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Antioxidant Activity of Aspirin Eugenol Ester for Aging Model of Mice by D-Galactose

[1]Jianyong Li, [2]Yuanguang Yu, [1]Yajun Yang, [1]Xiwang Liu, [1]Jiyu Zhang, [1]Bing Li, [1]Xuzheng Zhou, [1]Jianrong Niu and [1]Xiaojuan Wei
[1]Key Lab of New Animal Drug Project, Key Lab of Veterinary Pharmaceutical Development, Ministry of Agriculture, Lanzhou, Institute of Husbandry and Pharmaceutical Sciences of CAAS, 730050 Lanzhou, Gansu Province, P.R. China
[2]Lanzhou Institute of Veterinary Sciences of CAAS, 730030 Lanzhou, P.R. China

Abstract: Antioxidant activity of Aspirin Eugenol Ester (AEE) for aging model of mice by D-galactose for 8 weeks was investigated. MDA and lipofuscin contents of mice serum and heart, liver were selected as indexes to reflect antioxidant activity. The results showed that the small doses of AEE can effectively remove free radicals caused by D-galactose and large doses of AEE can make free radicals level below the normal level. There were some relationships between dose and effect. Compared with precursor drug aspirin and eugenol, AEE also has stronger antioxidant activity.

Key words: Aspirin Eugenol Ester (AEE), antioxidant, mice, liver, heart, China

INTRODUCTION

Aspirin has been used as a drug for treatment of inflammation and fever for more than a century. The biochemical mechanism of action of aspirin has been described earlier (Vane, 1971; Flower *et al*., 1972; Vane and Botting, 1987). Aspirin produces its therapeutic (e.g., anti-inflammatory and analgesic) and side effect (e.g., gastrointestinal ulcers) via inhibition of Cyclooxygenase (COX) which is a key enzyme to catalyze prostaglandin formation (Vane, 1971). Recently, its use has been extended to prevention and treatment of cardiovascular diseases based on its anti-thrombotic action in platelets since inhibition of COX by aspirin blocks thromboxane A2 production which is crucial for blood clotting (Patrono, 1989). The anti-platelet effect of aspirin has been tested in various forms of coronary artery disease, pregnancy-induced hypertension and preeclampsia in angiotensin-sensitive primigravida at low dosage and showed positive results in most of the reports (Schoemaker *et al*., 1998; Wallenburg *et al*., 1986).

Eugenol is the main component of volatile oil extracted from dry alabastrum of *Eugenia caryophyllata* Thumb. Various therapeutic effects of eugenol have been demonstrated including antivirus, antibacteria, antipyresis, analgesia, anti-inflammation, anti-platelet aggregation, anticoagulation, antioxidation, anti-diarrhea, anti-hypoxia and antiulcer and inhibition of intestinal movement and arachidonic acid metabolism (Tragoolpua and Jatisatienr, 2007; Chami *et al*., 2005; Gill and Holley, 2004; Nagababu and Lakshmaiah, 1997; Hashimoto *et al*., 1988; Feng and Lipton, 1987; Raghavendra and Naidu, 2009). It is used to treat toothache, hepatopathy and gastrointestinal diseases.

However, the side effect of aspirin such as gastrointestinal damage is very serious and eugenol is irritative and vulnerable to oxidation. Chemically carboxyl group of aspirin and hydroxyl group of eugenol are responsible for these side effects and structural instability. Therefore, based on prodrug principal, aspirin and eugenol can be combined into Aspirin Eugenol Ester (AEE) with reduced side effect and increased therapeutic effect and stabilization. AEE is supposed to be decomposed into aspirin and eugenol by the enzymes after absorption which would show their original activities again and might act synergistically. The results of the acute toxicity and activities of AEE showed that the acute toxicity of AEE was less than the controls which was 0.02 times of aspirin and 0.27 times of eugenol. The subchronic toxicity of AEE showed that NOAEL of AEE was considered to be 50 mg/kg/day under the present study conditions. Moreover, its anti-inflammatory, analgesic and antipyretic effects were similar as aspirin and eugenol but lasted for a longer period (Li *et al*., 2010, 2011, 2012a, b; Ye *et al*., 2011). Thus, AEE is a promising drug candidate for treatment of inflammation, pain and fever and prevention of cardiovascular diseases with few side effects.

