sigma, Osterode, Germany) and the supernatant was used for determining SOD, GSH-Px and MDA levels. The homogenate (10%, w/v) was then further diluted 1:10 in normal saline and centrifuged at 2000 g for 10 min (1-15k, Sigma, Osterode, Germany) and the supernatant was used for determining CAT levels. Tissue protein content was determined with a bovine serum albumin (BSA) protein Assay Kit, with BSA as the standard. SOD, GSH-Px, CAT activity and MDA levels were measured following the instructions on the kits.

Histological assessment

Fresh liver and kidney were immediately cut into small pieces after removal (about 1×1×1 mm), fixed in 3% (v/v) glutaraldehyde for 1 h and rinsed 3 times with phosphate buffer ($0.1mol.L^{-1}$, pH 7.4). The samples were then fixed in 1% (w/v) OsO_4 for 2 h, rinsed 2 times with phosphate buffer, and dehydrated with a graded acetone series. The samples were infused in epoxypropane/ Epon 812 (50%/50%) solution for 2 h and then infused in Epon 812 again for 2 h in vacuum condition. The samples were embedded in Epon 812 and reacted at 8 ℃ overnight. Dried samples were cut into ultra-thin sections by Ultra-microtome (Leica UCT, Wetzlar, Germany). Ultrathin sections were collected on copper grids, double stained with uranyl acetate and lead citrate, and observed in a Tecnai G2 electron microscope (FEI, Eindhoven, Netherlands) at an acceleration voltage of 80 kV.

Statistical analysis

Results were expressed as mean ± SD and *t*-test was performed to assess the statistical significance (SPSS 11.5 for Windows). Significance of differences between the control

and treatment groups was determined at level $p < 0.05$ or $p < 0.01$.

Results and discussion

Analysis of RA

A calibration curve was prepared by plotting the peak areas of the rosmarinic acid standard samples. The standard curve gave a linear response for the rosmarinic acid concentrations and the peak areas in the range from 0.5-2 $mg.mL^{-1}$ (y=4E+07x-25048, r = 0.9991). The content of RA was 98.1% calculated from a standard curve. RA was then kept in the dark at -20 ℃ until tested.

Effect of RA on antioxidant enzymes activities and lipid peroxidation of treated mice

The levels of MDA and the activities of SOD, CAT and GSH-Px in the liver and kidney of normal control, aging control, and RA treated groups are given in Table 1. It could be found that the liver and kidney SOD, CAT and GSH-Px activity in AC group were all decreased than those in NC group ($p < 0.05$ or no significant). In addition, the liver and kidney MDA level was increased than those in NC group ($p < 0.05$). As shown in Table 1, treatment of RA produced significant ($p< 0.05$ or $p < 0.01$) increase in the activity of SOD, CAT and GSH-Px in the liver and kidney, with an decrease in MDA levels in the group treated with 200 $mg.kg^{-1}$ of RA compared to the aging control. It is notable that the activities of SOD, CAT and GSH-Px, and MDA levels in the liver and kidney of mice administered 200 $mg.kg^{-1}$of RA were near to those in NC group (Table 1). These results indicated that RA could improve the activities of antioxidant enzymes and reduce the MDA levels in aging mice.

Table 1 Effect of RA on antioxidant enzymes activities and MDA levels.

