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A Study of Oxidative Stress Markers when Using the Liposomal Antioxidant Complex

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ΛΕΞΕΙΣ - ΚΛΕΙΔΙΑ: Oxidative stress; Antioxidant activity; Liposome; Coenzyme Q_{10}; Quercetin.

SUMMARY

The aim of the study was to create the complex liposomal preparation containing Coenzyme Q_{10} and Quercetin and learn its antioxidant activity.

The liposomes were obtained the lipid film method followed by emulsification with PBS, extrusion and sterilizing filtration. The emulsion was lyophilized with lactose as a cryoprotectant. The ratio of Coenzyme Q_{10} and Quercetin in complex liposomal composition was 1 : 1. The particles size and the degree of inclusion were determined. Pharmacological study was carried out on Wistar line rats with experimental ischemic heart disease model. The samples of complex liposomal composition and monocompositions of Coenzyme Q_{10} and Quercetin were intravenously administered daily for 5 days at a dose of 10 mg/kg. Oxidative stress markers (levels of malondialdehyde, conjugated dienes, sulfhydryl groups, protein peroxidation, enzyme superoxide dismutase catalase and total antioxidant activity) were analyzed in blood serum and cardiac tissue of the rats.

The obtained complex composition was characterized by a particle size of up to 220 nm and at least 90.0 % of the included Coenzyme Q_{10} and Quercetin. A decrease in the level of lipid oxidation products and an increase in the activity of antioxidant system using the complex liposomal preparation containing Coenzyme Q_{10} and Quercetin compared to its monocompositions in liposomal form were shown.

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1 Introduction

In a number of diseases, including patients with ischemic heart disease (IHD), a decrease in the antioxidant activity (AA) and changes in the levels of the antioxidant defense system are observed, which is accompanied by an increase in the level of lipid oxidation products (LOPs)[1–3].

Currently, antioxidant drugs of various origin are used to reduce the level of LOPs. Of particular interest are exogenous products - Bioactive Flavonoid, for example, quercetin (Quer) or endogenous products, for example, coenzyme Q_{10} (CoQ_{10}).

According to AA, Quer is one of the known antioxidants that limit the processes of chain reactions of free radical oxidation, preventing excessive oxidation of lipids, proteins and nucleic acids, which protect cell membranes from damage by oxide agents. Quer has angioprotective, antioxidant, anti-inflammatory, wound healing and antiviral effects[4, 5].

An antioxidant, successfully used in cardiology, is CoQ_{10}, with respect to which extensive evidence has been accumulated and there is a long-term global experience of observing patients taking per os CoQ_{10}, confirming its safety and efficacy. CoQ_{10} is an endogenously synthesized compound that plays a special role in oxidative phosphorylation and ATP synthesis. CoQ_{10} reacting with free radicals leads to a decrease in the level of superoxide and, thereby, inhibits the LOPs processes of biomembranes and lipoproteins of the circulating blood, as well as the oxidation of the body's DNA and proteins[6, 7]. The content of CoQ_{10} in cardiomyocytes is higher than in the tissues of other organs, which is explained by the greatest energy needs of the myocardium. The CoQ_{10} level in blood plasma, in general, reflects its content in the tissues. A special property of CoQ_{10} is the ability to be recovered under the action of the enzyme systems of the body, while other antioxidants are oxidized irreversibly[8, 9].

CoQ_{10} dosage forms are represented by per os drugs. However, the bioavailability of these drugs is extremely low and does not exceed 2–3 %, which is associated with low solubility, and, therefore, with low adsorption in the body. Currently, the intravenous form of CoQ_{10} is absent in clinical practice. Most of the pharmacokinetic studies of CoQ_{10} were carried out with its intramuscular or per os administration. To increase the bioavailability of CoQ_{10}, it is necessary to create a drug with increased solubility in water. In such a strategy, the use of liposomal (LS) form of CoQ_{10} can be effective[7–10]. Many companies around the world are conducting research aiming at obtaining therapeutic LS drugs, and the arsenal of doctors is constantly enriched with drugs based on them[11, 12].

Considering the high antioxidant activity of CoQ_{10} and Quer and their effect on different levels of the antioxidant system, the creation of an LS complex antioxidant drug (LS-CAD) based on them is very promising.

The purpose of the study was to create a LS-CAD containing CoQ_{10} and Quer and study its antioxidant activity.

2 Materials and Methods

2.1 Materials

The researchers used Quer ($C_{15}H1_0O_7$) manufactured by PVP Sociedate Anonima (Brazil) and CoQ_{10} ($C_{59}H_{90}O_4$) manufactured by Hangzhou Huandong Medicine Group Kangrum Pharmaceutical Co., LtD (China); phospholipids (PL):egg phosphatidylcholine - EPC (EPC-Lipoid) and dipalmitoylphosphatidylglycerol - DPHG (Lipoid-PG) manufactured by Lipoid (Germany); lactose - Sigma Aldrich; PBS - KH_2PO_4, Na_2HPO_4.

