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Plasma circulating fibrinogen stability and moderate beer consumption

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Abstract

Moderate beer consumption (MBC) is cardioprotective: it positively influences plasma lipid levels and plasma antioxidant activity in beer-consuming individuals. The connection between MBC and blood coagulation is not clearly defined. Forty-two volunteers were equally divided into experimental (EG) and control (CG) groups following coronary bypass surgery. For 30 consecutive days, only patients of the EG consumed 330 mL of beer per day (about 20 g of alcohol). A comprehensive clinical investigation of 42 patients was done. Blood samples were collected before and after the investigation for a wide range of laboratory tests. The plasma fibrinogen was denatured with 8 M urea and intrinsic fluorescence (IF), hydrophobicity and differential scanning calorimetry (DSC) were used to reveal possible qualitative changes. After 30 days of moderate beer consumption, positive changes in the plasma lipid levels, plasma anticoagulant and plasma antioxidant activities were registered in patients of the EG group. In 17 out of 21 patients of the same group, differences in plasma circulating fibrinogen's (PCF), secondary and tertiary structures were found. The stability of fibrinogen, expressed in thermodynamic parameters, has shown that the loosening of the structure takes place under ethanol and urea denaturation. Also fluorescence stability of PCF was decreased. No changes in the lipid levels, anticoagulant and antioxidant activity or changes in PCF were detected in patients of CG. In conclusion, for the first time after a short term of moderate beer consumption some qualitative changes in the plasma circulating fibrinogen were detected: differences in the emission peak response, fluorescence intensity and all thermodynamic data. Together, with the decrease in the PCF concentration it may lead to an elevation of the blood anticoagulant activity.

Keywords: Beer; Plasma circulating fibrinogen; Secondary and tertiary structural changes

1. Introduction

Coronary artery disease (CAD) is the main cause of morbidity and mortality in Western industrial countries [1]. It has been unequivocally established that moderate alcohol consumption is cardioprotective: it positively influences plasma lipid levels, antioxidant and anticoagulant activities in patients suffering from CAD [2–6]. However, it is common knowledge that alcohol consumption has not only beneficial, but also adverse effects. Consumption of alcoholic beverages can have a negative influence on protein metabolism [7,8]. These negative changes also include plasma circulating proteins [9]. Our previous investigations have revealed some conformational changes in human serum proteins after short-term beer consumption [10–12]. Fibrinogen is one of the plasma circulating proteins. This protein is synthesized in liver and circulates in plasma at a concentration of 200 to 400 mg/dL. Fibrinogen plays an important role in blood clotting, fibrinolysis, cellular and matrix interactions, inflammation, wound healing, and neoplasia. These events are regulated to a large extent by fibrin formation itself and by complementary interactions between specific binding sites on fibrinogen and extrinsic molecules including proenzymes, clotting factors, enzyme inhibitors, and cell receptors. Fibrinogen is comprised of two sets of three polypeptide chains termed A alpha, B beta, and gamma that are joined by disulfide bridging within the N-terminal E domain. The molecules are elongated 45-nm structures consisting of two outer D domains, each connected to a central E domain by a coiled-coil segment. These domains contain constitutive binding sites that participate in fibrinogen conversion to fibrin, fibrin assembly,

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crosslinking, and platelet interactions (e.g., thrombin substrate, Da, Db, gamma XL, D: D, alphaC, gammaA chain platelet receptor) as well as sites that are available after fibrinopeptide cleavage [13].

The main reason of the high mortality from CAD is myocardial infarction due to thrombosis of coronary arteries [14]. Mennen et al. [15] claim that moderate drinking may lead to a decrease in PCF concentration and therefore to a decrease in mortality from CAD. Could moderate alcohol consumption lead also to qualitative changes in PCF? In order to disclose such possible changes electrophoresis (2-DE), circular dichroism (CD) and fourier transform - infrared (FT-IR) spectra were applied in our previous investigation [16]. In addition to FT-IR spectra, intrinsic fluorescence (IF), hydrophobicity and differential scanning calorimetry (DSC) were used.

