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Modulation of haemostatic function and prevention of experimental thrombosis by red wine in rats: a role for increased nitric oxide production

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1 The effects of ethyl alcohol and wine (red and white) on haemostatic parameters and experimental thrombosis were studied in rats; NO was evaluated as a possible mediator of these effects.

2 We found that red wine (12% alcohol) supplementation (8.4 ± 0.4 ml d^{-1} in drinking water, for 10 days) induced a marked prolongation of 'template' bleeding time (BT) (258 ± 13 vs 132 ± 13 s in controls; $P < 0.001$), a decrease in platelet adhesion to fibrillar collagen (11.6 ± 1.0 vs $32.2 \pm 1.3\%$; $P < 0.01$) and a reduction in thrombus weight (1.45 ± 0.33 vs 3.27 ± 0.39 mg; $P < 0.01$).

3 Alcohol-free red wine showed an effect similar to red wine. In contrast, neither ethyl alcohol (12%) nor white wine (12% alcohol) affected these systems.

4 All these effects were also observed after red wine i.v. injection (1 ml kg^{-1} of 1 : 4 dilution) 15 min before the experiments.

5 The effects of red wine were prevented by the NO inhibitor, N^{ω}nitro-L-arginine-methyl ester (L-NAME). L-arginine, not D-arginine, reversed the effect of L-NAME on red wine infusion.

6 Red wine injection induced a 3 fold increase in total radical-trapping antioxidant parameter values of rat plasma with respect to controls, while white wine and alcohol did not show any effect.

7 Our study provides evidence that red wine modulates primary haemostasis and prevents experimental thrombosis in rats, independently of its alcohol content, by a NO-mediated mechanism.

Keywords: Haemostasis; platelet adhesion; bleeding; venous thrombosis; nitric oxide; plasma antioxidant capacity

Abbreviations: BT, 'template' bleeding time; NO, nitric oxide; L-NAME, N^{ω}nitro-L-arginine-methyl ester; TRAP, total radical-trapping antioxidant parameter

Introduction

The protective effect of a moderate daily consumption of alcoholic beverage on the risk of cardiovascular disease has been described by a number of epidemiological studies (Renaud and De Lorgeril 1992; Goldberg *et al*., 1995; Rimm *et al*., 1996; Hein *et al*., 1993; Gaziano *et al*., 1996). Several mechanisms have been proposed that mediate the protective effect of alcohol in ischaemic vascular disease. In particular, alcohol increases the prostacyclin/thromboxane ratio (Landolfi & Steiner, 1984) and decreases platelet aggregability (Renaud *et al*., 1992); moreover, it increases the release of plasminogen activator and lowers the levels of fibrinogen (Veenstra *et al*., 1990; Laug, 1983; Ridker *et al*., 1994).

The possibility that the type of alcoholic drink, beside alcohol itself, influences the risk of cardiovascular disease has been suggested. The so called 'French paradox' proposed that low cardiovascular mortality rate in France was due, at least in part, to regular consumption of wine, more than of beer (Renaud and De Lorgeril 1992). The Copenaghen City Heart Study showed among wine but not beer or spirit drinkers a lower cardiovascular mortality, similar to that observed in moderate alcohol drinkers (Groenbaek *et al*., 1995). Lately, results of a large prospective study performed in Eastern France, appeared to confirm the association between the regular consumption of alcohol, mostly wine, and lower mortality for coronary artery disease (Renaud *et al*., 1998).

From epidemiological studies, whether the beneficial effects of alcohol consumption have to be ascribed to wine in a substantial way remains an unresolved issue. It is also unknown whether red or white wine equally contribute to the potential benefits of alcohol. The observation that red wine administration to healthy subjects increased plasma high-density lipoprotein cholesterol and apolipoprotein A-1 plasma concentrations while white wine did not (Lavy *et al*., 1994), suggested that other components in red wine rather than its alcoholic content might play a role in cardiovascular prevention.

Red wine contains many other compounds that might influence the process of thrombosis (Rice-Evans *et al*., 1997). Polyphenolic red wine components inhibited platelet aggregation and platelet-leukocyte interactions (Bertelli *et al*., 1995; Pellegrini *et al*., 1996; Pace-Asciak *et al*., 1995; Rotondo *et al*., 1996, 1998). Flavonoids and other red wine ingredients, which were recognized as strong antioxidants and oxygen free radical scavengers, enhanced in *in vitro* and in *ex vivo* experiments, the generation of nitric oxide (NO), a platelet inhibitor and vasodilator (Fitzpatrick *et al*., 1993; van Acker *et al*., 1995; Andriambeloson *et al*., 1997). Fitzpatrick *et al*. showed that

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wine and other grape products induced a NO-dependent relaxation of aortic rings (Fitzpatrick *et al*., 1993). Later, Andriambeloson *et al*. identified polyphenol compounds and leucocyanidol as responsible for this NO-dependent effect (Andriambeloson *et al*., 1997). However, no data are available on the relevance of such mechanisms *in vivo*.

The aims of this study were the following: (1) To establish whether ethyl alcohol and wines (red and white) would exert comparable effects on haemostatic parameters and on experimental thrombosis; (2) To evaluate whether removal of alcohol from wine would also remove its potential effects on haemostasis and thrombosis and (3) To determine the role of NO production as a possible mediator of the effects of alcoholic beverages on haemostasis and thrombosis.

We provide here evidence that red wine, but not white wine or ethyl alcohol administration to rats induces NO production *in vivo* and tentatively suggest a clinically-relevant mechanism to explain, in part, the protective effects of moderate red wine consumption on cardiovascular disease in man.

