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# 大薊 藥鍼液의 지질과산화 및 CYP 억제에 미치는 影響

## ; 활성산소자유기 및 CYP 매개의 동맥경화 치료를 위한 천연약물 개발의 기초 평가

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## Suppression of Lipid Peroxidation and CYP Isozymes activities by *Circium japonicum* Herbal-acupuncture Solution ; Basic Study for Screening of Medicinal Herb on Reactive Oxygen Radical and CYP-Mediated Atherosclerosis

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**Abstract**

**목적** : 藥鍼液의 지질과산화 예방 및 cytochrome P450과의 상호 작용에 있어서 大薊의 역할은 과거 연구가 거의 없었다. 따라서 본 실험에서는 大薊 藥鍼液이 지질과산화를 예방하고, 심혈관계질환 유발에 밀접한 연관이 있는 cytochrome P450의 직접적인 저해 효과를 검토 하고자 한다.

**방법** : 大薊 藥鍼液이 지질과산화를 억제하는 정도를 평가하기 위하여 세포막을 구성하는 불포화지방산의 일종인 linoleic acid를 대상으로 지질과산화 진행 시간과 大薊 藥鍼液의 농도에 의존적인 저해 효과를 실험하였다. 또한 실험쥐의 간조직을 이용하여, 강제적인 과산화를 유도한 후 이를 방어하는 효능을 검토하였다. 그리고 cytochrome P450을 구성하는 그룹의 1A1, 1A2 및 2E1의 활성을 각각 EROD, MROD, p-nitrophenol, aniline 방법으로 측정하였다.

**결과 및 결론** : 大薊 藥鍼液은 세포막 구성의 불포화 지방산인 linoleic acid의 산화를 시간 및 처리 농도에 의존적으로 억제하였고, 실험쥐의 조직 과산화를 유의성 있게 저해하였다. 또한 aryl hydrocarbon receptor (AHR)을 활성화 시켜 polycyclic aromatic hydrocarbons (PAHs)에 의한 심혈관계 질환 유발 인자로 알려진 cytochrome P450 1A1 및 1A2의 발현을 일부 저해 하였으며, 특히 체내에 흡수된 알콜 대사에 관여하는 P450 2E1을 강하게 억제 시켰다.

**Key words** : 대계 약침액, 지질과산화, cytochrome P450, EROD, 심혈관계질환

## Ⅰ. Introduction

Increased production of reactive oxygen species (ROS) has been implicated in the pathogenesis of cardiovascular diseases such as atherosclerosis, restenosis, hypertension and heart failure[1,2] as well as the role of ROS. ROS are key components the integration in atherosclerotic events[3] and the generation of large amounts of ROS that they may induce increased

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lipid peroxidation (LPO) and subcellular damage such as DNA fragmentation[4]. Therefore, in recent years, there has been a global trend toward the development of LPO-attenuation targeted natural product in herbs or oriental medicine[5-7]. In other pathway, polycyclic aromatic hydrocarbons (PAHs) have been known to induce atherosclerosis[8]. It has been reported that the metabolic activation of PAHs by cytochrome P (CYP) 450 is an important step for PAH-induced atherosclerosis. PAHs are generally known to produce the toxic effects through the activation of aryl hydrocarbon receptor (AHR)[9]. However, detailed mechanisms responsible for the toxicities have remained unknown. AHR is a basic helix-loop-helix (bHLH) protein belonging to the Per-Arnt-Sim (PAS) family of transcription factors. CYP 1A1 is an enzyme well known to bioactivate carcinogenic compounds such as B[*a*]P one of the typical PAHs[10]. The expression of CYP 1A1 is induced by PAHs including B[*a*]P and other xenobiotics via AHR. Recently, several reports have suggested that the metabolic activation of PAHs by CYP isozymes is a necessary step for PAH-induced atherosclerosis[11].

Aqua-acupuncture solution were widely used in traditional medicine[12]. We demonstrated that the *Circium japonicum* aqua-acupuncture solution (CJAS) shows effectively free radicals scavenging activities on various exposure condition of oxidative stress[13]. However, the mechanisms of how CJAS bio-medical activities in the preventive effects of LPO and inhibition of CYP isozymes on *in vitro* condition remains unclear.

