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Antioxidative activity of flavonoid rich extract of *Oenothers odorate* Jacquin on oxidation of low density lipoprotein

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Abstract

There is growing interest in understanding the role and mechanisms of flavonoid as antioxidant on LDL. The antioxidative activity of flavonoid typically present in *Oenothers odorate* Jacquin was investigated *in vitro* using a human LDL oxidation assay. In present work, LDL was incubated with increasing concentrations of extracts of *Oenothers odorate* Jacquin and LDL oxidation was started by adding $CuSO_4$ to the media. Substances in leaves extracts of *Oenothers odorate* Jacquin are capable of inhibiting the initiation and the propagation of LDL oxidation. They inhibit LDL oxidation, monitored by thiobarbituric acid-reactive substances(TBARS), as well as modification as shown through direct measurement of electrophoretic mobility, diene conjugates. Inhibition is a dose dependent effect that becomes already apparent at concentration of extracts as low as 40μg/mL. Inhibition is almost complete at 80μg/mL. Extracts of *Oenothers odorate* Jacquin were more potent antioxidative activity than either ascorbic acid and *dl*-α -tocopherol.

Key words – Low density lipoprotein(LDL), antioxidative activity, *Oenothers odorate* Jacquin, flavonoid

Introduction

Oxidative modification of low density lipoprotein(LDL) that alter physicochemical and biological properties of the particles, are thought to play a central role in atherogenesis[1,3,17]. Several lines of evidence support a role for oxidized LDL in atherosclerosis and for its *in vivo* existence. Oxidized LDL was shown to exist in the atherosclerotic lesion and it possesses several different atherogenic properties including cells, cytotoxicity, thromobosis and inflammatory processes [2,12,18,21,23]. The exact mechanism which LDL undergoes oxidation *in vitro* is not yet fully clear but there is hardly and doubt that involves free radical peroxidation of LDL lipids [9,13,14]. Antioxidants have been shown to decrease atherosclerosis lesion formation in animal models and inhibited LDL oxidation [4,5,9]. Hence, the importance of LDL associated antioxidants in the prevention of LDL oxidation and thereby possibly the prevention of formation of atherosclerotic plaques becomes rather evident [14,15,18].

There is growing interest in understanding the role and mechanisms of flavonoids as antioxidant of atherosclerosis. Therefore, the intake of vegetable foods and beverages that contain flavonoid as antioxidant potentially protects against atheroscleosis [7,9]. It has been postulated that the high content of flavonoid in red wine might be responsible for the beneficial effects of moderate drinking on coronary heart disease [8]. The main natural polyphenols of wine are flavonoids produced by healthy

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plants. Some have already been found *in vivo* and additional evidence suggests that they act as antioxidant [5,22]. Higher intake of flavonoid have also been associated with decreased incidence of vascular diseases.

Our interest has been focused on plants which are known to contain much flavonoid. Following these considerations, present work was carried out to determine the antioxidative activity of extract of *Oenothers odorate* Jacquin on $CuSO_4$-mediated LDL oxidation.

Materials and Methods

Isolation of antioxidative substances from *Oenothers odorate* Jacquin

Air dry leaves(500g) were extracted three times with hot 70% aqueous acetone(liquor ratio 1 : 5). The combined acetone extracts was filtered and concentrated, and the concentrate(~0.1L) was treated with chloroform (3× 300 mL), and then with diethyl ether(7 times). The combined ether extract(~2.0L) were dried with sodium sulfate, concentrated to a volume of 20mL, and chromatographed on the silica gel(2㎝×15㎝) column and eluted with moist diethylether. Diethylether layer was concentrated with vaccum.

Lipoproteins

Human LDL was isolated by ultracentrifugation [11], and dialyzed extensively against 0.9% (w/v) NaCl, 0.004% (w/v) EDTA, pH 7.4. Prior to oxidation, LDL was dialyzed against phosphate-buffered saline, pH 7.4 to remove the EDTA.