Methods

Animals

Male Sprague-Dawley rats, from Mario Negri Sud breeding facility, weighing 300–370 g were used for this study, housed under controlled conditions and fed a standard diet *ad libitum*. Procedures involving animals and their care were conducted in conformity with the Institution guidelines that are in compliance with national (D.L. n. 116, G.U. suppl. 40, February 18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358,I, Dec. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication N. 85-23, 1985 and Guidelines for the Use of Animals in Biomedical Research., Thromb Haemost 58, 1078-1084, 1987).

Experimental design

Oral administration Rats were supplemented with drinking water for 10 days, with red wine (12% alcohol by volume), white wine (12% alcohol by volume) or ethyl alcohol (12%). Three different drinking solutions were prepared by adding one part of red wine, white wine or ethyl alcohol to three or seven parts of drinking water. Since rats drank an average of 32 ± 3 ml d^{-1} of these solutions, the daily amount of wines or ethyl alcohol was the following: 8.4 ± 0.4 or 4.2 ± 0.2 ml d^{-1} of red wine, 8.4 ± 0.4 ml d^{-1} of white wine and 8.3 ± 0.4 or 4.1 ± 0.3 ml d^{-1} of ethyl alcohol.

In some experiments alcohol-free red wine was administered at a daily amount of 8.8 ± 0.2 ml in drinking water for 10 days. Alcohol-free red wine was obtained by red wine lyophilization and resuspension in an equal volume of drinking water.

BT, *ex vivo* platelet studies, experimental thrombosis induction and laboratory analyses were performed after 10 days of supplementation. Groups of 15 animals were studied for each experiment.

To test the role of NO, a group of animals (ten rats) was supplemented for 10 days with red wine, containing 1.0 g l^{-1} of L-NAME, an inhibitor of NO formation, or with L-NAME alone diluted in drinking water.

Intravenous (i.v.) administration One part of red wine, white wine, ethyl alcohol or alcohol-free red wine was diluted in three parts of saline solution (NaCl 0.9%). One ml kg^{-1} of this solution was injected i.v. in rats and experiments were performed after 15 min (this time interval was chosen on the basis of preliminary experiments, data not shown). Groups of 15 animals were used.

To investigate the role of NO on intravenous injection of red wine, groups of animals (ten rats) were pretreated with L-NAME, at the dose of 30 mg kg^{-1}, i.v. Since the inhibitory effect of L-NAME was detected only when it was given before red wine administration and lasted no more than 20 min, we administered L-NAME 2 min before wine injection (preliminary experiments, data not shown). Fifteen minutes after the treatments, BT was measured or blood was drawn from the heart for *ex vivo* platelet studies and laboratory analyses.

When venous thrombosis was studied, L-NAME or saline solution were also infused in the femoral vein after inferior vena cava ligature for the whole experiment. The infusion led to a reduction in thrombus weight, due to continuous washing of the thrombus or to blood dilution at the site of thrombus formation.

L-arginine, the precursor of NO synthesis in vascular endothelium and its stereoisomer D-arginine, were used to reverse the effect of L-NAME on red wine-induced effects. Pretreatment with L-NAME (30 mg kg^{-1}, i.v.) 2 min before wine injection was followed by the injection of L-arginine or D-arginine (300 mg kg^{-1}) 5 min after (Remuzzi *et al*., 1990).

To rule out a role of prostacyclin on red wine effects, aspirin was used at a dose (5 mg kg^{-1}, i.v.) effectively inhibiting vascular cyclo-oxygenase (Cerletti *et al*., 1986).

'Template' bleeding time BT was measured as described (Wollny *et al*., 1997). Briefly, rats were placed in a plastic cylinder with several openings from one of which the animal's tail was protruding. A standardized device was applied longitudinally on the dorsal part of the tail between 6 and 9 cm from the tip, taking care to avoid large veins. Immediately after injury, the tail was placed into a cylinder with isotonic saline solution at 37°C. BT was measured in seconds from the time when the tail was surgically cut until bleeding stopped completely (no rebleeding within 30 s).

Platelet preparation After oral or i.v. treatment, blood was drawn from the heart into plastic syringes containing 3.13% sodium citrate (1 : 9 v v^{-1}) from rats anaesthetized with sodium pentobarbital (40 mg kg^{-1}), injected intraperitoneally. Suspensions of washed platelets were prepared as previously described (Rotondo *et al*., 1997). Platelet-rich plasma (PRP) was obtained by consecutive centrifugation at $200 \times g$ for 15 min and then at $800 \times g$ for 3 min at room temperature. Then PRP was removed and pooled, the residual blood sample was centrifuged at $2000 \times g$ for 10 min to obtain platelet-poor plasma (PPP). The PRP platelet number was adjusted to 3×10^5 platelets μl^{-1} with autologous PPP.

Platelet adhesion to fibrillar collagen Ex vivo platelet adhesion was carried out as described (Radomski *et al*., 1987). Briefly, washed platelet samples (0.75×10^8 platelets in 0.25 ml) were incubated in duplicate in an aggregometer (Elvi Logos, Milano, Italy) at 37°C and stirred at 900 r.p.m. EDTA (5 mM) was added to prevent platelet aggregation. After 5 min, collagen (50 μg ml^{-1}) was added and the samples were stirred for a further 15 min. Platelets were counted optically using a Burker chamber, after sample dilution by the Unopette system (Becton-Dickinson, N.J., U.S.A.), before and 15 min after collagen addition and a difference in platelet count was taken as an index of their adhesion to collagen.

Platelet aggregation Platelet aggregation in PRP was induced by collagen and ADP as previously described (Remuzzi *et al*., 1990; Di Minno *et al*., 1979). The threshold-aggregating concentration was defined as the lowest concentration of aggregating agent, which induced irreversible platelet aggregation, starting within 3 min of addition of aggregating agents to PRP.

