

Wines and grape juices as modulators of platelet aggregation in healthy human subjects

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Abstract

To test the hypothesis that red wine, by virtue of its relatively high concentration of polyphenols, is more protective against atherosclerosis and coronary heart disease (CHD) than white wine, and that grape juice enriched in one of these, *trans*-resveratrol, may share some of these properties, studies were performed on 24 healthy males aged 26–45 years. Each consumed the following beverages for periods of 4 weeks: red wine, white wine, commercial grape juice and the same grape juice enriched with *trans*-resveratrol. Apart from the last beverage, 2 weeks abstinence was maintained before commencing the schedule. Blood was taken at the beginning and end of each schedule to determine plasma thromboxane B₂ (TxB₂) concentration and the IC₅₀ (concentration required for 50% aggregation) for ADP and thrombin-induced platelet aggregation. White wine ($P < 0.05$) but not red wine increased the IC₅₀ for ADP. Both wines increased the IC₅₀ for thrombin ($P < 0.02$ and $P < 0.001$, respectively) and also lowered plasma TxB₂ concentrations ($P < 0.01$ and $P < 0.025$, respectively). Neither grape juice altered ADP-induced aggregation or TxB₂ concentrations, but the commercial juice lowered the IC₅₀ for thrombin ($P < 0.001$) whereas the resveratrol-enriched juice caused a dramatic increase ($P < 0.001$). In vitro experiments demonstrated that the aggregation of fresh washed human platelets by ADP and thrombin was moderately reduced by both grape juices, strongly by red wine and not at all by white wine. The synthesis of TxB₂ by platelets from labelled arachidonate was stimulated by commercial grape juice, slightly enhanced by resveratrol-enriched juice and strongly inhibited by red wine with white wine having little effect. Platelets from subjects consuming the commercial juice had a higher ratio of cyclo-oxygenase to

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lipoxygenase product formation and those consuming the resveratrol-enriched juice a lower ratio than during the control period. We conclude that *trans*-resveratrol can be absorbed from grape juice in biologically active quantities and in amounts that are likely to cause reduction in the risk of atherosclerosis. The failure of red wines (which have a 20-fold excess of polyphenols over white wines) to show any advantage suggests that, *in vivo*, ethanol is the dominant anti-aggregatory component in these beverages which are more potent than grape juices in preventing platelet aggregation in humans.

Keywords: Alcohol; Arachidonate metabolism; Atherosclerosis; Coronary heart disease; Cyclo-oxygenase; Eicosanoid production; Grape juice; Lipoxygenase; Platelet aggregation; Thromboxane B₂; Wine

1. Introduction

Many epidemiological studies have established a reduced incidence of death from coronary artery disease and atherosclerosis among moderate consumers of beverage alcohol by comparison with abstainers [1–3]. A significant component of this cardio-protective effect can be accounted for by the well-known ability of ethanol to increase the serum concentration of high-density lipoprotein cholesterol [4,5]. Another potential site for the beneficial action of ethanol is the circulating platelets, the aggregation of which it is able to inhibit in response to certain stimuli, but not others [6,7]. Renaud and colleagues have demonstrated a correlation between moderate alcohol consumption and reduced platelet aggregation during their studies on a healthy human population [8]. An issue of considerable nutritional interest centres upon the potential role of factors other than ethanol in alcohol-containing beverages which may favourably modulate the biochemical processes that promote coronary vascular disease. Among these beverages, wine has attracted the most attention because of its association with the “Mediterranean Diet”, as well as a body of epidemiological literature suggesting that it may be more beneficial in lowering coronary mortality rates than other forms of beverage alcohol [9–12]. Because of its relatively high content of polyphenolic compounds [13], red wine has been postulated to be more effective than white wine in providing such protection [14]. A number of investigations, both *in vitro* [15,16] and *in vivo* [17,18], have shown that such polyphenols can block the oxidation of low-density lipoprotein (LDL), an event which is considered by some to be crucially important in the initiation and propagation of arterial damage leading to atherosclerosis [19,20]. The anti-aggregatory actions of certain of these polyphenols, especially resveratrol

and quercetin, upon platelets from human subjects have been demonstrated *in vitro* by ourselves and others [21–23].

In 1990, Seigneur and colleagues reported that among a range of alcoholic beverages administered to human volunteers over 2-week periods, ethanol increased and red wine decreased ADP-induced platelet aggregation whereas white wine had no effect; none of the beverages altered the synthesis of thromboxane A₂ (TxA₂) by platelets from the individual subjects [24]. These results have never been confirmed or extended, despite their obvious relevance to the debate surrounding the relative merits of specific alcohol-containing beverages as well as ethanol in general. The notion that commercial grape juice, like red wine, may contain bio-active polyphenols in sufficient concentrations to accomplish a favourable modulation of platelet aggregation and eicosanoid production has never been tested, despite the social and economic advantages of this beverage over those containing ethanol. The present investigations were therefore undertaken to examine and compare the effects of red and white wines with those of commercial grape juice and a grape juice formulation enriched in *trans*-resveratrol, a tri-hydroxystilbene found in red but not white wines and known to have strong anti-aggregatory effects *in vitro* [21–23], upon healthy male subjects.

