Solid herbal extract of *Primula veris* L. improves morphofunctional condition of rats' myocardium in chronic alcohol intoxication

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ABSTRACT

Background and aim: Chronic alcohol intoxication (CAI) induces heart damage. One of the promising ways of its treatment involves the administration of herbal medicinal products. The purpose of this study was to explore the effect of solid herbal extract of Primula veris L. (PVSHE) on the morphofunctional changes in rats' myocardium after CAI.
Experimental procedure: CAI was simulated for 24 weeks. Loading testing was used to assess the functional condition of the heart, the functional assessment of mitochondria was based on the polarographic determination of oxygen consumption rate and determination of the indices of lipid peroxidation and antioxidant enzymes activity. We performed a microscopic examination of the left ventricle following the standard protocol of histological processing and h&e staining.
Results and conclusion: PVSHE restricts the toxic effects of ethanol on the heart which was indicated by a higher rise in the rates of myocardial contraction (by an average of 3.9 times, $P < 0.05$) and relaxation (2.6 times under volume load, $P < 0.05$), LVP (by an average of 1.7 times, $P < 0.05$) and MISP (by an average of 1.5 times, $P < 0.05$). PVSHE caused an improvement in the functional state of rats' cardiac mitochondria exposed to CAI, which was demonstrated by on average 1.3–1.4 times ($P < 0.05$) as high RCR as compared to the control group. The histological examination of the myocardium of the animals treated with PVSHE showed the increase in the volume fraction of cardiac myocytes, and a 31.2% ($P < 0.05$) decline in the interstitial volume. Therefore, PVSHE has a protective effect on the heart after CAI.
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1. Introduction

Alcohol has a marked deleterious effect on the heart. Chronic intake of large amounts of ethanol increases the risk of arterial hypertension, cardiomyopathy, cardiac rhythm disorders, and coronary heart disease.[1] Although the findings of a number of studies have demonstrated that infrequent intake of small amounts of alcoholic beverages is associated with a low risk of cardiovascular pathology, ethanol can cause severe pathophysiological disturbances such as mitochondrial dysfunction, oxidative stress, hypoxia, apoptosis, as well as pathomorphological changes in cardiac and vascular cells.[2,3]

The key element in the myocardial dysfunction is altered cell bioenergetics resulting in decreased cardiac myocyte contractility. Alcohol causes a decline in the mitochondrial membrane potential, activity of respiratory chain complexes and damages mitochondrial DNA.[4] The modified mitochondria are less effective, a decreased ATP synthesis is observed in them, there is an increase in electron

List of abbreviations

CAI	chronic alcohol intoxication
h&e	hematoxylin and eosin staining
PVSHE	solid herbal extract from *Primula veris* L.
LVP	left ventricular pressure
MISP	maximum intensity of structural performance
MDA	Malone dialdehyde
RCR	respiratory control ratio
GPx	glutathione peroxidase
SOD	superoxide dismutase
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
ROS	reactive oxygen species
LPO	lipid peroxidation

escape in the respiratory chain and a surge in reactive oxygen species (ROS), inducing lipid peroxidation (LPO) and damage to various cell structures.[5] All of these result in cardiac failure, severe disability and lethal outcomes. Therefore, a search for new methods of correcting myocardial damage induced by chronic alcohol intoxication appears to be of great importance today.

Herbal medicinal products, which are unique multi-agent complexes of biologically active substances, are regarded as especially promising.[6] Of particular interest are plants of the genus primrose type (Primula L.), of the primrose family (Primalaceae Vent.), namely spring primrose (Primula veris L.). Traditionally, Cardiodoron® drops, which contain a liquid extraction from recently cut flowers of cowslip (Primula veris L., flos recens), thirstle (Onopordum acanthium, flos recens), henbane (Hyoscyamus niger, herba recens) are used as a therapeutical agent. The drug is administered to treat nervous diseases, circulatory failure and desynchronosis. The findings of a promising multicenter non-interventional study have demonstrated that Cardiodoron® drops have a positive medicinal effect, are well tolerated and may be recommended for treating functional cardiovascular disorders and/or sleep disorders as part of a combination therapy or alone.[7]

The solid herbal extract from *Primula veris* L. (PVSHE) contains flavones including polymethoxyllated flavones, which are effective antioxidants, showing antineoplastic, anti-inflammatory and antibacterial properties as well as cardio- and immunoprotective action.[8] Considering traditional use of Cardiodoron in circulatory failure and our previous studies when cardioprotective, endothelioprotective, antioxidant, antiplatelet, and anticoagulant effects of L-isoproterenol in chronic heart failure were detected,[9,10] we consider necessary to study of PVSHE effects on the morphofunctional changes in rats' myocardium after chronic alcohol intoxication exposure.

2. Material and methods

2.1. Animals

The experiments were conducted on female Wistar rats weighing 280–320 g aged 10 months (at the beginning of the study). They were obtained from the Stolbovaya breeding station (Moscow region). The animals were housed under standard vivarium conditions in accordance with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (The European Convention, 1986) and the European Union Directive (EU Directive 2010/63/EU). They had free access to water and food and 12 h of

daylight. The protocol of the experimental study was reviewed by the Regional Independent Ethics Committee (SI Volgograd Medical Research Centre) (protocol N. 2034-2017 of 15.09.2017).

To simulate chronic alcohol intoxication (CAI) 10-month-aged rats were given 10% (vol/vol) ethanol solution sweetened with sucrose (50 g/l) as a single source of drinking for 24 weeks.[11] To make this solution, 95% ethanol was used (ZAO RFK, Russia). The amount of the consumed solution was measured and the solution was replaced once daily at the same time.

Intact animals (n = 25) received drinking water throughout 24 weeks of the experiment. After alcohol intake was discontinued, the rats exposed to CAI (n = 68) were divided into groups: control group – females after CAI exposure (n = 24) receiving normal saline 0.1 ml per 100 g of weight orally; and two experimental groups – females after CAI exposure receiving PVSHE at a dose of 30 mg/kg (n = 23) and mildronate, its comparator (AO Grindex, Latvia), at a dose 50/mg kg (n = 21). Normal saline and the investigated agents were administered intragastrically (gavage) once daily for 14 days starting from the next day after CAI exposure was discontinued. Subsequently, cardiac inotropy was explored in one set of animals using load tests (9, 8 and 7 rats from the respective groups), functional state of cardiac mitochondria and lipid peroxidation intensity and antioxidant enzyme activity were investigated in another fraction of the animals (8, 8 and 7 rats from the respective groups), whereas a third group underwent morphological and morphometric examination of the heart (7 animals each group).