Our group hypothesized that CJAS has preventive effects on oxidative stress induced by LPO and inhibitory activity on CYP isozymes-mediated atherosclerosis.

## Ⅱ. Materials and Methods

### 1. Materials

All the plastics were obtained from Falcon Labware (Becton-Dickinson, Fraanklin Lakes, NJ). Chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, Mo), and other chemicals used were analytical grade and obtained from either Merck (Merck KGaA, Germany) or Junsei (Junsei Chemical Co., Ltd., Japan).

### 2. Preparation of CJAS Extracts

*Circium japonicum* was purchased from a Dongguk Korean Medicine Hospital and confirmed by comparison with descriptions of characteristics at laboratory of Diagnostics, college of oriental medicine, Dongguk University in Kyungju. The extract was prepared that a 60 g sample of crude plant was cut into small pieces and then added 500 mL of distilled-water. The detail description of CJAS extracts were according to the method on our previous report (Lee *et al.*)[13].

### 3. Ferric thiocyanate assay

The lipid preoxidation preventing activity analysis using ferric thiocyanate (FTC) was

slightly modified to the method by Osawa *et al*[14]. 1 mg of the CJAS and CJWS (compared with *Circium japonicum* water extract) were dissolved in 0.1 mL of distilled water, and 2.9 mL of a 2.51% linoleic acid solution in absolute ethanol and 9 mL of a 50 mM potassium phosphate buffer (pH 7.4) were added. The mixture was incubated at 40℃ in a dark screw-cap tube, and then during the incubation time aliquot of 1 mL from the mixture. The aliquot mixture were diluted with 9.7 mL of 75% ethanol, followed by the addition of 0.1 mL of 30% ammonium thiocyanate. Correctly, after the solution incubated at room temperature for 3 min, added by 0.1 mL of 20 mM ferrous chloride ($FeCl_2 \cdot nH_2O$) in 3.5% hydrochloric acid (HCl). Oxidation of the linoleic acid was monitored (for the red color develop) spectrometrically by measuring absorbance at 500 nm over a 24-hr period. The level of lipid peroxidation prevention by each samples (CJAS, CJWS and positive L-ascorbic acid) were calculated from the absorbance ratio to that of a control without any addition. The time scanning of CJAS extract tested ranged from 0 to 72 hours.

## 4. Preventive Effect of Linoleic Acid Oxidation on CJAS

Preventing effects of CJAS on linoleic acid oxidation was carried out according to the method of Emmons *et al.*[15] with slightly modifications. 3 mg of $\beta$-carotene was dissolved in 30 mL of chloroform, and 3 mL was added to 40 mg of linoleic acid and 400 mg of Tween 40. Chloroform was removed under a stream of nitrogen gas in $LN_2$ protected basket. After added 100 mL of distilled water, and the solution was gently mixed. Aliquots (3 mL) of the $\beta$-carotene /linoleic acid emulsion were mixed with 0.05 mL of various concentration CJAS (ranged from 0.005 to 0.05 mg/mL) and incubated in a water bath at 50℃. The blank sample contained same volume of solvent in place of the CJAS, and then the absorbance was measured at 470 nm. Reference positive sample were used vitamin E at a final concentration from 0.05 mg /mL. The preventive effects of CJAS are expressed as percent inhibition relative to the blank after 60 min incubation using from the following equation:

$$PE = [DRc \mid DRs] / DRc \times 100$$

* PE is the preventive effect of the CJAS
* DRc is the degradation rate of the control (=ln(a/b)/60)
* DRs is the degradation rate in the presence of the sample (=ln(a/b)/60)