Cell culture

Transformed mouse macrophage, J774 cells were maintained in Ham's F-10 supplemented with 10%(v/v) fetal calf serum, $NaHCO_3$(2g/L) and 4mM Hepes pH 8.1. A series of antibiotics was included in the medium. The cells were cultured routinely in large dishes (90㎜ diameter) in 10mL of medium and plated out into smaller dishes (60㎜ diameter) containing 2mL of medium for experimentation. Cultures were maintained in a humidified incubator at 37 ℃ and the medium changed every 48hr.

Oxidation of LDL

Two different methods were used to examine the effect of novel compound on the oxidation of LDL. In the first method, LDL(100㎍ protein/mL) was incubated in the presence of 5μM $CuSO_4$ in phosphate-buffered saline, pH 7.4 at 37℃ for 18 h [6]. In the second method, LDL(100㎍ protein/mL) was incubated with J774 macrophges in Ham's F-10 culture medium for 24 hr at 37℃. To examine the effect of antioxidant on LDL oxidation, sample were added to the LDL containing solutions at the beginning of the incubation period. The oxidation of LDL was stopped by the addition of EDTA(final concentration of 10μM)and placing the lipoproteins on ice [15].

Assay of thiobarbituric acid-reactive substances (TBARS)

TBARS levels were determined spectrophotometrically [25]. 0.1 mL aliquots of post incubation mixture and also tetramethoxypropane standards were added 1 mL of 20% trichloroacetic acid and 1 mL of 1% thiobarbituric acid containing EDTA. Tubes were placed in a boiling water bath for 30min. After cooling, tubes were centrifuged at 1,500×g for 15min. Absorbance of the supernatant was measured at 532㎚.

Detection of conjugated dienes

The formation of conjugated dienes associated with oxidized LDL was measured by monitoring the absorption at 234㎚ using a UV-VIS spectrophotometer[6]. Briefly, 1mL of a LDL solution (100㎍ LDL, protein/mL) in phosphate buffered saline, pH 7.4 was incubated with 5μM $CuSO_4$ at 37℃ in both the presence or absence of samples, and then the absorbance at 234㎚ was measured every 30min. The formation of conjugated dienes in control solutions containing antioxidant in the absence of

LDL and 5μM $CuSO_4$ was also determined.

LDL gel electrophoresis

Electrophoresis of oxidized and native LDL was carried out on agarose gel in barbital buffer, pH 8.6. The agarose plates were then stained with nile red [10]. Results are expressed as relative electrophoretic mobilities compared with the migration of native LDL.

Dertermination of cellular protein

Cell protein was measured according to Lowry's method [17] using bovine serum as standard.

Statistics

Data in the figures are given as mean±SEM. Statistical significance was examined through one-way analysis of variance and Dunnett's multiple range test [24]. Significant differences were accepted at $P<0.05$.

Results and Discussion

Inhibition effect on the human LDL

Fig. 1 shown that oxidation of LDL by mouse macrophages was in a time-dependent manner and the production of TBARS reached a plateau after 24 h of incubation. Therefore, all data relation to macrophages presented here were obtained following 12~60 h incubation. The production of TBARS for 36 h in the presence (LDL+cells) or absence (LDL alone) of the macrophages was 3.93±0.02 nmol and 0.87±0.02 nmol MDA equivalent/mg protein, respectively. Increasing the number of macrophages per dish increased the production of TBARS.

As shown in Fig. 2, extracts of *Oenothers odorate* Jacquin in dose- dependently inhibited LDL oxidation by the macrophages with concentrations of 20, 40, 60, 80 μg/mL. Extracts of *Oenothers odorate* Jacquin at the maximal concentration tested have an apparent antioxidative effect on the cells as determined by TBARS.

