

Vasorelaxatory Effect of *Vitis vinifera* Extract on Rat Aorta

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Abstract

Reports have shown the antioxidant, hypotensive, hypolipidemic and vasodilatory effects of grape (*Vitis vinifera* L.) seed extract. We have recently shown the relaxatory effect of grape leaf extract on rat uterus and reduction of frog heart rate and contractility. The aim of the present study was to investigate the relaxant effect of *Vitis vinifera* leaf hydroalcoholic extract (VLHE) on rat thoracic aorta contractions induced by phenylephrine and KCl and the role of aorta endothelium on this action. Rat aorta was removed and placed in an organ bath containing Krebs-Henseleit solution and aorta contractions were recorded isometrically.

The results demonstrate that VLHE (0.125-2 mg/ml) reduces the endothelial intact aorta reconstructed by phenylephrine (1 μ M) dose-dependently ($P < 0.0001$). Extract induced the same response in endothelial denuded aorta, but in a much lesser extent. The IC_{50} for both groups were 0.45 ± 0.08 and 1.73 ± 0.23 mg/ml, respectively. However, the contractile responses of these groups were similar. VLHE (0.125-2 mg/ml) reduced the contractions induced by KCl (80 mM) dose-dependently ($P < 0.0001$). The relaxatory effect of VLHE on KCl-induced contractions was less than those evoked by Phenylephrine. Vasorelaxatory effect of VLHE on intact aorta was attenuated by nitric oxide synthase inhibitor (L-NAME, 100 μ M) and guanylate cyclase inhibitor (methylene blue, 10 μ M) significantly, but was unaffected by atropine (1 μ M).

The results suggest that the greatest vasorelaxant effect of VLHE on rat aorta is endothelium-dependent and nitric oxide (NO) and cGMP are mostly responsible but cholinergic receptors are not involved.

Keywords: *Vitis vinifera*; Leaf, Rat; Aorta; Endothelium; Nitric oxide; cGMP.

Introduction

Vitis vinifera (grape) is a perennial woody vine native to Asia Minor and then introduced in Europe and other continents (1). Different parts of this plant, particularly the fruits, used in folk medicine. Among the most interesting constituents responsible for the therapeutically

properties of the plant, procyanidins are used for the treatment of microcirculatory disorders (1). The leaves of *Vitis vinifera* (Vitaceae) is used in traditional food (dolmathes) in some Mediterranean countries and in folk medicine used for the treatment of diarrhea and vomiting (2). It has been shown that despite a similar distribution of other coronary risk factors such as high blood pressure, serum cholesterol, and cigarette smoking, the mortality of coronary heart disease in France and Switzerland was

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lower compared with that in other industrialized countries. From analyzing World Health Organization data (3), it has been suggested that, the phenomenon (French paradox) might be because of the frequent consumption of wine in France and Switzerland in addition to the potentially important effects of a Mediterranean-style diet (4, 5). Grape skin produces endothelium-dependent aorta relaxation possibly by its flavonoids (quercetin) via increasing cGMP (6), as grape seed extract increases NO and cGMP (7). Also it is reported that grape seed extract induces the human artery relaxation dependent on endothelium (8). Anti-tumor, anti-cataract and anti-atherosclerosis activities of grape seed extracts are also reported (9-12). However, the grape seed extract is not toxic even after twelve months consumption (13). The scavenging free radicals by flavonoids (e.g. quercetin) are reported (14) and quercetin reduces blood pressure in rat (15). The same result has been reported on grape skin extract (16). It is reported that grape leaves protect liver against alcohol intoxication (17). Our previous studies showed the relaxatory effect of grape leaves extract on KCl and oxytocin-induced contractions in rat uterus (18) and it is concluded that the voltage dependent calcium channels are involved. The same extract reduces the rate and contractility of isolated frog heart but cholinergic receptors are not involved (19). Since there is a possibility that some of the grape seed constituents exist in grape leaves and collection and extraction of grape leaves is cheaper and easier, therefore, the aim of the present study was to investigate the effect of *Vitis vinifera* hydroalcoholic leaf extract (VLHE) on isolated rat aorta.