## 5. Animals and Preparation of Tissue Fractions[16]

Male Sprague-Dawley rats (weight 150-200 g) were obtained from the Orient Bio corporation (Seongnam, GyeongGi-Do). All animals were housed in plastic cages with wired floors and allowed free access to food and water throughout the adaptation period. They were maintained in a room under controlled conditions (12 h light and dark cycle, set temperature 24 ± 2℃, and supplement of 60% humidity). Rats were randomly assigned to each experimental or control group (six animals for a groups). How-

ever, oxidation study was obtained normal liver protein from homogenized and were induced cytochrome P450 1A1 and 1A2 with β-naphthoflavone for three days (*i.p.* injection of 80mg/kg body weight). And then, pyrazole-treated (four daily rat *i.p.* injection of 200 mg/kg body weight) liver fraction were used to study the effects of CJAS on cytochrome P450 2E1 activities. The animals were killed by cervical dislocation 24 hr after the last inducible-treatments. Hepatic tissue was homogenized (Teflon Plotter Elvehiem Homogenizer, Glas-Col, USA) in 0.15 M KCl buffer (pH 7.0) and high centrifuged at 12,000 rpm for 20 min. The supernatant was discard and microsomal pellets were obtained by ultra-centrifugation (Beckman coulter, USA) at 36,000 rpm for 60 min. The microsomes were finally re-suspended in the Tris-HCl solution (pH 7.4), containing 0.25 M of sucrose in water. In this experimental steps were maintained at 4℃. The liver homogenized fraction or microsomal protein concentration were determined by using a bicinchoninic protein kit using bovine serum albumin as the standard. Liver fraction and microsomal protein were stored at -86℃ deep freezer until use.

## 6. $FeCl_2$-ascorbic Acid Stimulated Lipid Peroxidation in Rat Liver Homogenate

The preventive effect of CJAS on rat liver homogenate stimulated $FeCl_2$-ascorbic acid induced lipid peroxidation was determined by the slightly modification of Lin *et al.*[17] The reaction mixture containing 0.5 mL of 7.5 mg/mL liver homogenate, 0.1 mL of Tris-HCl buffer (pH 7.2), 0.05 mL of 0.1mM ascrobic acid, 0.05 mL of 4 mM $FeCl_2$ and 0.05 mL of various concentrations of CJAS, and using the positive control by vitamin E. It was then incubated for 60 min at 37℃, added after incubation 0.9 mL of distilled water and 2 mL of thiobarbituric acid (TBA). Then, the solution was shaken vigorously, and the mixture was heated for 30 min in a boiling water bath at 100℃. After cooling on ice, 5 mL of *n*-butanol was added and the mixture was then mixed gently. The *n*-butanol layer was separated by centrifugation at 3,000 rpm for 10 min. The supernatant was collected and measured by spectrophotometry at 532 nm.

## 7. Total cytochrome P450 contents

The activities of CYP 1A1, 1A2 and 2E1 enzymes were determined by the modified methods of Burke *et al.*[18], Dicker *et al.*[19] and Christopher *et al.*[20]

### (1) Inhibition of Cytochrome P450 1A1 and 1A2

Inhibitory effects of β-naphthoflavone-induced cytochrome P450, including 1A1 and 1A2 using different substrate assay system. For assays of 7-ethoxyresorufin O-deethylation (EROD) and 7-penthoxyresorufin O-depenthylation (PROD) activities, incubations contained 200 μL microsomal protein (for EROD test, 0.2 mg/mL; and MROD, 1 mg/mL), 10 μL of substrates (prepare stock solutions 0.1 mg/mL), 100 μL of BSA, 20 μL of 0.25 M $MgCl_2$, 10 μL of 500 μM dicoumarol, and various concentrations of sample, made up to a final volume of 610

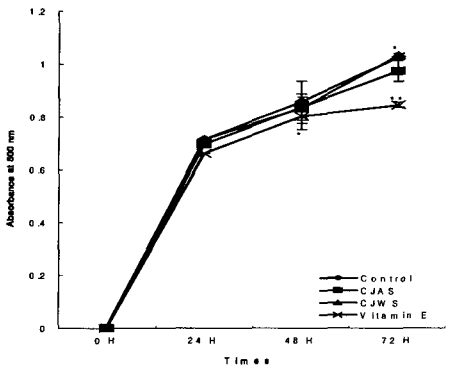


Fig. 1. Preventive effect of CJAS on hydroxyl radical-mediated linoleic acid oxidation. The concentrations tested 1 ㎎/㎖. Absorbance of the extracts from CJAS by FTC method with increasing incubation time, * p ⟨0.05, and ** p ⟨0.01 ; compared with the control group.