2.2. Preparation of PVSHE

The technology of obtaining herbal medicines depends directly on the chemical composition of the plant. The method of extraction, purification of the final product has a significant impact on the composition and therapeutic properties of phytopreparations.

The object of our research was the above-ground part, the herb Primula veris L., as the richest in the content of biologically active compounds and annually renewable morphological part of the plant.

Our earlier studies of the chemical composition of the spring primrose herb showed the presence of not only hydrophilic (polyphenols, phenolic acids, hydroxycinnamic acids, etc.) but also lipophilic biologically active compounds, such as polymethoxylated flavonoids, which are chemotaxonomic markers of primrose plants, diterpenes, and fatty acid derivatives. Our own experimental studies have shown that the optimal type of extractant for the extraction of hydrophilic and lipophilic compounds was 70% ethyl alcohol solution. Optimal extraction parameters were selected: the degree of grinding of raw materials - 1–3 mm; the ratio of raw materials-extractant 1:10. The extraction method - triple fractional maceration, the method of purification from ballast substances – sedimentation.

A solid extract of spring primrose was obtained by thickening aqueous-alcoholic extract obtained according to our developed technology. The technology of dense extract production included the following stages:

1. Preparation of herbal raw materials;
2. Preparation of the extractant;
3. Obtaining the primary extraction;
4. Purification of the hood from ballast substances;
5. Evaporation.

We have determined the component composition of biologically active compounds of the studied phytopreparation. Using the methods of NMR 1H-, NMR 13C-spectroscopy, 1H-1H COSY, 1H-13C HSQCED NMR correlation spectroscopy, NMVS, chromatography-

mass spectrometry established that the phenolic compounds of the extract under study include flavonoid compounds, including flavones (apigenin, luteolin and their glycosides), polymethoxylated flavones, flavonols (quercetin, kaempferol and their glycosides), flavanonols (dihydroquercetin), simple phenols (arbutin), tannins (tannin, catechin, epicatechin, epigalocatechin gallate), coumarins (umbelliferone, coumarin), phenolic acids (gallic, salicylic acids), hydroxycinnamic acids (cinnamic, caffeic, ferulic, chlorogenic, neochlorogenic acids). The composition of triterpene saponins was studied by thin-layer chromatography, spectroscopy, spectrophotometry methods; ursolic acid, aescin was determined. The research results showed that the dominant groups of biologically active substances in the resulting preparation, which are important for the purposes of standardization, are rutin and aescin. The content of the sum of flavonoids in terms of rutin in the samples of the drug is not less than 5.0%, saponins in terms of escin is not less than 10.0%.[9]

2.3. ECG recordings

The Lead II ECG (50 mm/s of velocity and sensitivity of 20 mm/mV) traces were recorded to analyze heart rate, QRS interval duration, QRS amplitude and p-wave duration. Skin-safe (non-invasive) electrodes were fixed on the animal's paws and were connected to POLY-SPECTRUM-8/V Veterinary Digital ECG System (Neurosoft, Russia).

2.4. Functional tests

To assess the functional condition of the heart the following load tests were used: volume load test (administration of saline solution 0.3 ml per 100 g of body weight intravenously), adrenoreactivity test (introduction of adrenalin 0.1 ml per 100 g of body weight intravenously at a 10^{-7}g/l dilution) and maximum isometric load test (occlusion of the ascending aortic arch for 30s).

2.5. Functional assessment of mitochondria

The hearts of the narcotized rats were removed and washed with ice-cold normal saline. All further manipulations were conducted at a temperature of 4 °C. The hearts were cut into small pieces and then again washed with ice-cold saline. After that they underwent homogenization with a Potter-Elvehjem homogenizer (Potter-Elvehjem) and a teflon pestle. Mitochondrial fractions were obtained by the standard method of differential centrifugation in a sucrose medium.[12]

We performed polarography using a Clark-type electrode from Handsatech Oxytherm System (Hansatech Instruments, UK) to estimate the rate of oxygen consumption by mitochondria. The functional assessment of mitochondria was based on the protocol described by I. R. Lanza.[12] Previously, all the solutions were thermostated for 20 min at 33 °C. The reagents used to make solutions were manufactured by Sigma-Aldrich, USA.

The rate of oxygen consumption was expressed in nmolO$_2$/min/mg of protein and calculated in the metabolic states according to Chance: V1 − rate of endogenous respiration; V2 − rate of substrate-dependent respiration; V3 − rate of oxygen consumption by mitochondria when an oxidation substrate and ATP are added (state of oxidative phosphorylation); V4 −rate of mitochondrial respiration in the presence of a substrate after oligomycin, an ATPase inhibitor, is added; V of uncoupling − a proton ionophor carrying protons across the membrane eluding the ATP synthase channel was added to the mitochondria (FCCP uncoupler). We

calculated the respiratory control ratio (RCR) (V3/V4 ratio) to evaluate respiration and phosphorylation.[13]

2.6. Determining the indices of lipid peroxidation and activity of antioxidant enzymes in cardiac mitochondria

The method of assessing the MDA content was based on the identification of TBA-reactive substances, which form when 0.7% solution of thiobarbituric acid is boiled with isolated mitochondria in an acidic medium (with 1.3% H$_3$PO$_4$ added).[13] The optical density of the solution was measured at 532 nm using a Helios γ spectrophotometer (Thermo Electron Corporation, UK). The level of MDA was expressed in μmol/mg of protein.[13]

We analyzed the loss of peroxide in the reaction mixture compared to the blank assay (without mitochondrial suspension) to determine catalase using Helios γ spectrophotometer at a wavelength of 410 nm.[13]

The assessment of glutathione peroxidase (GPx) activity was based on the reaction of glutathione oxidation by *tert*-butyl hydroperoxide. We elucidated the level of reduced glutathione in the supernatant with the help of 0.4% 5,5′-dithiobis(2-nitrobenzoic acid) using a Helios γ spectrophotometer (Thermo Electron Corporation, UK) at 412 nm.[13]

To explore superoxide dismutase activity (SOD), we relied on V. A. Kostyuk's method (1989).[14] The concentration of protein in the samples was assessed using a Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA).

