# Antiatherogenic Effect of Grape Flavonoids in an *Ex Vivo* Model

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The effects of grape phytoestrogens on cholesterol accumulation were studied in primary culture of human blood monocytes incubated with blood serum from postmenopausal women obtained before and 2, 4, and 6 h after single intake of plant components of grapes. Phytoestrogens from grape seeds, pressed out grapes, and fermented grape ridges prevent cholesterol accumulation in cells and can be regarded as prospective components for the development of natural preparations for the prevention of atherosclerosis in postmenopausal women.

**Key Words:** *phytoestrogens; atherosclerosis, postclimacteric period; human monocyte culture; intracellular accumulation of cholesterol*

Women of reproductive age are less liable to clinical manifestations of atherosclerosis in comparison with men of the same age. The risk of cardiovascular diseases increases in women of the postmenopausal age, which is attributed to decreased estrogen level [9]. It was assumed that hormone replacement therapy (HRT) reduced cardiovascular mortality among postmenopausal women [8]. However, recent prospective randomized placebo-controlled studies showed that HRT did not reduce the risk of atherosclerotic involvement of vessels in postmenopausal women [12]. Moreover, estrogen treatment can lead to untoward side effects such as venous thrombosis and induction of hormone-dependent mammary and endometrial tumors [3].

It is therefore interesting to investigate the effects of plant-derived compounds structurally similar to estrogens and possessing estrogen-like properetes, so-called phytoestrogens (PE) [1], on the development of atherosclerosis in postmenopausal women. Phytoestrogens, ingredients of foodstuffs (cereals, bean, vegetables, fruit, and medicinal plants), produce no side effects, and hence, can be regarded as prospective compounds (alternative to HRT) for the treatment and prevention of atherosclerosis in women during the postmenopausal period.

The well-known cardioprotective effect of red grape wine (the so-called "French paradox") is largely attributed to the presence of grape PE in wine [5]. The main grape PE are flavonoid polyphenols (catechin, epicatechin, quercetin, kempferol, lutein), stilbene resveratrol, and procyanidins (flavanol polymers). Very high antioxidant activity of grape PE, 20-fold higher than that of vitamin C and 50-fold higher than that of vitamin E [6], and their antiinflammatory and antiaggregant effects, along with estrogen-like activity [14], make them an interesting object for studies of their antiatherogenic effect, particularly in women during the postmenopausal period. However, the majority of studies

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were carried out *in vitro*, while *in vivo* effects are little studied and mainly on animals.

Previous studies showed that serum from atherosclerotic patients induces cholesterol (CH) accumulation in cell culture, *i.e.* produces an atherogenic effect [2]. Reduction of the serum atherogenicity is regarded as a pathogenetic approach aimed at prevention of CH accumulation in the vascular cells, the key stage of atherosclerosis at the cell level [11].

We studied the effect of grape PE on reduction of atherogenicity of the serum from postmenopausal women *ex vivo*.

## MATERIALS AND METHODS

Components of grapes (*Vitis vinifera L.*) derived from products of its technological processing during wine production were used in the study: dry water-ethanol extract from grape seeds, crushed grape seeds, crushed fermented grape ridges (fruit stems), and crushed dry pressed-out grapes (Oeno-Consulting SRL) in doses of 1000, 500, 250, 100, and 50 mg. The reference drug was commercial preparation Phytosoybean (Arkopharma, Laboratories Pharmaceutiques) recommended for menopausal women and used in a daily dose of 35 mg soybean flavonoids (its cardioprotective effects were demonstrated not once in studies of PE, including *in vivo* experiments [4]).

Six volunteers aged 47-61 years in the postmenopausal period with atherogenic sera were selected for the study. Serum atherogenicity was evaluated by its capacity to induce significant accumulation of intracellular CH in primary culture of human blood monocytes in comparison with control cells incubated without serum. Serum was collected before and 2, 4, and 6 h after single dose of plant products with high level of PE.