Experimental

Animals and tissue preparation

Healthy male Sprague Dawley rats (185-220 g) were supplied by the University Animal Facility and the protocol was approved by the Committee of Ethics in Research (Jondi Shapur Ahwaz University of Medical Sciences). Animals were kept at 20-24°C under 12 h light-dark cycle, and were allowed free access to tap water and commercial chow. Animals were anaesthetized by ketamine (50 mg/kg, ip) and

exsanguinated. Thoracic aorta was rapidly removed and cleaned of extraneous connective and fatty tissue in ice-cold Krebs-Henseleit solution and cut into rings 5 mm long. Isometric contractile responses were determined by placing the rings in an organ bath (10 ml) containing Krebs-Henseleit solution (37°C, pH 7.4) of the following composition (mM): NaCl 118, KCl 4.7, CaCl₂ 2.52, MgSO₄ 1.64, KH₂PO₄ 1.18, NaHCO₃ 7 and glucose 5.5 and gassed with oxygen. Tissues were then maintained under 1 g initial tension and allowed to equilibrate for 1 h during which bath solution was replaced every 15 min. All the dissection procedures were performed with extreme care to protect the endothelium from inadvertent damage. Aortic rings were suspended horizontally between two stainless steel hooks. One of the hooks was fixed to the chamber wall, while the other was attached to an isometric force transducer (UF1 Harvard Transducer, UK) and to an ink-writing curvilinear polygraph (Universal Harvard Oscillograph, UK). In some aortic rings, the endothelium was removed by gently rubbing the inner surface with a rough steel needle, and used as the denuded endothelium aorta. Body weight of animals in all protocols was matched.

Extraction

Vitis vinifera leaves were collected from the campus of the Jondi Shapur Ahwaz University of Medical Sciences in April 2003. The leaves were identified by Mr. Parishani from the Department of Biology of Shahid Chamran Ahwaz University. A voucher specimen was submitted to the herbarium of the same university. The leaves were dried under shade, powdered and extracted by maceration with a 70% hydro alcoholic solution for 72 h at ambient temperature. Extract was filtered and then, solvent was evaporated at room temperature (20). The extraction ratio was 19% of dried leaves and the extract was kept in 4°C until use.

Drugs

N^o-nitro-L-arginine methyl ester (L-NAME), atropine sulfate, phenylephrine hydrochloride, acetylcholine chloride and methylene blue were purchased from Sigma (USA) and mineral solutes

from Merck (Germany). All the chemicals were dissolved in Krebs-Henseleit and volumes added to bath were less than 2% of the organ bath volume and solutions were made daily.

Experimental protocols

In all experimental groups, after equilibration, the presence of a functional endothelium was assessed on the basis of relaxation evoked by the endothelium – dependent dilator acetylcholine (1 μ M) in aorta rings precontracted with phenylephrine (1 μ M). Acetylcholine in intact endothelium aortae induced more than 60% relaxation of the phenylephrine-induced contraction (21). To evaluate the effects of the *Vitis vinifera* leaf extract on the contractile responses, in a first and second series of experiments, the effects of non-cumulatively increasing concentrations of the extract (0.125-2 mg/ml) on the contractile responses to phenylephrine (1 μ M) or KCl (80 mM) were studied. Once the plateau of the KCl- or phenylephrine-induced contractions was achieved, a specific concentration of extract was used. The role of NO and cGMP was determined using the nitric oxide synthase inhibitor (L-NAME, 100 μ M for 2 min) and guanylate cyclase inhibitor (methylene blue, 10 μ M for 30 min) respectively. All the mentioned concentrations are the final concentrations present within the tissue bath.

Statistical analysis

Results are expressed as the mean \pm SEM of percentage reduction in aortic ring tension from phenylephrine or KCl precontracted aorta. Statistical analysis was performed using the Student's t-test or one- and two-way analysis of variance (ANOVA). *P*-values which were less than 0.05 considered statistically significant and *n* represents the number of animals in each group.