2.2 Pharmacological Studies

The experimental part of the studies was carried out on the basis of the Department of Biochemistry of Kharkiv National Medical University. The object of the study were 30 Wistar line rats weighing 150–180 g, which were kept in standard vivarium conditions. The following groups of animals were used: 1. Intact rats (n = 6); 2. Rats with experimental IHD (n = 6); 3. Rats with experimental IHD, which were administered with LS form of Quer (n = 6); 4. Rats with experimental IHD, which were administered with LS form of Q_{10} (n = 6); 5. Rats with experimen-

tal IHD, which were administered with LS-CAD (n = 6).

IHD model was carried out according to the following method[13]: 0.1 ml of 0.1 % solution of adrenaline and 1 ml of 2.5 % suspension of hydrocortisone acetate were subcutaneously administered daily for 7 days. After simulating the IHD, the test samples were intravenously administered daily for 5 days at a dose of 10 mg/kg.

Blood samples were obtained from tail vein. Cardiac tissue samples were obtained by the cardiac puncture in anesthetized rats using the following techniques: heart was washed in ice-cold 0.9% solution NaCI, weighed and homogenated with 3-times volume of the solution. The homogenate was filtered and precipitated at 10,000 g for 10 minutes. The supernatant was used for the study.

The animals were kept under standard conditions in accordance with regulations of the National Research Council (2010). All procedures were adhered to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and protocol was approved by the local animal ethics committee.

2.3 Determination of peroxidation and antioxidant protection products

Malondialdehyde (MDA) was determined by thiobarbituric acid reaction. The absorbance of coloured complex was measured spectrophotometrically at 532 nm[14]. Conjugated dienes (CD) were extracted with the heptane-isopropanol mixture and determined spectrophotometrically at 233 nm[14, 15]. Activity of superoxide dismutase (SOD) was determined spectrophotometrically at 450 nm. The method is based on inhibition of nitroblue tetrazolium reduction in the non-enzymatic system phenazine methosulfate and NADH[16]. Activity of catalase was determined spectrophotometrically. The method is based on the capability of hydrogen peroxide to form a resistant stained complex with ammonium molybdate with the maximum absorbance at 410 nm[17]. Determination of SH-groups and glutathione is based on the reaction of the sulfhydryl group with 5,5-dithiobis-(2-nitrobenzoic acid), which is resulting in a yellow-coloured thionitrophenyl anion in equimolar amounts with the maximum absorbance at 412 nm[14]. Determination of ATP is based on the capability of to phosphorylate glucose in the presence of hexoinase. The amount of ATP is equimolar to NADPH formed in the glucose-6-phosphate dehydrogenase reaction[18]. Determination of protein peroxidation (PP) is based on the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine to form a protein-bound 2,4-dinitrophenylhydrazone, which can be detected spectrophotometrically at 370 nm[19]. Total antioxidant activity (TAA) in blood serum was determined spectrophotometrically as degree of inhibition of LOPs (thiobarbituric acid reactive substances) production in yolk lipoproteins. Lipid peroxidation in yolk lipoproteins suspension was induced by the addition of $FeSO_4$[20].

2.4 Obtaining of Liposomes

LS form of Quer and CoQ_{10} [21, 22] were obtained on a previously developed technological platform. LS-CAD were obtained by the method of "lipid film" followed by the emulsification with PBS (pH 6.5–6.8). The resulting emulsions of multilamellar vesicles were extruded on a Microfluidizer extruder (Microfluidics, USA) to obtain multilamellar LS with a size of 150–220 nm. The resulting emulsions were subjected to sterilizing filtration through a cascade of PALL Suppor (USA) filters. The ratio of antioxidants in LS form was 1 : 1. The emulsion was placed in 20 ml vials and lyophilized (Martin Christ 2-6-D) in the presence of a lactose cryoprotectant.

2.5 Analytical Studies

The determination of the LS particle size was carried out on a Malvern Zetasizer Nano ZS nanosizer (UK). The size of particles was measured using a semiconductor laser at a wavelength of 375 nm and a temperature of 30 °C.

The assay of impurities and the content of Quer and CoQ_{10} was performed by HPLC on a Zhimadzu LC 20 chromatograph according to the method[23, 24].