Group	SOD (U.mg protein^{-1})	CAT (U.mg protein^{-1})	GSH-Px (U.mg protein^{-1})	MDA (nmol.mg protein^{-1})
Liver				
NC (Normal control)	177.14±6.48^{c}	9.58±0.33^{c}	475.88±42.14	1.65±0.13^{c}
AC (Aging control)	169.04±7.38	8.98±0.58	455.36±48.56	1.78±0.13
RA (50 $mg.kg^{-1}$)	169.18±6.80	9.15±0.40	454.48±29.66	1.69±0.07
RA (100 $mg.kg^{-1}$)	172.20±8.93	9.25±0.26	473.90±31.89	1.66±0.06
RA (200 $mg.kg^{-1}$)	179.22±13.46^{a}	9.54±0.34^{a}	489.57±43.18^{a}	1.61±0.04^{a}
Kidney				
NC (Normal control)	104.74±3.46^{c}	5.95±0.31	64.58±4.32	0.63±0.02^{d}
AC (Aging control)	100.82±3.55	5.80±0.27	60.27±5.48	0.67±0.04
RA (50 $mg.kg^{-1}$)	100.37±7.24	5.88±0.33	61.18±3.29	0.67±0.06
RA (100 $mg.kg^{-1}$)	103.18±2.07	5.98±0.27	62.64±3.95	0.65±0.04
RA (200 $mg.kg^{-1}$)	104.30±3.63^{a}	6.07±0.24^{a}	65.20±2.53^{a}	0.64±0.02^{b}

Data are mean ± SD (n = 10). $^{a}p<0.05$ *vs*. AC group, $^{b}p<0.01$ *vs*. AC group; $^{c}p<0.05$ *vs*. AC group, $^{d}p<0.01$ *vs*. AC group.

The antioxidant enzymes SOD, CAT and GSH-Px have complementary activities in the antioxidant defences system. [21] All the changes of enzyme activities in the cellular level contribute to the levels and/or activities of the same enzymes in the extracellular compartment. [22] MDA level is an index of lipid peroxidation. [23] The production of ROS increases the peroxidation of lipid. In our study, in normal control and RA treated mice, the lipid peroxidation was significantly lower, which indicates that RA is able to prevent lipid peroxidation by scavenging free radicals in aging mice. SOD is considered to be an inducible key enzyme; it catalyzes the dismutation of O^{2-} into oxygen and H_2O_2, thus decreases ROS generation and oxidative stress. [24] GSH-Px is considered to be an essential factor in defense against oxidative tissue damage and cellular function by reduction of H_2O_2 to H_2O. [25] Increased activities of SOD and GSH-Px have been reported to scavenge the superoxide radicals to protect the tissue damage by free radicals. [26] CAT is a heme containing enzyme catalyzing detoxification of H_2O_2 to water and oxygen. [27] According to the results in this study, a decrease in lipid peroxidation, i.e., decreased MDA level with concomitant increase in SOD, CAT and GSH-Px activity was observed both in liver and in kidney at a dose of 200 $mg.kg^{-1}$ of RA treatment compared to AC group. These results suggest that RA can exert its ROS protective effect during the process of mice aging.

RA is known to possess marked antioxidant properties as a reactive species scavenge. [28, 29] In our study, RA supplementation elevated SOD, CAT, and GSH-Px activity and reduced MDA production in liver and kidney. Meanwhile, RA can cause dose-dependent changes of antioxidant enzymes. [30] Soobratee *et al* mentioned that gallic acid and rosmarinic acid were the most potent antioxidants among the simple phenolic and hydroxycinnamic acids, respectively. The mechanism by which the rosmarinic acid exerts its antioxidant activity is probably due to its hydroxyl group's structure, but it is not the only factor in determining the potency of their activities. [31] The capacity to inhibit hydroxyl radical is based mainly on the combination of conjugated structures in the polyphenolic skeletons, especially the o-dihydroxyphenol or catechol structure, and also the presence of a carboxylic group. [32] Del Baño *et al*. demonstrated that the presence of two catechol structures conjugated with a carboxylic acid group in rosmarinic acid increases its antioxidant activity in aqueous media. [33] The authors also stress that the presence of a catechol group in the aromatic ring (C11-C12) of the rosemary phenolic diterpene skeleton is probably the most important structural element in the antioxidant activity of these compounds. In addition, [34] Renzulli *et al* showed that the antioxidant activity of rosmarinic acid is mainly due to its redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides.