2.7. Histopathological evaluation

We performed a microscopic examination of the left ventricle following the standard protocol of histological processing and h&e.[15] The biomaterial sampling was performed within 30 min after the functional tests were over. The muscular tissue blocks 0.5 × 0.8 cm in size were fixed in 10% neutral buffered formalin for 24 h. After being washed the biomaterial was dehydrated and subsequently treated with xylol and impregnated with paraffin. Microtoming (5−6 μm) was performed with a rotatory microtome (Thermo Fisher HM340E, USA). We used the Thermo Scientific Polysine slides (USA). Subsequent to deparaffinization and rehydration, the myocardial microslides were stained with hematoxylin (NPF Abris+, Russia) and 0.5% hydroalcoholic solution of eosin (OOO Labiko, Russia).

The morphometric examination involved digitalized microphotographs of the rats' myocardium processed with MCview software (LOMO-microsystems, Russia). To meet the representativeness criterion, morphometric measurements were taken in 10 randomly chosen fields for each section. We used the semi-quantitative method with a 1-4-point scale to assess the pathohistological changes.[16,17]

2.8. Statistical analysis

The findings were presented as the mean value ± standard error of the mean (S.E.M.). The Shapiro-Wilk test was used to check the normality of distribution. To conduct the statistical analysis we employed the GraphPad Prism 9 software (GraphPad Software, San Diego, USA), Student's test (for pairwise comparisons for normally distributed data) and one-way ANOVA test for multiple comparisons with a subsequent Tukey post hoc test (for multiple comparisons for normally distributed data) and Kruskal-Wallis with Dunn's test (for multiple comparisons for data whose distribution is different from normal). The differences were considered statistically significant when P < 0.05.

3. Results

3.1. Effects of PVSHE on the parameters of ECG

The CAI + Saline group displayed significant (P < 0.0001) increase of heart rate compared to the intact rats. Moreover, were detected the increased QRS interval by 1.5 times (P < 0.0001) in comparison to the intact animals where the QRS amplitude was lower by 1,2 times (P < 0.005). In rats of the CAI + Saline group, PQ interval was 1.3 times longer than in intact animals.

The heart rate in the animals receiving PVSHE and mildronate after CAI exposure, was on average 1.1 (P < 0.005) times lower than those in the control group. Duration of the QRS interval, in animals of the CAI + PVSHE was less, but did not differ significantly from the control group. In the experimental groups animals receiving PVSHE and mildronate after CAI QRS amplitude on average was 1.3 times (P < 0.0001) higher than in females CAI + Saline rats. The PQ interval in the animals receiving PVSHE after CAI exposure, was 1.4 (P < 0.0001) times higher than those in the control group, in those receiving mildronate - 1.3 (P < 0.0001) times higher. Duration of P wave of all animal groups did not differ significantly (Table 1).

3.2. Assessment of the cardiac functional reserve in the animals receiving PVSHE after CAI exposure

In response to a higher preload the females after CAI exposure showed rises in both + dP/dt and LVP, which were 4.2 (P < 0.05) and 1.5 (P < 0.05) times as low in the intact animals. PVSHE had a positive effect on the studied parameters: a surge in +dP/dt max, -dP/dt max and LVP in response to a higher preload were 10.4 (P <0.0001), 2.6 (P < 0.005) and 1.7 (p <0.05) times as high as the similar indices in the control group rats, respectively. Mildronate had a less noticeable effect on the cardiac contractility parameters: increases in the rates of contraction, relaxation and LVP were 6.2 (P < 0.0005), 1.9 (P <0.005) and 2.3 (P < 0.05) times higher than the findings in the negative control group (Fig. 1).

Adrenoreactivity test revealed that CAI exposure resulted in a considerable decrease in heart contractility: the negative control animals displayed a lower maximum increase in response to norepinephrine administration compared to the similar indices in the intact group and were +dP/dt max 2.1 (P < 0.0001), -dP/dt 1.3 (P < 0.05) and LVP 1.6 (P < 0.005) times as low than intact animals. Alcoholized animals receiving PVSHE demonstrated a surge in the contraction rate, the relaxation rate and increase in LVP on average 1.7 (P < 0.05) times higher compared to the values of the control group rats (Fig. 2). Mildronate had an effect comparable with PVSHE (Fig. 2): increases in +dP/dt max, -dP/dt max and LVP in females receiving the agent were 2 (P < 0.005), 1.4 (P < 0.05) and 1.5 (P < 0.05) times as high in contrast to the females after CAI exposure receiving normal saline, respectively.

Rises in +dP/dt max and LVP in the control group females exposed to maximum isometric load were 0.7 (P < 0.005) and 1.7 (P < 0.0001) times lower against similar indices in the intact group females (Fig. 3). PVSHE promoted increase in the rate of contraction and LVP compared to the control group on the 5th second of the occlusion of the ascending part of the aorta (1.4 times (P < 0.05) and

1.6 times (P < 0.0005), respectively). The experimental group females receiving mildronate showed was 1.6 times (P < 0.0005) as high as in the animals after CAI exposure (Fig. 3).

The animals exposed to CAI receiving normal saline showed an apparent decline in cardiac inotropy on the 30th second of the occlusion of the ascending part of the aorta. +dP/dt max, -dP/dt max and LVP in the control group females were 2.5 (P < 0.005), 1.6 (P < 0.05) and 2.3 (P < 0.0001) times lower than the values of the intact animals, respectively (Fig. 4).

The rats exposed to CAI receiving PVSHE demonstrated on average, contributed to a 2.1 times rise (P < 0.0001) in +dP/dt max and LVP in the alcoholized animals, receiving Mildronate - 1.7 times and 2.1 times (Fig. 4).

When calculating an increase in the maximum intensity of structural performance (load per unit of myocardial mass), we found that this index in the animal group exposed to CAI was on the 5th and on the 30th second of the occlusion of the ascending part of the aorta 1.6 and 2 times higher than in the intact group animals, respectively (P < 0.0001). A rise in the groups of females receiving the compounds under study and mildronate, their comparator, after alcoholization was on average 1.5–1.8 times higher than in the control group (P < 0.005) (Fig. 5).

3.3. Effect of PVSHE on the functional state of cardiac mitochondria in rats exposed to CAI

The functional state of cardiac mitochondria also deteriorated as a result of chronic ethanol intake (Fig. 6). The rate of endogenous respiration in the control group rats it was 1.5 times as low (P < 0.0001) than in intact animals. In the experimental groups females receiving PVSHE and mildronate which was on average 1.2–1.3 times (P < 0.0001) higher than in the negative control group.