Table 1. Results of the study of the AA using the IHD model: rat blood serum, 10 mg/kg daily, 5 injections

Indices	MDA, μmol/l	CD, μmol/l	SOD, RU/sec/l	Catalase, μmol/l	SH Groups mmol/l	TAA, %	PP, U/mg protein
Control	1.996 ± 0.09	48.92 ± 2.1	32.92 ± 1.38	21.14 ± 1.00	12.2 ± 0.43	52.58 ± 2.1	0.116 ± 0.005
IHD	5.46 ± 0.22	90.33 ± 3.8	40.10 ± 1.73	29.53 ± 1.35	6.05 ± 0.24	35.73 ± 1.58	0.31 ± 0.011
IHD + Quer	3.44 ± 0.15	65.04 ± 2.75	51.19 ± 2.02	39.57 ± 1.5	7.83 ± 0.21	55.63 ± 2.38	0.113 ± 0.004
IHD + Q10	2.99 ± 0.14	60.32 ± 3.0	56.44 ± 2.6	47.19 ± 2.02	9.68 ± 0.42	58.62 ± 2.67	0.162 ± 0.007
IHD + Quer + Q10	1.77 ± 0.056	42.14 ± 1.85	60.04 ± 2.56	43.65 ± 2.105	8.75 ± 0.33	76.22 ± 2.94	0.0766 ± 0.0035

The impurities in Quer (kaempferol, isorhamnetin) amounted to not more than 2.0 % and the impurities in CoQ_{10} amounted to not more than 1.0 %, which corresponds to the data specified by the manufacturer.

The determination of the statistical reliability of the experiment results and the suitability of chromatographic systems were carried out in accordance with the State Pharmacopoeia of Ukraine.

3 Results and Discussion

Studies using HPLC demonstrated the stability of the components in the process of obtaining LS. The amount of Quer impurities in the LS-CAD did not change from the original Quer (up to 2.0 %). The amount of CoQ_{10} impurities in the LS-CAD is increased to 1.2 % (the original content of CoQ_{10} was 0.93 %). The lyophilized LS-CAD is of yellow-green color with the content of water not exceeding 5.0 %. The obtained LS-CAD samples contain at least 90.0 % of the included Quer and CoQ_{10} with a particle size of up to 220 nm. The animals were administered with the samples of 10.0 mg/kg.

Tables 1 and **2** present the data obtained using the IHD model in rats. The obtained data confirm changes in the AA system in this pathology, consistent with the literature data[25, 26].When studying the mechanisms of development of IHD and its complications, the main factors leading to IHD pathogenetic mechanisms were established, and clinical criteria, methods of diagnosis and treatment were developed. Moreover, great importance is attached to the study of oxidative stress in IHD. The AA system was established to be responsible for protection against the effects of oxidative stress. Disorders in the antioxidant system play a crucial role in the development of IHD. The main levels of the AA include: glutathione, ascorbate, CoQ_{10}, SOD, catalase and other compounds. For example, glutathione is involved in the detoxification of xenobiotics and metabolic products, exert influence on the activity of enzymes, regulates the exchange of eicosanoids and prostaglandins, has an impact on the synthesis of nucleic acids and performs a number of other functions in the body[27]. Determination of the AA system (glutathione, SOD and catalase) in IHD has significant prognostic value and may contribute to the study of the mechanisms of disease development, allowing to justify the use of drug correction, in particular, by antioxidant drugs[28–30].The results of the study of the AA of LS-CAD samples using the IHD model in the serum and cardiac muscle of animals are shown in **Tables 1** and **2**.

Table 2 Results of the study of the AA using the IHD model: rat cardiac tissue, 10 mg/kg daily, 5 injections

Indices	MDA mmol/g protein	CD mmol/g protein	SOD RU/g protein	Catalase μmol/g protein	Glutathione mmol/g protein	ATP mmol/g protein
Control	1.806 ± 0.07	14.87 ± 0.68	100.08 ± 4.65	25.25 ± 1.05	89.46 ± 3.75	3.10 ± 0.123
IHD	3.967 ± 0.15	27.79 ± 1.26	117.13 ± 5.13	30.89 ± 1.46	61.09 ± 2.85	1.52 ± 0.06
IHD + Quer	2.35 ± 0.11	19.58 ± 0.87	138.31 ± 5.65	44.18 ± 2.03	65.12 ± 2.45	1.81 ± 0.08
IHD + Q10	1.83 ± 0.065	18.17 ± 0.89	140.3 ± 5.8	49.58 ± 2.23	81.04 ± 4.0	2.134 ± 0.09
IHD + Quer + CoQ10	1.00 ± 0.047	9.9 ± 0.42	152.88 ± 7.1	53.92 ± 2.45	74.04 ± 3.68	2.074 ± 0.09