2. Methods

2.1. Beverages

The white and red wines were from the Niagara region of Ontario and contained *trans*-resveratrol concentrations of less than 10 µg/l and 4 mg/l, respectively, as measured by a direct-injection GC-MS procedure [25]. The latter value is a little higher than that of most Ontario red wines, but in the range encountered in those from the Bordeaux and Burgundy regions of France [26]. Both wines had an alcohol concentration of 12% by volume. The commercial grape juice was a widely available and popular North American product with a total sugar content approximating 160 g/l. To this, an extract of the filter-cake formed of skins and pips after crushing the grapes was added to produce a second grape juice enriched in *trans*-resveratrol to a concentration of approximately 4 mg/l. The procedure (unpublished) used to produce the resveratrol extract was developed and carried out by Dr. Leroy Creasy, Department of Fruit and Vegetable Science, Cornell University, Ithaca, New York.

2.2. Subjects

Twenty-four healthy males aged 26–45 years (students, academic staff or hospital employees) gave informed consent to participate in this study

which was approved by the Human Experimentation Committee of the University of Toronto. All were screened by interview and a series of clinical and laboratory procedures including blood pressure measurement, full blood count, urinalysis, lipid profile and biochemical tests for renal and liver function. None were receiving any medication. A family history of diabetes, hyperlipidemia and hypertension was excluded. All were consuming an unrestricted, typically North American diet and took alcoholic beverages in a range of 7–21 drinks (12 g alcohol/drink) per week.

From the point of entry into the study, all agreed to maintain constant diet and exercise and to refrain from any form of medication. Throughout the study, all subjects submitted a complete account of dietary intake over a 24-h period on the same day of the week each week. This was analyzed for carbohydrate, protein, saturated fat, monounsaturated fat, polyunsaturated fat and cholesterol. Every alternate week, weight and blood pressure were recorded at a clinic visit and a questionnaire relating to exercise, drinking, health and medication was completed.

After a baseline blood sample, all subjects abstained from alcohol for 2 weeks. They were then placed on a beverage regime which included either grape juice or wine (Fig. 1). For those receiving grape juice, two different formulations were employed; each was given in a volume of 500 ml per day for a period of 4 weeks with no break in between, and the commercial juice was always given first. All subjects continued their abstention throughout this further 8-week period. Those receiving wine consumed both red wine and white wine (375 ml per day with food) of equal alcohol content (12% by volume), each for a period of 4 weeks separated by a 2-week period of total abstinence. During the consumption of the wines, no other form of alcohol was permitted. Blood for analysis was collected at the beginning and end of each 4-week period of beverage consumption after a 14-h fast (overnight). Half of the subjects began with the grape juices and half with the wines. During the wine consumption protocol, half the subjects began with red wine and the other half with white. Most subjects completed the described protocol, but intercurrent illness (colds and influenza) requiring aspirin or anti-inflammatory agents led to the exclusion of some from part of the overall procedures. Blood pressure, body weight and exercise remained constant throughout the study period and the estimated intake of the various food groups listed above varied by less than 10% between the 4-week periods of beverage consumption.

2.3. Preparation of plasma and platelets

Blood was obtained by venipuncture and collected in a syringe containing 0.13 M sodium citrate (1:9 (v/v) to blood). Platelet-rich plasma (PRP)

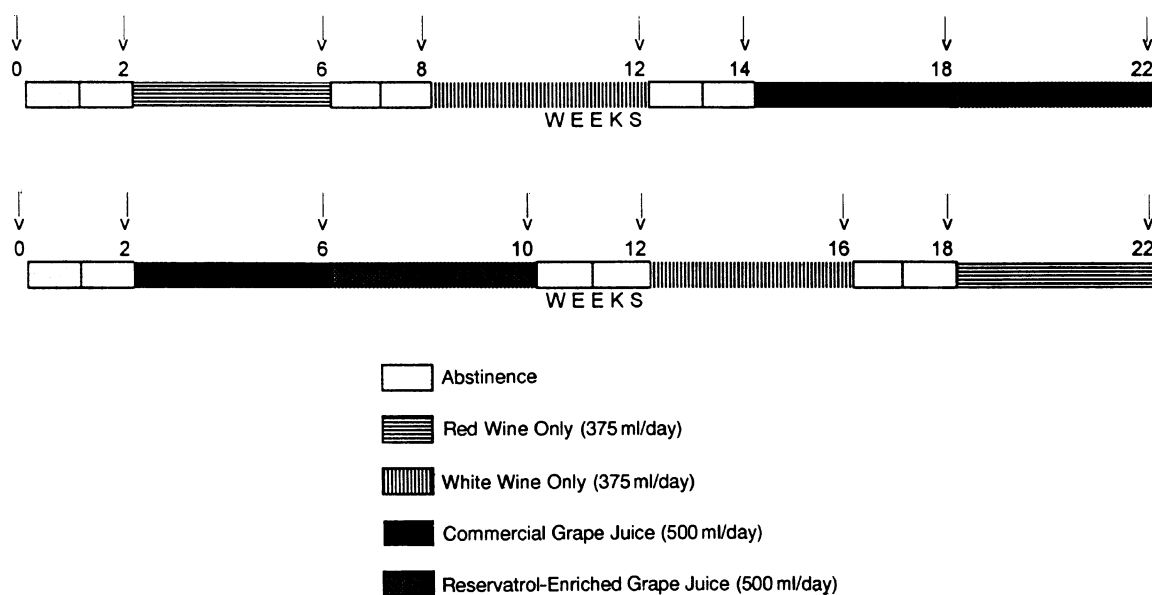


Fig. 1. Chart showing beverage schedule. Times of blood sampling are indicated by arrows, the numbers being the weeks of study at the end of which fasting blood was taken for analysis.

was immediately prepared by centrifugation of blood at $140 \times g$ for 15 min. Most of the PRP was removed and platelet-poor plasma (PPP) was then prepared by centrifuging the residual blood at $900 \times g$ for 5 min. The concentration of platelets in the PRP was determined in a cell counter (Coulter Electronics, Burlington, Ontario, Canada).