Results and Discussion

Effect of VLHE on precontracted aorta by phenylephrine and the role of endothelium

Intact (*n*=8) and denuded endothelium (*n*=9) aorta with functional endothelium (75.1 \pm 4% and 5.3 \pm 3.5% respectively) were used. As figure 1

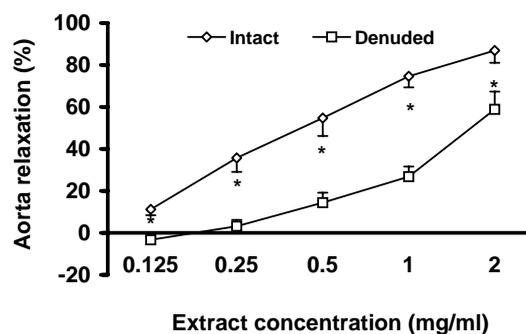


Figure 1. The vasorelaxant effect of *Vitis vinifera* hydroalcoholic leaf extract (VLHE) on intact (*n*=9) and denuded (*n*=8) endothelium rat aorta precontracted by 1 μ M of phenylephrine (ANOVA, *P*<0.0001). * Shows that *P*<0.01 (t-test) at same concentrations of VLHE for the two groups.

shows, the extract (0.125, 0.25, 0.5, 1 and 2 mg/ml) relaxed the phenylephrine-contracted (1 μ M) tissues in both aorta groups dose-dependently and significantly (*P*<0.0001). The IC₅₀ values were 0.45 \pm 0.08 and 1.73 \pm 0.23mg/ml respectively. The statistical analysis (two-way ANOVA) indicates that the vasorelaxation effect of the two groups are different (*P*<0.0001) and also present in all extract concentrations. However, the phenylephrine-induced contractions are not different (*P*=0.17). In figure 6 (A₁, A₂) the role of endothelium on the vasorelaxatory effect of VLHE could be seen.

Effect of L-NAME on vasorelaxatory effect of VLHE

Intact endothelium aorta was contracted by phenylephrine (1 μ M) and then relaxed by VLHE at 0.5 and 1mg/ml in two separate protocols.

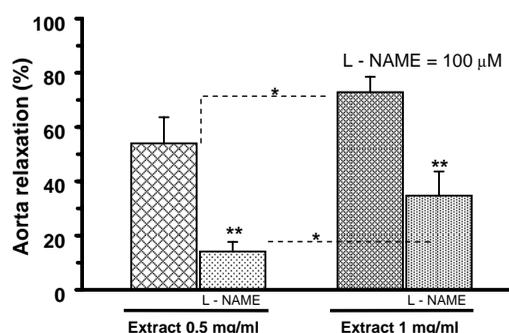


Figure 2. Effect of VLHE (0.5 and 1mg/ml) on intact endothelium aorta (*n*=6) precontracted by phenylephrine (1 μ M) in the absence and presence of L-NAME (100 μ M, for 5min). * *P*<0.05 and ** *P*<0.01 show the statistical comparison (t-test) between the indicated groups.

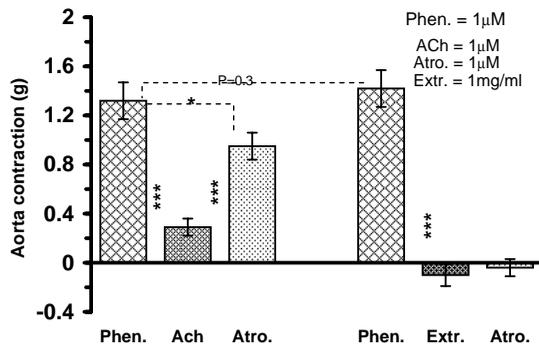


Figure 3. In intact endothelium aorta (n=7), ACh (1μM) reduced the phenylephrine-induced contraction, and atropine (1μM) returned aorta contraction but not completely. As could be seen, the vasorelaxatory effect of VLHE (1mg/ml) on phenylephrine-induced contraction is not affected by atropine. However, the phenylephrine-induced contractions in these two protocols are not significantly different. * P<0.05 and *** P<0.0001 show the statistical comparison (t-test) between the indicated groups.

After washing the tissue and at least a 15 min recovery period, tissue was pretreated with L-NAME (100 μM) for 5 min and then the same protocol was repeated. As could be seen in figures 2 and 6 (D), the VLHE vasorelaxatory effect is reduced by L-NAME significantly (both, P<0.01, n=6)

Effect of atropine on vasorelaxatory effect of VLHE

Intact endothelium aorta (n=7) was contracted by phenylephrine (1 μM). Acetylcholine (1 μM) relaxed the aorta (P<0.0001), but atropine (1 μM) attenuated this relaxation (P<0.0001).

