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POLYPHENOLS OF RED GRAPES IN WINE AND CONCENTRATES FOR USE IN REHABILITATION TECHNOLOGIES

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Abstract

In recent years, as cardiovascular mortality is increasing, the interest in studying the influence of polyphenol-rich grape products (PRGP) on cardiovascular risks is constantly growing. The estimation of a safe and effective dose of PRGP deserves special attention, because an excessive consumption can lead to endothelial dysfunction and oxidative stress. The hygienic and curative properties the young branches of grapevine, leaves, berries, juice and wine are used in traditional medicine for a long time. The curative properties of grapes are known to be due to the presence of biologically active grape polyphenols, which are accumulated in grapeskin, pulp, and seeds, etc. Polyphenols are extracted during alcoholic fermentation and determine the antioxidant status and biological activity of wines and other grape products. Here our objective was to analyze polyphenols in red wine (RW) and extracts from red grapes (EPG) and to compare the influence of fixed concentration of polyphenols on cardiovascular parameters, lipid peroxidation (LPO) and lipid metabolism at ischemic heart disease (IHD) and essential hypertension (EH) in the course of the SPA and resort-based treatment of 259 patients. The clinical trials of therapeutic and prophylactic properties of the experimental samples of red wine and extracts of polyphenols from red grapes showed that the use of these products as part of complex therapy contributes to the significant improvement of lipid metabolism, i.e. total cholesterol was reduced by 22 % and the atherogenic index decreased by 10 %, to a reduced free radical oxidation (end products of LPO decreased by 30.3 % when using the RW, and by 32.3 % in case of EPG), as compared to the patients from control groups who were not provided with PRGP in addition to a standard rehabilitation. The normalization of cardiovascular indexes also occurred. In the most patients, the tolerance to physical activity increased by 22.4 % compared to the control group. The clinical effect was achieved during a 14-day course at a daily dose of 3.6 ml/kg for RW, and of 0.45 ml/kg for EPG. The mechanisms of action of these polyphenolic products and the possibility of their use for primary and secondary prevention of disorders in patients with cardiovascular disease are discussed.

Keywords: grape polyphenols, antioxidant activity, ischemic heart disease, hypertension, red wine, extract of polyphenols from red grapes

Products of grape processing with a high content of polyphenols obtained from grape pulp exhibit unique bioactive properties and may be used in pharmacology [1-3]. Red grape wines containing on average 2.5 g/dm^3 of poly-

phenols, are recommended at a dose of 150-300 ml/day by American nutrition experts for reducing risk of cardiovascular diseases [4] that is explained by a beneficial effect on cardiometabolic factors [5-7]. Cardioprotection effect of polyphenols is associated with the vitamin P activity of the tannin-catechin complex that enhances the strength of capillary walls [3], the similar effect of anthocyans [3] and fortifying effect of procyanidins which inhibit collagen-destructive enzymes and reduce aortic elastin-associated cholesterol [3]. Antioxidative activity of wine correlates to the content of polyphenols in grapes, and there is a synergy of effects of polyphenols in the product [8, 9].

Controlled and regular wine consumption is considered favorable for mitigating a threat of cardiovascular accidents [5-7, 10], although alcohol at doses of more than 31 ml/day may negatively affect cardiovascular system [11-13]. Therefore, it is necessary to identify effective and safe doses of wines and other polyphenol-enriched products of grape processing. It should be noted that the content of polyphenols in these products is not controlled by domestic and foreign standards. Such an uncertainty makes it difficult to assess grapes and determine quality requirements for primary producers and breeders, as well as elaborate regulations for manufacturing and introducing the functional products in rehabilitation.

Polyphenols of red grapes in wines and concentrates are composed of two primary groups of flavonoid and non-flavonoid substances [2, 8, 14-16]. Monomeric flavonoids are glycosides of anthocyans (delphinidin, malvidin, cyanidin, petunidin), quercetin and its glycoside, (+)-D-catechin, (–)-epicatechin. Oligomeric flavonoids (procyanidins) consist of several (up to six) condensed catechin units (catechin, epicatechin and their dimers); polymeric procyanidins contain seven and more catechin units. Oligomeric and polymeric forms of flavonoids constitute a greater labile part of total polyphenols in bioactive red wines and concentrates. Among non-flavonoid polyphenols, there are hydroxybenzoic acids (gallic, syringic) and hydroxycinnamic acids (caftaric, cautaric), chlorogenic acid and trans-resveratrol [16].