2.4. Platelet aggregation

Platelet aggregation was determined on the freshly prepared platelets using the PAP-4 aggregometer (VWR Scientific, Buffalo, NY, USA). The absorbance of the PPP was measured, whereupon samples of PRP containing 3×10^5 platelets were transferred to siliconized cuvettes and the volume was adjusted to $500 \mu\text{l}$ with Buffer A (137 mM NaCl, 1 mM KCl, 0.42 mM NaH_2PO_4 , 0.5 M MgCl_2 , 5.5 mM glucose and 20 mM HEPES (pH 7.4)). Buffer was chosen as diluent in place of PPP to avoid any substances in the latter that might interfere with the assay. The platelet suspension was stirred at 800 rev./min at 37°C in the aggregometer for 1 min. Aggregation was induced using solutions of thrombin (32, 64 or 96 mU/ml) or ADP (2, 6 or $10 \mu\text{mol/l}$) dissolved in Buffer A. Light transmission was recorded until equilibrium was attained. The change in optical density due to aggregation was expressed as the percentage of the difference in optical density between PRP and PPP. Dose response curves were constructed for each agonist, and the amounts of each required for 50% aggregation (IC_{50}) was calculated. This value was a measure of the aggregatory potential of the platelets; increases represented an anti-aggregatory response and decreases a pro-aggregatory response in com-

paring samples drawn after periods of abstinence or beverage consumption. The response to ADP was monophasic, irreversible, associated with degranulation and, although independent of intracellular ADP stores, is best characterized as “secondary”.

Plasma separated during preparation of the platelets was stored at -70°C for up to 12 weeks and thromboxane B_2 (TxB_2) concentrations were determined by gas chromatography with mass spectrometric detection [27].

2.5. *In vitro* experiments

The response of fresh washed human platelets to ADP and thrombin in the presence of varying volumes of red wine and grape juices, and the effects of red wine and juices upon the synthesis by platelets of TxB_2 and hydroxyheptadecatrienote (HHT) from labelled arachidonate were measured by thin layer chromatography as previously described [23]. The latter two compounds are stable products of the cyclo-oxygenase pathway which are derived from the powerful but unstable aggregation promoter TxA_2 which is rapidly converted to the stable product TxB_2 .

2.6. *Ex vivo* experiments

From 12 of the subjects, fresh PRP prepared from blood taken before and after consuming the commercial and resveratrol-enriched grape juices was incubated with labelled arachidonate and the synthesis of TxB_2 and 12-HETE was measured over a 20-min period [23]. The reactions were linear over this time period and permitted accurate inhibition values because conversion of arachidonate was near-maximal. The results were expressed as a ratio of $\text{TxB}_2/12\text{-HETE}$ in order to assess the relative activities of the cyclooxygenase (TxB_2) and lipoxygenase (12-HETE) pathways. Increases and decreases in the ratio were interpreted as favouring pro- and anti-aggregatory modifications in arachidonate metabolism, respectively.

2.7. *Statistics*

The means \pm S.D. for all assays were calculated. The statistical significance of differences between the means for basal and intervention periods in the same subjects was evaluated by the paired *t*-test and the *P*-values cited were obtained with this test. ANOVAR was also performed as a confirmatory procedure and validated the results of the paired *t*-test in all instances where significant differences were demonstrated with the latter.

3. Results

3.1. Human experiments

With ADP as agonist, no significant changes in platelet aggregation were observed in response to 4 weeks consumption of the two grape juices. White wine demonstrated a significant anti-aggregatory effect with this agonist ($P < 0.05$). During white wine consumption, the IC_{50} for ADP increased in 14 subjects, decreased in 3 and was unaltered in 2 (Fig. 2A). The mean IC_{50} for ADP increased by 17% overall during consumption of red wine, being increased in 15 subjects, reduced in 5 and unaltered in 2; these changes were on the threshold of statistical significance ($t_0 = 1.94$; $0.05 < P < 0.06$).

With thrombin as agonist, the commercial grape juice demonstrated a highly significant reduction in IC_{50} ($P < 0.001$). The IC_{50} for thrombin was reduced in 17 subjects, increased in 3 and unaltered in one (Fig. 2B). When the commercial juice was followed by the resveratrol-enriched juice, there was a marked rebound ($P < 0.001$), with IC_{50} values increasing in 22 subjects, unaltered in 1 and showing no decrease in any (Fig. 2C). The mean IC_{50} value after 4 weeks on this enriched juice was approximately 24% above the initial control value prior to starting the commercial juice, but on an individual basis this increase occurred in only half the subjects and the difference was therefore not statistically significant. However, bearing in mind the significant fall after consuming the commercial juice and before the resveratrol-enriched juice was taken, it is quite likely that a significant increase above the control value would have been demonstrated if this juice had been administered first, or if a 2-week drying-out period had followed consumption of the commercial juice as was the case with the two wines.