The rate of substrate-dependent respiration when ATP was added for Complex I in the animals exposed to CAI was 1.6 times as low (P < 0.0001) as in the intact females. The rate of oxygen consumption in the animals receiving PVSHE after CAI exposure, was 1.3 (P < 0.0001) times higher than those in the negative control group, in those receiving mildronate - 1.4 (P < 0.0001) times higher.

The rate of substrate-dependent respiration when both Complexes I and II in rats exposed to alcoholization this index was 1.6 times (P < 0.0001) lower than intact animals. In the experimental groups animals receiving PVSHE and mildronate which on average was 1.4 times (P < 0.0001) higher than in the females exposed to CAI. Oxygen consumption after ATP and rotenone were added to inhibit Complex I performance and singularly stimulate.

respiratory chain Complex II OCR(ADP)II) was 1.6 times (P < 0.0001) lower in the control group females. In the experimental groups receiving PVSHE and mildronate the rates of substrate-dependent respiration were 1.5 (P < 0.0001) and 1.4 (P < 0.0001) times higher than in the negative control group. No significant differences in the respiration rate in a non-phosporylated state were observed across the groups.

Respiratory control ratios for separately functioning Complexes I and II in rats exposed to CAI were 1.6 times (P < 0.0001) as low as in the intact animals. PVSHE and mildronate enhanced the functional state of cardiac mitochondria in rats after CAI exposure which is

Table 1
Effects of PVSHE on the parameters of ECG.

Animal groups	Heart rate	QRS interval, s	QRS amplitude, mV	P-wave duration, s	PQ interval, s
Intact rats (n = 9)	361.2 ± 35.5	0.041 ± 0.004	0.313 ± 0.05	0.02 ± 0.008	0.054 ± 0.01
CAI + Saline (n = 9)	417.4 ± 35.2****	0.061 ± 0.015****	0.256 ± 0.11**	0.01 ± 0.013	0.07 ± 0.01****
CAI + PVSHE (n = 7)	384.7 ± 34.1**	0.054 ± 0.009	0.332 ± 0.05****	0.02 ± 0.012	0.051 ± 0.01****
CAI + Mildronate (n = 7)	382.4 ± 50.6***	0.063 ± 0.011	0.343 ± 0.08****	0.03 ± 0.010	0.056 ± 0.01****

****P < 0.0001, ***P < 0.0005, **P < 0.005, *P < 0.05 versus intact rats or versus CAI + Saline (Kruskal-Wallis test with Dunn's test).
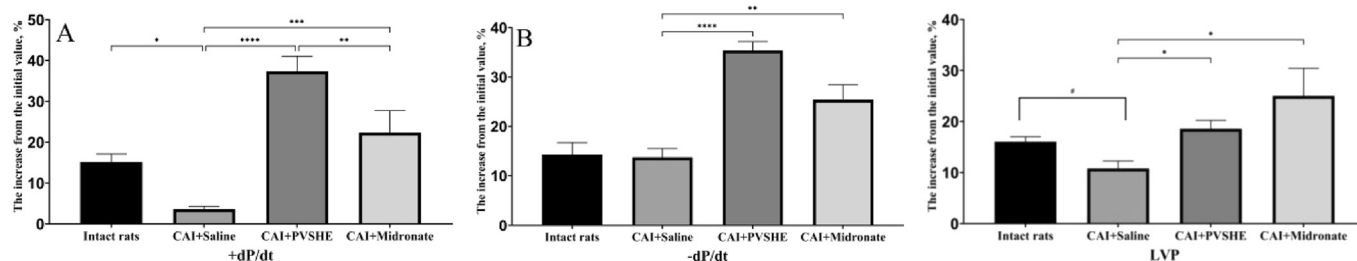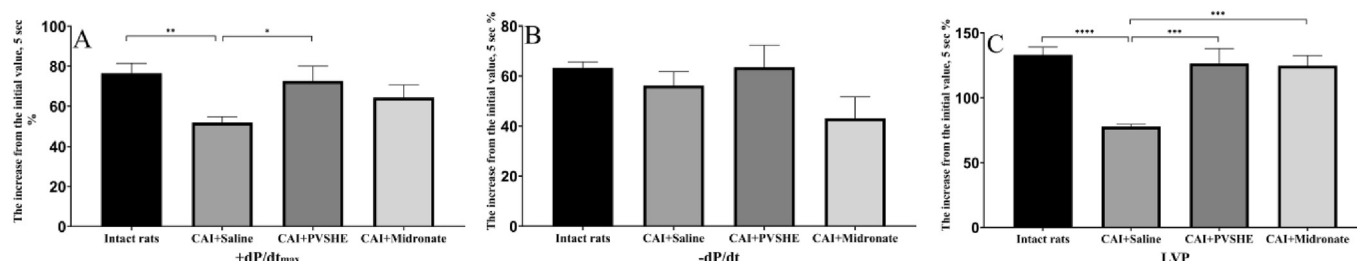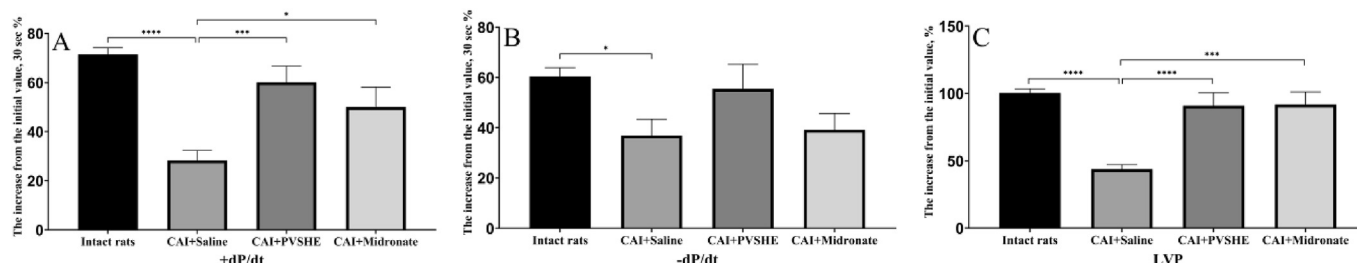
Fig. 1. The effects of PVSHE on myocardial contractility in the rats exposed to CAI after volume load). The increase shown from the initial value is expressed in percent for + dP/dt (A), -dP/dt (B) and LVP (C). ****P < 0.0001, ***P < 0.0005, ** P <0.005, *P < 0.05 versus intact rats or versus CAI + Saline (Tukey test).
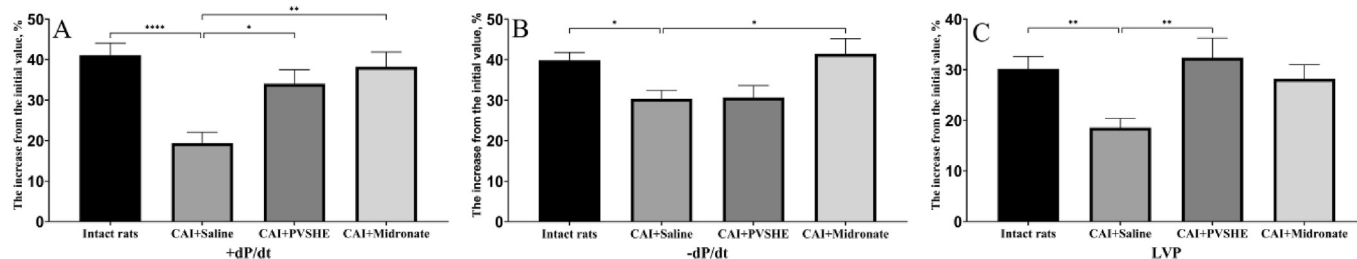
Fig. 2. The effects of PVSHE on myocardial contractility in the rats exposed to CAI after testing for adrenoreactivity. The increase shown from the initial value is expressed in percent for + dP/dt (A), -dP/dt (B) and LVP (C). ****P < 0.0001, ***P < 0.0005, **P < 0.005, *P < 0.05 versus intact rats or versus CAI + Saline (Tukey test).