Experimental thrombosis Experimental thrombosis was induced by ligature of the inferior vena cava (Reyers *et al*., 1989). Rats were anaesthetized with sodium pentobarbital (40 mg kg^{-1}, bolus IP) and the rat abdomen was surgically opened on the median line. After a careful dissection, a tight ligature (with cotton thread) was placed around the inferior vena cava, just below the left renal vein. Two hours later, the abdomen was reopened under anaesthesia, the thrombus, if present, was removed, washed in distilled water, blotted on filter paper and placed in a desiccator; 24 h later, the dry weight of the thrombus was recorded.

Laboratory analysis

Fibrinogen was measured according to Clauss method; 200 μl of diluted plasma were incubated with 200 μl of thrombin (100 U ml^{-1}, Ortho Diagnostics, N.J., U.S.A.) and clotting time was measured by an electromagnetic coagulometer. The fibrinogen levels were calculated using a standard curve.

Procoagulant activity was assessed by a one-stage clotting assay (Napoleone *et al*., 1997); 100 μl of plasma were placed at 37°C and after 30 s, 100 μl of 25 mmol l^{-1} $CaCl_2$ at 37°C were added and the time of clot formation was recorded.

Euglobulin clot lysis time (ECLT) was assayed as previously described (Johnson *et al*., 1964).

Ethanol analysis in whole blood

Ethanol analysis in whole blood was performed by Headspace gas chromatography with capillary column (Correa & Custodio Pedroso, 1997) (Perkin Elmer GC-Autosystem XL chromatograph, fitted with CP-Wax 57 CB fused silica capillary column 50×0.25 mm I.D.; df = 0.2 mm; Chrompack, The Netherlands). The column temperature was initially set at 40°C (0 min) and then programmed at 7°C min^{-1} to 100°C for 4 min. The injector and the flame ionization detection (FID) system were at 210°C. Helium (He) was used as carrier gas at 1.2 ml min^{-1}. Air and He were set at 450 μl min^{-1}, respectively. The split rate was 1/50. The integrator was used with attenuation setting of 2.

Ethanol aqueous working solutions were prepared from 4% (v v^{-1}) ethanol standard solutions (Carlo Erba, Rodano, MI, Italy). The internal standard (analytical grade, 99.5% purity) n-propanol was purchased from Riedel-de Haën, (Seelze, Germany).

One μl of internal standard was added to 200 µl of whole blood in a 2 ml glass vial. The vial was rapidly sealed with silicone rubber septum cap and an aluminum crimp seal and incubated for 30 min at 70°C. The upper gas phase was homogenized three times by pulling and pushing the vapour phase using the injection syringe. After this, a homogenized 100 μl gas aliquot was withdrawn through the rubber cap with a 100 μl gas-tight Hamilton syringe (model 1705N, Supelco) and injected directly into the gas chromatograph. This syringe was equilibrated at 50°C, in order to prevent internal condensation on the walls (Tangerman, 1997).

Calibration curves were performed in whole blood spiked with ethanol standard to obtain concentrations ranging from 0.016 to 3.156 g l^{-1}.

Whole blood was obtained from rats ($n = 6$) ten days after red wine supplementation and 5 and 15 min after its intravenous injection.

Characterization of red and white wines

Red wine (1995 Montepulciano D'Abruzzo 12% alcohol by volume) and white wine (1996 Trebbiano D'Abruzzo 12% alcohol by volume) were kindly supplied by Cantina Miglianico, Miglianico, Italy.

Samples of red and white wine were analysed by HPLC (Varian 9010 Solvent delivery system, with Varian 9065 Polychrom diode array detector), following the method described by Goldberg *et al*. (1996), slightly modified as follows: 10 ml aliquots of freshly opened wine bottles were filtered through Minisart 0.45 mm (Sartorius) filter pad and stored at 4°C, protected against direct light. Analyses were completed within a week. Samples of 20 μl of filtered wine were directly injected into the column (ODS Hypersil 5 mm, purchased by Sigma-Aldrich) and eluted with the following gradient: solvent A: acetic acid, solvent B: methanol, solvent C: bidistilled water. Zero time conditions: 5% A, 15% B, 80% C; flow rate: 0.4 ml min^{-1} for 5 min, 5% A, 20% B, 75% C; flow rate 0.5 ml min^{-1} for 30 min; 5% A, 45% B, 50% C for 10 min.

Every run was followed by 10 min equilibrium period with the zero time solvent mixture prior to injection of the next sample.

Total phenols were analysed according to the Folin Ciocalteu method (Goldberg *et al*., 1996), using gallic acid as the standard, and the results are given as gallic acid equivalents (GAE). Total flavonoids, total anthocyanins, free anthocyanins, and the difference between total flavonoids and total anthocyanins, here indicated as non-anthocyanin-flavonoids, were estimated colorimetrically according to Di Stefano *et al*. (1989), calibrating against (+)-catechin and expressing results as (+)-catechin equivalents for total flavonoids and non-anthocyanin-flavonoids; cyanidin chloride was used as standard for anthocyanin determination.

Tannin content was determined following the method described by Serafini *et al*. (1997) and here slightly modified. Lyophilized wine samples were rehydrated with bidistilled water and the calibration curve was made using tannic acid ranging from 0.78 to 12.5 mg l^{-1}.

Phenolic composition of red and white wine

As shown in Figure 1, red wine contained a higher amount of phenolic compounds than white wine. The prominent peaks corresponded to protocatechin acid and gallic acid, identified with numbers 1 and 2 respectively, (+)-catechin and (−)-epicatechin, (peaks 3 and 4 respectively), *Trans*-Resveratrol (peak 5) and rutin (peak 6), were present only in red wine. This difference in polyphenolic content between red and white wine is more evident from the results reported in Table 1; the total phenol content in red wine exceeded more than two times that of white wine; total flavonoid content in red wine was more than six times and tannins were 12 times higher than in white wine. The content of other substances reported in Table 1 for white wine were below the detection limit and are indicated as < 20 mg l^{-1}.

