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Full Length Research Paper

A pilot study for evaluation of antioxidant activity of resveratrol from *Polygonum cuspidatum* in senescence-accelerated mice

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The current study was undertaken to measure the antioxidant activity and free radical scavenging ability of resveratrol from *Polygonum cuspidatum* in senescence-accelerated mice (SAM). The SAM were administered with resveratrol by introgastric gavage at doses of 25, 50 and 100 mg/kg·bw for 2 months. The activities of the antioxidant enzymes namely superoxide dismutase (SOD), catalase (CAT) and oxidation products of malondialdehyde (MDA) in the serum and liver of the SAM were determined. Also, the gene expression of SOD in mice liver was investigated by real time reverse transcriptase–polymerase chain reaction (Real time RT-PCR). The results showed that the SOD and CAT activities in the serum and liver increased significantly, while the MDA level declined distinctly. Real time RT-PCR indicated that the change of SOD mRNA was the same as the modification of SOD activity in mice liver. In conclusion, this study demonstrated that resveratrol had potential antioxidant activities and provided scientific support for the empirical use of resveratrol as an antioxidant for various diseases caused by oxidative stress. Meanwhile, the SAM represented an appropriate animal model to evaluate the antioxidant activities of natural products.

Key words: Resveratrol, antioxidant enzymes, malondialdehyde, SOD mRNA, senescence-accelerated mice.

INTRODUCTION

The involvement of reactive oxygen and free radicals in the pathogenesis of many human diseases, including cancer, aging and coronary heart diseases is increasingly being recognized (Moskovitz et al., 2002). Reactive oxygen species (ROS) in the forms of superoxide anion ($\cdot O_2^-$), hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2) are constantly formed in the human body by normal metabolic processes or from exogenous factors and agents (Lih et al., 2001). The production of ROS in excess breaks a balance between oxidants and antioxidants in the body (Ames, 1979). Therefore, much attention has been focused on the use of antioxidants to protect the body from reactive oxygen species and free radicals. Natural sources of antioxidants has gained increasing interest in the safety and no toxic side effects as compared with the synthetic antioxidants (Claudio and Hector, 2000; Goli et al., 2005; Balasundram et al., 2006). In this concern, many sources of natural antioxidants have been investigated, including plants and microorganisms (Bandoniene et al., 2000; Arai et al., 2002). In conventional Chinese medicine, *Polygonum cuspidatum* is being incorporated as a primary component in numerous nutraceutical products due to its elevated intensity of trans-resveratrol. Trans-resveratral is a kind of polyphenolic trans-stilbene which is known to contribute largely to antioxidant potential. It protects against the molecular effects of lipid peroxidation, free radicals and ROS, and it also delays the progress of many chronic diseases (Lai et al., 2001; Gulcin et al., 2003). Resveratrol may have the inverse correlation between red wine consumption and incidence of cardiovascular disease reported in epidemiological studies (the French paradox). Therefore, resveratrol will

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play an important role as antioxidative agent. The advances of biomedical research depend to a considerable extent, on the availability of relevant and appropriate experimental animal models, particularly animals that have not been subjected to experimental manipulation (Toshio et al., 1997). This is especially true for an aging model since aging progresses are insidious and irreversible (Takeda et al., 1981, 1994). The SAM strains, consisting of 14 senescence-prone inbred strains (SAMP) with accelerated senescence and age-associated pathologies, and 4 senescence-resistant inbred strains (SAMR) with normal aging (Toshio, 1999), meet most criteria for the use of mammalian models for aging research such as life table data, short life span, genetic characteristics, etc. Mice have a innate advantage for studies of the age-associated pathologies and genetics of aging because of the richness of genetic information and the similarity of mouse and human genetics (Sprott, 1994).

Therefore, the SAM model, as a biogerontological resource, will play an important role in elaborating and solving various problems of aging. The aim of this pilot study was to evaluate the antioxidative effect of resveratrol at doses that are feasible for supplying more scientific evidences for the research and development of resveratrol in the field of pharmaceutical and functional foods.

MATERIALS AND METHODS

Resveratrol (purity ≥97%) was purchased from Tianjin Jianfeng Natural Product Co., Ltd, (Tianjin, China), dissolved in 0.1% Carboxymethyl, Cellulose Sodium (CMC) and stored at 4°C in the dark at concentrations of 25, 50 and 100 mg/ml.