Innes, 1998, had proposed to use beer instead of wine as cardioprotective beverage [17]. According to this investigator, effectiveness, low cost, acceptable adverse-event profile and single-dose dispensers are the basis for replacing red wine by beer. Therefore, beer was chosen for this investigation.

As far as we know, there are no studies, which described the connection between moderate beer consumption and possible qualitative changes in plasma circulating fibrinogen.

2. Materials and methods

2.1. Beer

Beer samples were used in this study. Their major components were proteins (5.2 g/L), total sugars (20.5% on dry substance), alcohol (5.1% volume) and total polyphenols (345 mg/L), epicatechin (65.5 mg/L) and quercetin (0.95 mg/L).

2.2. Fibrinogen

Fibrinogen was precipitated from human serum by methanol, purified by sequential DEAE anion exchange chromatography, dialyzed against water for 72 hrs and lyophilized. Proteins were dissolved in 0.01 M phosphate buffer, pH 7.2. Human plasma fibrinogen (F4883) was purchased from Sigma Chemical Co., St. Louis, USA, as well as all the other chemicals.

2.3. Subjects

The study population was recruited from patient volunteers, who had previously undergone coronary bypass surgery due to CAD in the Institute of Cardiology of the University Medical Center, Rehovot, Israel. The subjects gave written, informed consent to a protocol approved by the responsible Institutional Committee on human experimentations based on the Helsinki Declaration of 1975 as revised in 1983.

148 male patients between the ages of 39 and 73 years were examined. All of them had undergone bypass surgery due to three-vessel CAD. 42 nondrinkers between the ages of 41 and 72 years, also suffering from hypercholesterolemia, were chosen for further investigation.

2.4. Study design

Forty two patients were randomly divided into experimental (EG) and control (CG) groups, 21 in each group. All had consumed the same diet rich in vegetables and fruits and limited quantities of fats as recommended for CAD patients. For 30 consecutive days this diet was supplemented once a day by 330 mL beer (about 20 g of alcohol) for EG patients. During the 30 days period, the CG patients drank 330 mL of mineral water Netivot instead of beer. Beer and mineral water were consumed during lunches. An assigned member of the investigation team checked daily the consumption of diet, lifestyle and physical activity of the patients. During the investigation period there were no treatment complications and all patients completed this trial.

2.5. Clinical methods

Before and after completion of the investigation every one of the patients of both groups was examined. Systolic and diastolic blood pressure, heart rate and weight were registered. A wide range of laboratory tests were performed.

2.6. Laboratory methods

Blood samples a day before and a day after investigation were collected after an overnight fast. Lipids, tocopherols, proteins, PCF and the prothrombin time (PT), Factor VIIag, Factor VIIc and plasminogen activator inhibitor (PAI) tests were determined as previously described [5,6].

In order to reveal possible qualitative changes of PCF after beer consumption, the following procedures were applied.

2.6.1. Fluorescence measurement

Intrinsic fluorescence was done using a Model FP-770 Jasco-Spectrofluorometer (Japan Spectroscopic Co., Ltd., Hachioji City, Japan). Fluorescence emission spectra were taken at excitation wavelengths of 295 nm and 274 nm and recorded over the frequency range from the excitation wavelength to a wavelength of 500 nm [10]. The temperature of the samples was maintained at 30°C using a thermostatically controlled cell holder. Protein solution with concentration of approximately 0.015% was used. Deionized distilled water was used throughout. 8M urea, as the maximal concentration for denaturation, was added to the protein solution. The percent of denaturation was determined after incubation of protein with denaturants for 1 hr.

The magnitude of protein denaturation was calculated using the following equation: the percent of denaturation (%D) = $(I_o\text{-}I_1)/I_o x100$, where I_o and I_1- fluorescence intensity of fibrinogen in the absence and presence of urea, respectively.

2.6.2. Hydrophobicity

Hydrophobicity (S_o) was determined by 1-anilino-8-naphthalenesulfonate (ANS)-fluorescence probe measurements with 0.01 M phosphate buffer, pH 7.0, from 0.001 to 0.02% protein concentrations at λex 357 nm. The fluorescence intensity was measured at 513 nm. Protein hydrophobicity index was calculated as the initial slope of fluorescence intensity vs. protein concentration (%) plot [12].