Histopathological assessment

Histological assessment of hepatic tissues in AC group showed many lipid droplets accumulated in the cytoplasm, exerted slight swollen of the mitochondria and the mitochondrial cristaes fuse, reduce or disappear (Fig. 2A, 2B). In contrast, the ultra-structural picture of liver treated with RA revealed that changes were characterized by a considerable reduction of the lipid droplets in the cytoplasm. In addition, the cristaes of the mitochondria were arranged closely. (Fig. 2C, 2D). Liver

plays a key role in lipid metabolism. Hepatic histological damage refers to the excessive accumulation of lipids within hepatocytes due to the imbalance between lipid formation and lipid degradation. In this study, ultrastructure studies of liver indicated that the administration of RA can increases fatty degeneration of hepatocytes when it compared with the AC group. Cell ageing is accompanied with a change in a number of cellular biochemical parameter. Biochemical findings of MDA also showed significant decrease in its level after RA treatment in our studies. Dysfunctional mitochondria in aged mice are characterized by increased content of oxidation products of phospholipids, proteins and DNA, decreased membrane potential, and increased size and fragility. [35] RA treatment seems to be positive effect on the inner mitochondrial membrane. The inner membrane of the mitochondrion folds inwards, forming the cristae. This folding allows a greater amount of membrane to be packed into the mitochondrion.

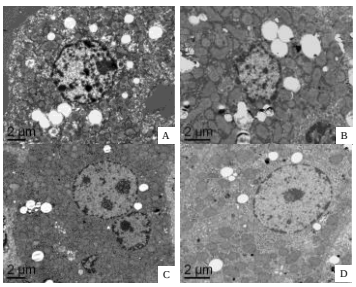

Fig. 2 Transmission electron micrograph of liver of AC group (A, B) and treated with RA (200 mg.kg^{-1} per day) (C, D) for 30 days. (A, C: Magnification ×4200; B, D: Magnification ×6000).

Histopathological studies of the kidney for the group treated with RA 200 mg.kg^{-1} per day revealed that treatment with RA induces structural alterations (Fig. 3C, 3D). Renal tissue of RA treated mice showed presence of scanty microvilli of variable height in renal tubular epithelium, whereas microvilli arranged orderly and closely in AC group (Fig. 3A, 3B). Renal tissue of RA treated mice also showed the changes in its subcellular distribution and shape of mitochondria. Besides that, renal tissue of RA treated mice showed increased population of polymorphic mitochondria with dense matrix.

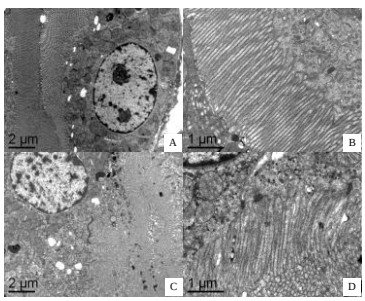

Fig. 3 Transmission electron micrograph of kidney of group AC (A, B) and treated with RA (200 mg.kg^{-1} per day) for 30 days (C, D). (A, C: Magnification ×6000; B, D: Magnification ×16500).

Oxidative stress plays a key role in the pathophysiology during the kidney aging, which induces the overproduction of ROS. [36] Matsushima *et al* have reported that free radical scavengers could prevent renal failure through attenuation of tubular damage, enhanced regenerative response of tubular cells and preservation of renal blood flow. [37] However, in this work, as shown in Fig. 3, several lysosomal bodies in renal tissue of RA treated mice were observed, which means the degenerative activity. That is, RA may have caused different degrees stress in the renal tissue. In this case, on the one hand RA treatment could increase the antioxidant enzymes activities in kidney; on the other hand it may be caused the degenerative change in renal tissue. Aging is a complex process that involves progressive, degenerative changes in multiple organ systems. Each assay has its own limitations and is based on single aspects of single organs, so a combination of assays results in comprehensive and specific information on the aging process should be considered in our next work.

Conclusion

RA effectively scavenged the free radicals, altered the antioxidant enzymes and inhibits lipid peroxidation *in vivo* studies. It may be helpful in preventing or slowing the progress of various oxidative stress-related aging.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Notes

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