Monocytes were isolated from human blood by gradient centrifugation in Ficoll-Paque as described previously [2]. The cells were cultured in growth medium 199 supplemented with 2 mM L-glutamine, antibiotics (Gibco Europe, Paisley), and 10% test serum at 37°C and 5% $CO_2$ for 3 h. Control cells were incubated in a medium containing 10% fetal calf serum (Gibco Europe, Paisley). Cholesterol was extracted from cells 3 times with hexan-isopropanol mixture (3:2) and measured using enzyme immunoassay kits. Intracellular protein after 0.1% NaOH hydrolysis was evaluated after Lowry. The content of CH in cells was presented as $M \pm m$ from 4 parallel measurements and expressed in μg/mg protein.

The content of CH in control cells incubated without test serum was taken for 100%. Serum causing significant accumulation of intracellular CH in comparison with control cells was considered as atherogenic. Atherogenicity was expressed in percent of control. In order to evaluate the antiatherogenic effect, the initial accumulation of cell CH surpassing the control level was taken for 100%. The data were statistically processed using Student's *t* test.

## RESULTS

The content of CH in control cells incubated without test serum varied from 34.4±0.8 to 57.6±1.6 μg/mg protein and was taken as 100%. Incubation of cells with the test serum increased intracellular CH in cultured monocytes from 47.6±1.3 to 78.8± 3.2 μg/mg protein (1.3-1.5-fold increase in CH content) in comparison with the control.

Single dose of soybean isoflavonoids (Phytosoybean preparation) decreased serum atherogenicity by 28±20% after 2 h, by 38±14% ($p<0.05$) after 4 h, and by 30±19% of initial level after 6 h.

Single intake of dry grape seed extract in doses of 50-1000 mg significantly decreased serum atherogenicity after 2, 4, and 6 h by 67±6, 66±5, and 71±6% of the initial level, respectively ($p<0.05$). Antiatherogenic effects of grape seed extract in doses of 50-1000 mg were virtually the same. The minimum effective (optimum) single dose producing the antiatherogenic effect was found: 100 mg (Table 1).

Antiatherogenic effect of crushed dry pressed-out grapes was also noted for all doses. Serum atherogenicity decreased by 44±6, 41±15, and 58±8% of the initial level ($p<0.05$) 2, 4, and 6 h after intake of dry pressed-out grapes, respectively. Antiatherogenic effect of dry pressed-out grapes in doses of 250-1000 mg was virtually the same; the optimum single dose is 250 mg (Table 1).

Crushed fermented grape ridges significantly reduced serum atherogenicity in all studied doses by 28±5, 37±9, and 43±8% of initial level ($p<0.05$) 2, 4, and 6 h after intake, respectively. Antiatherogenic effects of the doses of 250-1000 mg were virtually the same; the optimum single dose is 100 mg (Table 1).

Crushed grape seeds in a dose of 1000 mg did not appreciably reducted serum atherogenicity 2, 4, or 6 h after intake. Hence, the atherogenic effects of lower doses of this component of grapes was not evaluated.

Hence, the most pronounced antiatherogenic effect was exhibited by dry extract of grape seeds at the minimum effective dose of 100 mg. The antiatherogenic effect of dry pressed-out grapes and fermented grape ridges was lower, their minimum effective doses being 250 and 100 mg, re-

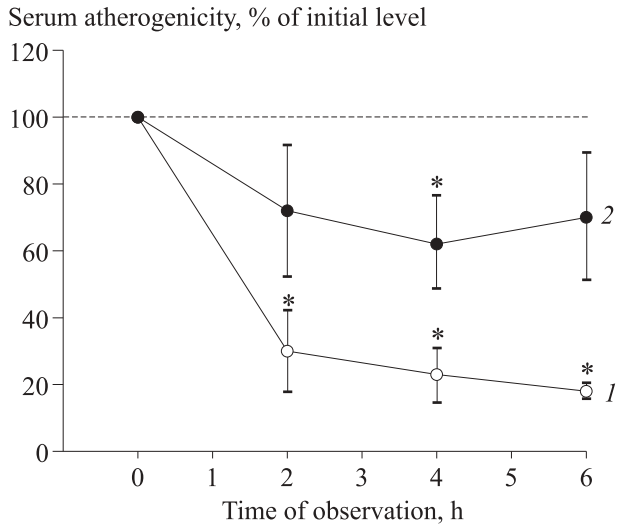


**Fig. 1.** Comparative characteristics of antiatherogenic effects of dry grape seed extract in the minimum effective dose (*1*) and of Phytosoybean preparation (*2*). *Significant reduction of serum atherogenicity ($p<0.05$).