2.6.3. Differential Scanning Calorimetry (DSC)

The extent of denaturation in solid proteins was estimated on a Perkin Elmer DSC System DSC Mettler using GraphWare TA-72 (Mettler-Toledo, GmbH, Schwerzenbach, Switzerland). Portions (4 to 6 mg) of solid proteins were transferred into preweighed aluminum pans and sealed. Proteins were mixed with urea in proportion of 1:1. An empty pan was used as the reference. The pans were heated in the calorimeter at 5°C/min over the range of 30 to 140°C. All thermodynamic parameters were found. The denaturation temperature (Td) and changes in the enthalpy of denaturation (ΔH) were computed from the thermograms [18]. To show the effect of proteins during denaturation, standard human fibrinogen was examined by the same method.

2.7. Statistics

To verify the statistical significance of the studied parameters means (M) of 3 times analyzed samples ± SD was defined. Differences between groups were established using 2-way ANOVA. The *P*-values of <0.05 were considered significant.

3. Results

3.1. Clinical data

Heart rate, systolic and diastolic blood pressures and weight of the patients of both groups before and after completion of the investigation were without significant differences. The mean weight before the investigation was 75. 7 ± 1.9 and 75. 8 ± 1.9 and after the investigation - 75. 9 ± 1.8 and 75. 7 ± 1.8 for the experimental and control groups, respectively.

3.2. Laboratory data

Before the investigation the levels of TC, LDL-C, HDL-C and TG were 8.07 ± 0.8 and 8.06 ± 0.8, 6.03 ± 0.6 and 6.01 ± 0.6, 1.28 ± 0.2 and 1.29 ± 0.2 and 2.25 ± 0.3 and 2.26 ± 0.3 mmol/L for patients of the EG and CG, respectively. The differences were not significant. The changes in the plasma lipid levels after completion of the trial are summarized in the Table 1. As can be seen, a significant decrease in the level of the plasma TC, LDL-C and TG in patients of the EG group vs. CG group was registered. An increase in the plasma HDL-C in patients of the EG group was not significant.

Table 1
Plasma lipids in both groups of patients after investigation (mmol/L)

Diets	TC	LDL-C	HDL-C	TG
EG	6.78 ± 0.7[1]	4.98 ± 0.5[1]	1.39 ± 0.2[1]	1.91 ± 0.3[1]
CG	8.08 ± 0.8[2]	6.02 ± 0.6[2]	1.29 ± 0.2[1]	2.27 ± 0.3[2]

[1] Values are means ± SD, n = 19.
[2] Means in columns without letters in common differ significantly ($P < 0.05$).
[3] Abbreviations: CG, control group; EG, experimental group; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; TC, total cholesterol; TG, triglycerides.

As in the previous investigation [5], after 30 days of beer consumption a significant decrease in the PT, F VIIag, F VIIc and PAI tests was found (Table 2). A significant increase in the levels of total and α-tocopherols was registered only in patients of the EG group (from 22.6 ± 2.1 and 18.3 ± 1.7 to 27.1 ± 2.2 and 22.7 ± 2.1 μmol/L for total and α-tocopherols, respectively: an improvement in the antioxidant and anticoagulant activities in the beer-consuming patients.

There were no significant changes in the quantitative levels of proteins. However, an insignificant decrease in the level of PCF was registered.

In 19 out of 21 patients of the EG group typical qualitative structural changes in PCF were detected by intrinsic fluorescence, hydrophobicity and differential scanning calorimetry.

Typical changes in PCF of patient A (EG group) are presented below.

