ISSN 2304-3415, Russian Open Medical Journal
2018. Volume 7. Issue 4. Article CID e0405
DOI: 10.15275/rusomj.2018.0405

Original article

Biological effects of grape polyphenols processing products in experimental metabolic syndrome

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Received 15 November 2017, Revised 4 September 2018, Accepted 9 September 2018

Abstract: *Objective* — Biological effects of grape processing products (GPP) rich in polyphenols are still not studied completely. The aim of the investigation was to study co-interactions between peroxidation lipid oxidation (PLO) and nonspecific proteases and their inhibitors activity in the animals with the model of metabolic syndrome (MS) corrected with GPP.
Material and Methods — The study was performed on 54 white male rats of the weight of 180-200 g. The animals of experimental groups were fed by the standard menu and were given 10% fructose solution instead the drinking water during 8 weeks, which promoted development of MS. "Enoant", "Enoant-premium" and "Fenocor" were used as GPP in dosage of 2.5 ml/kg during 4 weeks just after 5th week. The PLO was estimated according to the concentration of thiobarbituric acid active products (TBA-AP), ceruloplasmin (CC), catalase-like activity (CLA), peroxidase-like activity (PLA) and superoxide dismutase activity (SOD). Trypsin-like activity (TLA), elastase-like activity (ELA), alfa-1-antitripsin activity (ATA) and acid-stable inhibitors activity (ASA) were estimated.
Results —MS demonstrated rise of TBA-AP by 50.4 % ($p<0.01$) and TLA by 19.5% ($p<0.01$), and drop of ATA, SOD, CLA, PLA in comparison with the control. The GPP (especially "Fenocor") led to the block of pathogenetic link of MS because of suppression of PLO and proteases' activity and increase of their inhibitors.
Conclusion —There is a tight connection between PLO and proteases' activity in MS. "Fenocor" could be used as MS-modified remedies acting positively on key links of MS.

Keywords: polyphenols, grape processing products, antioxidants.

Cite as Kubyshkin AV, Fomochkina II, Ogai YA, Shramko YI, Aliev LL, Chegodar DV, Chernousova IV. Biological effects of grape polyphenols processing products in experimental metabolic syndrome. *Russian Open Medical Journal* 2018; 7: e0405.

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Introduction

Biological activity of grape processing products which are rich in polyphenols as well as their benefits of the human health are well known and worth of studying. It was first shown in Toulouse, France, that cardiovascular mortality stayed the lowest in Europe despite the high level of dietary saturated fats. It was named "a French paradox" [1].

It was proved later that the paradox is caused by the cardioprotective action of polyphenols in red wines that are the traditional beverages of an average Frenchman. Polyphenols are supposed to act as antioxidants that neutralize free radicals, decrease oxidative enzymes' activity and reduce peroxide lipids' levels in blood serum [2]. Considering the latter facts, it would be practical to investigate the influences of grape processing products on the condition and metabolism of fat tissue.

Metabolic syndrome (MS) is dramatically important problem in both Russian Federation and the whole world. According to WHO data about 40-60 million people in Europe suffer from MS resulted from urbanization, changing of lifestyle and obesity [3, 4]. MS is considered a complex of co-interacted physiological, biochemical and metabolic factors which combination significantly increase the risk both cardiovascular pathology and diabetes mellitus type II [5, 6]. The basic components of MS are insulin resistance, visceral obesity, atherogenic dyslipidemia, endothelial dysfunction, hereditary predisposition, hypertension, hypercoagulability and a chronic stress [7]. The key factor of MS's pathogenesis is chronic inflammation that is characterized by the abnormal adipocytokines' liberation. The most important adipocytokines are tumor necrotic factor α (TNF-α), interleukin-1 (IL-1), IL-6, leptin, adiponectin, hepatocytes' growth factor, insulin-like growth factor-1, acute-phase proteins, matrix metalloproteases 2 and 9 etc. [8, 9]. The interaction of clinical and biological components of MS promotes the development of pro-inflammatory condition, which leads to the appearance, and persistence of atherosclerosis.