Kits chemicals

Commercial kits used for determination of SOD, CAT and MDA were purchased from Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China). Commercial kits used for RevertAid^{TM}First Strand cDNA Synthesis (Fermentas, MBI) were purchased from Beijing Biomed Co., Ltd of Biotechnology (Beijing, China). The forward and reverse primer sequences for SOD and GAPDH were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The following chemicals were also used: Trizol (Invitrogen Corporation, Carlsbad, CA, USA); SYBR Green Real-time PCR Master Mix (Toyobo, Japan); Agarose (Promega Corporation Madison, WI, USA). All other chemicals and solvents were of analytical grade.

Experimental design

The senescence accelerated male mice (3-month-old, 28±2 g body wt) were used in this experiment. The mice were housed under normal laboratory conditions (22°C ±2°C, 12/12 h light-dark cycle) with free access to standard rodent chow and water *ad libitum*. After adaptation for a week, the mice were distributed randomly into five groups of 10 animals each: SAM-R1 in normal group (NG), SAM-P8 in model group (NG), SAM-R1 in model group (MG), SAM-P8 of low dose of resveratrol-treated group (RESL), SAM-P8 of moderate dose of resveratrol-treated group (RESM) and SAM-P8 of high dose of resveratrol-treated group (RESH). The NG and MG were allowed free access to standard feed, 0.1% CMC water; RESL, RESM and RESH were all allowed free access to standard feed, water and treated, respectively with resveratrol dissolved in 0.1% CMC by introgastric gavage at doses of 25, 50 and 100 mg/kg•bw.

After the 8-week treatment, mice were killed by cervical dislocation. The blood samples were collected for biochemical analysis. The liver was removed, weighed, immediately frozen in liquid nitrogen and then stored at -80°C for the measurement of SOD, CAT, MDA and Real-time RT-PCR for SOD mRNA expression analysis.

Identification and quantitation of the resveratrol from *P. cuspidatum*

The purified compound was identified and quantified by reverse phase high-performance liquid chromatography (HPLC). The HPLC system consisted of a Shimadzu HPLC (Model LC-20AT) equipped with ultraviolet detector (Model SPD-20A) (Shimadzu, Kyoto, Japan). Separation was performed on a Lanbo Kromasil C18 column (250 × 4.6 mm internal diameter, 5 μm) (Lanbo, Tianjin, China), using CH_3CN / H_2O = 43: 57 (v:v) as the mobile phase, at a flow-rate of 1.0 ml/min and an column temperature of 30°C. Resveratrol and the compound purified from *P. cuspidatum* were injected into HPLC in turn under the same conditions. The results (Figures 1 and 2) showed that the major component of the sample was resveratrol because the elution time for the peak that appears in Figures 1 and 2 was the same. The results showed that the percent purity of the sample was 97.30% as calculated from the peak height.

Determination of SOD activity

The determination of SOD activity was performed using commercial kits. The assay for total SOD activity was based on its ability to inhibit the superoxide anion free radical of O^{2-} generated by the xanthine-xanthine oxidase system (McCord and Fridowich, 1969). The SOD activity was expressed as U/mg protein.

Determination of CAT activity

CAT activity was measured spectrophotometrically at 405 nm by detecting H_2O_2 consumption (Naik et al., 2003). Whole blood was diluted 100 times prior to measurement. The tissue was homogenized with the buffer type used in homogenization and centrifuged at 2500 rpm for 10 min to obtain the supernatant. The reaction mixture contained 1 ml H_2O_2 and 2 ml test sample. The CAT activity was expressed as U/mg protein.

Determination of MDA level

The MDA levels of serum and liver were determined by using the Ohkawa (1979) technique following the instructions of commercial kits. This method was based on the reaction of thiobarbituric acid with malonyl dialdehyde (MDA). The absorbance was read at 532 nm. MDA levels were expressed as nmol /mg protein.