With both white and red wine, a significant increase occurred in the IC_{50} for thrombin. The mean percentage increase (49%) was somewhat higher with red wine ($P < 0.001$) than for white wine ($P < 0.02$) which showed a mean increase of 31%. Analyzing the data for individual subjects, 19 increased their IC_{50} for thrombin with a decrease in 2 during white wine consumption (Fig. 2D), whereas with red wine 20 manifested an increased IC_{50} and 3 a decrease (Fig. 2E). Thus, for practical purposes, the two wines showed equivalent anti-aggregatory effects using this agonist.

The mean plasma TxB_2 concentrations fell by 12% during consumption of the commercial juice and by 29% with the resveratrol-enriched juice; the data demonstrated very wide variance and these differences were not statistically significant. By contrast, white wine consumption reduced plasma TxB_2 concentrations by 52.5% ($P < 0.01$) and red wine by 59.4%

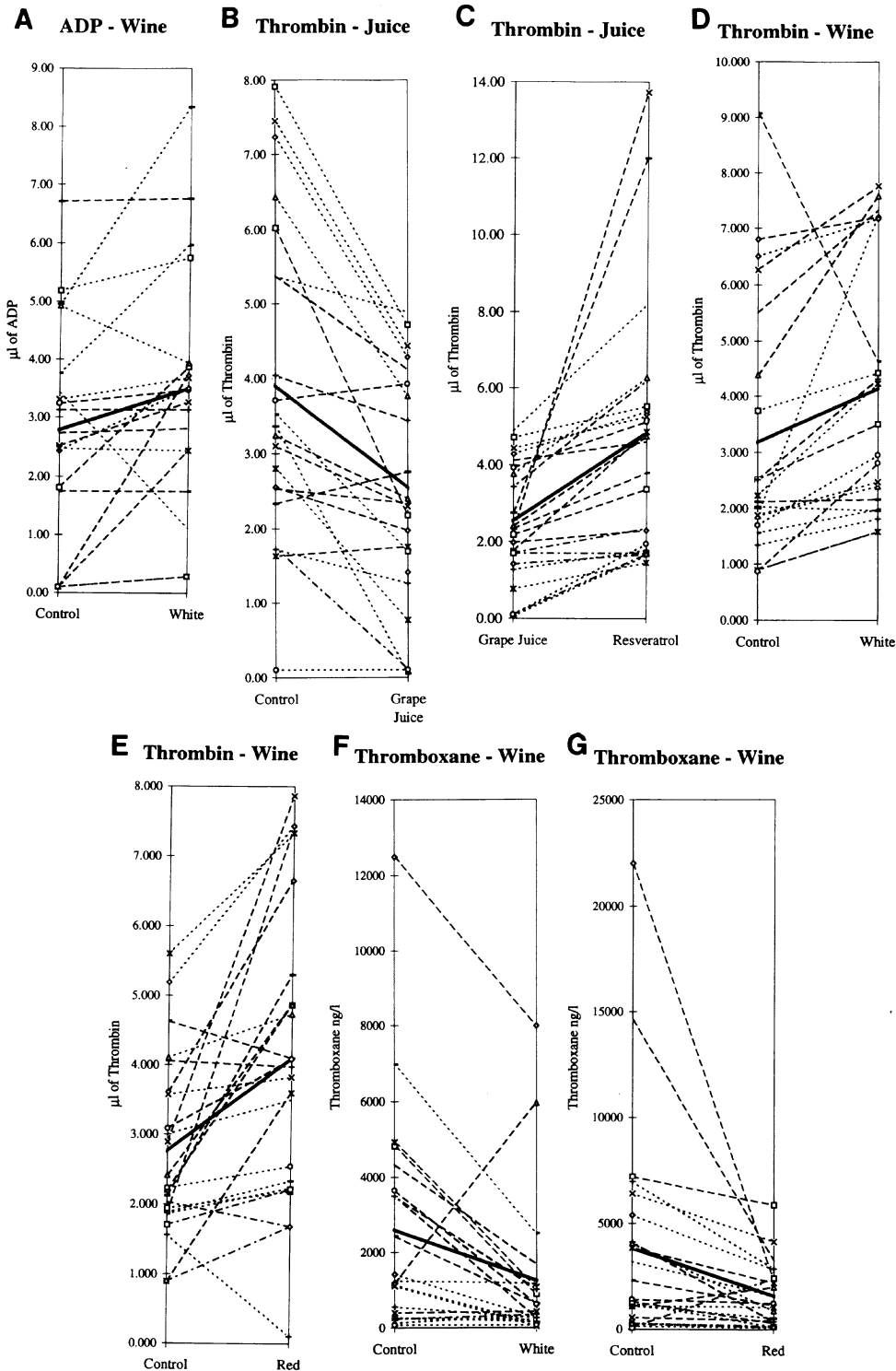


Fig. 2. Changes in platelet aggregation (IC_{50}) and TxB_2 concentration of individual subjects before and after 4 weeks on different beverages. The ADP and thrombin solutions used in the aggregation experiments were 2, 6, 10 $\mu\text{mol/l}$ and 32, 64, 96 mU/ml, respectively (final concentration) from which the IC_{50} was calculated as μl of stock required (10^{-3} M and 500 mU/ml, respectively). (A) Change from abstinence to white wine (ADP induction). (B) Change from abstinence to commercial juice (thrombin induction). (C) Change from commercial juice to resveratrol-enriched juice (thrombin induction). (D) Change from

($P < 0.025$). During white wine consumption, 17 subjects decreased and 4 increased their plasma TxB_2 concentrations (Fig. 2F), while for red wine the equivalent numbers were 22 and 1, respectively (Fig. 2G). Both wines seemed to be equally anti-aggregatory by this criterion.