3.3. Intrinsic fluorescence

Fibrinogen at 295 nm showed the maxima (nm) at 337.8 and 331.7 with a shift of 6.1 nm and a decrease in intensity

Table 2
PT, Factor VIIc and Factor VIIa (%) and PAI ($uamL^{-1}$) in patients of both groups of the trial

	PT	F VIIc	F VIIa	PAI
CG	97.2 ± 2.6[a]	94.9 ± 2.5[a]	94.7 ± 2.5[a]	7.1 ± 0.4[a]
EG	90.3 ± 2.3[b]	88.4 ± 2.3[b]	88.1 ± 2.2[b]	6.1 ± 0.3[b]

[a]Values are means ±SD, n − 19.
[b]Means without letters in common differ significantly ($p < 0.05$).
[c]Abbreviations used: CG, control group; EG, experimental group; Factor VIIc, Factor VII coagulation; Factor VIIag, Factor VII antigen; PAI plasminogen activator inhibitor; PT, prothrombin time.

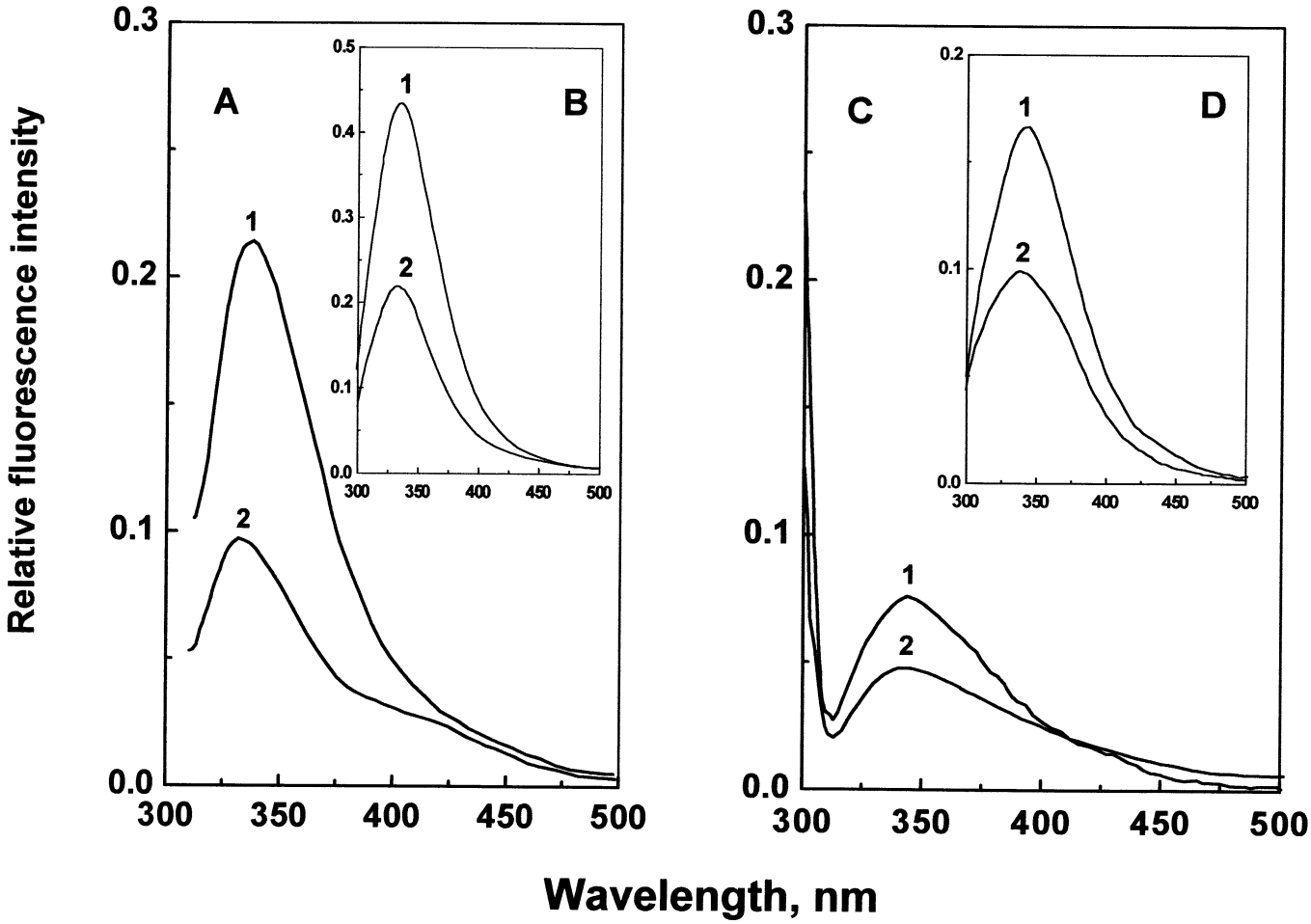

Fig. 1. Effect of fibrinogen denaturation at λ_{exc} 295 nm: A, curve1, native before beer consumption (BC), A, curve 2, after BC. At λ_{exc} 274 nm: B, curve 1, native before BC; B, curve 2, after BC. Denaturation with urea at λ_{exc} 295 nm: C, curve 1, before BC; C, curve 2, after BC. At λ_{exc} 274 nm: D, curve 1, before BC; D, curve 2, after BC. All samples were dissolved in 0.01 M phosphate buffer at pH 7.2. The protein concentration 0.01 mg/mL at 30°C.

of 2.3 times (Fig. 1A, curves 1 and 2). At 274 nm showed the peaks (nm) at 334.5 and 332.1 with a shift of 2.4 nm and a twofold decrease in the intensity twice (Fig. 1B, curves 1 and 2). Fibrinogen before and after BC (Fig. 1C, curves 1 and 2) showed peaks at 343.6 and 340.6 nm and a decrease in FI of 1.7 times. At 274 nm the peaks were at 344 and 337 with a shift of 6 nm and a decrease in FI of 1.5 times (Fig. 1D, curves 1 and 2). There is a clear shoulder at 308 nm at 274 nm that corresponds to tyrosine