It is known that the impairment of the balance between pro-inflammatory and anti-inflammatory mediators is a leading mechanism founded on both metabolic and cardiovascular consequences [10]. In accordance with the modern investigations, MS is a programmed disease with epigenic genes' modification under the

exposure to the oxidative stress. It is assumed that the study of imbalance in antioxidative oxidative system (AOS-HEOX) could reveal the new pathogenetic mechanisms of MS, determine their early predictors and propose remedies or /and dietary recommendations able to delay or even turn back epigenic changes [11].

Together with peroxidation lipid oxidation (PLO) it is important to study the role of proteases and their inhibitors in the mechanisms of MS [12]. Nowadays the role of matrix metalloproteinase 9 in the development both MS and its complications is proved [13, 14]. Therefore, proteases might be an appropriate target for the therapy of MS. At the same time, the suppression of proteases by natural plants' components may be a promising method against MS's complications. In the view of previously mentioned, the estimation of the role of PLO, antioxidants, nonspecific proteases and their inhibitors (PIN) in mechanisms of MS is the problem of great value. The research in the named branch will allow creating the new remedies/prophylactic measures both against MS and for the natural control mechanisms' restoration.

Hence, the aim of the investigation is to study co-interactions between PLO and nonspecific proteases and their inhibitors activity in the blood serum of the animals with the model of MS and to estimate the action of grape processing products' polyphenols on the named parameters.

Material and Methods

Study design

The experimental study was performed on 54 white male rats of the weight of 180-200 g and of the age of 10-12 weeks. The animals were maintained in the standard conditions.

The publication data proved that the most reliable model of MS is the fructose-induced one [15]. Thus, the animals of the experimental groups were fed by the standard menu and were given 10% fructose solution instead the drinking water during 8 weeks. The standard menu for the animals of control and experimental groups consisted of 60-65% dry food (grain, oat flakes, bread, and crackers) and 35-40% of juicy food (carrots, cabbage leaves, lettuce, etc.); every rat consumed 20-25 g of food per day. The control group and non-corrected group were drinking water in the dosage of 0.1 ml during the same period.

Five groups of animals were formed: 1st – control group (basic routine) (n=8), 2nd – MS of 8 weeks (n=10), 3rd – MS of 8 weeks and "Enoant" (from 5th to 8th week) (n=12), 4th – MS of 8 weeks and "Enoant-premium" (from 5th to 8th week) (n=12), 5th – MS of 8 weeks and "Fenocor" (from 5th to 8th week) (n=12).

The criteria of MS were: abdominal obesity, hyperglycemia, hypercholesterolemia and the tendency to decrease in high-density lipoproteins [16].

The rats were sacrificed under chloroform anesthesia in accordance with the International recommendations for Biomedical Research using Animals (1985) and the Rules of laboratory practice in the Russian Federation (Protocol No. 267 of the Ministry of Health of the Russian Federation, June 19, 2003). The experiments were carried out according to the permission of the Academic Council of the Crimean Medical Institute (Act No. 103 of 30.11.77). The animal maintenance was approved by the Institutional Committee on Bioethics and is consistent with the Guidelines for the Care and Use of Laboratory Animals published by the US NIH (No. 85-23, revised 1985).

Polyphenols used in the study

"Enoant", "Enoant-premium" and "Fenocor" (RESSFUD LLC, Yalta, Russia) were used as polyphenol grape processing products in the dosage of 2.5 ml/kg (0.05 ml for the each animal). The dosage was inserting together with 0.05 ml of water daily orally through the probe during 4 weeks after 5th week of MS development.

According to phytochemical investigations oxybenzoic acids and flavonoid polyphenols in the "Enoant", "Enoant-premium" and "Fenocor" composition are predominantly represented gallic acid, catechol, epicatechin, quercetin and procyanidins represented in *Table* 1 [17, 18].

The choice of the named grape processing products (GPP) was inspired with some circumstances. It was shown recently, that polyphenolic antioxidant activity of "Fenocor" concentrate due to suppression of lipid peroxidation and oxidative modification of proteins leads to the blockage of oxidative stress – an important pathogenetic mechanism of cellular membrane damage in histotoxic hypoxia [19]. At the same time, there is a minority of modern research in the correction of complex disorders, such as MS, with GPP from red grapes. In the research [20] some positive effects of white wines in MS are discussed. However, it was not investigated how they affect metabolic parameters in MS, which would be useful for the correction of the patients with named diagnosis. Furthermore, the modern data prove, that polyphenols start to act effectively from the concentration of 20 g/l [21-23], whereas used white wines contain only from 1.70 to 0.44 g/l of polyphenols. That is why, research in the complex exposure of red grape polyphenols becomes highly important nowadays.

