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# Grape intake reduces thrombin generation and enhances plasma fibrinolysis. Potential role of circulating procoagulant microparticles

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**Abstract**

Phytochemicals contained in grapes down-regulate several prothrombotic pathways *in vitro*. We evaluated the effect of grape consumption on coagulation and fibrinolysis in healthy volunteers. Thirty subjects were enrolled: 20 were given grape (5 g/kg body weight/day for 3 weeks), while 10 served as controls. Blood samples were taken at baseline (T0), at the end of the grape diet (T1) and after 4-week wash-out (T2). Grape intake caused a significant decrease of the procoagulant and inflammatory responses of whole blood and/or mononuclear cells to bacterial lipopolysaccharide at both T1 and T2. At plasma level, grape diet decreased thrombin generation at T1 and T2, largely through a reduction in the number and/or activity of procoagulant microparticles. This anticoagulant effect resulted in the formation of clots that were more susceptible to fibrinolysis, mainly because of a lesser activation of thrombin activatable fibrinolysis inhibitor. No difference in any variables was detected in controls at the time points considered. In conclusion, chronic grape consumption induces sustained anticoagulant and profibrinolytic effects with potential benefits for human health.


*Keywords:* Grape; Coagulation; Thrombin; Fibrinolysis; Microparticles

## 1. Introduction

Cardiovascular disease (CVD) represents a leading cause of mortality and morbidity worldwide and a major health problem with a huge social and economic impact. In the past few decades, there has been increasing interest in dietary approaches as an economic, safe and effective adjuvant to the common pharmacologic strategies aiming at reducing the risk of CVD and preventing its major complication, that is, thrombosis. In particular, derivatives of grapes (e.g., grape juice, grape extracts and wine) have been demonstrated to reduce cardiovascular mortality and to protect against common risk factors for atherosclerosis (hypertension, diabetes, hyperlipidemia and oxidative stress) in numerous human studies [1–3]. Such therapeutic potential is mainly due to the anti-inflammatory, antioxidant and antithrombotic properties of the polyphenolic compounds (flavonoids, phenolic acid, resveratrol) contained in grapes [1–3]. The antithrombotic activity relies on various mechanisms, which include impairment of platelet activation, enhancement of nitric oxide release by endothelial cells and inhibition of coagulation [1,2,4–6]. Moreover, our group has reported that skin extracts from two Italian table grapes inhibit the expression of tissue factor (TF), the main trigger of coagulation, by human blood mononuclear cells (MNC) [7], and that no single polyphenol is able to inhibit TF synthesis as efficiently as the crude grape extracts, suggesting a synergistic effect of compounds present in the extracts [8].

Coagulation and fibrinolysis are strictly intertwined processes. Excessive activation of the coagulation cascade can profoundly impact the propagation and stability of formed thrombi mainly through the impairment of endogenous fibrinolysis. Thrombin, the main coagulation enzyme, is critical for clot stabilization, which is accomplished through several mechanisms including activation of FXIII [9], formation of thin and compact fibrin fibers [10] and activation of thrombin activatable fibrinolysis inhibitor (TAFI) [11,12]. TAFI is a plasma procarboxypeptidase that, upon conversion to the active form by thrombin and other enzymes, removes the binding sites for plasminogen from partially degraded fibrin, ultimately delaying fibrinolysis. To our knowledge, no study has investigated the effect of grape on thrombin formation and thrombin-dependent fibrinolysis inhibition, nor has such evaluation been made *in vivo* in human subjects. This study was undertaken to investigate the influence of chronic grape consumption on the coagulation–fibrinolysis axis in healthy volunteers through functional assays of pathophysiological relevance.

## 2. Materials and methods

### 2.1. Reagents

The following reagents were used: single-chain recombinant tissue plasminogen activator (t-PA) (Boehringer Ingelheim GmbH, Florence, Italy); prothrombin time (PT)

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reagent (human thromboplastin, Recombiplastin) and activated partial thromboplastin time (aPTT) reagent (HemosIL, Instrumentation Laboratory, Milan, Italy); potato tuber carboxypeptidase inhibitor (PTCI), bovine fibrinogen and *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) (Sigma, Milan, Italy); fluorogenic thrombin substrate Z-Gly-Gly-Arg-7-Amino-4-MethylCoumarin (ZGGR-AMC), thrombin calibrator and reptilase (Diagnostica Stago, Asnieres, France); cell culture medium RPMI-1640 (Euroclone, Milan, Italy); and Lympholyte-H cell separation medium (Cedarlane Laboratories, Hornby, Ontario, Canada). Small unilamellar phospholipid vesicles, composed of 20% phosphatidylserine, 40% phosphatidylcholine and 40% phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL, USA), were prepared by sonication.