Clinical studies of patients with chronic diseases have revealed some differences in the effectiveness of enotherapy [17, 18]. In particular, it has been found that the cardioprotection activity depends on the structure and concentration of polyphenols, their bioavailability, and patients' diet and individual characteristics. Thus, there is grate necessity for more representative clinical studies of effects of polyphenols contained in red wines and concentrates.

We are the first who developed formulae and manufacturing technologies for grape wines and concentrates having identical compositions and amounts of polyphenols for therapeutic application and estimated experimentally their cardioprotection activity.

The objective of the study was to obtain products for enotherapy and concentrates with standardized contents of polyphenols using red grapes, and to investigate their efficacy in the sanatorium-and-spa treatment (SST) of cardiovascular diseases.

Technique. Red table wine Zdorovye was produced from mature Saperavi grape with total sugars of at least 170 g/dm^3. Fermentation with floating cap was performed in a vinificator at 25-27 °C for 4.5 days with a control for concentration of total polyphenols in the substrate ready for bottling (at least 2.5 g/dm^3). In preparing an extract of polyphenols, fermented Cabernet Sauvignon grape pulp undergone a water-alcohol extraxtion with further dealcoholization under vacuum to the ethanol content of 10.5-15.0 vol.% and the concentration of total phenolic substances of at least 20.0 g/dm^3. Experimental products were produced from grapes harvested in 2015. Samples of commercial wines from red

grapes (Cabernet Sauvignon, Merlot and Saperavi; vintage of 2014) were used to compare the polyphenol composition.

The composition and quantity of polyphenols were analyzed by the high-performance liquid chromatography (HPLC) using an Agilent 1100 chromatograph with a diode array detector (Agilent Technologies, USA). Chromatographic column Zorbax SB-C18 (2.1 × 150 mm) filled with silica gel with bonded octadecylsilyl phase (sorbent particle size of 3.5 μm) was used for separation. The eluent consisted of A (methanol) and B (0.6 % trifluoroacetic acid aqueous solution). Chromatography was performed in the gradient mode with varying the content of component B as follows: 0 min — 8 %; 0-8 min — 8-38 %; 8-24 min — 38-100 %; 24-30 min — 100 %; eluent flow rate was 0.25 ml/min; injection volume 1 μl. Separation of fractions was recorded at 280 nm for gallic acid, (+)-D-catechin, (–)-epicatechin and procyanidins, at 313 nm for derivatives of hydroxycinnamic acids, at 371 nm for quercetin, and at 525 nm for anthocyans. For identification of substances, retention time was compared with that of standards [19, 20]; for quantitation we used calibration curves for individual substance solutions. Aanthocyan contents were expressed as equivalent of malvidin-3-O-glucoside chloride, caftaric acid — as equivalent of caffeic acid, polymeric and oligomeric procyanidins — as equivalent of (+)-D-catechin. Gallic acid, caffeic acid, (+)-D-catechin, malvidin-3-O-glucoside chloride, quercetin dihydrate, isoquercitrin (Fluka Chemie AG, Switzerland) and trans-resveratrol, (–)-epicatechin, syringic acid (Sigma-Aldrich, Switzerland) served as standards. All measurements were made in 3 replicates. For the consistency of results, amounts of substances were expressed in milligrams per 1 kg of dry husk weight.