3.2. *In vitro* experiments

We have previously demonstrated profound inhibition of platelet aggregation in response to both ADP and thrombin by the wine phenolics resveratrol and quercetin but not with any of the other polyphenols tested [23]. Dealcoholized red wine also blocked aggregation in response to both agonists [23]. The lack of a significant effect of red wine consumption upon ADP-induced aggregation as well as the failure of resveratrol-enriched grape juice to demonstrate an anti-aggregatory effect with this agonist were therefore surprising. Our previous investigations had also shown that resveratrol, but no other wine polyphenol, inhibited the production of plasma TxB_2 from arachidonate by fresh human platelets and this was also accompanied by reduced synthesis of HHT, a stable intermediate of the cyclo-oxygenase pathway [23]. Dealcoholized red wines demonstrated reduced synthesis of HHT and plasma TxB_2 in proportion to their resveratrol content. It was therefore surprising that red wine failed to show a significant superiority over white wine in reducing plasma TxB_2 concentration in the human volunteers. To clarify these unexpected findings, we performed a number of *in vitro* experiments. When fresh washed human platelets were stimulated with ADP and thrombin, under conditions that would enable us to observe both activation and inhibition, the commercial and resveratrol-enriched grape juices both inhibited aggregation by approximately 50% (Fig. 3). Red wine virtually abolished ADP-stimulated and thrombin-induced aggregation (Fig. 3) whereas white wine had almost no effect (not shown). When fresh washed human platelets were incubated with [^{14}C]arachidonate in the presence of 0, 50 and 100 ml of the various beverages (to reach 0%, 5% and 10% by volume), red wine demonstrated a marked inhibition on the synthesis of both TxB_2 and HHT (Fig. 4). The commercial grape juice enhanced the synthesis of both compounds, but the resveratrol-enriched juice demonstrated inhibitory effects (Fig. 4). White wine, in the volumes used for the red wine experiments, had no consistent effect upon platelet

Fig. 2. (Continued)

abstinence to white wine (thrombin induction). (E) Change from abstinence to red wine (thrombin induction). (F) Change from abstinence to white wine (plasma TxB_2 concentration). (G) Change from abstinence to red wine (plasma TxB_2 concentration).

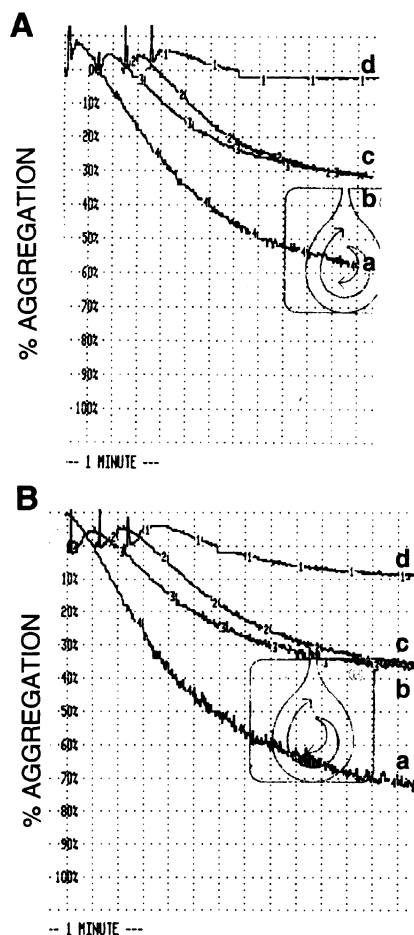


Fig. 3. Aggregation of human platelets in response to different beverages. Samples containing 3×10^5 platelets in 0.5 ml buffer were incubated at 37°C for 1 min with $10 \mu\text{l}$ of the following compounds: a, buffer (control); b, control grape juice; c, grape juice supplemented with 20 mg/ml resveratrol; d, red wine. Cells were then stimulated to aggregate with $10 \mu\text{M}$ ADP (Panel A) or 120 mU/ml thrombin (Panel B). Aggregation was measured in the PAP-4 Aggregometer (VWR Scientific). Figures are actual traces on chart paper; the central symbol has no significance.

eicosanoid formation, slight activation occurring at the lower concentration and slight inhibition at the higher concentration (data not shown).

3.3. *Ex vivo* experiments

As shown in Fig. 5, the ratio of $\text{TxB}_2/12\text{-HETE}$ produced from arachidonate by PRP increased after 4-weeks consumption of commercial juice (Panel A; $P < 0.02$) and decreased after 4 weeks of resveratrol-enriched juice (Panel B; $P < 0.01$). As the denominator of the ratio, 12-HETE served as an “internal control” against which relative changes in TxB_2 production could more easily be discerned, since the former is not affected by resveratrol over the range of concentrations employed [23].