Total Radical-trapping Antioxidant Parameter (TRAP)

TRAP assay was performed by measuring in plasma the rate of peroxidation induced by 2,2′-diazobis(2-amidinopropane)dihydrochloride (ABAP) through the loss of fluorescence of R-Phycoerythrin (R-PE) (Ghiselli *et al*., 1995). Briefly, the reaction mixture consisted of 1.5×10^{-8} M R-PE in 75 mM phosphate buffer, pH 7.0. Eighty μl of freshly prepared and diluted plasma (1 : 6) or any other reagent were added to 2.0 ml final volume, and the resulting solution was maintained at 37°C for 5 min in fluorimeter cuvettes. The oxidation reaction was started by adding ABAP to a final concentration of 4.0 mM, and decay of R-PE fluorescence was monitored every 5 min on Perkin-Elmer LS-5 Luminescens Spectrometer equipped with thermostatically controlled cell holder; monochromators were operating at excitation wavelength 495 nm and emission wavelength 575 nm. When plasma was added to the reaction mixture, a period of complete protection of R-PE was observed. The length of this lag-phase (T) was directly related to total plasma antioxidant capacity. To quantify the TRAP, the T produced by plasma was compared to the T produced by a known amount of Trolox (a water-soluble analogue of vitamin E), according to the following proportion: Trolox concentration (μmol l^{-1}) : T Trolox = X : T Plasma.

Table 1 Quantification of red and white wine antioxidant components

	Red wine (mg l^{-1})	*White wine* (mg l^{-1})
Total phenols*	1110 ± 15	458 ± 7
Total flavonoids†	462 ± 5	75 ± 4
Non coloured flavonoids†	336 ± 8	< 20
Total anthocyanins‡	56 ± 3	< 20
Free anthocyanins‡	36 ± 3	< 20
Tannins^{+}	6.2 ± 0.1	0.5 ± 0.1

Expressed as *mg l^{-1} of Gallic Acid; †(+)-Catechin; ‡Cyanidin Chloride; $^{+}$Tannic Acid; mean ± s.d., $n = 4$.