Mass concentration of phenolic substances in the products studied was identified colorimetrically. To an aliquot of 1 cm^3 of a sample diluted to 10^{-2} the Folin-Ciocalteu reagent (1 cm^3) and sodium carbonate (10 cm^3) were added, then the mixture was diluted to 100 cm^3 with distilled water and allowed at room temperature (20±0.5 °C) for 30-40 min. Optical density was measured against the control in a cuvette with 10 mm optical path length at λ = 670 nm. The mass concentration of phenolic substances (C, mg/dm^3 expressed as equivalent of gallic acid) was calculated using the calibration curve as C = F × OD, where, C is mass concentration of phenolic substances, F is dilution factor, OD is optical density. The calibration curve was constructed as described [21]. The arithmetical mean of two measurements with the allowable discrepancy of no more than 33 mg/dm^3 for the range of 3000-20000 mg/dm^3 was taken as a final result. The measurement error limit for mass concentration of phenolic substances at P = 0.95 for this range was ±39 mg/dm^3. The measurements were made in 3 replicates. Antioxidative activity of samples was assessed amperometrically with trolox antioxidant as standard (Tsvet Yauza 01-AA apparatus, Khimavtomatika, Russia) [22].

Clinical studies (Ai-Petri sanatorium, May-October 2016; protocol approved by a local ethics committee) involved 259 patients aged from 30 to 80 years old with ischemic heart disease (IHD, n = 96) and hypertension (HT, n = 163). Among them, 40 patients without carbohydrate metabolism disorders, comparable in age and sex, were control groups (IHD, n = 20; HT, n = 20). The diseases were diagnosed for 2-15 years, in IHD there was a stable effort angina of no more than Functional Class III (FC) in a treadmill test (as recommended by the Canadian Cardiovascular Society), chronic heart failure (CHF) of no more than Stage IIa (according to the classification of Vasilenko-Strazhesko), CHF FC I-III. All enrolled patients signed the informed participation consent forms. The treatment group of patients with IHD included 34 (44.7 %) males and 42 (55.3 %) females, with a median age of 61.0 years old (46.0 and 77.0, respectively). A total of 47 pa-

tiens with IHD had stable angina without myocardial infarction; the average FC of angina was 1.98±0.60; 24 patients with IHD had HT as a concomitant disease. The treatment group with HT (grade I-II arterial hypertension) included 105 (73.4 %) males and 38 (26.6 %) females, with a median age of 57.5 years old (37.0; 75.0); 23 patients with HT had IHD as a concomitant disease. Initially, the treatment and control groups with the same disease had no differences in significant parameters ($p < 0.05$).

All the groups were prescribed with similar non-drug treatment (climatotherapy, exercise therapy, massage, balneotherapy, instrumental physiotherapy, etc.) [18] with regard to individual indications and medicines according to the standards approved: isosorbide dinitrate 20 mg twice daily (in the IHD group), lisinopril 20 mg once daily (in the HT group), acetylsalicylic acid 75 mg once daily, amlodipine 5 mg once daily, bisoprolol 2.5-5.0 mg once daily, atorvastatin 10 mg once daily. In the treatment groups, daily intake of polyphenolic products of grape processing (PPGP) (10 mg of total polyphenols per 1 kg of body weight) as wine Zdorovye (3.6 ml/kg per day) or polyphenol concentrate (0.45 ml/kg per day) were added to dinner and supper diets for a 14-day course. No PPGPs were given to the patients in the control groups.

Examinations at the beginning and end of the treatment course (day 0 and day 14) included the assessment of complaints, arterial blood pressure (ABP) dynamics by N.S. Korotkov's method, functional status of cardiovascular and respiratory systems (spirographic, rheographic, electroencephalographic and electrocardiographic indices) using DKh-NT Poli-SPEKTR+ diagnostic suite (Spektromed-Ukraine, Kharkov), hemodynamic cardiac function, i.e. stroke (SVB) and minute volume of blood (MVB), total peripheral resistance (TPR) and exercise tolerance (using the treadmill test). Hematologic study included complete blood count, glucose analysis, blood chemistry, i.e. C-reactive protein, lipid profile, total bilirubin, diene conjugates (DC), determination of prothrombin index (PTI) and fibrinogen. Concentration of C-reactive protein was determined by the enzyme-linked immunosorbent assay [23], for other hemostatic profile Technology-Standard kit (Russia) was used. Lipid peroxidation and blood antioxidant activity of were estimated [24].