Corresponding Author: Jianyong Li, No. 335, Jiangouyan, Qilihe District, 730050 Lanzhou, China

Many studies have demonstrated that with animal age increasing and the vitality and quantities declining in endogenous antioxidant enzymes, aging and several diseases may be caused by reduction of the organism antioxidative capacity and the accumulation of lipid peroxidation product (Blander *et al*., 2003). Such as diabetes, cancer, Ischemia-Reperfusion Injury (IRI), Amyotrophic Lateral Sclerosis (ALS), Alzheimer's Disease (AD), artery Atherosclerosis sclerosis (As), Parkinson's Disease (PD), etc. are confirmed to be related with the free radical (Schulze-Osthoff *et al*., 1992; Sun, 1990; Granger, 1988; Ogata *et al*., 2000). Moreover, excessive amounts of free radicals may also cause easily intestinal mucosa damage and permeability increase and make digestive system function abnormal and enterogenous infection (Chen *et al*., 2006). Recently, it has been well known that local generation of various Reactive Oxygen Species (ROS) plays a significant role in the formation of gastric ulceration associated with NSAID therapy (Bandyopadhyay *et al*., 1999; Hassan *et al*., 1998). So, antioxidants may be used to prevent NSAIDs induced gastric ulcers. This experiment is mainly to study antioxidant activity of AEE through the aging model in order to clarify its mechanism in prevention and curing of many diseases with few side effects and provide the theory basis for its further clinical trials.

MATERIALS AND METHODS

Chemicals and reagents: Aspirin Eugenol Ester (AEE), transparent crystal (purity: 99.5% with RE-HPLC) was prepared in Key Lab of New Animal Drug Project of Gansu province, Key Lab of Veterinary Drug Development of Agricultural Ministry, Lanzhou Institute of Husbandry and Pharmaceutical Sciences of CAAS. Aspirin, eugenol and 2-Thiobarbituric acid were bought from Sigma-Aldrich company. CMC-Na (carboxyl methyl cellulose sodium), chloroform, methanol, quinine sulfate, sodium dodecyl sulfate, n-butyl alcohol, pyridine were supplied by Tianjin Chemical Reagent Company (Tianjin, China). D-galactose was supplied by Shanghai HeBao chemical Company, Ltd. (Shanghai, China). Phosphotungstic acid was supplied by China Ling Lake Chemical Reagent Company.

Animals: A total of 140 mice of both sexes with clean grade (Certificate No.: SCXK (Gan) 2008-0075), weighing 20~30 g were purchased from the animal breeding facilities of Lanzhou University (Lanzhou, China) and housed individually to allow recording of individual feed consumption and to avoid bias from hierarchical stress. They were housed in plastic Macrolon cages of appropriate size with stainless steel wire cover and chopped bedding. Light/dark regime was 12/12 h and living temperature is 22±2°C with relative humidity of 55±10%. Standard compressed mice feed from the animal breeding facilities of Lanzhou University and drinking water were supplied *ad libitum*. The study was performed in compliance with the guidelines for the care and use of laboratory animals as described in the US National Institutes of Health. Animals were allowed a week quarantine and acclimation period prior to start of the study.

Dosing: The high, medium and low-doses were selected as 9, 18 and 36 mg kg^{-1} BW, respectively based on recommending dose of preliminary studies in mice. AEE dose suspension liquids and aspirin dose suspension liquid were prepared in 0.5% of CMC-Na. Eugenol dose suspension liquid was prepared with tween-80 into 0.32 g L^{-1}. Mice were randomized into seven groups (n = 20 mice each, male:female = 1:1): three test groups, an aspirin group, an eugenol group, an aging model group and a normal vehicle group as control. With the exception of subcutanenous administration of physiological saline as 10 mL kg^{-1} in normal control group, all other groups were administrated subcutanenously in back with 10% D-galactose physiological saline as 10 mL kg^{-1}. AEE, aspirin and eugenol were administered intragastrically in each mice based on individual daily body weights once daily with the volume of 20 mL kg^{-1} for 8 weeks. Model group and control group were also administrated intragastrically with distilled water as the same method.

Study design: At 2 h after the final administration, blood was taken from the eyes of the mice in each group and the serum was separated. All mice from each group were euthanized by exsanguinations in femoral artery and then hearts and livers were taken in physiological saline and made into 10% of the homogenate by grinding. These homogenates were kept at −70°C refrigerator.