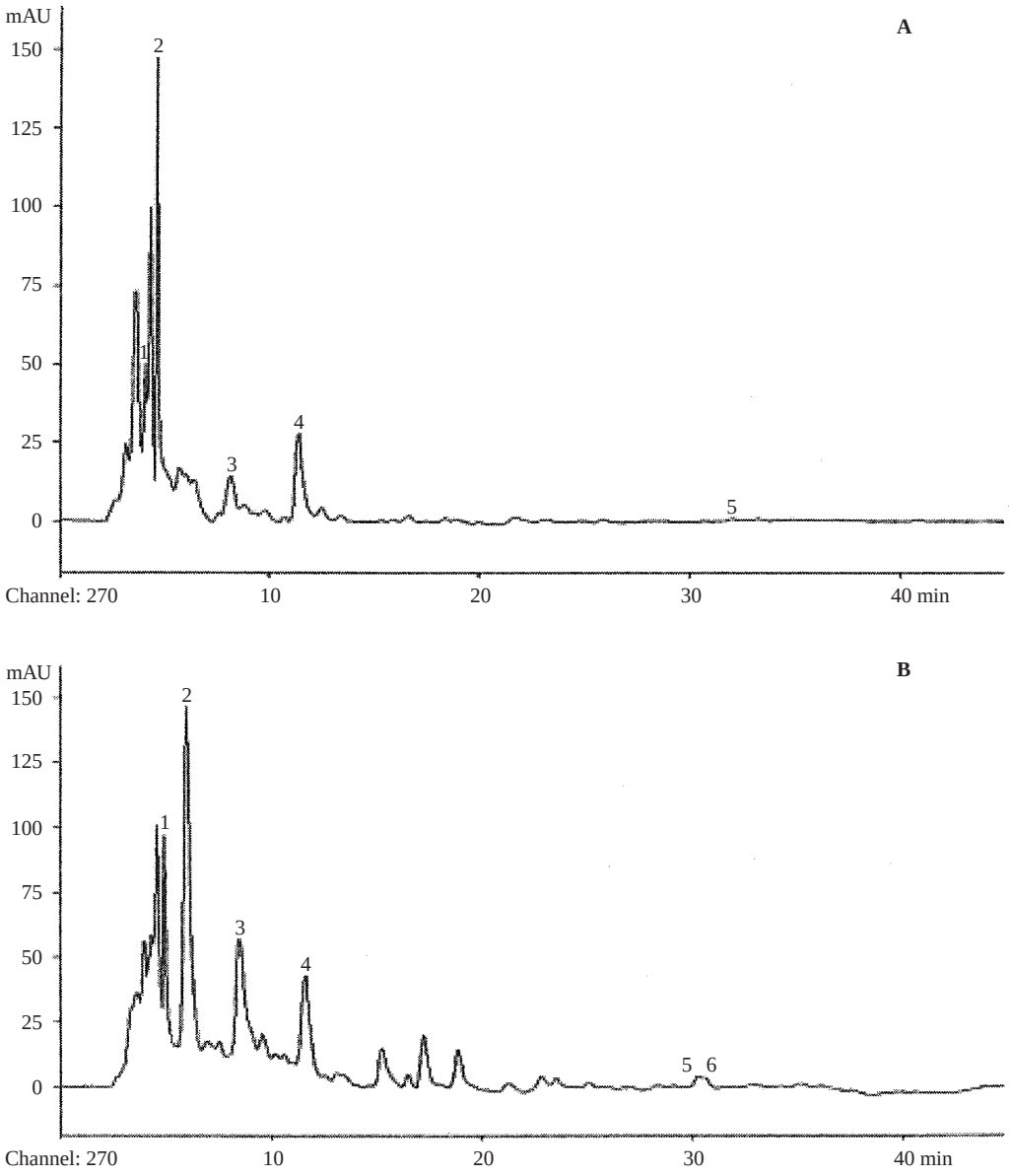

Figure 1 Comparison between HPLC chromatograms at 278 nm of white and red wines used. Chromatogram (A) refers to white wine (Trebbiano d'Abruzzo 1996); chromatogram (B) to red wine (Montepulciano d'Abruzzo 1995). Identified componds are: 1. Prothocatechuic acid; 2. Gallic acid; 3. (+)-Catechin; 4. (−)-Epicatechin; 5. *trans*-resveratrol; 6. Rutin.

Drugs

L-NAME, L-arginine and D-arginine (Sigma Chemical Co. St. Louis, MO, U.S.A.), aspirin (Flectadol 1000, Maffioni Spa, Milan, Italy) and collagen (Semmelweis Milano, Italy) were dissolved in saline. 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) was purchased from Aldrich Chemical Co. (Milwaukee, MI, U.S.A.); 2,2′-azobis-(2-amidinopropane) dihydrochloride ABAP was a gift from Dr A. Ghiselli (National Institute of Nutrition, Rome, Italy) and R-Phycoerythrin (R-PE) from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ethyl alcohol was from Carlo Erba, Milan, Italy.

Statistical analysis

All the results are presented as mean ± s.e.mean. The analysis of Variance (ANOVA CR) was used for the comparison among groups, followed by Dunnett test for multiple comparison. Statistical significance was defined at $P<0.05$.

Results

Oral administration

Haemostatic parameters Neither alcohol nor white wine administration, at the same doses and equivalent concentrations, showed any effect on BT. In contrast, red wine supplementation significantly prolonged BT in rats, in a dose-dependent manner. Alcohol-free red wine also showed a significant effect, even if slightly lower than the corresponding dose of red wine (Table 2).

As shown in Table 2, *ex vivo* platelet adhesion to fibrillar collagen was significantly decreased in rats given red wine compared to controls. The effect was dose-dependent and was not observed in animals supplemented with equivalent doses of ethyl alcohol or white wine.

Ex vivo platelet aggregation responses induced either by collagen or ADP, were not changed following red wine administration (data not shown).

Fibrinogen levels, one-stage clotting assay and ECLT values were not altered in any treatment group (data not shown).

Experimental thrombosis Ethyl alcohol and white wine administration induced a slight, however non significant reduction in thrombus weight (Table 2). Red wine, in contrast, showed a significant antithrombotic activity, by reducing thrombus weight up to 58% (Table 2). A similar effect was observed when alcohol-free red wine was supplemented.

Table 2 Effect of 10 day supplementation with ethyl alcohol, white wine, red wine or alcohol-free red wine

Treatment (ml d^{-1})	*BT* (s)	*Platelet adhesion* (%)	*Thrombus weight* (mg)
Control	132 ± 13	32 ± 1.3	3.3 ± 0.4
Ethyl alcohol			
4.1 ± 0.3	126 ± 7	33 ± 3.1	2.1 ± 0.3
8.3 ± 0.4	120 ± 4	36 ± 2.2	2.3 ± 0.2
White wine			
8.4 ± 0.4	141 ± 14	29 ± 2.3	2.5 ± 0.4
Red wine			
4.2 ± 0.2	176 ± 10‡	21 ± 1.0†	1.4 ± 0.3†
8.4 ± 0.4 ml	258 ± 13‡	12 ± 1.1‡	1.4 ± 0.3†
Alcohol-free red wine			
8.8 ± 0.2	213 ± 10‡	17 ± 1.5‡	1.8 ± 0.3*

*$P<0.05$; †$P<0.01$; ‡$P<0.001$ vs control; means ± s.e.mean, $n=15$.

Role of NO production The NO synthase inhibitor, L-NAME, administered at the concentration of 1.0 g l^{-1}, which did not show any effect *per se*, completely abolished the prolongation of BT, the decrease in platelet adhesion and the reduction in thrombus weight induced by red wine (Figure 2A, B and C). L-arginine administration (10 g l^{-1}, in drinking water) for 10 days, significantly prolonged BT (126 ± 8 vs 207 ± 17 s, $P<0.001$); therefore, it could not be used to revert the effects of L-NAME.

Intravenous administration

Haemostatic parameters When ethyl alcohol or white wine were administered at the dose of 1 ml kg^{-1} of 1 : 4 dilution i.v.,