emission (even two distinct peaks at 305 to 309 and 336.5 nm). This means that the distance between tyrosine and tryptophan residues is enough to receive two emission peaks for these two residues. Emission wavelength at 336.5 nm describes the folded protein structure where tryptophan residues are situated in the interior of protein molecule. The change in the intensity of fibrinogen samples after beer consumption can be explained by the structural changes of proteins.

After the denaturation with 8 M urea unfolding of protein takes place, and tryptophan residues are displaced to a more polar environment. Such displacement gives a shift to lower energy in the maximum of emission and quenching of fluorescence intensity. A decrease in fluorescence intensity and a shift in the maximum of emission, which reflected unfolding of proteins, was observed. Comparison of the fractions before BC gives the following order: globulin > albumin > fibrinogen [11]. Our results are in accordance with others who have shown that the structural alteration of human fibrinogen determined by fluorescence cleaved and reduced to 42% of fluorescent intensity relative to native fibrinogen [19].

3.4. Hydrophobicity

The hydrophobicity of fibrinogen before beer consumption was 8.47 and after was 12.43. Increase in surface hydrophobicity (an indicator of protein stability) was reversibly correlated with the decrease in protein stability. Partially unfolded proteins denaturated with 8 M urea (21.32 before and 27.02 after beer consumption) also showed alternations in their structure. The surface hydrophobicity of plasma protein was increased by urea. This result suggests that the surface hydrophobicity of serum