When studying the AA of the monopreparations of Quer and CoQ_{10} LS forms in blood serum of rats with IHD model (**Table 1**), a decrease in the content of MDA (by 37.0 %) and CD (by 28.0 %) in blood serum with the introduction of Quer and a decrease in the content of MDA (by 46.0 %) and CD by (33.2 %) with the introduction of CoQ_{10} were shown as compared with animals with IHD (P<0.005). At the same time, the use of LS-CAD allowed to reduce the amount of MDA by 69.0 % and CD by 53.25 % almost to the level of intact control (P<0.001). The use of monopreparations allowed to increase the AA close to the control level of 55.63 % and 58.62 % (with IHD - 35.73 %). When using LS-CAD, the value of the AA was 76.22 % (with the initial AA being at the level of 52.58 %) (P<0.005). In addition, a higher AA for LS-CAD was established when determining PP. When determining the content of SH groups in blood serum, the AA of the studied drugs was established. However, using the LS form, it was not possible to achieve intact control values. Apparently, the AA of lipids depends both on the amount of antioxidants used (Quer and CoQ_{10}) and on their interaction. In addition, it is necessary to take into account the possibility of influencing the AA by substances that themselves do not have an anti- or prooxidative effect, but are capable of enhancing or inhibiting the action of antioxidants under the experimental conditions. At the same time, a higher antioxidant activity of CoQ_{10} was established as compared with Quer.

When studying the AA of the monopreparations of Quer and CoQ_{10} LS forms in cardiac tissue of rats with IHD model (**Table 2**), a decrease in the content of MDA by 40.80 % and CD by 29.5 % in cardiac tissue with the introduction of Quer and a decrease in the content of MDA by 54.0 % and CD by 34.6 % with the introduction of CoQ_{10} were shown as compared with animals with the IHD model. At the same time, the use of LS-CAD allowed to reduce the amount of MDA by 75.0 % and CD by 64.4 % (P<0.005) almost to the level of intact control.

The data obtained (**Tables 1** and **2**) demonstrate an increase in SOD and catalase levels both in blood serum and in cardiac muscle on the IHD model. These data are consistent with the results available in the literature. For example, Quer and dihydroquercetin drugs, which reduce the content of LOPs or inhibit their accumulation during the hypoxic action, increase the activity of antioxidant enzymes by eliminating the peroxide metabolism products that inhibit them. It is also possible that flavonoids can cause a direct effect on enzymes, interacting with amino acid radicals of the polypeptide chain and, thereby, changing the conformation of the protein molecule[28, 29, 32], which contributes to a change in the properties of the enzyme. This may be due to an increase in SOD and catalase activity above the control level[28]. It was shown that 1–2 days after myocardial infarction in

humans, the SOD content increases by 2.1 times as compared with the control (healthy people), which may be connected with its compensatory allosteric activation in the conditions of hyperproduction of superoxide anion radical that activates SOD and simultaneously inhibits catalase. In 10–14 days, the SOD activity decreases, but, nevertheless, its content was 1.4 times higher than that of control[32]. It was established that powerful antioxidants not only have a high function of "trapping" free radicals, but also increase the enzymatic activity of SOD. The use of CoQ_{10} caused a significant increase in endothelium-dependent relaxation of the brachial artery due to, as suggested by the authors, an increase in SOD activity in humans with IHD[31].

The obtained data (**Tables 1** and **2**) demonstrate the improved efficiency of LS-CAD that caused by effect of Quer and CoQ_{10} on the different levels of the antioxidant system. Quer reacts with hydrogen peroxides in the presence of peroxidases, reduces the concentration of hydrogen peroxide and prevents cell damage. According to the data the concentration of glutathione in cardiac tissue is restored and leads to the antioxidant effect of Quer. Even when low concentrations of Quer are used, the glutathione antioxidant system is activated and oxidative stress is reduced. The glutathione system is an important component of antioxidant activity, that neutralizes lipid peroxides and maintains SH-groups of proteins in functional reduced form. CoQ_{10} is a component of the mitochondrial respiratory chain and it is involved in oxidative phosphorylation process. CoQ_{10} is an endogenous antioxidant and antihypoxant with an effective antiradical effect and protects the lipids of biological membranes from LOPs, protects the DNA and body proteins from oxidative modification. The protective function of CoQ_{10} in IHD is determined by its participation in the energy metabolism of cardiomyocytes and antioxidant properties. The using of liposomal CoQ_{10} leads to decrease MDA concentration to the level of intact control, but using of complex drug significantly reduces the level of MDA.

4 Conclusions

Thus, the obtained data demonstrate the effectiveness of the antioxidant activity of LS-CAD containing Quer and CoQ_{10} in LS form when administered intravenously. A decrease in LOPs and an increase in the activity of the AA system using the LS-CAD in rats with experimental IHD model in compared as with monopreparations in LS form was shown. When evaluating the study of markers of oxidative stress, it is necessary to consider the possible influence of free radical reactions on membrane lipids, proteins, DNA and carbohydrates. □

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