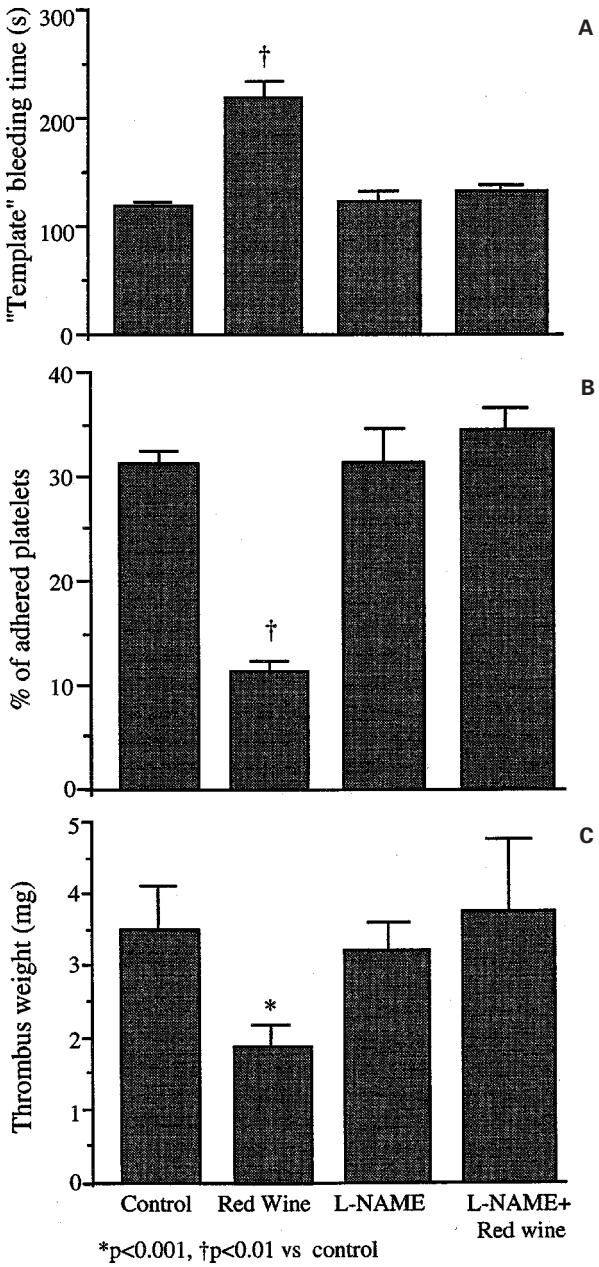

Figure 2 Effect of NO inhibition by L-NAME (1.0 g l^{-1} day^{-1} for 10 days) on (A) BT prolongation, (B) platelet adhesion to fibrillar collagen and (C) thrombus weight induced by red wine (8.8 ± 0.2 ml day^{-1}); 10 days supplementation.

no change in BT was observed (161±14 and 151±3 s, respectively; $P=0.1$).

Red wine, in contrast, significantly prolonged BT in rats if compared with controls (133±4 vs 284±10 s, $P<0.001$).

Intravenous injection of red wine also inhibited platelet adhesion to fibrillar collagen (13±3 vs 33±1%, $P<0.01$). Alcohol-free red wine showed similar effects (133±4 vs 260±11 s, $P<0.001$).

Fibrinogen levels, one-stage clotting assay and ECLT values were not altered in any treatment group (data not shown).

Experimental thrombosis Thrombus weight after inferior vena cava ligature was not modified after acute injection of ethyl alcohol or white wine (3.4±0.3 vs 3.30±0.8 and 3.21±0.8 mg, respectively). In contrast, red wine and alcohol-free red wine reduced by about 50% thrombus weight (3.4±0.3 vs 1.6±0.3 and 1.8±0.4 mg, respectively, $P<0.05$).

Role of NO production The effects of red wine on BT and platelet adhesion were completely abolished by L-NAME pretreatment. Administration of L-arginine substantially reversed the effect of L-NAME, while D-arginine did not. Neither L-NAME nor L-arginine or D-arginine *per se* modified the BT or platelet adhesion. L-NAME totally prevented also the antithrombotic activity of red wine, an effect prevented by L-arginine but not by D-arginine (Table 3).

Evaluation of cyclo-oxygenase involvement BT prolongation and thrombus weight reduction induced by the i.v. injection of red wine were unchanged by aspirin administration at the dose of 5 mg kg^{-1} (259±14 vs 284±10 s and 0.86±0.22 vs 0.84±0.24 mg, respectively, $n=10$). As previously shown, aspirin *per se* did not affect either BT (133±15 vs 133±4 s, $n=10$) or thrombus weight (2.8±0.5 vs 2.8±0.6 mg, $n=10$).

TRAP assay

Figure 3 shows plasma TRAP values measured in samples taken 15 min after treatments. TRAP did not change significantly after alcohol (163±43.5 vs 281±28 μM, $n=10$) or white wine (178±46 μM, $n=10$) administration. Red wine injected rats had TRAP values about three times higher than control animals (493±59 μM, $n=10$).

Ethanol analysis in whole blood

Ethanol concentration in whole blood was 11.2±2 μg ml^{-1} after 10 day oral administration of 8.4±0.4 ml d^{-1} red wine in drinking water (mean±s.e.mean, $n=4$). Intravenous administration of 1 ml kg^{-1} of 1:4 diluted red wine resulted in blood ethanol concentrations of 32.5±3 and 7.9±1.4 μg ml^{-1}, respectively 5 and 15 min after injection (mean±s.e.mean, $n=3$).

Discussion

There is epidemiological evidence that red wine can be beneficial in reducing the risk of cardiovascular disease above that expected from its alcohol content (Renaud and De Lorgeril, 1992; 1998; Gronbaek *et al*., 1995). An antithrombotic activity of red wine has also been demonstrated in experimental models of thrombosis (Demrow *et al*., 1995).