spectively. Crushed grape seeds exhibited no antiatherogenic effect. Lower antiatherogenic effects of dry pressed-out grapes and fermented grape ridges in comparison with the effect of grape seed extract and absence of crushed grape seeds effect seem to be due to lower bioavailability of active components and to different content of PE in different components of grapes. The greatest amount of polyphenols (60-70%) is extracted from grape seeds, 28-35% from grape peel, and less than 10% from the pulp [14]. Antiatherogenic effects of grape seed extract, dry pressed-out grapes, and fermented grape ridges was significantly higher in comparison with the effect of Phytosoybean preparation. Comparative data on antiatherogenic effects of dry grape seed extract in the minimum effective dose and Phytosoybean are presented in Fig. 1.

The decrease in serum atherogenicity under the effect of grape preparations was revealed on a cell model of primary culture of monocytes/macrophages. Using this model, we demonstrated the antiatherogenic effect of grape preparations at the cellular level (on the cells directly involved in atherogenesis). On the other hand, the model was used *ex vivo* and the biological effect of grape PE was evaluated after their absorption in the gastrointestinal tract, possible biotransformation, and release of active metabolites. Grape PE are presented mainly as glycosides, esters, or polymers; they cannot be adsorbed in the native form and are subjected to hydrolysis by gastrointestinal enzymes and/or enteric microflora [10]. Therefore, the detected antiatherogenic effect seems to be due to the effects of active metabolites.

The key point of atherogenesis is CH accumulation in arterial intimal cells [7]. It was previously shown that serum from patients with atherosclerosis is characterized by an atherogenic potential (causes lipid accumulation in cell culture [2]) due to the presence of multiply modified LDL [15]. The antiatherogenic effect of grape PE can be due to suppression of uncontrolled uptake of modified LDL

**TABLE 1.** Antiatherogenic Effects of Minimum Effective Doses of the Studied Grape Components ($M\pm m$)

| Grape component | Group; time after preparation intake, h | | Intracellular CH content, μg/ml | CH accumulation, % of control |
|---|---|---|---|---|
| Dry seed extract, 100 mg | Control | | 49.5±1.9 | 100±4 |
| | Experiment | before intake | 69.4±3.1* | 140±6* |
| | | after 2 h | 54.4±1.9$^+$ | 110±4$^+$ |
| | | after 4 h | 53.1±1.7$^+$ | 107±3$^+$ |
| | | after 6 h | 52.7±1.5$^+$ | 106±3$^+$ |
| Dry pressed-out grapes, 250 mg | Control | | 49.6±2.0 | 100±4 |
| | Experiment | before intake | 67.1±2.1* | 135±4* |
| | | after 2 h | 56.9±3.5$^+$ | 115±7$^+$ |
| | | after 4 h | 58.6±3.8 | 118±8 |
| | | after 6 h | 54.1±2.4$^+$ | 109±5$^+$ |
| Fermented ridges, 100 mg | Control | | 49.7±1.3 | 100±3 |
| | Experiment | before intake | 65.1±2.0* | 131±4* |
| | | after 2 h | 62.8±4.5 | 126±9 |
| | | after 4 h | 57.2±2.9$^+$ | 115±6$^+$ |
| | | after 6 h | 54.6±3.4$^+$ | 110±7$^+$ |

**Note.** $p<0.05$: significant *accumulation of CH; $^+$reduction of serum atherogenicity.

by cells and inhibition of intracellular CH accumulation. It is also possible that antiatherogenic activity of grape PE is due to their antioxidant properties. It was shown that grape PE inhibit LDL oxidation, and that due to the presence of different polyphenols in grapes their antioxidant activities are synergistic [13]. However, other mechanisms, e.g. inhibition of free radical production by macrophages and of some cell metabolism enzymes by grape PE cannot be excluded [14].

Hence, PE from the studied grape components, such as grape seed extract, pressed-out grapes, and fermented grape ridges, possess an antiatherogenic effect and decrease *ex vivo* serum atherogenicity, thus preventing CH accumulation in cultured monocytes; they can be regarded as prospective components for the development of natural preparations for direct antiatherosclerotic therapy of women during menopause.

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