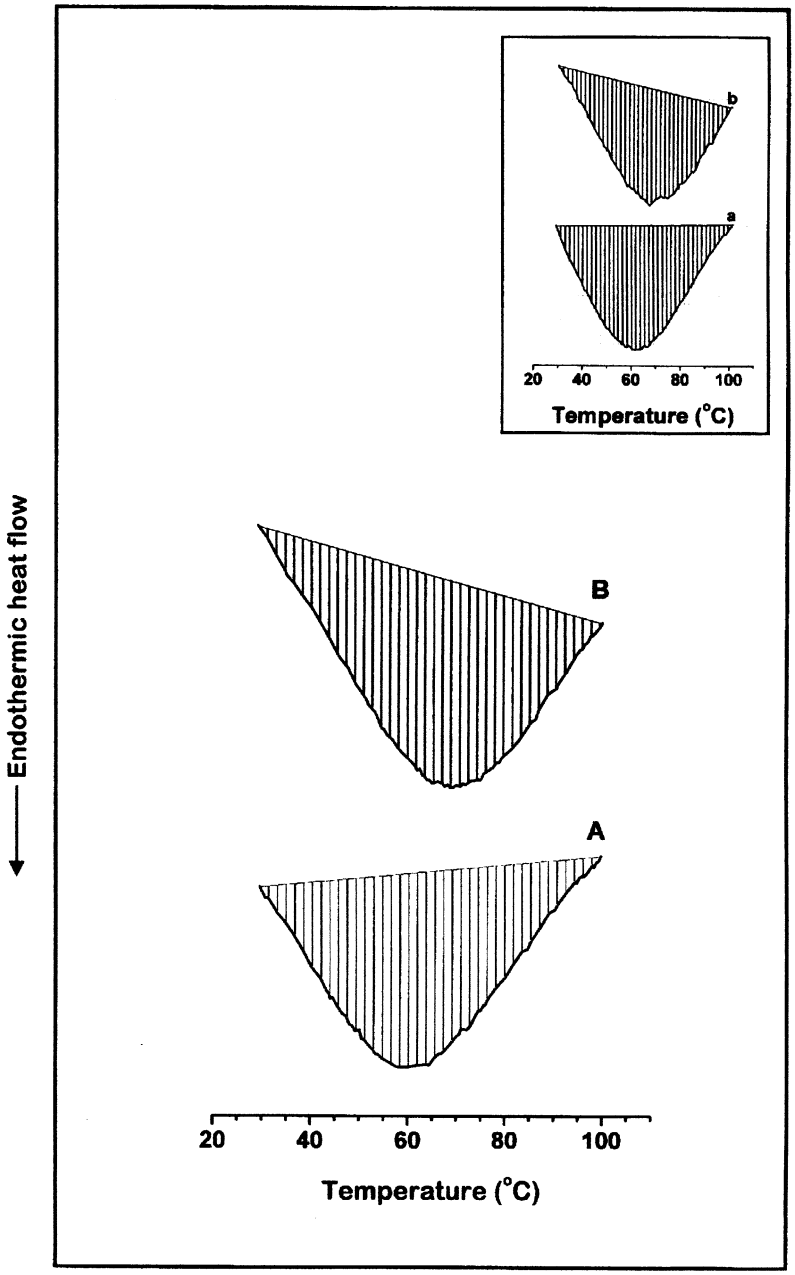

Fig. 2. Differential scanning calorimetry thermograms of fibrinogen before BC (B) and after BC (b); denatured with urea: before BC (A) and after BC (a).

protein is simple to increase and denaturation occurs easily during the treatment with urea. Current hypotheses suggests that surface hydrophobicity is one of the characteristics of the protein most likely to define its surface behavior and consequently its emulsifying properties [20].

3.5. DSC-thermograms and thermodynamic data

The DSC scans for native and denatured fibrinogen are shown in Fig. 2. Broadening of the peak, as well as decrease in ΔH indicates denaturation. Increase in the temperature and time of heat-treatment caused a decrease in enthalpy and slight shift in Td to the lower value with the addition of denaturants. The thermal stability depends mostly on the number of hydrogen bonds [21]. Disordering of the system takes place upon heating. A considerable number of protein molecules shift to a state that contributes much less to the unfolding transition, causing a significant decrease in the calorimetric enthalpy.

With the enthalpy ΔH (kcal/mol) of native fibrinogen (1081) and the ethanol denatured one (1055), we can evaluate approximately that 272 and 263 of hydrogen bonds

were broken during the conformational transition of native protein and of the previously denatured ones with ethanol after beer consumption (Fig. 2A and Fig. 2a). During denaturation with urea, the enthalpy (Fig. 2B and Fig. 2b) of native fibrinogen and the ethanol denatured one were 1010 and 517 and the hydrogen bonds 251 and 128 respectively, showing the decrease in the stability of fibrinogen after denaturation with ethanol and urea. These results are in harmony with others, who demonstrated that hydrogen bonding is the main stabilizing force in protein stability [22, 23]. In the case of fibrinogen denatured with urea about 51% of hydrogen bonds were ruptured during treatment. Saito and Taira [18] also reported the influence of hydrogen bond disruption on enthalpy changes in DSC. Two temperatures during the denaturation of bovine fibrinogen were similar to the ones found in this study: an important transition peak was observed at 78.8°C which is attributed to the C-terminal parts of fibrinogen and the transition at 57°C, which was found to be irreversible [24] and the human standard was with the peaks around 64.2°C and 58°C.