Lipofuscin measurement: About 1 mL 10% of the heart or liver homogenate was mixed with 1 mL Chloroform-methanol (v:v, 2:1), shaked for 3 min and centrifuged at 3000 rpm for 10 min. The liquid in chloroform layer was transferred into desiccated tube and the liquid volume was added with chloroform until 5 mL. Absorption intensity (F) was measured with 850 Hitachi fluorescence spectrophotometer at λex = 365 nm and λem = 435 nm.

Malondialdehyde (MDA) measurement

MDA measurement in tissue: In 0.1 mL liver or heart homogenate, 0.5 mL 3.0% sodium dodecyl sulfate, 1.5 mL

20% acetic acid buffer solution, 1.5 mL 0.8% Thiobarbituric Acid (TBA) and distilled water were add until 4 mL, respectively. The liquid was shaked up and covered. After heating at 95°C for 60 min, it was cooled to approximately 25°C and added with 5 mL n-butanol-pyridine (v:v 15:1), then shaked up and centrifuged at 3500 rpm for 15 min. Absorption intensity (F) of the supernatant was measured with 850 Hitachi fluorescence spectrophotometer at λex = 515 nm and λem = 553 nm. As above method, F of blank tube with distilled water and standard tube with 0.2 mL 10 nmol mL^{-1} MDA were also measured. The results calculated:

$$\text{MDA nmol/mg wet tissue weight} = 2.0\times\left(\frac{F_u-F_0}{F_s-F_0}\right)\times\left(\frac{10}{0.1}\right)$$

F_u, F_s and F_0 are absorption intensity (F) of test tube, standard tube and blank tube, respectively. About 2.0 is the amount of MDA standard fluid. About 10/0.1 is to convert the result into content per gram tissue.

MDA measurement in serum: In 0.1 mL serum, 4.0 mL 0.40% sulfuric acid and 0.5 mL 10% phosphotungstic acid were add until 4 mL, respectively. The liquid was shaked up and kept at room temperature for 5 min. After centrifuging at 3500 rpm for 15 min, the sediment was added with 2.0 mL 0.40% sulfuric acid and 0.3 mL 10% phosphotungstic acid. Then it was shaked up and centrifuged, the sediment was suspended in the solution of 4.0 mL distilled water and 1 mL TBA acetic acid (0.67% TBA was mixed with the same volume of acetic acid). The suspended solution was heated at 95°C for 60 min and cooled. After 5 mL n-butanol was added, it was shaked up and centrifuged at 3500 rpm for 15 min. With exception of 0.1 mL, 1 nmol mL^{-1} MDA used in standard tube, other procedure was carried out as MDA measurement in tissue. The results calculated:

$$\text{MDA nmol/mg protein} = \frac{0.1\times(F_u-F_0/F_s-F_0)\times 1}{\text{Protein content (mg)}}$$

F_u, F_s and F_0 are absorption intensity (F) of test tube, standard tube and blank tube, respectively.

Statistics: All data are expressed as mean±Standard Deviation (SD). The differences of ratios of organ weight to body weight were analyzed using ANOVA with LSD or Dunette's test (SPSS 12.0 Software, USA). Other data were tested using ANOVA with repeated measures built in General Linear Model (SPSS 12.0 Software, USA) and inter-group comparisons were made using the Multivariate of General Linear Model. The $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The results were shown in Table 1 and 2. In model group compared with blank group, MDA and lipofuscin content increased significantly and there was significant difference ($p<0.01$). This showed that the oxidation resistance of mice in model group was significantly lowered. Compared with eugenol group, aspirin group and AEE group, the decline of MDA and lipofuscin content in aging mice are significant, of which in high dose AEE group was most remarkable. In AEE groups, MDA and lipofuscin content with the increase of AEE dose were lowered significantly ($p<0.01$) and there also were positive dependence relationship between AEE dose and effect. Compared with eugenol group and aspirin group, the oxidation resistance in high dose AEE group was stronger.