Morphological methods

In both control and experimental groups' body mass and circumference of the abdomen were measured for the proving of abdominal obesity. In addition, the weight of liver and kidneys, the morphological study of the named organs were performed after animal's euthanasia.

Biochemical assays

In blood serum glucose and main cholesterol level, high-density lipoproteins and triglycerides levels were measured for the confirmation of MS.

Also, the measurement of free radical oxidation and antioxidants markers as well as inflammatory markers (nonspecific proteinases and their inhibitors) was performed in blood serum and hemolysates:

i) The intensity of PLO in blood serum was estimated according to the number of thiobarbituric acid active products (TBA-AP) [24, 25].

ii) Antioxidant potential of blood was estimated with catalase-like activity (CLA), peroxidase-like activity (PLA), ceruloplasmin concentration (CC) and superoxide dismutase activity (SOD) in blood serum [26]. CLA was determined by the registration of hydrogen peroxide residues after incubation of the biological samples in pH 7.4 and 25°C according to the concentration of stained molybdenum-catalase complex. [27]. CC was estimated by Revin's modified method based on p-phenilenediamine oxidation in ceruloplasmin presence with the reaction's stop by sodium fluoride solution and

the measurement of optical density in 540 nm. SOD was measured in model system of superoxide anions formation in the conditions of the reduced form of nicotinamide adenine dinucleotide phosphate-NADP ($NADP_2$) and phenazimethsulphate interaction. The ability of SOD to compete in superoxide anions was revealed by the stage of nitro blue tetrazolium's inhibition of restoration up to hydrazine tetrazolium [28].

iii) Proteolytic enzymes and their inhibitors activity were studied with trypsin-like activity (TLA), elastase-like activity (ELA), alfa-1-antitripsin activity (ATA) and acid-stable inhibitors activity (ASA) by enzymatic methods [29].

iv) The level of serum protein was measured by Loury.

All investigations were performed with the equipment undergone the metrological tests and expert examination in the appropriate center (Certificate № 021/12 on 12.12.2012).

Statistical analysis

Statistical investigations were performed with variative methods by estimation of arithmetic mean (M), error of arithmetic mean (m), parametric Student test (t). The reliable data were considered that when P<0.05.

Results

The study results have been demonstrated the presence of key symptoms of MS in in the blood serum of animals with the model of MS in comparison with the control one. Experimental animals were shown reliably higher mass of fat tissue and inner organs (P<0.05); both circumference of the abdomen and body mass were increased; raised levels of glucose, cholesterol and triglycerides were noticed, whereas high-density lipoproteins demonstrated the tendency to decrease. Also, the activation of PLO takes place, which was proved by the reliable rise of TBA-AP by 50.4 % (p<0.01) (*Figure* 1). This was an evidence of intensification of PLO as a risk factor of diabetes mellitus and cardiovascular pathology development, which coordinates with the modern research data [30].

Together with the growth of PLO secondary products a reduction of AOS compounds activity, such as SOD by 14.9% (p=0.04) took place (*Figure* 1). These data evidence an upward trend in the consumption of AOS because an excessive accumulation of oxidative-modified molecules. Besides this, the reliable decrease of ceruloplasmin by 37.6% (p<0.001) in comparison with the control group was revealed (*Figure* 1). Hence, in experimental MS both PLA intensification and a decrease of AOS together with ceruloplasmin level rise take place, which prove the activation of AOS to higher level.

The investigations showed at the same time that the 8-week-drinking of 10% fructose solution led to reliable growth of proteases and the decline of their inhibitors - TLA by 19.5% (p=0.010) and ELA – by 17.8 % (p<0.001) of control group (*Figure* 2).

As a result, the data evidence the activation of both proteases and oxidants and the decline of both their inhibitors and AOS in MS. Also, the changes both in AOS and PIN have a tendency to go in the same direction.