Measurement of SOD gene expression in the liver

Total RNA isolation and reverse transcription

Total RNA was isolated from the liver of mice by trizol reagent and

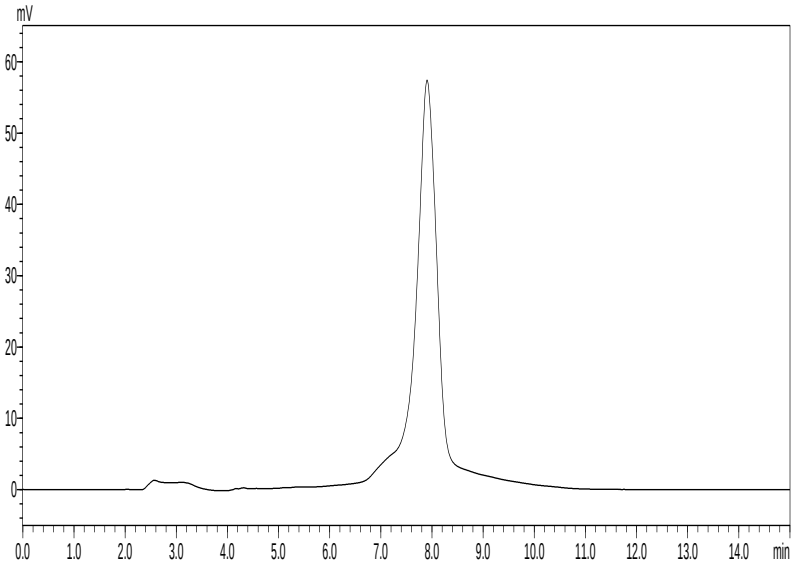

Figure 1. HPLC analysis of authentic resveratrol.

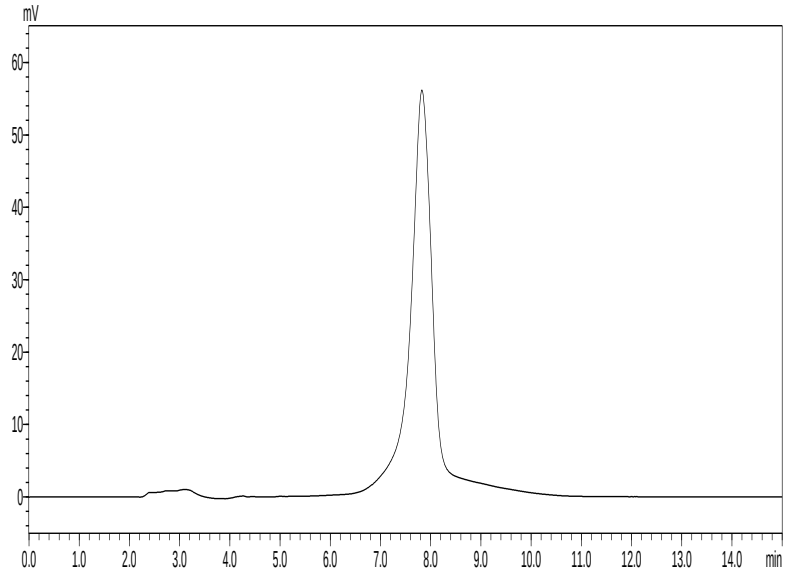

Figure 2. HPLC analysis of compound purified from *Polygonum cuspidatum.*

quantified by spectrophotometry. The integrity of the extracted total RNA was checked by agarose gel electrophoresis, and verified by the presence of the 28S, 18S rRNA bands. The quantity and purity of RNA was determined by absorbance reading at 260 and 280 nm. The ratio of OD_{260} to OD_{280} was higher than 1.8 to show a high purity of RNA. Total RNA was reverse transcribed into first-strand complementary DNA (cDNA) with RevertAid^{TM} FirstStrand cDNA Synthesis Kit (Fermentas, EU) (Mostafa et al., 2011).

Real-time RT-PCR for SOD mRNA expression analyses

Gene expression at mRNA level was analyzed with quantitative real-time RT-PCR method by SYBR green using an LightCycler System (Roche company, Switzerland). The sequences of the primers were designed using information contained in the public database in the GeneBank of the National Center for Biotechnology Information (NCBI). Primer sequences which were used for the GAPDH (the housekeeping gene) and SOD (the target gene) amplification are shown in Table 1. PCR reactions were performed under following conditions: Cycling was started with an activation step at 95°C for 30 s, and amplification program was repeated 40 times (denaturation, 95°C for 5 s; annealing at 60°C for 15 s, extension at 72°C for 15 s) with fluorescence measurement 72°C for 10 min (Mostafa et al., 2011).

The relative expression level of SOD gene in the liver of

Table 1. Sequences of primers designed for real-time PCR.