### 2.2. Subjects and study design

The study was carried out in adult healthy subjects in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the ethical committee of the University of Bari, and written informed consent was obtained from participants before starting the study. Exclusion criteria were as follows: chronic diseases (CVD, hypertension, gastrointestinal disorders, type 2 diabetes, other metabolic and endocrine diseases), clinical history of thrombosis or bleeding, thrombocytopenia, abnormal coagulation profile, dyslipidemia, pregnancy, use of medications (e.g., steroid, contraceptive pill, anticoagulants), antioxidant or vitamin supplements, alcohol consumption (≥20 g alcohol/day), vegetarian or otherwise restrictive diets and allergy to grape fruits. The experimental design was carefully explained to the volunteers prior to the trial. The study group (grape group) consisted of 20 healthy subjects who were asked to intake, on top of their usual diet, 5 g of grape per kg body weight per day for 21 days, preferably at mid-morning and mid-afternoon between meals, after which they had to follow a grape-free diet for 4 weeks. Our sample of 20 participants provided 80% power to detect net changes in any of the studied biomarkers of 15%–40%, depending on within-subject correlation of the outcome. A control group was studied in parallel to evaluate possible time-related changes of biomarkers under investigation. It consisted of 10 subjects, who were selected to match age and sex of the grape group. Control subjects were asked to continue their usual diet but to abstain from eating grape. During the whole study period, all participants were asked to fill out a diet diary to ensure that there were no major changes in the dietary patterns. Blood samples were taken before starting the grape diet (T0), at the end of the 21-day diet (T1) and after the 4-week wash-out period (T2). All samples were withdrawn in the morning between 8–10 a.m. Volunteers entered the study in groups of three, two belonging to grape group and one to the control group.

### 2.3. Grape variety

The grape used in this study was a black table grape cultivar (Autumn Royal), grown in the Apulian region and planted in the same trial site at Council for Agricultural Research and Economics, CREA-VE, (Azienda Lamarossa, Turi, BA, Italy). This variety was chosen because of its period of aging and its relevant and well-balanced antioxidant, micronutrient and phytochemical composition [8]. During harvest time (September), fresh grape was distributed to the participants who were instructed to keep grape servings in their home refrigerator at 4°C until consumption.

### 2.4. Blood collection and preparation of platelet-poor plasma, washed MNC and microparticles)

Peripheral venous blood (30 ml) was collected by venipuncture into 3.8% trisodium citrate (9:1 blood/citrate ratio) after overnight fasting. Part of the blood was kept as is for the assay of whole blood (WB) and MNC procoagulant activity (see below). The remainder was used to prepare platelet-poor plasma (PPP) and microparticles (MPs). MNC were isolated from blood by density gradient centrifugation on Lympholyte-H as described [13] and suspended in serum-free RPMI-1640 at the concentration of $3\times10^6$ cells/ml. Extracts of MNC were prepared as reported [14]. Briefly, 1 ml MNC was centrifuged at 500*g* for 10 min at room temperature, and the resulting pellet was suspended in 0.5 ml Tris-buffered saline (TBS; 20 mM Tris, 150 mM NaCl, pH 7.5) containing 0.5% Triton X-100. The sample was then placed on a rotating plate for 45 min, after which it was centrifuged at 15,000*g* for 30 min at 4°C to remove cellular debris. The supernatant was collected and frozen at −80°C until assay.

PPP was prepared by double centrifugation at 3000*g* for 10 min at room temperature and stored at −80°C until assay. MPs were isolated from plasma by high-speed centrifugation at 18,000*g* for 30 min at 4°C [15]. All measurements reported below were performed blinded to participant identifying information.

### 2.5. Routine laboratory assays

Blood cell count, hemoglobin, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides and glucose were measured according to standard laboratory techniques.

### 2.6. WB and MNC procoagulant and proinflammatory activity

The procoagulant activity of WB and isolated MNC was evaluated on unstimulated and LPS-stimulated samples. Two aliquots of blood (12 ml each) were incubated at 37°C for 2 h with saline (unstimulated) or LPS (1 μg/ml, stimulated), respectively. Afterward, 1 ml was withdrawn from each sample for WB procoagulant assay and the remainder was used for MNC and plasma preparation as detailed above. For determination of WB procoagulant activity, 200 μl blood was transferred into prewarmed tubes containing 100 μl $CaCl_2$ (25 mM), and the clotting time was recorded manually with the tilt tube technique [13]. For the assay of isolated MNC, 100 μl cell suspension was added to prewarmed tubes containing 100 μl pooled normal human plasma, after which 100 μl $CaCl_2$ (25 mM) was added and clotting time determined manually as reported above. The time between blood collection and procoagulant assay never exceeded 5 h. The amount of TF antigen in MNC extracts was assayed by ELISA (Imubind Tissue Factor; Sekisui Diagnostics GmbH, Pfungstadt, Germany), according to the manufacturer. Baseline levels of interleukin-1β (IL-1β) were measured in fresh plasma by Quantikine human IL-1β HS Immunoassay (R&D Systems, Minneapolis, MN, USA). IL-1β release by blood cells was measured in plasma samples obtained from LPS-stimulated blood (2 h at 37°C) by IL-1β Quantikine ELISA (R&D Systems).