Fig. 3. The effects of PVSHE on myocardial contractility in the rats exposed to CAI undergoing a maximum isometric load test (5 s occlusion). The increase shown from the initial value is expressed in percent for + dP/dt (A), -dP/dt (B) and LVP (C). ****P < 0.0001, ***P < 0.0005, **P < 0.005 *P < 0.05 versus intact rats or versus CAI + Saline (Tukey test).

Fig. 4. The effects of PVSHE on myocardial contractility in the rats exposed to CAI undergoing a maximum isometric load test (30 s occlusion). The increase shown from the initial value is expressed in percent for + dP/dt (A), -dP/dt (B) and LVP (C). ****P < 0.0001, ***P < 0.0005, **P < 0.005 *P < 0.05 versus intact rats or versus CAI + Saline (Tukey test).

implicated in on average 1.3–1.4 (P < 0.0005) times higher respiratory control ratios compared to the control group (Fig. 7).

3.4. Indices of the oxidant/antioxidant system in cardiac mitochondria of rats receiving PVSHE

The animals exposed to CAI showed a twice higher level of MDA (P < 0.0001) in heart mitochondria and 1.4 times higher SOD activity (P < 0.0001) in contrast to the intact group. In females receiving PVSHE after CAI exposure MDA concentration was 1.3 times lower (P < 0.005), whereas no significant differences in

enzyme activity were observed compared to the control group. The animals to which mildronate was administered after CAI demonstrated a greater activity of glutathione peroxidase and superoxide dismutase against the negative control group (P < 0.05), moreover, the MDA level was also significantly higher (Table 2).