Primer	Forward (5'–3' orientation)	Reverse (5'–3' orientation)	Product size
GAPDH	GGAGAAACCTGCCAAGTATG	GGAGTTGCTGTTGAAGTCG	124 bp
SOD	CTCGTCTTGCTCTCTCTGGTC	CTTCTGCTCGAAGTGGATGG	130 bp

Table 2. Effect of different doses of Res on liver antioxidant status in mice.

Group	SOD (U/mg pro)	CAT (U/mg pro)	MDA (nmol/mg pro)
NG	150.10±7.83**	11.23±0.90**	0.53±0.03**
MG	125.29±6.93	7.45±0.69	0.83±0.02
RESL	130.49±7.23**	9.07±0.64*	0.73±0.03**
RESM	139.26±6.38**	10.09±0.76**	0.65±0.02**
RESH	148.87±6.27**	8.92±0.67	0.62±0.04**

Note: *: indicates p<0.05 when compared with model group, there was significant differences; **: indicates p<0.01when compared with model group, there was significant differences.

resveratrol-treated group to that of the vehicle group were calculated by $2^{-\Delta\Delta CT}$ method (Liva and Schmittgen, 2001). Delta CT is the difference in threshold cycle (CT) values corresponding to SOD and GAPDH genes and ΔΔCt is (ΔCt resveratrol treated) - (ΔCt vehicle treated). The quality and the corrected size of the PCR products were checked by electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide.

Statistical analysis

All calculations were performed by using SPSS 13.0 statistical software. Data were analyzed with one-way ANOVA. P-values <0.05 were considered to be statistically significant and P-values < 0.01 as highly significant. Results were expressed as means ±S.E.M.

RESULTS

Effects of resveratrol on antioxidative status and MDA level of mice liver

As shown in Table 2, the presented data showed a significant decrease in SOD activity in the MG as compared with the NG (P<0.01) in the liver. The resveratrol–treated groups had greater effect (P<0.01) than the MG.

The levels of CAT activity were lower in the MG than the NG (P<0.01) in the liver of mice (Table 2). Resveratrol-treated groups restored the CAT level more significantly than the MG, especially RESM treatment (P<0.01). However, ANOVA data did not show a significant difference from the RESH (P>0.05).

Resveratrol did significant decrease in MDA level of liver (Table 2) in the NG as compared with the MG (P<0.01). The effect of the resveratrol-treanted groups was stronger than that of the MG (P<0.01).

Effects of resveratrol on antioxidative status and MDA level of mice serum

As shown in Table 3, the SOD activity decreased remarkably in the MG as compared with the NG in the serum of mice (P<0.01). Resveratrol-treated groups restored the SOD activity more significantly than the MG, especially RESM and RESH treatment (P<0.01).

The presented data in Table 3 showed a significant decrease in CAT activity in the serum of the MG as compared with the NG (P<0.05). The RESL resulted in a remarkable elevation of SOD activity (P<0.05); RESM had greater effect than the MG (P<0.01).

The results in Table 3 demonstrated that the MDA level of serum was significant difference between the MG and the NG (P<0.01). The MDA level of MG was significantly higher than any resveratrol-treanted groups following (RESL, RESM and RESH, P<0.01).

Effects of resveratrol on the expression of SOD mRNA in the mice liver

The real-time PCR method using SYBR Green I fluorescence dye has been successfully applied for the quantity of SOD gene expression. It demonstrated single bands corresponding to the expected product sizes of cDNAs for SOD (130 bp) and GAPDH (124 bp). As shown in Table 4, the ratio of SOD to GAPDH was significantly greater in the resveratrol-treated groups as compared with the MG in mice liver. The result showed that resveratrol-treated groups expressed high levels of SOD mRNA. This increase was produced in a dose-dependent manner. Change of SOD mRNA was the same as the modification of SOD activity by resveratrol in mice liver.

Table 3. Effect of different doses of Res on serum antioxidant status in mice.

Group	SOD (U/ml)	CAT (U/ml)	MDA (nmol/ml)
NG	102.09±2.48**	0.91±0.04*	3.00±0.22**
MG	68.32±2.36	0.69±0.03	4.89±0.25
RESL	85.54±1.63*	0.79±0.03*	4.12±0.20**
RESM	88.30±2.18**	0.82±0.04**	3.80±0.14**
RESH	99.02±2.45**	0.88±0.03	3.51±0.08**

Note: *: indicates p<0.05 when compared with model group, there was significant differences; **: indicates p<0.01when compared with model group, there was significant differences.