μ L with 50 mM Tris-HCl buffer (pH 7.5). The reaction solution were preincubated for 3min in a shaking water bath maintained at 37℃. Reactions were started by the addition 60 μ L of nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH) generating system (2.5 U glucose 6-phosphate dehydrogenase, 40 μ L of cofactor solution containing $NADP^-$ and glucose 6-phosphate) and were allowed to incubated with shaking for 4 min at 37℃, prior to quenching with 2 volumes of ice-cold methanol. Precipitated protein was removed by centrifugation (VISION VS-5500CF, Korea) for 20 min at 4,000 rpm. The fluorescent product, resorufin was measured at an excitation wavelength of 550 nm and an emission wavelength of 585 nm using a fluorescence spectrophotometer (BIO-TEK SFM25, USA).

### (2) Inhibition of Cytochrome P450 2E1

The activity of CYP 2E1 was measured as the rate of *p*-nitrocatechol formation from *p*-nitrophenol and *p*-aminophenol formation from a aniline, respectively. In this tests, 1.5 mg/mL of microsomal protein were used final volume 0.75 mL containing reaction mixture. Then, NADPH-generating system was mixed well, and incubated for 5 min at 37℃ in water bath. After added various concentrations of sample and the mixture was incubated for 20 min at 37℃ shaking water bath. The stop reaction by adding stop solution. Reference sample was used 0.25 μ M of diallyl sulfide. Results have been expressed as % inhibition as the *p*-nitrocatechol and *p*-aminophenol formation.

## 8. Statistical Analysis

The data were analyzed for statistical significance using a variance (SigmaPlot, version6.1 for Windows) and Student's *t*-test. Values were expressed as the mean ± S.D. and less than $P<0.05$ were considered to be significant.

# Ⅲ. Results and Discussions

## 1. Time-dependent Inhibition of Lipid Peroxidation by CJAS

The preventive effect of the CJAS was measured using ferric thiocyanate (FTC) test. In this assay, which determines the amount of peroxide produced at the initial stage of lipid peroxidation, a lower absorbance indicates a higher level of resistance action. The results

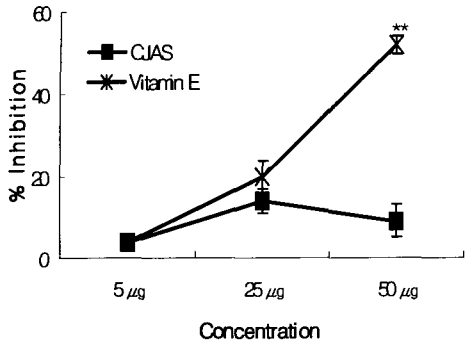


Fig. 2. Preventive effect of CJAS in the linoleic acid/β -carotene system. CJAS sample was used at a final concentration of 1 to 10 ㎍/㎖ and vitamin E was used at same concentration. Measurements were carried out in triplicate. Means and standard deviations are indicated. ** p <0.01 ; compared with the control group. ; compared with the control group.

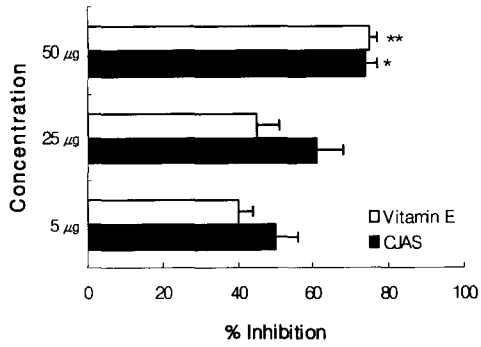


Fig. 3. Preventive effects of CJAS and vitamin E on MDA production in rat liver homogenate induced by $FeCl_2$-ascorbic acid *in vitro*. MDA data are presented as mean ± S.D. (n=3), values within the same column with different superscript letters are significantly different at p <0.05 (* p <0.05, and ** p <0.01 ; compared with the control group).

shows that CJAS was changes in the absorbance for each times during 72 hr of incubation at 40℃ in comparison with vitamin E, but the CJWS was not effectively against lipid peroxidation (Fig. 1.). The absorbance of control increased in proportion to the incubation time, and the absorbance of others also increased with time-dependent.