The data was analyzed by variation statistics methods using the Mann-Whitney U-test. The mean (M), standard deviations ($\pm\sigma$), medians (Me) and quartiles ($Q_{25\ \%}$, $Q_{75\ \%}$) are provided. Differences among the values were considered statistically significant at $p < 0.05$.

Results. Polyphenols in wines and concentrates produced from Cabernet Sauvignon, Merlot and Saperavi red grapes (originated from commercial vineyards in various southern regions of Russia) were represented by groups of flavonoid and non-flavonoid substances in the monomeric, oligomeric and polymeric forms that was consistent with the literature data [2, 4]. Trans-resveratrol was found in neither commercial nor experimental samples, or it was detected in concentrations less than 1.0 mg/dm^3 that was likely associated with seasons of 2014-2015 good for winegrowing and winemaking. Integrated indices for polyphenols determined by the HPLC and using the Folin-Ciocalteu reagent, were different, however, the antioxidant activity characterizing the potential of biological activity varied in proportion to the content of polyphenols in wines and concentrates, as noted previously [8].

Experimental samples of red wine used in the clinical studies contained at least 2.5 g/dm^3 of polyphenols, and those of concentrate (extract of grape polyphenols) contained at least 20.0 g/dm^3 of polyphenols, i.e. the samples were identical to commercial products in composition and concentration of polyphenols (Table 1).

1. Composition of polyphenols in commercial and experimental samples of the medicinal wine and concentrates produced from red grapes ($M \pm \sigma$)

Commercial							Experimental	
1	2	3	4	5	6	7	8	9	10
Anthocyans, g/dm^3									
Total anthocyans									
20.3±0.4	23.8±0.5	23.4±0.6	133.3±2.7	167.5±3.8	556.2±12.4	18.9±0.4	28.5±0.6	22.8±0.6	235.1±7.5
Flavones, g/dm^3									
Quercetin-3-O-glycoside									
8.5±0.2	15.9±0.3	11.5±0.3	15.7±0.3	36.9±0.8	9.8±0.2	3.1±0.1	3.5±0.1	19.0±0.1	17.4±0.6
Quercetin									
2.8±0.1	1.6±0.03	1.2±0.03	0.3±0.1	4.1±0.1	0.7±0.2	49.6±1.1	81.2±1.1	3.4±0.1	5.8±0.2
Flavan-3-ols, g/dm^3									
(+)-D-catechin									
34.7±0.7	44.8±0.9	26.8±0.6	60.8±1.2	83.5±1.9	58.6±4.0	177.6±4.0	208.5±5.1	18.4±0.4	880.3±28.1
(–)-Epicatechin									
34.5±0.7	47.4±0.9	29.7±0.7	52.9±1.1	78.8±1.8	71.2±1.6	118.4±2.7	127.3±3.1	28.7±0.7	435.2±13.9
Oxycinnamic acids, g/dm^3									
Caftaric acid									
45.6±0.9	58.0±1.1	44.3±1.1	29.9±0.6	52.7±1.1	69.6±1.6	11.7±0.3	16.9±0.4	149.8±3.7	12.1±0.4
Cautaric acid									
7.5±0.2	10.0±0.2	7.4±0.2	3.5±0.1	5.4±0.1	11.8±0.3	1.8±0.0	2.4±0.1	5.6±0.1	3.4±0.1
Oxybenzoic acids, g/dm^3									
Gallic acid									
39.3±0.8	42.6±0.8	33.8±0.8	78.1±1.6	67.8±1.5	63.8±1.4	341.1±7.7	465.2±11.3	66.0±1.6	127.3±4.1
Syringic acid									
7.0±0.1	5.3±0.1	9.0±0.2	8.0±0.2	4.0±0.1	4.3±0.1	22.6±0.5	26.2±0.6	11.7±0.3	5.3±0.2
Proanthocyanidins, g/dm^3									
Oligomeric proanthocyanidins									
187±4	222±4	200±5	221±5	222±0	212±5	603±14	1614±39	319±8	1625±52
Polymeric proanthocyanidins, g/dm^3									
3045±61	3723±73	3525±84	2068±42	2072±47	2380±53	28155±634	38436±932	4670±114	39943±127
Integrated indices									
Total phenolic substances (by HPLC), g/dm^3									
3.43±0.10	4.20±0.10	3.91±0.10	2.67±0.50	2.79±0.06	3.44±0.08	29.50±0.70	41.01±1.00	5.30±0.10	43.30±1.40
Total phenolic substances (by Folin-Ciocalteu), g/dm^3									
4.35±0.11	4.56±0.11	4.25±0.10	3.89±0.10	3.85±0.08	4.13±0.08	18.51±0.49	21.81±0.59	3.02±0.10	21.50±0.80
Antioxidant activity (trolox), g/dm^3									
2.36±0.06	2.75±0.07	2.38±0.70	2.38±0.70	2.49±0.06	2.69±0.06	24.72±0.73	36.48±0.92	1.72±0.10	33.74±1.20