3.5. Histopathological examination of changes caused by PVSHE in rats' heart myocardium after CAI exposure

No noticeable pathological changes were revealed in the myocardium of the intact animals. Miscroscopically, it was
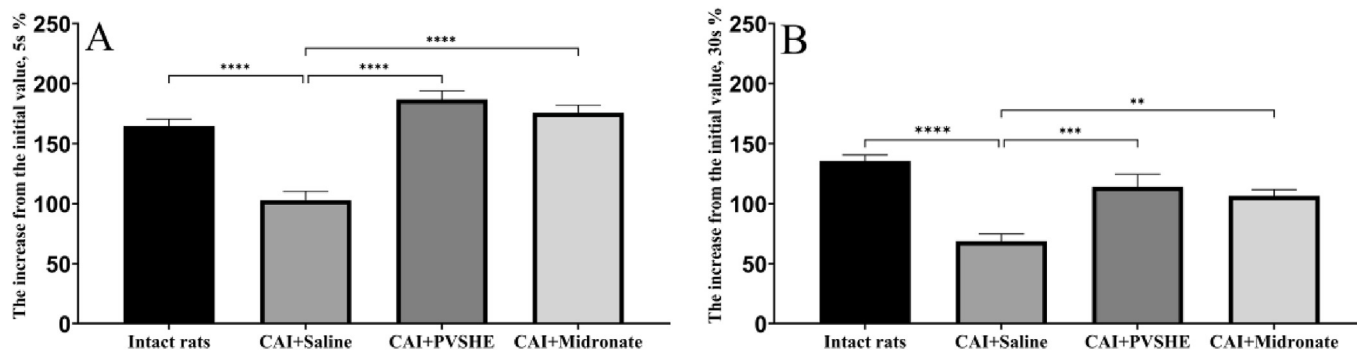
Fig. 5. The effects of PVSHE on MISP. The increase of MISP shown from the initial value is expressed in percent for 5th s (A) and 30th s (B) after occlusion. Data are presented as mean ± S.E.M. ****P < 0.0001, ***P < 0.0005, **P < 0.005 *P < 0.05 versus intact rats or versus CAI + Saline (Tukey test).
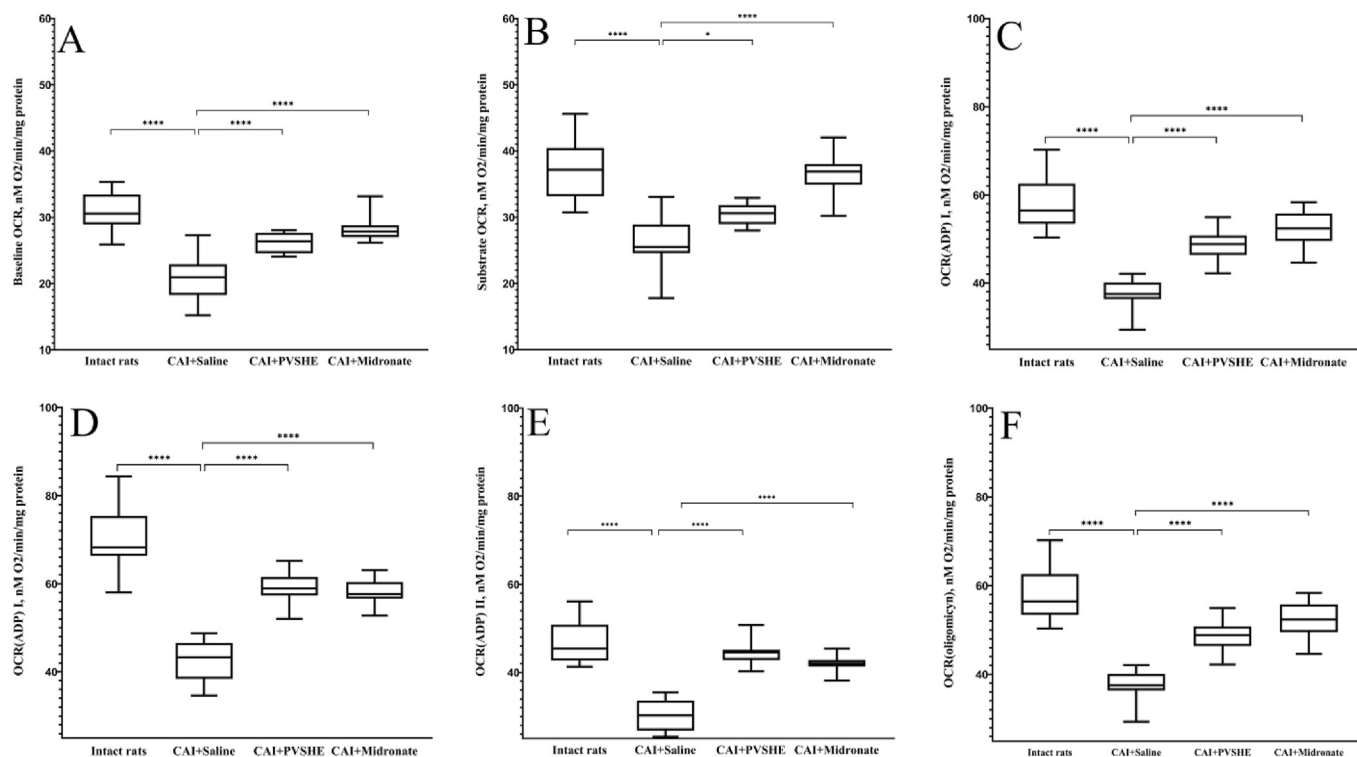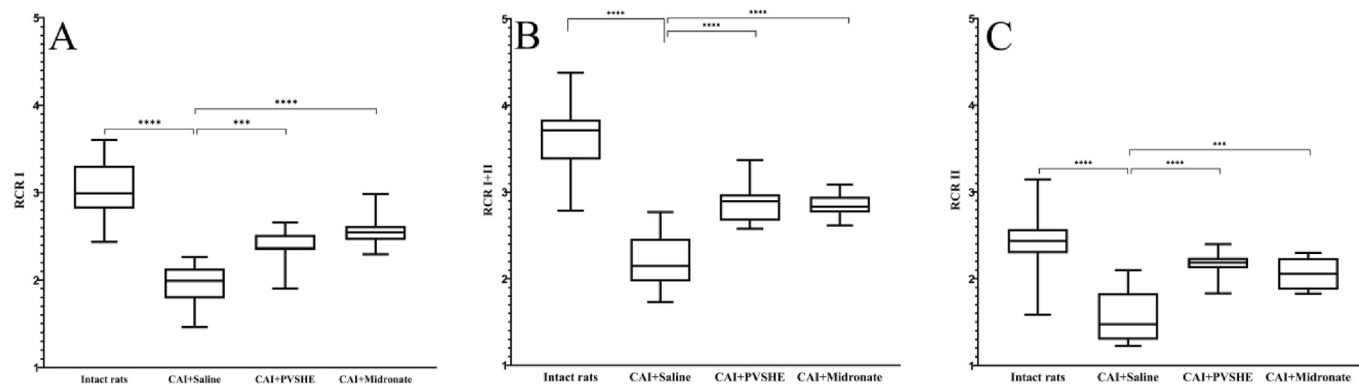

Fig. 6. Effect of PVSHE on the respiratory function of isolated mitochondria from the heart cells of rats exposed to CAI. Images represent the oxygen consumption rate after adding the solutions to the polarographic cell: A) baseline OCR − 100 μl (0.5−1 mg protein/ml) of mitochondrial suspension; B) substrate OCR − 100 μl 5 mM malate/5 mM glutamate; C) OCR $_{ADP\ I}$ − 100 μl 200 μM ADP; D) OCR $_{ADP\ I\ +\ II}$ − 100 μl 5 mM succinate; E) OCR $_{ADP\ II}$ − 100 μl 0.5 μM rotenone; F) OCR $_{oligomycin}$ - 100 μl 2 μg/μl oligomycin; ****P < 0.0001, ***P < 0.0005, *P < 0.05 versus intact rats or versus CAI + Saline (Tukey test).

presented as muscle fibers with well-defined cross-striated sarcoplasm, nuclei showing weak polymorphism. Cardiac myocytes were separated with thin layers of loose fibrous tissue (Fig. 8, A).

Diffuse edema of myocardial stroma causing accumulation of transudate in perivascular and intermuscular spaces was observed in the animals exposed to CAI. Cardiac myocytes differed in size, which was indicated by the fact that the cross-sectional area of cardiac myocytes was 9.2% larger (P < 0.05) with reference to the intact rats. Cardiac myocytes were tortuous, sometimes cross-striation was lost, with foci of plasmolysis and muscular fiber fragmentation. Loose eosinophil deposits were observed in cardiac myocyte cytoplasm. Round and oval nuclei showing diffuse hyperchromia were located in the central and peripheral portions of myocyte cytoplasm (Fig. 8, B). The morphometric analysis revealed a significant decrease in the volume fraction of cardiac

myocytes alongside with a 57.1% increase (P < 0.05) in the volume fraction of interstitial tissue in animals exposed to CAI compared to the healthy females. The alcoholized animals receiving PVSHE showed less pronounced changes. It was proved by preserved cross-striation of eosinophil sarcoplasm, absence of tortuosity in cardiac myocytes, which were separated with thin layers of loose fibrous tissue (Fig. 8, C). Decreased fibrous changes in the myocardium were also implicated in a higher volume fraction of cardiac myocytes and a significant decline (by 31.2%, P < 0.05) in volume of interstitial tissue. It should be noted that the volume fraction of the vascular component in the rats of the studied group was 33.3% lower than in the control group animals. Mildronate, a comparator, had a less noticeable protective effect: signs of irregularly pronounced edema of myocardial stroma, degenerative changes in cardiac myocytes were still present (Fig. 8, D).