## 2. Inhibition of β-Carotene/Linoleic Acid Oxidation by CJAS

In the results shows that CJAS inhibited β -carotene/linoleic acid emulsion system. This reaction using the discoloration of β -carotene is widely used, because β -carotene is extremely susceptible to free radical-mediated oxidation. Free radicals are often generated as byproducts of biological reaction or from exogenous factor. However, β -carotene is discolored easily by oxidation of linoleic acid, because its double bonds are sensitive to oxidation. CJAS was tested at a final concentration of 50 μ g/mL, and vitamin E was compared with under the same conditions. As the shown the results, CJAS had a normally against lipid peroxidation, but not concentration dependent. Moreover, CJAS exhibited weak resistantly effect of lipid peroxdation in this assay.

## 3. CJAS Inhibits $FeCl_2$-ascorbic Acid Induced Malondialdehyde Production

The effect of CJAS on malondialdehyde (MDA) production in rat liver homogenate induced by $FeCl_2$-ascorbic acid *in vitro*. Results presented that the inhibition of MDA formation increase with increasing concentration of CJAS and vitamin E. At concentrations of 5 to 50 μ

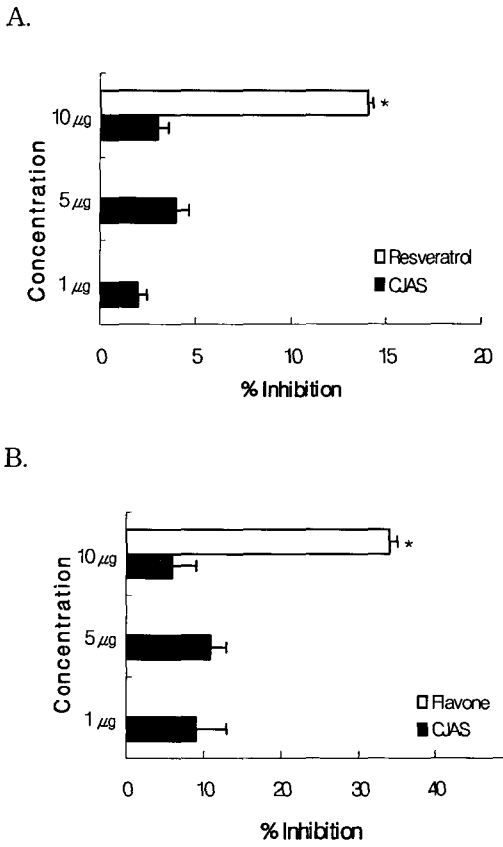


Fig. 4. Inhibitory activities of CJAS and positive controls (EROD; resveratrol and MROD; flavone) on A: cytochrome P450 1A1 and B: 1A2, respectively. Cytochrome P450 1A1-mediated EROD and 1A2-mediated MROD activities by using liver microsome derived from $\beta$ -naphthoflavone-treated rats. The concentration of CJAS samples tested ranged from 1 to 10 ㎍/㎖. Experimental details are described in the Materials and Methods section. The results are the means of three separate experiments.
* : $p < 0.05$ as compared to control.