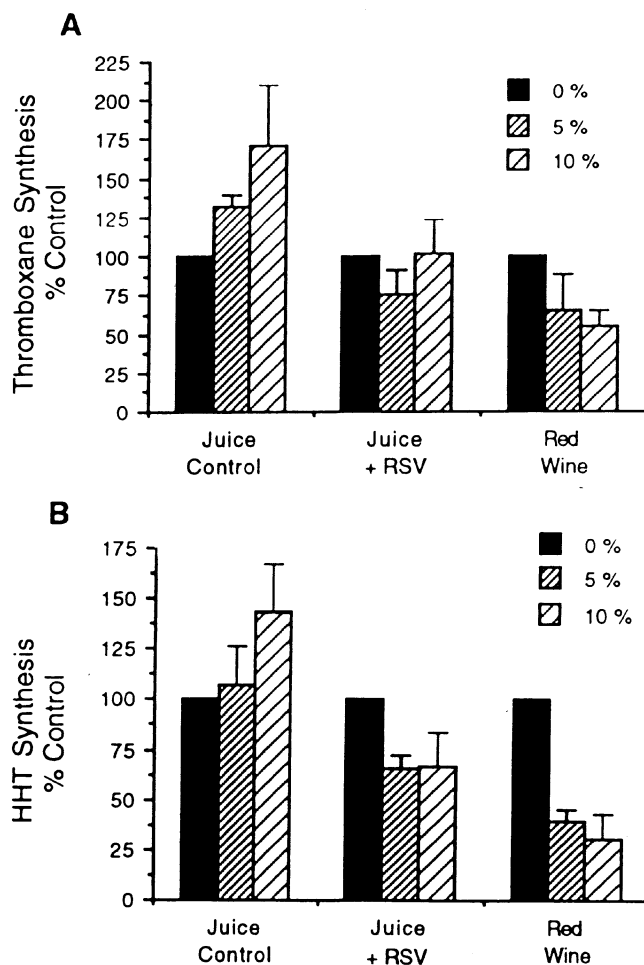


Fig. 4. Synthesis of TxB_2 by platelets incubated with grape juice and red wine. Samples containing 75×10^7 platelets in 1 ml buffer were incubated with the indicated concentrations of buffer (no addition), control grape juice, grape juice supplemented with 20 mg/ml resveratrol, or red wine. [^{14}C]Arachidonic acid (100 000 counts/min in 10 mg) was added to each sample, followed by stimulation with thrombin (160 mU/ml). This mixture was shaken at 37°C for 20 min. Lipids were extracted with ethyl acetate as described and separated by TLC. Spots corresponding to TxB_2 (Panel A) and HHT (Panel B) were scraped and counted. The final beverage concentrations by volume are indicated as follows: ■, 0%; ▨, 5%; ▩, 10%. Each point represents the mean \pm S.D. of three experiments and is expressed as the percentage of TxB_2 or HHT present with no addition.

This supports the notion suggested by the *in vivo* and *in vitro* results that whereas commercial grape juice enhances TxB_2 production, adding resveratrol to the juice reverses this effect.

4. Discussion

Many investigators have examined the effect of alcohol upon platelet aggregation. The results are not consistent because a number of different

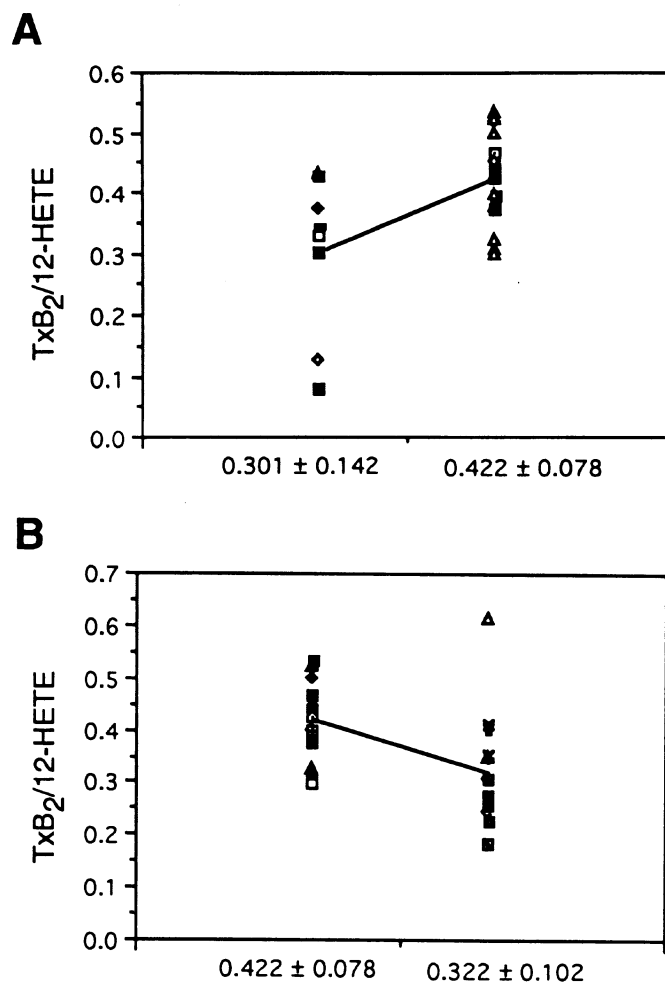


Fig. 5. Synthesis of TxB₂ from subjects drinking grape juice. PRP was prepared from the blood from each subject as described. Samples containing 75×10^7 platelets in 1 ml buffer were incubated with [¹⁴C]arachidonic acid (100 000 counts/min in 10 mg). Samples were stimulated with thrombin (160 mU/ml) and incubated at 37°C for 20 min. Lipids were extracted with ethyl acetate as described and separated by TLC. Spots, corresponding to TxB₂ and 12-HETE were scraped and counted. Panel A: commercial grape juice; Panel B: resveratrol-enriched grape juice. In each panel, the first set of points are data at the end of 2 weeks of abstinence, and the second are data obtained after 4 weeks consumption of the respective juice.

models and variable conditions have been employed. PRP and washed isolated platelets have been used and several species of animals as well as humans have been studied. Human investigations have been performed in chronic alcoholics on presentation and after abstinence, and healthy subjects have been tested during and after acute alcohol administration as well as after long-term chronic consumption. A range of agonists have been used to measure aggregation and different statistical approaches have been employed to analyse the data. The overall picture is therefore rather confusing, but a number of generalizations are possible (see Ref. [7] for review).