The application of polyphenol grape processing products in experimental MS promoted the reverse development of the key symptoms of MS in experimental animals in comparison with the group without correction. Thus, after 4 weeks of "Enoant", "Enoant-premium" and especially "Fenocor" application the experimental animals demonstrated reliable decrease in levels of glucose cholesterol and circumference of the abdomen, whereas high-density lipoproteins demonstrated the tendency to increase.

"Fenocor" demonstrated the most significant exposure on the studied data. First of all, reliable changes were noticed in the intensity of PLO. TBA-AP in the group of "Fenocor" exposure were slumped by 34.0% (p=0.811), which was slightly lower the control meanings, whereas "Enoant" and "Enoant-premium" reached changes only by 19.4% (p<0.001) and 30.4% (p=0.273) (*Figure* 1).

Except PLO, "Fenocor" also demonstrated obvious exposure on AOS-HEOX parametres. It led to the significant rise of SOD by 38.3% (p=0.004), decrease of CC by 15.3% (p=0.004) in comparison with the MS group without correction. The changes of the named data in both groups of "Enoant" and "Enoant-premium" were negligible (*Figure* 1).

"Fenocor" application demonstrated highest level of positive dynamic of proteolytic enzymes in blood serum as well. Thus, ELA had the maximum fall in the "Fenocor" application – by 26.3% (p<0.001) and TLA dropped in the same group of experimental animals by 22.5% (p=0.043) lower the group without correction (*Figure* 2). To compare with, in the application of "Enoant" TLA was higher the control data (p=0.899) (*Figure* 2). ELA was reduced unreliably under "Enoant" exposure by 7.9% (p=0.800), that exceeded the control data by 8.5% (p=0.057). "Enoant-premium" initiated the drop of TLA in the group with MS by 18.4% (p<0.01), that was lower the control by 2.4% (p=0.705); ELA was lower one the group without correction by 13.7% (p<0.001) (*Figure* 2).

The corrective influence of "Fenocor" worked also for the increase of PIN. "Fenocor" increased ATA by 26.7% (p<0.001) and ASA – by 21.3% (p=0.141) in comparison with the group without correction. At the same time, "Enoant" in experimental MS led to rise of ATA by 15.9% (p<0.001) in comparison with the group without correction (*Figure* 2). ASA in the named group was higher by 8.4% (p=0.553) of the group without correction. "Enoant-premium" had an analogous action on the named parameters in rats with MS.

Hence, the analysis of the findings after the application of polyphenol grape processing products in experimental MS is arrived to the conclusion that "Enoant", "Enoant-premium" and especially "Fenocor" exposure blocks the activation of AOS-HEOX and nonspecific proteinases and promotes the improvement of antioxidative and inhibitory potential in experimental MS.

Discussion

Cardiovascular pathology is the leading reason of the whole-world mortality and is considered the result of combined cardiological and metabolic risk factors [31]. When the number of named factors exist in an organism simultaneously, they promote the development of MS what increase, in its' term, the possibility of cardiovascular pathology. It is proved, that the main reason of MS is obesity, whereas pathogenesis of MS is based on PLO activation, pro-inflammatory influences and endogenous intoxication [32, 33].