Several studies have shown a positive correlation

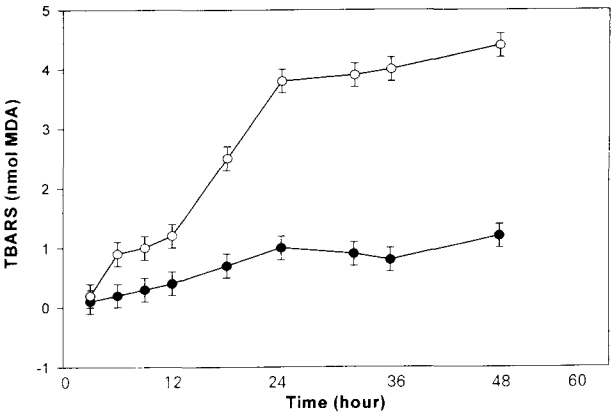

Fig. 1. Time courses of LDL oxidation by mouse macrophages.
LDL(100 μg protein/mL) was incubated in Ham's F-10 medium at 37℃ in the presence (3.8×10^6 cells, ●—●)or absence (LDL alone, ○—○) of macrophages for various time intervals. The LDL oxidation was estimate by the formation of TBARS and expressed in nmol equivalent MDA as described in Methods. The data are means of duplicate determinations from a representative experiment.

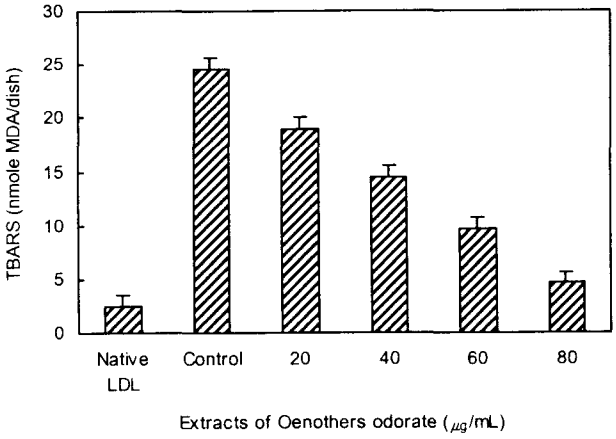

Fig. 2. Dose-dependent inhibition of macrophage induced LDL oxidation by extract of *Oenothers odorate* Jacquin.
LDL(100 μg protein/mL) was incubated for 24hr with macrophages in Ham's F-10 medium in the presence of extract of *Oenothers odorate* Jacquin or absence(control) of the test agent. The LDL oxidation was estimated by the formation of TBARS and expressed in nmol equivalent MDA as described in Methods. TBARS produced in cell-free conditions(medium+LDL) was subtracted from dose of the dishes containing macrophages. Each point is the mean±S.E.M. of 3-5 experiments done duplicate, while the data for α-tocopherol are the average of two experiments done duplicate.

between plasma concentration of vitamin E and C and risk of cardiovascular disease [7,15].

Flavonoids, phenolic compounds of vegetable origin commonly included in the diet, have antioxidative properties [15,16], and therefore, may be suitable for decreasing LDL susceptibility to oxidation and for preventing cordiovascular diseases. In fact, the French peradox has been attributed to the regular consumption of red wine [14].

Higher intake of dietary flavonoids have been associated with decreased risk of LDL oxidation. These evidence prompted us to study the LDL oxidation inhibiting properties of one species, *Oenothers odorate* Jacquin commonly and daily used in large quantites as natual product.

Inhibition of the propagation of LDL oxidation by extracts of *Oenothers odorate* Jacquin

In these experiments, *Oenothers odorate* Jacquin extracts were added at time 0 or at 15, 30, 60, 90, 120 and 180 min after the addition of Cu^{2+} to the LDL preparations. Then, the incubations were allowed to continue to the rest of the 180min period. Using conjugated dienes and TBARS, as shown in Table 1 *Oenothers odorate Jacquin* extracts inhibited the propagation of Cu^{2+} induced LDL peroxidation almost completely during the first two hours. Our data show that leaves extracts of *Oenothers odorate* Jacquin are capable of inhibiting the initiation of LDL oxidation.

Finally, the present shown that extracts of *Oenothers odorate* Jacquin inhibits not only the initiation of LDL inhibition but also the propagation. Propagation of lipid oxidation is a process less dependent on copper ions. When initiation of lipid peroxidation in LDL was allowed to occur even during two hours, addition of *Oenothers odorate* Jacquin inhibited subsequent propagation for the rest of the 180min inhibition period. This is, however, speculative. Further studies would be needed on the nature of the substances implicated to clarify their possible effects *in vivo*.