From *in vitro* experiments, it has been established that the major effect of ethanol is to block the stimulation of phospholipase A₂ induced by agonists such as collagen and thrombin [28,29] and thereby to prevent the mobilization of arachidonate required for the generation of TxA₂. In line with this notion, ethanol has little effect upon platelets in the presence of an adequate source of arachidonate, but it potentiates the effect of aspirin which blocks the conversion of released arachidonate to TxA₂ [30]. Direct inhibition of platelet cytosolic (and membrane-associated) phospholipase A₂ by ethanol has been demonstrated [31].

Earlier reports indicated that ethanol activated phosphoinositide-specific phospholipase C in human platelets, thereby initiating an increase in IP₃ leading to raised cytosolic Ca²⁺ and a series of phosphorylation events mediated by calmodulin-dependent protein kinases and protein kinase C [32,33]. By contrast, ethanol reduced IP₃ formation induced by thrombin in rabbit platelets [34]. It was subsequently shown that ethanol did not activate phospholipase C in human platelets but appeared to inhibit Ca²⁺-mediated granule centralization, thereby blocking the secretory effects of thrombin [35].

ADP causes primary aggregation of platelets at physiological Ca²⁺ concentrations. At lower Ca²⁺ concentrations this is followed in human platelets by a wave of secondary aggregation associated with TxA₂ production, which barely occurs in rabbit platelets [36]. It is to be expected that ethanol would inhibit secondary but not primary ADP-induced aggregation and this has been borne out in numerous *in vivo* and *in vitro* studies of human platelets [28,29,37–39].

Fairly high concentrations of ethanol (around 100 mmol/l) are required to inhibit TxA₂ production by platelets *in vitro* [40,41]. Administration of 50 g/day for 16 days had no effect on platelet TxB₂ production [24] whereas 1.5 g/kg given acutely led to a reduction of serum TxB₂ concentrations 2 and 4 h later [42]. The present data demonstrate, for the first time, a reduction in plasma TxB₂ concentrations in response to 4 weeks of moderate alcohol consumption, suggesting that reduced TxA₂ production accompanies long-term alcohol intake. Despite the dramatic ability of the red wine polyphenols, quercetin and resveratrol, to inhibit platelet TxB₂ production *in vitro* [23] as well as the inhibition shown by red wine but not white wine in the present *in vitro* experiments (Fig. 4), there was no difference discernible between red and white wine. This surprising result might be due to differences between the short-term (*in vitro*) and medium-term (*in vivo*) effects of ethanol and resveratrol. It is also possible that ethanol, in the present dosage, accelerates the clearance of TxB₂ from the plasma without influencing its rate of production.

The *in vitro* studies demonstrated inhibition of the cyclo-oxygenase

pathway of arachidonate metabolism by the resveratrol-enriched juice, with the commercial juice showing some stimulatory properties (Fig. 4). Although the plasma TxB_2 concentration tended to fall after 4 weeks of consuming resveratrol-enriched juice (but not commercial juice), this reduction was not statistically significant. It should, however, be recalled that these measurements were carried out at least 15 h after the end of the consumption period. They are therefore compatible with the notion that resveratrol reduces TxB_2 production in vivo as well as in vitro. Further support comes from the ex vivo experiments demonstrating a fall in the ratio of $\text{TxB}_2/12\text{-HETE}$ production from arachidonate in platelets of these subjects at the end of the resveratrol-enriched grape juice consumption period, in contrast with the increase in this ratio observed after completing the regular grape juice schedule (Fig. 5). This would explain why the commercial juice promotes and the resveratrol-enriched juice inhibits thrombin-induced platelet aggregation (TxA_2 -dependent). It is puzzling that neither juice exercised a significant effect upon ADP-induced aggregation since the “secondary” but not the primary phase of this event is TxA_2 -dependent. Nevertheless, the difference between the two juices suggests that, in the quantities administered (equivalent to the resveratrol concentration of most French and Canadian wines), sufficient resveratrol is absorbed to exercise a significant effect on eicosanoid metabolism and platelet aggregation. It is possible that these effects would have been more dramatic if the assays had been conducted during the absorptive or post-absorptive phase instead of after an overnight fast.