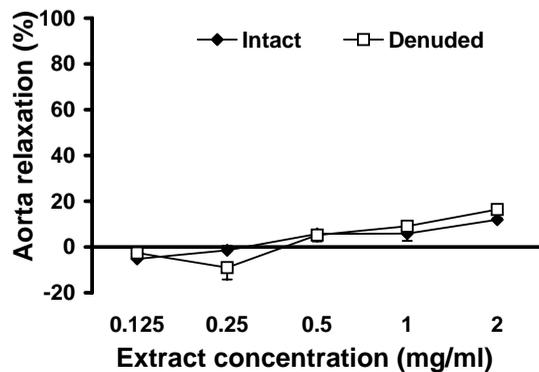


Figure 5. The vasorelaxant effect of *Vitis vinifera* hydroalcoholic leaf extract (VLHE) on intact (n=7) and denuded (n=7) endothelium rat aorta precontracted by 80mM of KCl (ANOVA for each group, P<0.0001). But, these two groups are not significantly different. The spasmogenic effects of low doses of VLHE have been shown.

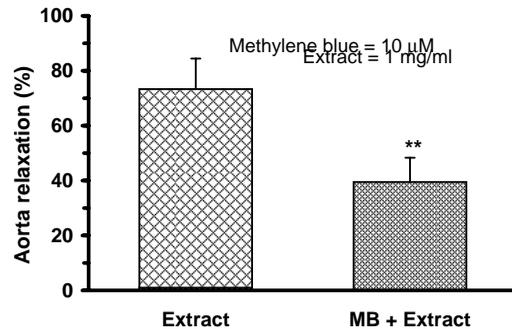


Figure 4. Effect of VLHE (1mg/ml) on intact endothelium aorta (n=9) precontracted by phenylephrine (1μM) in the absence and presence of methylene blue (10μM, for 30 min). * P<0.02 shows the statistical comparison (t-test) between these two protocols.

After washing and a 20 min recovery period, aorta was contracted by phenylephrine 1 μM, which abolished by VLHE (1mg/ml) as shown in figures 3 and 6 (C). But, VLHE-induced relaxation was unaffected by atropine (1 μM).

Effect of methylene blue on vasorelaxatory effect of VLHE

Intact endothelium aorta (n=9) was contracted by phenylephrine (1 μM) and then relaxed by 1mg/ml of VLHE (73.3±11.1%). After refreshing the bath solution and at least a 15 min recovery period, tissue was pretreated with methylene blue (10 μM) for 30 min and then the same protocol was repeated. As shown in figures 4 and 6 (E), the relaxatory effect of VLHE is reduced by methylene blue (P<0.02).

Effect of VLHE on KCl-induced aorta contraction and the role of endothelium

Intact (n=7) and denuded endothelium (n=7) aorta with functional endothelium (69.8±2.8% and 5±3.2% respectively) were contracted by KCl (80 mM). After the reaching of contractile responses to a plateau, extract (0.125, 0.25, 0.5, 1 and 2 mg/ml) was added to bath, non-cumulatively. As figure 5 shows, in both groups, the KCl-induced contractions are attenuated by VLHE dose-dependently (one-way ANOVA, P<0.001 and P<0.01 respectively). VLHE, in lower concentrations, however, induced small contractions. Statistical analysis (two-way ANOVA) indicates that these relaxatory responses are not different. But, in both aorta,

the relaxatory effect of VLHE on phenylephrine-induced contraction is stronger than the relaxation caused in the KCl-induced contractions, as seen in figure 6 (B₁, B₂), compared to A₁ and A₂.