Note. Wine brands and appellations (grape variety geographical indication): 1 — Cabernet, 2 — Merlot, 3 — Saperavi (Massandra); 4 — Cabernet, 5 — Merlot, 6 — Saperavi (Kuban); concentrates: 7 — Enoant, 8 — Enoant Premium; 9 — red table wine Zdorovje (Saperavy variety), 10 — extract of grape polyphenols (Cabernet Sauvignon variety); the samples are described in the *Technique* section. HPLC — high-performance liquid chromatography.

The experimental samples have passed clinical testing in the sanatorium. Initially, complaints in the IHD groups generally included typical angina attacks (78.7 %), decrease in memory (68.2 %), rapid fatigue (62.4 %), palpitation (36.1 %), headache (34.6 %), dizziness (30.5 %). After the SST course with PPGP intake, the number of angina attacks reduced almost twice in 84.4 % of patients ($p < 0.05$). In 24.3 % of patients the angina FC changed from II to I by the end of the treatment course. The majority of patients (87.3 %) showed decreased fatigue and increased exercise tolerance: the permissible exercise stress increased by 22.4 % ($p = 0.002$), and the recovery time decreased by 16.4 % ($p = 0.01$). Positive dynamics and clinical effectiveness of the concentrate and the medicinal wine did not differ. Efficacy of the basic treatment course was somewhat lower: cardiac pains decreased only in 52.7 % of patients, palpitation complaints decreased in 34.2 % of patients, fatigue reduced in 55 % of patients. A number of clinical symptoms reduced or disappeared, with improving general condition, in the majority (83 %) of patients with HT in the treatment group who received the PPGP. In the control group (basic SST course), the clinical symptoms and complaints decreased only in 44 % of patients.

Positive effects of PPGP were observed in IHD and HT by objective parameters characterizing the cardiovascular system status (Table 2).

2. Dynamics of heart rate (HR)**, systolic and diastolic blood pressure** (SBD and DBD) **in patients with ischemic heart disease and hypertension after the sanatorium-and-spa treatment course with administration of products of red grape processing with high content of polyphenols**

Group	HR, beats/min		SBD, mm Hg		DBD, mm Hg	
	at entry	at discharge	at entry	at discharge	at entry	at discharge
Ischemic heart disease						
Control (n = 20)	72±8; 72 (67; 77)	69±5; 68 (67; 71)	138±17; 143 (134; 146)	128±14; 130** (118; 140)	87±8; 85 (80; 95)	83±7; 80 80; 85)
RTW (n = 30)	71±9; 69 (65; 78)	66±5; 67** (63; 70)	137±12; 140 (131; 144)	123±11; 120** (115; 130)	87±7; 85 (80; 95)	81±7; 80** (80; 85)
EGP (n = 30)	73±7; 75 (67; 778)	66±7; 63** (60; 69)	137±12; 135 (131; 140)	124±9; 125** (116; 130)	88±8; 88 (80; 95)	81±5; 80** (80; 85)
Hypertension						
Control (n = 20)	78±8; 79 (74; 83)	74±6; 74 (70; 79)	156±18; 160 (140; 175)	137±8; 138** (135; 140)	94±10; 95 (90; 100)	87±6; 95** (84; 91)
RTW (n = 30)	75±8; 78 (70; 80)	71±7; 73** (65; 78)	157±13; 155 (146; 170)	131±16; 133** (116; 140)	94±10; 95 (85; 99)	82±9; 80** (75; 90)
EGP (n = 30)	77±6; 78 (74; 80)	70±6; 69** (65; 74)	156±19; 163 (136; 170)	129±11; 130*, ** (120; 135)	96±10; 95 (90; 105)	84±7; 85** (80; 90)