In contrast to the in vitro experiments where only red wine inhibited platelet aggregation in response to both thrombin and ADP, consumption of either for 4 weeks significantly increased the IC_{50} for thrombin. White wine consumption also increased the IC_{50} for ADP ($P < 0.05$) whereas with red wine this increase just failed to reach statistical significance ($P < 0.06$). This is especially surprising in light of the in vitro anti-aggregatory response of resveratrol and quercetin previously demonstrated [23]. One explanation is that the effects of red wine polyphenols may occur acutely during the absorptive phase and fall off with diminishing serum concentrations in the post-absorptive phase. Another explanation is that the effects of ethanol may be maximal, with no additional increment in anti-aggregatory activity occurring when polyphenols are added. A third possibility is that polyphenols are active in vitro, but may not be absorbed in biologically active amounts from the human intestinal tract. Some evidence against this notion is provided by the demonstration of increased plasma anti-oxidant activity in human volunteers following the ingestion of red but not white wine [17,18]. Waterhouse and colleagues have also described increments in the serum concentrations of

wine polyphenols in human subjects after oral administration [43]. Additionally, the differential effects of commercial and resveratrol-enriched grape juices on platelet aggregation are consistent with the intestinal absorption of resveratrol in biologically significant amounts.

Previous reports have yielded conflicting results concerning the relative effects of various alcoholic beverages upon platelet aggregation. Renaud and associates, in population surveys based upon the alcohol consumption admitted by the participants, described an anti-aggregatory response to ADP stimulation (both primary and secondary) but not to thrombin among alcohol consumers, utilising the odds ratio for high platelet response as the index of sensitivity to aggregation [8]. Acute administration of alcohol increased ADP-induced aggregation within 1 h, but this had fallen below baseline 15 h later [44]. By contrast, withdrawal of alcohol from alcoholic subjects [45] and from rats [46] changed their status from hypo- to hypercoagulable consistent with a rebound increase in platelet aggregability. In the rats, red wine or addition of grape seed tannins to the alcohol abolished this rebound hypercoagulability [46]. In the experimental model closest to our own, Seigneur et al. [24] reported that alcohol increased, red wine decreased and white wine had no effect on ADP-induced platelet aggregation, but their data indicate much higher baseline aggregation prior to red wine consumption.

Two other potential confounders deserve comment. Firstly, the alcoholic beverages were given in a fixed amount and not according to body weight which ranged in our subjects from 58 to 94 kg (mean 76 kg). This variation in dosage must have contributed to the wide variance in observed values, although use of the paired *t*-test would have partially offset this undesirable effect. Secondly, platelet aggregation is very sensitive to diet and platelet lipid composition, with saturated fat [47–49] and cholesterol [50, 51] being especially influential. These potential effects were minimized by closely monitoring the weight and diet of our volunteers whose participation was contingent upon maintenance of a consistent diet throughout the study. The motivation and status (students, staff, hospital employees) of the subjects are further reasons to believe in their compliance.

It may, at first sight, appear that regular commercial grape juice causes an increased tendency towards platelet aggregation. However, even if this proves to be so, it does not necessarily imply that grape juice is more deleterious in this respect than other fruit juices or soft drinks devoid of alcohol which have not been tested by us or others for their aggregatory potential. We are currently attempting to elucidate the mechanism of this effect which seems more likely to be due to the sugar content of the juice than to any unique grape constituent. On the other hand, there is a clear

benefit in enhancing the resveratrol concentration of grape juices. Since this is a natural product of the grape and is already being consumed in significant amounts by drinkers of red wine, there should be no legal or ethical objection to the commercial production and sale of grape juices in which it is enriched, particularly as its potential health benefits have already been clearly demonstrated in animal and in vitro experiments [21–23] and its lack of toxicity to human liver cells has been demonstrated at concentrations many order of magnitudes higher than those that are likely to arise from beverages containing the concentrations used in this study [52].

In conclusion, we have been unable to confirm the findings of Seigneur et al. that red wine was more effective than white wine in reducing platelet aggregation and the plasma TxB_2 concentrations in healthy human volunteers [24]. The superior effect of red wine in protecting against coronary disease mortality which has previously been proposed [14] and the unconfirmed report by Klurfeld and Kritchevsky that red wine is more effective than white wine and spirit alcohol in reducing the incidence of atherosclerosis in cholesterol-fed rabbits [53] cannot be due to greater inhibition of platelet aggregation over the medium-term, although the effects of acute short-term administration were not tested. Thus, despite their anti-aggregation properties in vitro, the ability of red wine phenolics to inhibit platelet aggregation does not appear to confer significant benefit in vivo. It is inherently likely that the anti-aggregatory effects of alcoholic beverages will therefore depend upon their ethanol content, at least in those beverages containing 10% or more ethanol by volume.

At this point, the superior anti-atherosclerotic potential of red wine cannot be dismissed, since the present investigations have examined only one possible mechanism: reduced platelet aggregation and thromboxane production. The antioxidant effects of red wine phenolics have been amply demonstrated in vitro [15,16] and more recently in vivo [17,18] and their protection of LDL against oxidation would be expected to confer significant protection against coronary heart disease beyond that which can be ascribed to ethanol alone, since the latter has no anti-oxidant properties. Further, it has been reported that certain red wine polyphenols, including resveratrol, can reduce lipoprotein synthesis [54–56] and the production of inflammatory mediators [21,23,24] in experimental animals. Favourable modulation of nitric oxide production by red wine phenolics has also been reported during in vitro incubation of segments of rabbit aorta [34] and a very recent population survey based on response to questionnaires seems to demonstrate a much lower incidence of CAD and all-cause mortality among wine drinkers (colour-unspecified) than among those who habitually consume spirits or beer [57]. However, until the differen-

tial effects of alcoholic beverages upon each of the above mechanisms are systematically tested in human subjects, the notion that red wine is the most cardioprotective alcoholic beverage must be regarded as unproven, in line with the conclusion reached by Klatsky and Armstrong [58].

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