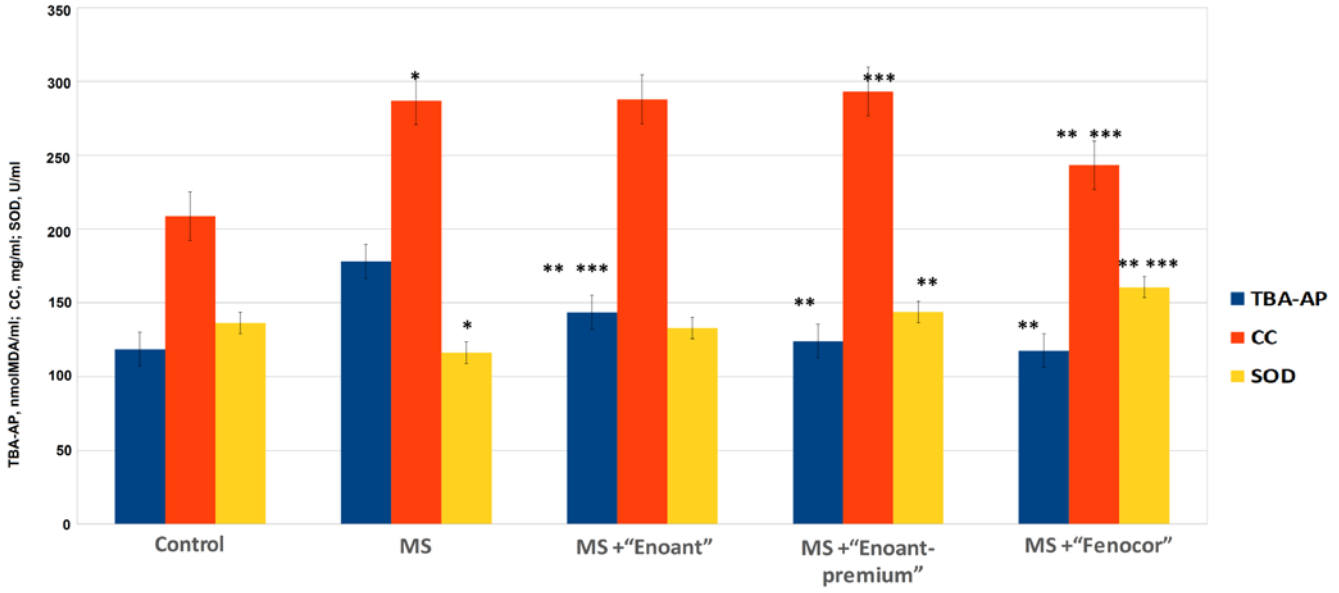

Figure 1. The influence of polyphenol grape processing products on the rats' blood PLO and AOS-HEOX homeostasis in experimental MS.

Control – control group; MS – group with modelling metabolic syndrome; MS+"Enoant" – group with modelling metabolic syndrome, correcting by "Enoant"; MS+"Enoant-Premiym" – group with modelling metabolic syndrome, correcting by "Enoant-Premium"; MS+"Fenocor" – group with modelling metabolic syndrome, correcting by "Fenocor".

TBA-AP, number of tiobarbituric acid active products; CC, ceruloplasmine concentration; SOD, superoxide dismutase activity; nmolMDA/ml – concentration of malone dialdehyde in nanomol per ml; U/ml, inhibitors unit per 1 ml.

p<0.05: * – the reliability in comparison of the MS group with the control group; ** – the reliability in comparison of the correcting groups with the MS group; *** – the reliability in comparison of the correcting groups with the control group.