4. Discussion

It is wide-spread knowledge that moderate alcohol consumption can lead to favorable biochemical changes, which are indicators of improved prevention of CAD [2,3,5,6]. The results of the present investigation are in accordance with the data of the above-mentioned authors: a significant decrease in the plasma TC, LDL-C, TG, and an increase in total and α-tocopherols were observed. Therefore, as in our previous investigations of patients with normocholesterolemia [16], we have found that even short-term moderate beer consumption improves lipid metabolism and increases the antioxidant activity also in CAD patients with hypercholesterolemia. The above-mentioned findings correspond with the data of others [25], who have found a coronary heart risk reduction in a predominantly beer-drinking population. And even an acute ingestion of beer increases the plasma antioxidant capacity in humans [26].

However, the main cause of the mortality from CAD is thrombosis of the coronary arteries and as a consequence myocardial infarctions, which in many cases can be fatal [15]. Therefore, the main aim of this study was to assess the influence of short-term moderate beer consumption on the blood coagulation and on the PCF, which is an important link in this process. For this purpose PT, Factor VIIag, Factor VIIc and PAI tests before and after completion of the trial were studied.

According to Renaud and Lorgeril [3], the main positive effect of alcohol consumption is prevention of blood coagulation. So, how are these parameters affected by short-term moderate beer consumption in this investigation? We found a significant decrease in PT, F VIIag, F VIIc and PAI levels in beer-consuming patients after completion of the trial. Also, these results correspond with others [3]. However, the decrease in the level of PCF was not significant and, therefore we cannot support the claims of others who have found a significant decrease in plasma fibrinogen in alcohol consuming laboratory animals and humans [27–30]. This discrepancy could be explained by the short-term beer consumption of our patients and by the quantity of the consuming beer (330 mL). Other authors, who have found a significant decrease in plasma fibrinogen level, used higher quantities of beer. So, in the investigation of Sierksma et al. [30], men consumed four glasses and women - three glasses of beer. Our conclusions correspond with other investigators, who have demonstrated that alcohol-induced changes in coagulation and fibrinolysis are connected to the quantity of the beverages used [31].

It must be underlined that only a few authors have found functional and structural changes in plasma proteins, which are connected to alcohol consumption [9–11]. The present investigation also reveals for the first time that plasma circulating fibrinogen can be affected even by short-term beer consumption.

Little, if any work has been devoted to the use of DSC in fibrinogen conformational studies before and after beer consumption. Denaturation precedes aggregation and provides the driving force for protein-protein interactions. It has been postulated that hydrogen bonding is the major attractive force in gels formed at temperatures $<$70°C. The detergents and denaturants destabilize hydrophobic interactions. Disulphide bonds are predominant and both hydrogen and hydrophobic interactions also contribute to the formation of plasma protein gels [18]. The thermal denaturation of fibrinogen expressed in temperature of denaturation (T_d °C) and enthalpy (ΔH, kcal/mol) showed similar changes in protein fractions, which were also characterized by fluorescence.

It can be concluded that even a short term moderate beer consumption in patients with CAD and hypercholesterolemia leads to an improvement in lipid metabolism, anticoagulant and antioxidant activities. For the first time some qualitative changes in the plasma circulating fibrinogen after a short term moderate beer consumption were detected: differences in the emission peak response, fluorescence intensity and all thermodynamic data, which could increase the anticoagulant activity.

Fibrinogen plays an important role in the coagulation process, therefore these finding have to be explored further.

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