Effects of *Oenothers odorate* Jacquin on LDL oxidation assay electrophoretic mobility

Table 2 shows the effect of *Oenothers odorate* Jacquin on the electrophoretic mobility of LDL submitted to oxidative modification by macrophage. A marked increase from 1.79±0.02 to 1.05±0.02 mm in the electrophoretic mobility of LDL incubated with macrophages for 24 h implies lipid peroxidation of LDL and an increase in negative charges on the LDL molecule. *Oenothers odorate* Jacquin reduced the relative electrophoretic mobility of LDL dose dependently. LDL oxidized by $CuSO_4$ displayed a greater electrophoretic mobility in agarose gels compared to native LDL. When LDL was incubated with 20 μg/mL

Table 1. Inhibition of the propagation of LDL lipid oxidation by extracts of *Oenothers odorate* Jacquin(E.O)

Conditions	TBARS (nmol, MDA/mL)	Diene conjugates (mol/mol apoB)
LDL	1.26±0.02	0.0
LDL*+Cu^{2+}	20.63±0.05	37.64±0.04
LDL*+Cu^{2+}+E.O. at 15min	2.33±0.02	2.48±0.03
LDL*+Cu^{2+}+E.O. at 30min	2.86±0.02	1.67±0.03
LDL*+Cu^{2+}+E.O. at 60min	3.12±0.03	2.77±0.02
LDL*+Cu^{2+}+E.O. at 90min	3.74±0.02	4.54±0.03
LDL*+Cu^{2+}+E.O. at 120min	6.30±0.03	9.90±0.03
LDL*+Cu^{2+}+E.O. at 180min	12.96±0.03	32.63±0.05

*LDL(100 μg, protein/mL) was incubated with 10 μmole/mL $CuSO_4$ and 60 μg/mL extracts of *Oenothers odorate* Jacquin(E.O) were added at the times indicated, then incubation was continued at 37℃ up to 180 min. Data are means±SEM of two seperated experiments run in duplicate.

Table 2. Effects of *Oenothers odorate*(O.E) Jacquin on LDL oxidation as assessed by electrophoretic mobility[a]

Incubation condition	Relative electrophoretic mobility (mm)
Native LDL	1.0
LDL + cell + vechicle(control)	1.79±0.02
LDL + cell + O. E 20 μg/mL	1.60±0.03
LDL + cell + O. E 40 μg/mL	1.28±0.03
LDL + cell + O. E 60 μg/mL	1.16±0.02
LDL + cell + O. E 80 μg/mL	1.05±0.02

[a]LDL (100 μg protein/mL) was incubated for 24 h in Ham's F-10 medium in 35-mm dishes containing macrophages in the presence or absence of *Oenothers odorate* Jacquin. The electrophoretic mobility of LDL was determined in agarose gel as described in the next. Results are means±SEM of three to five independent experiments.

Oenothers odorate Jacquin, the electrophoretic mobility of oxidized LDL was only slightly greater than native LDL. *Oenothers odorate* Jacquin inhibited the cell-induced oxidation of LDL as measured by lipoperoxide content of the electrophoretic mobility of LDL in agarose gels. Steinbrecher et al. [23] demonstrated that LDL can be modified by the addition of fatty acid peroxidation in the absence of cells. This modified LDL processes an enhanced electrophoretic mobility without the lipid constituents of LDL being oxidized. It is possible that oxidation of LDL mediated by macrophages in Ham's F-10 culture medium can also contribute to the modification of the LDL protein as determined by the enhanced electrophoretic mobility.