This study in the rat shows that red wine modified haemostatic parameters and prevented experimental thrombosis, independently of its alcohol content. White wine was ineffective while alcohol-free red wine was as effective as the original beverage, supporting the hypothesis that red wine components other than alcohol were responsible for the observed effects. The remarkable difference in phenolic components measured in red and white wine and the increased radical-trapping antioxidant activity in the plasma of animals

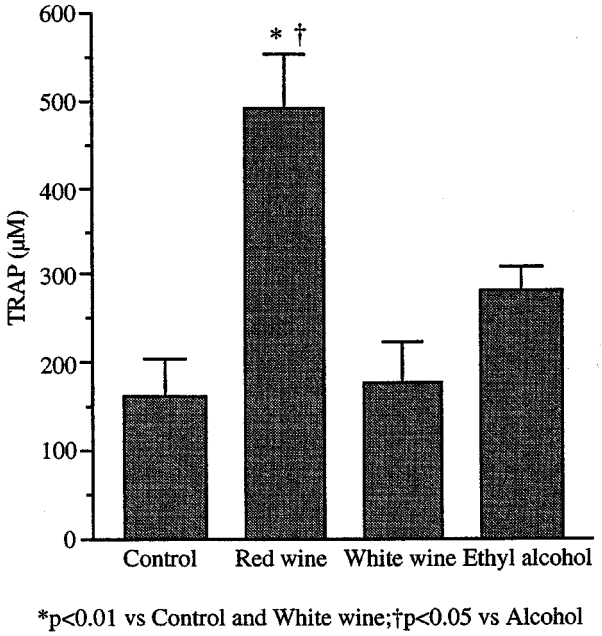

Figure 3 Total radical trapping antioxidant parameter (TRAP) of freshly prepared rat plasma 15 min after i.v. injection (1 ml kg^{-1} of 1:4 dilution) of red wine, white wine or ethyl alcohol.

Table 3 Effect of NO inhibition on BT prolongation, platelet adhesion and thrombus weight reduction induced by red wine in rats

Treatments	*BT* (s)	*Platelet adhesion* (%)	*Thromus weight#* (mg)
Control	132±13	37±2	1.7±0.3
Red wine	254±18†	18±3*	0.8±0.2*
Red wine+L-NAME	160±12^{+}	35±2^{+}	1.5±0.3‡
Red wine+L-NAME+L-Arginine	250±14†	20±2*	0.8±0.3*
Red wine+L-NAME+D-Arginine	136±15	36±3	1.6±0.2
L-NAME	124±8	35±1	1.8±0.2
L-Arginine	130±10	35±3	1.7±0.2
D-Arginine	131±12	33±3	1.5±0.3

#The experimental protocol for studying venous thrombosis has been reported and discussed under Methods. Red wine was injected i.v., at the dose of 1 ml kg^{-1} of 1:4 dilution; L-NAME was infused i.v., at the dose of 30 mg kg^{-1}; D-Arginine and L-Arginine were injected at the dose of 300 mg kg^{-1}. *$P<0.01$, †$P<0.001$ vs control, ‡$P<0.05$, $^{+}P<0.01$ vs red wine; mean±s.e.mean, $n=10$.

given red wine strongly support the hypothesis that red wine antioxidant polyphenols may be implicated.

Ethyl alcohol, at the same doses and equivalent concentrations of red wine, did not affect haemostatic parameters, measured as BT and platelet adhesion to fibrillar collagen. In contrast, it tended to decrease thrombus weight after inferior vena cava ligature; however, the effect was observed only after 10 days oral ethyl alcohol administration but not after acute intravenous injection. These findings confirm that long term alcohol consumption has antithrombotic properties, although suggesting a different mechanism from that of non-alcoholic red wine components. Whether non-alcoholic red wine components and alcohol interact in inhibiting thrombus formation remains to be determined.

This study also provides evidence that red wine induces NO production *in vivo* and may prevent experimental thrombosis by modulating NO-dependent haemostatic mechanism(s) such as platelet-vessel wall (collagen) interaction.

Red wine supplementation, either after 10 day oral or acute intravenous administration in rats, markedly prolonged BT and inhibited platelet adhesion to fibrillar collagen as well as significantly reduced thrombus weight after inferior vena cava ligature. In all cases, L-NAME, an inhibitor of NO formation, prevented the effects of red wine, indicating the involvement of NO in this process. The possibility to revert the effect of L-NAME by L-arginine, the precursor of NO synthesis in vascular endothelium but not by its stereoisomer, D-arginine, clearly strengthens the role of NO in red wine-induced effects.

The red wine ability to inhibit experimental thrombosis has already been reported (Demrow *et al*., 1995), as well as the induction of NO-dependent effect in *in vitro* studies (Fitzpatrick *et al*., 1993; van Acker *et al*., 1995; Andriambeloson *et al*., 1997). The novelty of our observation is that red wine and its alcohol-free component prevent thrombosis and modulate primary haemostasis *via* NO *in vivo*. This is, to our knowledge, the first *in vivo* evidence that NO has antithrombotic properties.

BT has been extensively used to evaluate primary haemostasis and particularly the contribution of platelets and vascular tone to the arrest of bleeding in humans and in animals. We recently reported that this test is modulated by NO production in an experimental model of haemolysis in rats, the effect of NO on BT being mediated by inhibition of platelet adhesion (Wollny *et al*., 1997). Therefore, we evaluated the adhesion of platelets to fibrillar collagen after both chronic and acute wine administration. In both cases, after red wine consumption, platelet adhesion to fibrillar collagen was markedly decreased, while no change in platelet aggregation was observed. L-NAME administration also reverted the effect of red wine on platelets.

Adhesion of platelets to the subendothelial matrix, after vessel damage, is a triggering mechanism of thrombus formation, therefore, platelet inhibition by red wine could, at least partially, explain the prevention of thrombus growth.

No effect was observed on platelet aggregation after either acute or chronic red wine administration. These findings are in agreement with previous data, that NO-inhibited platelet adhesion, but not aggregation, was involved in BT prolongation in rats (Remuzzi *et al*., 1990; Wollny *et al*., 1997).