According to the theory of free radicals theory when organisms age, the contents and activities of Superoxide Dismutase (SOD), GSH and Peroxidase (GSH-Px) in antioxidant system will be decreased. This make their antioxidant ability to drop and the free radicals produced

Table 1: The effect of AEE on lipofuscin content in aging model of mice by D-galactose

		Lipofuscin (absorption intensity F)	
Groups	Dose (mg kg^{-1})	Heart	Liver
Blank control	/	422.50±25.04**	458.45±23.48**
Model control	/	555.14±24.7††	658.44±47.49††
Eugenol	8	491.85±34.08**	543.32±13.93**
Aspirin	9	483.57±31.65**	537.21±22.29**
	9	497.33±33.90**††	552.00±35.18**††
AEE	18	461.57±21.13**††	517.42±32.19**††
	36	425.85±19.78$^{**\cdot\cdot\cdot\cdot}$	434.28±22.76$^{**\cdot\cdot\cdot\cdot}$

Compared with model group, $^{*}p<0.05$, $^{**}p<0.01$. Compared with blank group, $^{\dagger}p<0.05$ $^{\dagger\dagger}p<0.01$. Compared with eugenol group, $^{\cdot}p<0.05$, $^{\cdot\cdot}p<0.01$. Compared with aspirin group, $^{\cdot}p<0.05$, $^{\cdot\cdot}p<0.01$

Table 2: The effect of AEE on MDA content in Aging Model of mice by D-galactose

		MDA		
Groups	Dose (mg kg^{-1})	Serum (nmol mg^{-1} prot)	Liver (nmol mg^{-1} wet weight)	Heart (nmol mg^{-1} wet weight)
Blank control	/	4.67±0.22$^{**\cdot\cdot\cdot\cdot}$	3.61±0.27$^{**\cdot\cdot}$	5.86±0.12$^{**\cdot\cdot}$
Model control	/	5.91±0.25$^{\cdot\cdot\cdot}$	4.28±27$^{\cdot\cdot\cdot\cdot}$	7.30±0.19$^{\cdot\cdot\cdot\cdot}$
Eugenol	8	5.19±0.12**	3.78±0.13**	6.27±0.27**
Aspirin	9	5.05±0.21**	3.80±0.15**	6.49±0.22$^{**\cdot}$
	9	5.59±0.16$^{**\cdot\cdot\cdot\cdot}$	4.02±0.11$^{**\cdot\cdot}$	6.86±0.17$^{**\cdot\cdot}$
AEE	18	5.04±0.10**	3.83±0.12**	6.22±0.21$^{**\cdot}$
	36	4.52±0.13$^{**\cdot\cdot\cdot\cdot}$	3.57±0.11$^{**\cdot\cdot}$	5.77±0.20$^{**\cdot\cdot}$

Compared with model group, $^{*}p<0.05$, $^{**}p<0.01$. Compared with eugenol group, $^{\cdot}p<0.05$, $^{\cdot\cdot}p<0.01$. Compared with aspirin group, $^{\cdot}p<0.05$, $^{\cdot\cdot}p<0.01$

in body cannot be promptly cleared which caused multiple unsaturated fatty acid lipid peroxidation and generated Lipid Peroxide (LPO). LPO can decompose into Malondialdehyde (MDA). MDA can cause many of biological toxicity reaction and form such abnormal metabolism products as senile plaque and lipofuscin and cause organism ageing and a variety of diseases. Many scientific studies have confirmed that a lot of antioxidant compounds realized antioxidant action mainly through direct clear of excessive free radicals or by enhancing the antioxidant system to restrain lipid peroxidation.

D-galactose, as a chemical to cause aging, has been taken as one of the important ways of aging research (Chen, 1996). Under aldose reductase catalytic, galactitols produced from D-galactoses by reduction are accumulated in a cell and cannot be further metabolized which affects the normal osmotic pressure, making the cell swelling, function obstacle, metabolic disorder and results in the occurrence of aging. In this experiment, compared with blank control group, MDA and lipofuscin contents of mice serum and brain, liver in aging model group have been increased significantly after injecting D-galactose continuously for 8 weeks. Moreover, there were significant differences between them. Given the different doses of AEE, MDA and lipofuscin contents in all treatment groups were significantly lowered and there were some relationships between dose and effect.

CONCLUSION

The results showed that the small doses of AEE can effectively remove free radicals caused by D-galactose and large doses of AEE can make free radicals level below the normal level. This will be healthier to mouse organisms. Compared with precursor drug aspirin and eugenol, AEE also has stronger antioxidant ability. For its antioxidant mechanism, AEE may have great similarity with the precursor drug eugenol and aspirin. Eugenol removed free radicals mainly by phenolic hydroxy combining with oxygen free radicals (Exner *et al*., 2000). Aspirin cleared away free radicals by hydroxyl product generated through its metabolism and decomposition (Ogata *et al*., 2000). As a prodrug/codrug with aspirin and eugenol unit, AEE may play antioxidant activity by releasing the parent drug at the site of action in bodies.

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