Fig. 7. Effect of PVSHE on the Respiratory control ratio (RCR) of isolated mitochondria from the heart cells of rats exposed to CAI. Respiratory control ratio (according to Chance) calculated as: A) RSR I— OCR ADP I/OCR oligomycin; B); RCR I + II - OCR ADP I-II/OCR oligomycin; C) RCR II − OCR ADP II/OCR oligomycin. Data are presented as the box-and-whisker plot (5 and 95 percentile). ****P < 0.0001, ***P < 0.0005, *P < 0.05 versus intact rats or versus CAI + Saline (Tukey test).

Table 2
Effects of PVSHE on the mitochondrial oxidative status in the cardiac cells exposed to CAI.

Animal groups	MDA, µM/mg protein	Catalase, mg H_2O_2/min/mg protein	GPx, µM/min/mg protein	SOD, % inhibition/mg protein
Intact rats (n = 9)	15.9 ± 2.1	11.0 ± 2.5	3.5 ± 0.7	42.5 ± 4.1
CAI + Saline (n = 9)	30.1 ± 1.7****	12.0 ± 1.9	3.2 ± 0.5	29.4 ± 4.3****
CAI + PVSHE (n = 7)	23.6 ± 4.4**	11.1 ± 2.3	2.8 ± 0.8	29.1 ± 2.2
CAI + Mildronate (n = 7)	50.5 ± 3.8****	7.2 ± 1.7**	5.0 ± 0.5***	40.6 ± 6.5**

****P < 0.0001, ***P < 0.0005, **P < 0.005, *P < 0.05 versus intact rats or versus CAI + Saline (Tukey test).
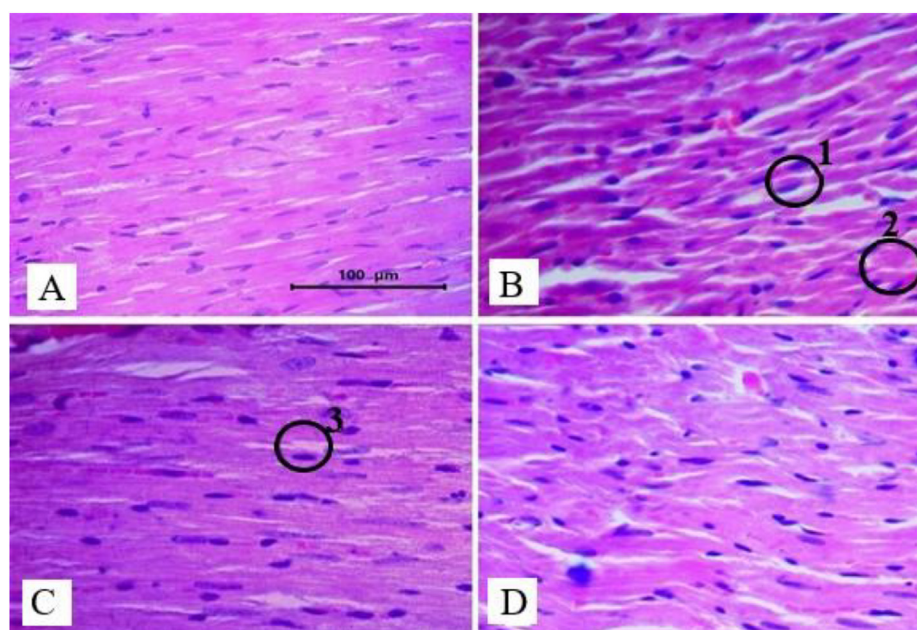
Fig. 8. Histological changes in the myocardium of the animals from the studied groups (H&E, x400). A − intact group; B − control group of animals exposed to CAI. The microphotographs show the groups of hypotrophic (1) and hypertrophic (2) cardiac myocytes; cardiac myocytes are tortuous, with patches where cross-striation is lost, with foci of plasmolysis and muscular fiber fragmentation present. C − experimental group of animals receiving PVSHE after CAI (30 mg/kg). No signs of tortuosity in cardiac myocytes, which were separated with thin layers of loose fibrous tissue, were present (3); D − group of rats exposed to CAI, which received mildronate (50 mg/kg). The myocardium of this group females still showed the signs of irregularly pronounced edema of myocardial stroma, degenerative changes in cardiac myocytes.

Morphometric analysis of myocardium demonstrated cardiac degenerative changes in the group of animals with chronic alcoholism. Cardiac myocyte volume and cardiac interstitial volume were significantly changed in the group of animals CAI + PVSHE: cardiac myocyte volume fraction (%) in the animals CAI + PVSHE was 92,7 ± 0,8 in comparison to 89,4 ± 1,2 (%) in the animals CAI + saline; interstitial volume fraction (%) was 7,7 ± 1,1 in the

animals CAI + saline and became 5,3 ± 1,1 in the group of animals CAI + PVSHE.

4. Discussion

Currently, the prevalence and severity of somatic complications caused by alcohol intoxication remain a matter of great concern.

Penetrating into the body ethanol is evenly distributed throughout it affecting practically all organs and tissues. One of the body systems, which is the most vulnerable to ethanol damage is the cardiovascular system, which accounts for high mortality rate associated with ethanol.[18]

In our study the control group animals exposed to CAI demonstrated a decline in cardiac inotropy. The fact that ethanol has a negative effect on the functional state of cardiac myocytes and heart output on the whole and on excitation-contraction coupling, oxidative stress development, apoptosis and cell metabolism, in particular, accounts for that.[19] Acute ethanol exposure induces changes in ion channel performance: fast sodium channel, L-type Ca2+- channel, inwardly rectifying potassium channels, etc. Effects are associated with changes in gap junctions and disturbances in cell-cell interactions.[20] Leakage of $Ca2+$ out of the sarcoplasmic reticulum caused by oxidative stress and Ca2+/calmodulin-dependent protein kinase II (CaMKII) is an important mechanism behind decreased myocardial contractility and arrhythmia development.[21]

Alcohol induces downregulation of the key genes of the extracellular matrix encoding titin, type III collagen and calponin, which cumulatively results in heart damage.