The present study was performed in order to investigate the possible vasodilatory effects of the *Vitis vinifera* hydroalcoholic leaf extract (VLHE) on rat thoracic aorta. It was observed that VLHE induced NO- and endothelium-dependent relaxation. Several lines of evidence support the idea that grape seed extract induces vasorelaxation (7, 8, 22), but the present study is the first report on vasodilatory effect of grape leaf. However, we have previously reported the relaxatory effects of grape leaf extract on rat uterus (18). The negative chronotropic and inotropic effects of the same extract has also been reported (19), but the effects on frog heart was unaffected by atropine. This suggests that cholinergic receptors are not involved. Phenylephrine is "a" α_1 -adrenergic agent, but its receptors do not exist in endothelial cells. Hence, these cells are unable to release vasoactive substances (23). Acetylcholine induces vasorelaxation via an endothelium-dependent mechanism (24). Binding ACh to vascular muscarinic receptors induces the release of NO from endothelial cells (5, 25). NO diffuses into adjacent vascular smooth muscle cells, activating the guanylate cyclase and increasing the cGMP production and finally, induces vascular relaxation (5). As figure 1 shows, the relaxatory effect of VLHF in denuded aorta is less than the intact aorta. Therefore this part of study shows that the vasorelaxatory effect of VLHE is endothelium dependent, as reported for grape seed extract (8). The similarity of phenylephrine-induced contractions in these two groups indicates that the reduction of contraction is not due to a modification in the contractility of aorta (26). The 3.8 times difference in IC₅₀ indicates the importance of functional endothelium in relaxatory effect of VLHE as reported on relaxation induced by grape seed extract in rat and human arteries (5, 8). It is reported that elevated cGMP by procyanidins induces relaxation in rat aorta, which is unaffected by atropine (27) and this report is compatible with our results. In the present study, methylene blue reduced the vasorelaxant effect of extract. This shows that

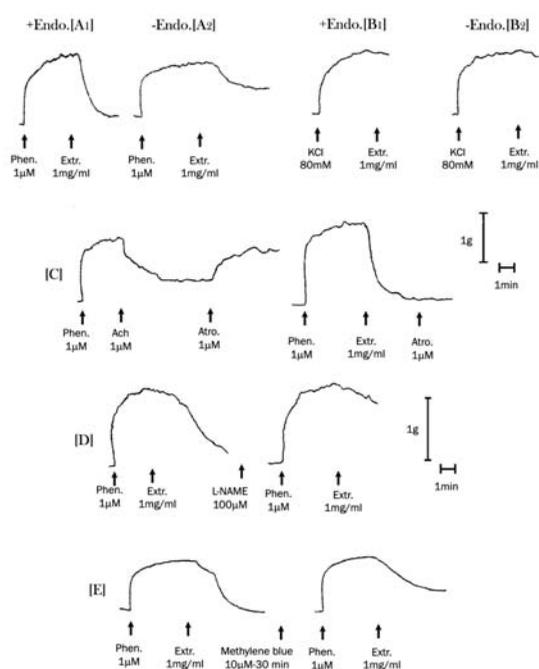


Figure 6. Presenting some of the typical recordings of vasorelaxant effects of VLHE in the present study. Horizontal and vertical bars indicate the time (1min) and force of aorta contraction (1g) respectively. Phen.: Phenylephrine, + Endo.: intact endothelium, -Endo.: denuded endothelium, Extr.: Extract, Atro.: atropine, Ach: acetylcholine.

cGMP is involved, as reported (27, 28). One of the flavonoids that could be responsible for this relaxatory response is quercetin, which has been found in *Vitis vinifera* leaf (29) and it is reported that the relaxatory effect of quercetin in rat aorta abolished by L-NAME (30). This study suggests that the involvement of NO and cGMP in the observed vasorelaxatory effects of VLHE.

Furthermore, the extract induced the same inhibitory effect on KCl-induced contraction in aorta with and without endothelium, although in a weaker extent. It is well documented that the contraction induced by high extracellular K⁺ concentration is due to depolarization followed by opening the voltage dependent calcium channels (31). It is suggested that substances which reduce the KCl-induced contraction in smooth muscle, block these channels (31). Since it is reported that cGMP causes hyperpolarization by the opening of potassium channels (32), therefore, the reason for this weaker relaxation could be due to the presence of high K⁺ concentration in the organ bath. It is reported that L-type voltage dependent calcium channels are the most abundant and important calcium channels

in rat aorta (31). Therefore, it could be suggested that the inhibitory effect of the extract could be due to, at least partly, the voltage dependent calcium channels (VDCCs) blockade as we have reported in our previous studies (18, 19). On the other hand, atropine diminished the relaxatory effect of acetylcholine, but the inhibitory effect of extract was unaffected, which indicates the lack of cholinergic activity in VLHE. Another possibility is that the extract contains the α_1 -adrenoceptor antagonists. On the other hand, the fast disappearance of VLHE vasorelaxatory effect by washing, suggests that this effect should be on the cell membrane rather than an intracellular event.

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