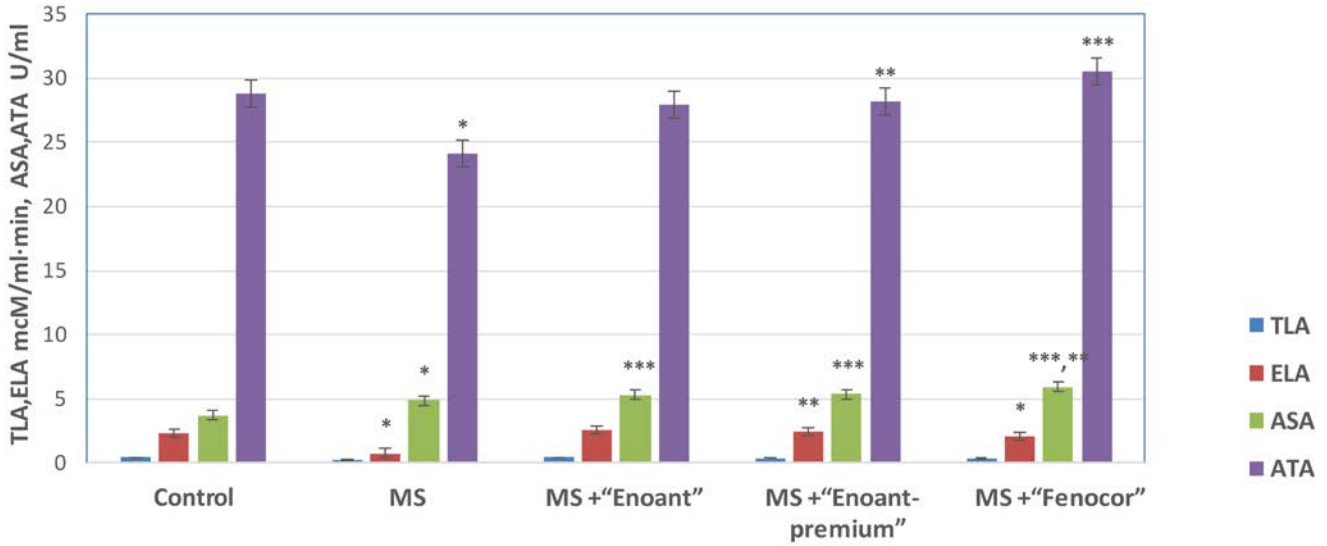

Figure 2. The dynamics of proteolytic enzymes and their inhibitors activity in rats with experimental MS.

Control – control group; MS – group with modelling metabolic syndrome; MS+"Enoant" – group with modelling metabolic syndrome, correcting by "Enoant"; MS+"Enoant-Premiym" – group with modelling metabolic syndrome, correcting by "Enoant-Premium"; MS+"Fenocor" – group with modelling metabolic syndrome, correcting by "Fenocor".

TLA, trypsin-like activity; ELA, elastase-like activity; ATA, alfa-1-antitripsine activity; ASA, acid-stable inhibitors activity; mcgM/ml•min, activity in micrograms in 1Mol per 1 ml in 1 minute; U/min, inhibitors unit per minute.

p<0.05: * – the reliability in comparison of the MS group with the control group; ** – the reliability in comparison of the correcting groups with the MS group; *** – the reliability in comparison of the correcting groups with the control group.

Table 1. Composition of polyphenols in experimental samples of the concentrates produced from red grapes (M±m)

Parameters	*Enoant*	*Enoat-Premium*	*Fenokor*
Total anthocyans, g/dm^3	18.9±0.4	28.5±0.6	-
Flavones, g/dm^3			
- Quercetin-3-O-glycoside	3.1±0.1	3.5±0.1	15.4±0.03
- Quercetin	49.6±1.1	81.2±1.1	10.2±0.2
Flavan-3-ols, g/dm^3			
- (+)-D-catechin	177.6±4.0	208.5±5.1	1752.6±35.1
- (-)-Epicatechin	118.4±2.7	127.3±3.1	1374.2±27.5
Oxycinnamic acids, g/dm^3			
- Caftaric acid	11.7±0.3	16.9±0.4	-
- Cautaric acid	1.8±0.0	2.4±0.1	-
Oxybenzoic acids, g/dm^3			
- Gallic acid	341.1±7.7	465.2±11. 3	1119.2±22.4
- Syringic acid	22.6±0.5	26.2±0.6	-
Proanthocyanidins, g/dm^3			
- Oligomeric proanthocyanidins	603±14	1614±39	4598±92
- Polymeric proanthocyanidins	28155±634	38436±932	172662±3455
Integrated indices			
- Total phenolic substances (by HPLC), g/dm^3	29.50±0.70	41.01±1.00	181.53±3.60
- Total phenolic substances (by Folin-Ciocalteu), g/dm^3	18.51±0.49	21.81±0.59	82.69±2.29
- Antioxidant activity (trolox), g/dm^3	24.72±0.73	36.48±0.92	196.22±4.92

Our findings have shown that in the group of animals with MS the imbalance in AOS-HEOX was observed. The latter could be connected with alimentary obesity. The main index shown the activation of PLO was TBA-AP rise together with the change of antioxidant status. It corresponds to later data of the increase of malone dialdehyde in MS [33]. The activation of PLO was accompanied by the decrease of CLA, PLA, and SOD, whereas CC was increased that may be estimated as the response to PLO activation.