### 2.7. Thrombin generation in plasma

Plasma thrombin generation was analyzed by the calibrated automated thrombinography (CAT) method as described by Hemker *et al.* [16]. Briefly, 80 μl of plasma was incubated for 5 min at 37°C in round-bottomed microplate wells in the presence of thromboplastin (1:10,000, final dilution, f.d., corresponding to approximately 0.6 pM of TF). Where specified, 5 μg/ml of synthetic phospholipid vesicles was added. Analysis was performed in a final volume of 120 μl and started upon the addition of 20 μl of a mixture containing 100 mM $CaCl_2$ and 2.5 mM ZGGR-AMC (fluorogenic thrombin substrate). Measurements were taken every 20 s over time in a Fluoroscan Ascent fluorometer (Thermo Scientific, Dreieich, Germany), and data were analyzed using the Thrombinoscope software (Thrombinoscope BV). The parameters calculated by the software were the lag time, thrombin peak, velocity index and endogenous thrombin potential (ETP). All samples and calibrators were run in duplicate.

### 2.8. Assay of procoagulant MPs

The activity of procoagulant MPs in plasma was assayed by the STA-Procoag-PPL (Diagnostica Stago, Asnières, France), a clotting test that specifically measures the ability of anionic procoagulant phospholipids (PPLs) to enhance the activation of prothrombin by factor Xa [17]. The assay was performed according to the manufacturer's instructions, with minor modifications. In brief, 25 μl of test plasma was added to 25 μl of manufactured citrated human plasma devoid of PPL and incubated at 37°C. Then, 100 μl of a mixture containing $CaCl_2$ and bovine factor Xa was added and the clotting time was determined manually with the tilt tube technique. Results are expressed as clotting time, which is inversely correlated with the amount of PPL present in plasma. All samples were tested in duplicate.

In another set of experiments, we tested the procoagulant activity of isolated MPs, obtained from plasma samples as reported above. In this case, the MP-rich pellet was suspended in half the original volume of a normal pooled plasma deprived of MPs by high-speed centrifugation, and the thrombin generation assay was performed as detailed above.

### 2.9. Plasma clot lysis assay

The lysis of TF-induced plasma clots exposed to exogenous t-PA was studied with a turbidimetric assay as described [18], with minor modifications. One hundred microliters of plasma, 10 μl thromboplastin (1:10,000, f.d.), 10 μl t-PA (30 ng/ml, final concentration, f.c.) and 20 μl TBS were added to microplate wells, after which the clotting reaction was started with 100 μl $CaCl_2$ (20 mM). The plate was incubated at 37°C, and the changes in optical density at 405 nm were measured every minute in a microplate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA). Clot lysis time was defined as the interval between the midpoint of the clear to maximum turbidity transition and the midpoint of the maximum turbidity to clear transition. Where indicated, experiments were performed in the presence of the specific TAFIa inhibitor PTCI (25 μg/ml, f.c.) in place of TBS.

### 2.10. TAFIa generation

Thrombin-induced TAFIa generation was assessed in plasma by a two-stage functional assay as previously described [19], with minor modifications. Plasma was defibrinated by reptilase (1:50, f.d.) for 1 h at 37°C. Then, a mixture similar to that used for clot lysis assay, except for the absence of t-PA, was prepared in a test tube and incubated at 37°C. After 15 min, an aliquot was withdrawn, mixed with hirudin (200 U/ml, f.c.; Abbott GmbH, Ludwigshafen, Germany) to stop TAFI activation, and kept on melting ice until tested. TAFIa activity was evaluated as the ability to prolong the lysis time of purified fibrin clots. Seventy-five microliters of sample was added to microplate wells along with 25 μl bovine fibrinogen (0.83 mg/ml, f.c.) and 10 μl t-PA (40 ng/ml, f.c.), and clot formation was induced by 10 μl reptilase (1:25, f.d.). The plate was read every minute at 405 nm at room temperature (to reduce the temperature-dependent TAFIa decay), and lysis times were calculated as described above. PTCI served as a reference for the absence of TAFI activity, and results were expressed as arbitrary units (AU/ml) based on the prolongation of lysis time over parallel samples supplemented with PTCI in the first reaction mixture.