BT in rats can also be prolonged by prostacyclin increase as a consequence of its ability to induce vasodilation and to inhibit platelet aggregation (Villa & de Gaetano, 1979). On the other hand, alcohol has been described to increase prostacyclin production (Landolfi & Steiner, 1984). The administration of aspirin, at a concentration totally inhibiting prostacyclin synthesis by vascular walls in rats (Cerletti *et al*., 1986), did not revert the effect of red wine in prolonging BT, thus suggesting that prostacyclin is not involved in such a mechanism. Alcohol and wine have also been reported to affect the haemostatic process by increasing the release of plasminogen activator and lowering the levels of fibrinogen (Veenstra *et al*., 1990; Laug, 1983; Ridker *et al*., 1994). In our experimental system, no changes in fibrinogen levels, clotting activities or in the total fibrinolytic capacity of plasma, were observed, after either wine or alcohol administration.

Increase in NO levels by red wine components has been described in *in vitro* and *ex vivo* experiments (Gryglewski *et al*., 1987; Andriambeloson *et al*., 1997). Wine contains a large number of compounds with antioxidant properties, including phenolic flavonoids, tannins, anthocyanins and natural antifungal compounds, such as *trans*-resveratrol (Rice-Evans *et al*., 1997). In particular, tannic acid, but neither resveratrol nor malvidin produced an endothelium-dependent relaxation of intact rat aortic rings, that was L-NNA-inhibitable, while quercetin-induced relaxation was not reversed by NO synthase inhibition (Fitzpatrick *et al*., 1993).

Chromatographic resolution of the wines used in our experiments confirmed that red wine contained greater amounts of antioxidant substances than white wine. In particular, the tannin content was 12 times higher in red wine than in white wine. The greater content in antioxidant substances of red wine as compared to white wine induced a higher antioxidant potential *in vivo* as it has been shown by the evaluation of the antioxidant capacity of rat plasma after wine intake. Animals given red wine showed a 4 fold increase in the TRAP capacity as compared to controls or animals given white wine or ethyl alcohol. These results strongly support the concept that the effect of red wine in prolonging BT, through a NO-dependent mechanism, can be mediated by its high content in antioxidant substances and tannins. However, further studies are necessary to specifically identify the molecular compounds responsible for the *in vivo* effect described here.

The mechanism of the NO increase is unknown. It is possible that red wine components decrease degradation of basal levels of NO, preventing its destruction by superoxides (van Acker *et al*., 1995). However, NO increase might also result from a stimulation of its synthesis by endothelial cells, as reported by *in vitro* experiments (Andriambeloson *et al*., 1997; Gryglewski *et al*., 1987). We have no evidence to support either mechanism; however, it can be conceived that both are acting in *in vivo* conditions.

The mechanism of platelet inhibition by red wine is different from that of other platelet-inhibiting substances, such as aspirin. Therefore, the effect of its moderate consumption in the prevention of coronary artery disease might be additive to that of aspirin. According to this hypothesis, Rotondo *et al*. (1996) showed that *trans*-resveratrol, a phenolic compound present in several red wines, inhibited human platelet aggregation induced by thrombin and cathepsin G also in the presence of aspirin.

Similarly to drugs interfering with primary haemostasis (Eristland *et al*., 1995; Steering Commitee of the Physicians' Health Study, 1989), red wine may increase the tendency to bleed. Some studies have shown that moderate alcohol drinkers had an elevated risk of subarachnoid haemorrhage (Stampfer *et al*., 1988; Donahue *et al*., 1986). Although this effect has not been specifically described for red wine, the mechanisms that mediate the protective effect of wine against ischaemic vascular disease might also increase the risk of bleeding. This possibility can simply be the counterpart of red wine antithrombotic activity, and in conditions of low risk for

thrombosis, or in hypertensive subjects it should be taken into careful consideration.

In conclusion, moderate red wine consumption induces, independently of its alcohol content, impairment of primary haemostasis and prevention of thrombosis in rats. These effects are mediated by a NO-dependent mechanism, possibly triggered by antioxidant substances present in red wine. Our study may offer experimental support and biological plausibility to the observed epidemiological protection from coronary artery disease associated with moderate red wine consumption.

Limitations of the study

Wine is a complex mixture of many different compounds, whose analysis is difficult and necessarily incomplete. We identified in the wines used for this study the major classes of components and showed differences in their concentrations between red and white wine; however, some substances, potentially responsible for the effect measured, could have been missed in our analysis. The effects of red wine described here should be interpreted as properties of a dietary component as a whole: extrapolation to pharmacological effects of some well-identified wine components is not allowed and needs further experiments.

'Wine' composition depends indeed on a wide spectrum of different conditions, such as the type of grape, the region where it is grown, the methods of grape cultivation and wine production. Therefore the reported effects of Montepulciano D'Abruzzo on haemostasis and thrombosis cannot be necessarily generalized to all red wines, but only to wines with similar characteristics. Flesch *et al*. lately demonstrated that only Italian and French red wines produced 'en barrique' (Bordeaux, Chateauneuf-du-Pape, Barolo) were able to induce a NO-dependent relaxation of rat aorta and human coronary arteries. In contrast red wines not produced 'en barrique' (Valpolicella, Ahr Spatburgunder), or white wines (produced 'en barrique' Rioja, Chardonnay, Mosel-Riesling) or ethanol did not show any effect (Flesch *et al*., 1998). Furthermore, Chateauneuf-du-Pape, but not white wine Chateau Villotte Bordeaux inhibited platelet activity and thrombosis in dogs (Demrow *et al*., 1995).

Finally, it should be noted that, following red wine lyophilization to remove alcohol, oxidation of certain constituents, such as volatile aromas, likely has occurred; therefore, it is conceivable that lyophilized versus non lyophilized red wine may differ more than by alcohol contents. However, such unidentified substances should not have major effects on the NO-dependent changes in haemostasis and thrombosis induced by red wine, since no statistically significant difference was observed in the effects of non-lyophilized and lyophilized red wine.

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