Table 4. Comparison of SOD gene expression levels in SAM liver.

Parameter	NG	MG	RESL	RESM	RESH
SOD/GAPDH	1.17±0.06**	0.65±0.05	0.96±0.07**	1.49±0.08**	1.67±0.07**

Note: *: indicates p<0.05 when compared with model group, there was significant differences; **: indicates p<0.01when compared with model group, there was significant differences.

DISCUSSION

Recent studies demonstrated that a number of plant products including polyphenols, flavonoids and terpenes and various plant extracts exerted an antioxidant action (Zhou and Zheng, 1991; Quinn and Tang, 1996; Seymour et al., 1996). Resveratrol has been reported to have many beneficial effects (Fremont, 2000), including potent antioxidative activity but low cytotoxicity *in vitro*.

In the present study, we expected resveratrol to show the similar effects in vivo due to their common free-radical scavenging activity. Free radicals can directly damage antioxidant enzymes such as SOD, and reduce their activities. SOD is a metalloenzyme containing either manganese (Mn) or copper (Cu) and zinc (Zn). SOD catalyzes the removal of superoxide radical O_2^-, which damage the cell membrane and biological structures (Posadas et al., 2009). In the present study, the activity of SOD was markedly increased in resveratrol-treated groups. This result showed that resveratrol had a significant antioxidant capacity.

Hydrogen peroxide plays a critical role in the process of aging. CAT, an enzyme enriched in hepatocytes and erythrocytes and predominantly located in peroxisomes, catalyzes dismutation of toxic hydrogen peroxide (Masaki et al., 1998). A high level of CAT activity has been shown to be protective against hydrogen peroxide toxicity. In this study, CAT activity in the serum and liver of SAM decreased remarkably in the MG as compared with the NG. The resveratrol-treated SAM increased CAT activity, further prevented the cells from the damaging effect of hydrogen peroxide.

Membranes and lipids are particularly susceptible to the oxidant process and to the peroxidative reaction induced by free radicals (Minotti, 1988). The MDA level is a secondary product of lipid peroxidation, and is used as an indicator of tissue damage (Ohkawa et al., 1979). In this study, the MDA level in the mice liver and serum of the NG was significantly lower than the MG. The resveratrol-treated groups had a significant effect. These data corroborate findings in earlier investigations where antioxidants and administration of a natural grape seed extract decreased lipid peroxidation in the brain of aged rats (Arivazhagan et al., 2000; Balu et al., 2005). The reduction of MDA level suggests that this compound resveratrol may have an antioxidant effect.

ROS may modulate the antioxidant enzyme activities by regulating the mRNA levels through activation of signaling pathways (Fulle et al., 2004). According to Franco et al. (1999), the induction of antioxidant enzyme mRNA levels coincides with increases in oxidative damage of proteins, supporting the postulated relationship between oxidative stress and antioxidant enzyme mRNA expression. In the present study, the level of SOD gene expression was quantified by the real-time PCR method (Simpson et al., 2000). The greatest advantage of this method is that it can be used with any pair of primers for any target, making its use less expensive than that of a probe. The increase of the quantity of SOD mRNA was more remarkable and obvious in resveratrol-treated groups. Similar changes were also observed in the activity of SOD in mice liver. This result showed that resveratrol had an effective antioxidant to increase the activity of SOD in mice liver which would be beneficial to neurons subjected to oxygen free radical damage.

In conclusion, this study shows antioxidant capacity of resveratrol from *P. cuspidatum* in senescence-accelerated mice (*in vivo*). The consumption of resveratrol increases the activities of superoxide

dismutase (SOD) and catalase (CAT) in the serum and liver of the mice, thus protecting the liver, and decreases the level of malondialdehyde (MDA) in the serum and liver of the mice, protecting this organ. Meanwhile, the quantity of SOD-messenger RNA was increased remarkably in resveratrol-treated groups. Further studies are required to determine the causes behind the distinct effects of the *P. cuspidatum* extract concentrations and it remains to be seen whether a similar protective effect can be demonstrated in aged humans. Thus, resveratrol from *P. cuspidatum* could be incorporated to the diet as a nutritional supplement, to augment the body's defenses against oxidative stress.

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