Simultaneously with of AOS-HEOX, the activity of PIN was studied. The linkage of PLO and PIN in MS is a topical problem, because of a positive correlation between metalloproteinases' activity and active forms of oxygen. Active forms of oxygen seem to be the promoters of proteases and, consequently, the improvement factors of the inflammatory process [34]. Our study revealed the reliable positive correlation between nonspecific proteases' activity and circumference of the abdomen, what indicated that MS promoted the active pro-inflammatory factors synthesis that initiated proteases' synthesis. Hence, both the literature data and our research's results allowed assuming, that hyperactivation of PLO initiates and PIN maintains and intensify metabolic disturbances making vicious circle of both humoral and cellular events.

Adaptive mechanisms in MS are not able to level the increased load, so they should be supported [35, 36]. The multiplying research of the appropriate remedies did not bring any positive results. The main reason of the fault is only search of pharmacological treatment, whereas the key stone of MS cure is the group of non-medicinal measures directed on BMI reduction, changing the life-style, etc [36]. That is why the prospective preventive measures could be the group of metabolic regulators, which increase morpho-functional stability of organs and systems. This can explain our choice of polyphenol grape processing products for the named correction. It is known, that polyphenol concentrates possess antioxidant, anti-stress and anti-atherogenic activity. Antioxidant action of polyphenol concentrates is caused by both flavonoids (anthocyanes, quercetin, rutin, catechines, epicatechine, leucoanthocyanes, and tannins) and non-flavonoids (gallic and syringic acids, resveratrol, caffeic and ellagic acids) [37, 38]. Used preparations of "Enoant", "Enoant-premium" and "Fenocor" demonstrated obvious antioxidant activity as well as a positive effect on AOS-HEOX. "Fenocor" performed the most effective effect on AOS-HEOX. Reduction of AOS compounds activity, such as superoxide dismutase, catalase and peroxidase, were noticed. For example, SOD level in MS was reliably gone down by 14.9% ($p=0.004$), ceruloplasmin – by 37.6% ($p<0.001$), SOD by 14.9% ($p=0.004$) in experimental MS was registrated. These data evidence an upward trend in the consumption of AOS due to an excessive accumulation of oxidative-modified molecules.

The applied data are in concordance with the number of research. Thus, in the study of modelling fructose-induced MS antioxidant effects were revealed in feeding with staffs reach in polyphenols [38]. One more study proved antihypertensial activity of oral usage of polyphenols from grape's pits with the maximum effect in the dosage of 375 mg/kg [39]. In addition, in randomized clinical study polyphenols demonstrated hypoglycemic effect and increase of both insulin synthesis and sensitivity of insulin receptors [40].

As it was mentioned before, active forms of oxygen seem to be the promoters of proteases and, consequently, the improvement factors of the inflammatory process [34], especially chronic one in MS [8, 9]. The application of grape polyphenols in our experiment developed the drop of trypsin and elastase activity in MS simultaneously with rise of ATA and ASA. That is why, together with positive effects in PLO, we are entitled to state about not only antiprotease but also anti-inflammatory effects of polyphenol grape processing products. The revealed protective features of "Enoant", "Enoant-premium" and especially "Fenocor" allow using polyphenol grape processing products as MS-modified remedies acting positively on key links of the syndrome.

Conclusion

The development of MS is accompanied by the activation of PLO and proteolysis (increase of TBA-AP by 50.4% and rise of TLA by 19.5% together with the drop in ATA and other antioxidant enzymes.

The application of polyphenol GPP "Enoant", "Enoant-premium" and especially "Fenocor" leads to block of the important pathogenetic link of MS-activation of PLO and inhibition of proteases in the increase of PIN.

The expressed protective action of polyphenol grape processing products in MS in animals is the basis of the inventions of new correctional methods for metabolic disturbances' improving.

Ethics Approval

Animal experiment was approved by the Bioethics committee of Crimea Federal University Center (Protocol № 8 from 15.03.2016) according to the permission of the Academic Council of the Crimean Medical Institute (No. 103 of 30.11.77). The study was approved by the Institutional Committee on Bioethics and is consistent with the International Guidelines for the Care and Use of Laboratory Animals published by the US NIH (No. 85-23, 1985) and Guide for the Care and Use of Laboratory Animals (2009).

Conflict of Interest: none declared.

Funding

This work was partially supported by the V.I. Vernadsky Crimean Federal University Development Program for 2015–2024.

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