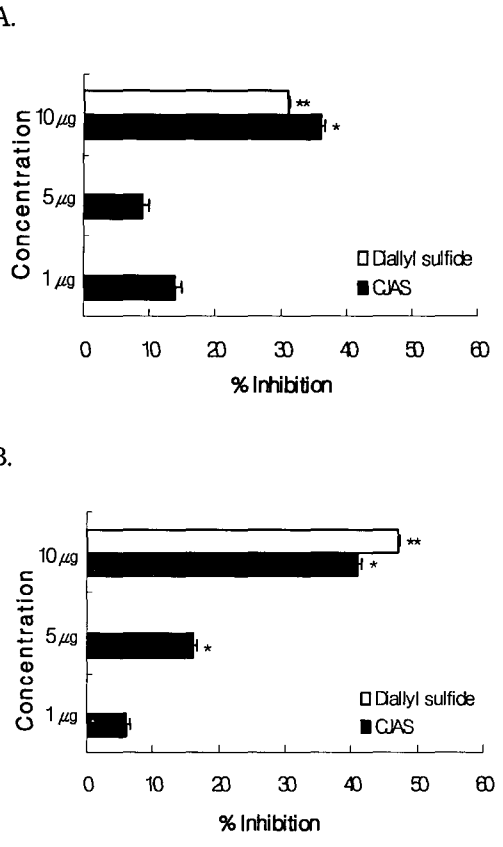


Fig. 5. Inhibitory activities of CJAS and diallyl sulfide on cytochrome P450 2E1. The effects of cytochrome P450 2E1-mediated A: *p*-nitrophenol and B: aniline hydroxylase activities using liver microsome derived from pyrazole-treated rats. The concentration of CJAS samples tested ranged from 1 to 10 ㎍/㎖. Experimental details are described in the Materials and Methods section. The results are the means of three separate experiments.
* $p < 0.05$, and ** $p < 0.01$ ; compared with the control group.

g/mL, CJAS displayed an against lipid peroxidation activity, with an inhibition rate varies from 50 to 74% (Fig. 3.). In particular, at a concentration of 5 to 25 $\mu$ g/mL, CJAS on the MDA formation was inhibited superior than vitamin E. In this results is interesting to note, because of it has been confirmed that vitamin E decreased atherosclerosis and delay death from myocardial infraction, presumably by inhibition lipid peroxidation. Also, free radicals scavenging ac-

tivity are well known to be a major against function of chronic diseases, such as heart diseases.

### 4. Inhibitory Effects of CJAS on Cytochrome P450 Isozymes *in vitro*

We examined was potent CYP 1A family inhibitor of the CJAS from various concentration sample. The CJAS weakly inhibited CYP 1A1 and 1A2, respectively, and no significant difference in EROD or MROD activities. But a comparable effect were exhibited strongly inhibited CYP 1A1 and 1A2 with resveratrol or flavone (Fig. 4A. and 4B.). However, the CYP 1A isoforms can activated a number of cancer or cardiovascular disease such as atherosclerosis. In other tests for detect of CYP 2E1, the CJAS were potent inhibitors of the activities used *p*-nitrophenol and aniline hydroxylase, primarily catalyzed by CYP 2E1 in pyrazole-induced rat liver microsomes. The inhibitory activity of CYP 2E1 using by another substrate assay systems, the CJAS were strongly inhibited superior than or similar to diallyl sulfide. Especially, at concentrations of 1 to 10 $\mu$ g/mL, CJAS displayed an inhibitory actions, with an inhibition rate varies from 14 to 36% (Fig. 5A.) in *p*-nitrophenol, and inhibitory effect of CJAS at 6 to 41% (Fig. 5B.) in aniline hydroxylase assay system, respectively.

## Ⅳ. Conclusion

The aqua-acupuncture extract of *Circium japonicum* was confirmed to show antioxidants and resistants of lipid peroxidation. In this study, our results suggest that the preventive effects of CJAS on the LPO have a high activities, which are comparable to vitamin E. Their MTT assay (data not shown) revealed that CJAS also contains a low cell toxicity on human hepatoma cell line HepG2. And then, CJAS may act as resistance agents because of their inhibitory activities not only toward CYP 1A family-isozymes *in vitro* but also strong and specific scavenging activity of CYP 2E1. However, further study should be investigated to identify the exact molecular mechanism of both the preventive effect of LPO and interaction of CYP-mediated atherosclerosis metabolism.

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