However, a number of studies have demonstrated that moderate consumption of alcohol, red wine for example, has cardioprotective effects. This action is associated with polyphenols having a potent antioxidant effect. They activate eNOS, increase NO production, and reduce morbidity and mortality caused by cardiovascular diseases.[22,23] It has been found that the PI3K/Akt signaling pathway plays a crucial role in mediating alcohol effects on the cardiac function. ROS activate this signaling pathway. Low doses of alcohol decrease the activity of Akt and simultaneously increase the expression of FOXO-3 protein in cardiac myocytes.[24] There is some evidence that cardioprotective effects of alcohol are also associated with the positive impact on the plasma lipid profile and homeostasis.[25] The study by Scrimgeour L.A. et al. (2017) has demonstrated that in moderate doses alcohol modulates the nucleus factor κ-B pathway in ischemic myocardium and decreases the number of anti-inflammatory cytokines, which may act as a mechanism underlying its cardioprotective action.[26]

To date, no convincing evidence on the causal relationship between alcohol consumption and the lower rate of cardiovascular diseases has been generated.[27] As no well-designed randomized trials testing the role of alcohol in cardiac protection have been conducted, the benefits of alcohol consumption are debatable.

Alcohol causes mitochondrial dysfunction, a decrease in the membrane potential and a rise in the ROS level.[4] Chronic ethanol consumption for 6 or 16 weeks has been found to increase the production of superoxide, a ROS marker, in male mice.[28] Y. Wang et al. (2015) have demonstrated that treating cardiac myocytes with alcohol for 24 h (50 мM, 100 мM, 200 мM) elevates ROS levels in a dose-dependent manner.[29]

However, regardless of the general opinion that oxidative stress provokes heart damage induced by alcohol intoxication, there have been some reports on the decreased level of H_2O_2 in isolated cardiac myocytes treated with low concentrations of alcohol.[24] It should also be noted that the current findings on the activity of antioxidant enzymes are conflicting: in some studies the activities of catalase and superoxide dismutase rose, whereas in others they did not change during long-term experimental alcohol intake.[4,30,31] Furthermore, the content of glutathione and glutathione peroxidase in the myocardium declined in response to chronic alcohol consumption.[4] Therefore, the role of oxidative stress in the pathogenesis of alcohol-induced myocardial damage has to be further elucidated to discover the roots of these apparent discrepancies.

In our study the control group animals developed a mitochondrial dysfunction, which was implicated in decreased rates of mitochondrial respiration and respiratory control ratio in the electron transport chain complexes. These changes are mainly associated with oxidative stress in mitochondria. ROS are continuously formed in a living cell. Their production is restricted by antioxidant system Naturally, ROS production and destruction are well-coordinated; an increase in pro-oxidants activates antioxidant enzymes to maintain the equilibrium. However, the upset equilibrium may provoke oxidative stress inducing macromolecular damage, which results in cell dysfunction and their death. The animals exposed to CAI developed oxidative stress which was demonstrated by the increased number of TBA-reactive products forming when the membranes were oxidized by ROS and decreased SOD activity compared to the intact group.

The microscopic examination of the myocardium revealed micromorphometric and ultrastructural changes in cardiac myocytes as well as qualitative and quantitative changes in the intercellular substance. The pathological changes discovered in our study are likely to be due to the toxic effects of ethanol and its metabolites. The latter are immediately involved in the mechanisms underlying intracellular metabolism disturbances and discirculatory episodes. Cumulatively, these disorders provoke cardiac muscle hypoxia and impaired heart contractility which is consistent with literature data.[32]

Electrocardiographic changes (prolongation of the QRS interval, decrease of its amplitude, prolongation of the PQ interval, and increased heart rate), along with functional tests, histological changes, mitochondrial dysfunction and rise in the ROS level indicate serious disturbances in the functioning of the heart caused by ethanol.

The investigated PVSHE restricts the toxic effects of ethanol on the heart which is indicated by a higher rise in the rates of myocardial contraction and relaxation, LVP and MISP when functional tests were conducted: volume loads, adrenoreactivity tests, and maximum isometric load. These effects may be primarily associated with the chemical composition of PVSHE, a rich combination of hydrophilic and lipophilic biologically active compounds, among which polyphenol substances, in particular flavonoids represented as aglycones and glycosides, polymethoxylated flavones such as flavonoid aglicones (apigenin, quercetin, kaempferol), flavonoid glycosides (cynaroside, rutin, hyperoside) and polymethoxylated flavones acting as chemotaxonomic markers for the primrose genus (8-methoxy-flavone, flavone, 3′,4′-methylendi-oxy-5′-methoxy-flavone) are of special importance.

Numerous studies have demonstrated that flavonoids can be referred to as non-enzymatic antioxidants able to directly or indirectly reduce or prevent cell damage induced by free radicals.[33] In our earlier studies PVSHE had a cardioprotective effect when a model of isoproterenol-induced cardiac failure was used. This was proved by a smaller number of deaths and the low level of markers in the blood serum of animals receiving the drug compared to the control group, higher increases in the rates of myocardial contraction and relaxation, LVP and MISP when adrenoreactivity, volume load and maximum isometric load tests were used, greater indices of mitochondrial functional state in contrast to the control group, restricted development of oxidative stress, and enhanced SOD activity.[7] The determination of rutin when PVSHE was intragastrically administered based on HPLC revealed that this glycoside is distributed in the heart and penetrates in mitochondria.[34] We may assume that flavonoids, flavonols and ferrulic acids which PVSHE contains immediately act as ROS traps and restrict the development of oxidative stress. This is indicated by a decrease (although insignificant) in the concentration of TBA-active products, whereas mildronate indirectly activates enzymatic

antioxidant defense and does not influence MDA production.

PVSHE restricts the toxic effects of ethanol on the heart which was indicated by a higher rise in the rates of myocardial contraction and relaxation, LVP and MISP when functional tests were conducted, normalization of heart rate, duration of the PQ interval and amplitude of the QRS on ECG recording.

However, this study has limitations. The investigation has not studied the functionality of myocardium in a non-invasive way, for example by echocardiography. This can determine the prospects of further research to expand our understanding of cardioprotective effect of PVSHE under chronic alcohol intoxication.

5. Conclusion

Thus, chronic alcoholic intoxication leads to mitochondrial dysfunction, stimulation of lipid peroxidation, damage to the morphological structure of cardiomyocytes, which eventually result in the reduction of myocardial contraction and myocardial relaxation rates, as well as of the left ventricular pressure when the load tests were conducted.

The solid extract from the primary herb of spring (*Primula veris* L.) has showed a pronounced cardioprotective effect after prolonged exposure to ethanol. The positive effect of PVSHE on myocardium is possibly due to the antioxidant effects of its flavonoids resulting in improved mitochondrial function, enhanced ATP production, normalization of ECG parameters increased cardiac inotropy, and limitation of pathohistological changes.

Declarations of competing interest

The authors declare no conflict of interest.

Acknowledgments

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