Note. RTW — red table wine, EGP — extract of grape polyphenols (the experimental products are described in the Procedure section). Values of $M\pm\sigma$, Me ($Q_{25\,\%}$; $Q_{75\,\%}$) provided.
*, ** $p < 0.05$ (differences to control groups and differences between parameters at entry and at discharge, respectively).

Furthermore, the IHD and HT groups reported the positive effect of PPGP on metabolism. So, the decrease in total cholesterol after administration of PPGP was more significant ($p < 0.05$) than that in the control group. The fact that the administration of PPGP allowed the improvement of some lipid metabolism indices which were especially disturbed in patients with IHD was an additional confirmation of the efficacy of polyphenols in its normalization ("French paradox").

Blood chemistry parameters (glucose level, total bilirubin, PTI) in the IHD and HT groups did not differ significantly at entry and at discharge and were essentially independent of the PPGP administration. SST in conjunction with PPGP contributed to reduction in C-reactive protein concentration (to less than 2.6 mg/l in all the groups) that was indicative of the suppression of subclinical inflammation and reduced risk of vascular complications. Efficacy of the medicinal wine and the extract of grape polyphenols was comparable to and exceeded that of the basic SST course.

3. Some lipid peroxidation indices in patients with ischemic heart disease and hypertension after the sanatorium-and-spa treatment course with administration of products of red grape processing with high polyphenol content

Group	Parameter	TBA-AP, nmole MDA/ml		CP, mg/l	
		at entry	at discharge	at entry	at discharge
Ischemic heart disease					
Control (n = 20)	M	194.52	158.79	301.42	268.14
	σ	26.04	29.53	57.29	50.60
RTW (n = 30)	M	186.97	143.49	325.03	233.13
	σ	34.82	23.59	55.27	38.83
EGP (n = 30)	M	185.15	139.97	317.74	223.58
	σ	31.23	24.40	72.61	60.28
Hypertension					
Control (n = 20)	M	178.00	149.26	241.41	224.86
	σ	28.21	22.65	39.67	27.64
RTW (n = 30)	M	177.17	141.26	229.75	242.30
	σ	27.03	23.19	22.59	33.18
EGP (n = 30)	M	184.00	148.10	244.14	231.84
	σ	26.79	26.48	30.50	46.81

Note. RTW — red table wine, EGP — extract of grape polyphenols (the experimental products are described in the Procedure section); TBA-AP — active products of thiobarbituric acid, MDA — malondialdehyde, CP— ceruloplasmin. Differences with the control are statistically significant at $p < 0.05$.

As to oxidant-antioxidant homeostasis (Table 3), in IHD and HT,

PPGPs helped reduce free-radical oxidation that was evidenced by the decrease in number of primary and secondary lipid peroxidation products and the reactivation of antioxidative enzymes.

Hence, the PPGPs as a part of a combined therapy for patients with HT and IHD both have the hypotensive action and improve the lipid metabolism, as well as the activity of the antioxidant system. It should be noted that the PPGPs helped improve the health status in patients with IHD to a greater extent than in those with HT. Central hemodynamics was improved in patients with both diseases.

Thus, the developed technology for manufacturing products from red grapes provides a standardized content of biologically active polyphenols. It has been found out that the products (red table wine Zdorovye and extract of polyphenols) have a curative effect in ischemic heart disease and hypertension and may be used in rehabilitation in addition to the basic sanatorium-and-spa treatment. The therapeutic dose shall correspond to known long-term performance of the total polyphenols and be about 3.6 ml/kg per day in enotherapy, and about 0.45 ml/kg per day for the extract of polyphenols (14-day course). For optimization of technologies for sanatorium-and-spa treatment and rehabilitation, the dosages should be further specified.

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