Comparative antioxidant effects of extracts of *Oenothers odorate* Jacquin and other antioxidants

As shown in Fig. 3, human LDL was oxidized by mouse macrophages, in the same concentrations of extracts of *Oenothers odorate* Jacquin and vitamin C, dℓ-α-tocopherol and the production of TBARS reached a plateau after 18h of incubation. Therefore, all data relating to macrophages presented here were obtained following 18 h incubation. The oxidation of LDL by J774 macrophages was completely inhibited in the presence of 80μg

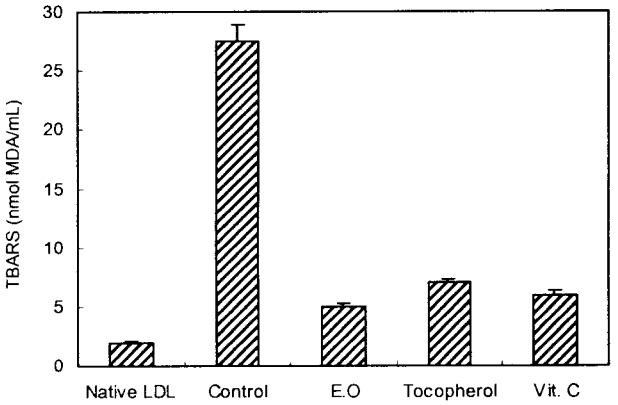

Fig. 3. Comparative antioxidative effect of extracts of *Oenothers odorate* Jacquin. (E.O), vitamin C and dl-α-tocopherol.
LDL (100 μg protein/mL) was incubated for 18 hr with macrophage, J774, in Ham's F-10 in the absence (control) or presence of *Oenothers odorate* Jacquin (E.O. 80 μg/mL), vitamin C (80 μg/mL) and *dl*-α-tocopherol (80 μg/mL) with 10 μmole/mL $CuSO_4$. Data points are means of a typical experiment (out of three)run in duplicate.

/mL *Oenothers odorate* Jacquin in the culture medium. In terms of mass the potencies of leaves extracts of *Oenothers odorate* Jacquin, vitimin C and dℓ-α-tocopherol are of the same magnitude. Moreover, in our system, leaves extracts of *Oenothers odorate* Jacquin exhibit a significantly higher effectivity as LDL antioxidants than either vitamin C and dℓ-α-tocopherol. Although the mechanism of oxidation is not known, LDL oxidation may involve cellular lipoxygenase. The inhibition of macrophage induced oxidation by antioxidants is consistent with the role of lipoxygenase [19]. Macrophages endocytose and degrade oxidatively modified LDL via scavenger receptors at a much greater rate than do native LDL [12,18] and this property was used to assess antioxidative activity of antioxidants during the oxidation period. Mouse peritoneal macrophage have been shown to oxidize LDL *in vitro* [13,20]. It was observed that protection by antioxidants against cell induced LDL oxidation may be, in part, through its capacity to scavenge free radicals. However, inhibition of Cu^{2+}-mediated LDL oxidation by antioxidant was less marked than the oxidation by macrophages. The dif-

ferences in antioxidative potency in the two systems might be due to the fact that oxidative modification is more complex than a simple free radical reaction. Moreover, the mechanism of LDL oxidation by macrophage still remains to be clarified.

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초록 : LDL 산화에 대한 달맞이꽃의 플라보노이드 추출물의 항산화 활성

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달맞이꽃 추출물로써 항산화활성을 알아보기 위하여 사람 low densty lipoprotein(LDL)에 대하여 실험하였다. 사람 LDL에 $CuSO_4$를 첨가한 후 배양하면서 달맞이꽃 추출을 일정 농도씩 증가시키면서 첨가하여 배양하였다. 달맞이꽃 추출물로서 LDL의 산화에 대한 개시와 연쇄 반응에서 thiobarbituric acid reactivy substances (TBARS)와 전기 영동 및 공액 2중 결합으로 측정하였다. 달맞이꽃 추출물을 LDL에 첨가한 후 macrophage J774로써 배양한 결과 TBARS값은 용량 의존형의 항산화 활성을 나타내었으며 전기 영동 이동상에서도 대조구에 비하여 각 시험구에서 LDL의 산화를 억제하였다. 또 공액 2중 결합에 대한 실험에서 용량 의존형 산화 억제 효과를 나타내었다. 달맞이꽃 추출물의 항산화 활성은 40 μg/ml의 농도에서도 항산화 효과가 있었으나 80 μg/mL의 농도에서는 거의 완전한 억제효과가 있었다. 그리고 비타민 C 및 *α-dl*-tocopherol과 비교한 결과 달맞이꽃 추출물이 약간 높은 항산화 활성을 나타내었다.