A Complex of Antioxidant Vitamins Effectively Inhibits Free-Radical Oxidation of LDL Phospholipids in Blood Plasma and Membrane Structures of the Liver and Myocardium

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Antioxidant effect of a complex preparation including antioxidant vitamins C, E, provitamin A and selenium was studied on the model of Cu^{2+}-initiated free-radical oxidation of LDL isolated from human blood plasma. The antioxidant effect of combined administration of α-tocopherol+ascorbic acid and α-tocopherol+β-carotene is far more pronounced that the antioxidant effect of individual components of these cocktails. Moreover, in the model system the combined action of all antioxidant components completely inhibited free-radical oxidation of LDL. A 30-day course of peroral administration of antioxidant vitamin cocktail and selenium to rats pronouncedly enhanced the antioxidant potential of liver and completely suppressed free-radical processes in the myocardium. It is suggested that preparations containing antioxidant vitamins and selenium can be perspective for prevention and complex therapy of atherosclerosis.

Key Words: *free-radical lipid oxidation; antioxidant vitamins; low-density lipoproteins; antioxidant tissue potential*

Oxidative modification of plasma LDL enhances their atherogenic potency and promotes their accumulation in monocytes-macrophages in the vascular wall, which leads to vascular lipidosis at the early stages of atherogenesis [1,2,4,12,13,15]. Therefore, the drugs preventing free-radical oxidation of LDL can be used for prophylaxis and complex therapy of atherosclerosis. Regular administration of the drugs containing antioxidant (AO) vitamins, such as vitamin C (ascorbic acid), vitamin E (α-tocopherol), and provitamin A (β-carotene) in order to improve AO status of the organism and to reduce the risk of the development of atherosclerosis seems to be quite reasonable, especially in Russia, where catastrophic deficiency in natural AO was demonstrated in many epidemiologic studies [7].

Although the complexes with AO-vitamins were repeatedly tested in various clinical trials during the last years, the results of these studies are controversial [9,10,12]. This can be explained by significant differences in the composition and concentrations of AO-components in the test preparations. Possible inversion of AO effect should be taken into consideration. We demonstrated this inversion in our *in vivo* experiments on rats [5]: β-carotene in low concentrations produced a pronounced AO effect in the liver and myocardium, while high doses of provitamin A produced a prooxidant effect in the same organs [5]. Thus, optimal concentrations and proportions of the AO-components complex AO-preparations should be chosen. Here we studied antioxidant efficiency of components of complex preparations containing AO-vitamins C and E, provitamin A, and selenium (Triovit and KRKA).

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MATERIALS AND METHODS

Donor venous blood obtained after overnight fast was stabilized with EDTA (1 mg/ml). The plasma was twice centrifuged in a NaBr density gradient at 42,000 rpm for 2 h (4°C, 50Ti angle rotor, Beckman L-8 centrifuge). LDL were collected and dialyzed at 4°C for 16 h [14]. Short period of centrifugation prevents oxidation of native LDL during isolation occurring during routine LDL isolation. Electrophoresis showed that LDL fraction was not contaminated by plasma proteins or other lipoproteins and was identical in particle size and composition to the LDL fraction isolated by the classical method of Lindgren [11]. Protein content was measured by the method of Lowry, and LDL concentration was adjusted to 50 μg protein/ml with 50 mM phosphate buffer (pH 7.4) containing 154 mM NaCl. LDL oxidation was induced with 30 μM $CuSO_4$, and accumulation of lipohydroperoxides was measured on a Hitachi 220A spectrophotometer at 233 nm in dynamics [8].

Ascorbic acid (100 μM, Serva), α-tocopherol (40 μM, Serva), and β-carotene (10 μM, Sigma) were added to the incubation medium both individually and in various combinations. The concentrations of AO vitamins were chosen according to their proportions in a complex preparation Triovit taking into account the optimal concentration of β-carotene in the incubation medium determined previously [6]. Ascorbic acid was diluted with water (pH of initial solution was adjusted to 7.0), and the liposoluble components (α-tocopherol and β-carotene) were dissolved in isopropanol (final concentration of isopropanol 1.5%). The control sample (free from AO-vitamins) and the sample with ascorbic acid contained the same amount of isopropanol. The experiments were repeated three times under standard conditions.

A special series of experiments was carried out on male Wistar rats (initial body weight 260±10 g) subdivided into 2 groups. The experimental rats (n=12) received (*per os*) Triovit suspension via an esophageal tube in a daily dose of 1 capsule/kg body weight for 30 days. One Triovit capsule contains 10 mg β-carotene, 40 mg α-tocopheryl acetate, 100 mg ascorbic acid, and 50 mg selenium as an yeast extract. The control rats (n=8) received 0.5 ml water through an esophageal tube.

At the end of the experiment the rats were narcotized and decapitated. The liver was perfused, and the heart was carefully rinsed with cold isotonic KCl saline. The liver and myocardium were homogenized with cooling in an Ultra-Turrax SDT-1810 microgrinder (Tekmar) (15 mg wet tissue/ml solution containing 154 mM NaCl and 50 mM K,Na-phosphate buffer, pH 6.0). The homogenates were incubated at constant shaking under aerobic conditions with 0.5 mM ascorbate and without Fe^{2+} [3,5]. Aliquots were taken every 1-5 min and the concentrations of LPO products reacting with thiobarbituric acid (TBA) were measured spectrophotometrically at 532 nm (Hitachi-557). The initial absorption of TBA-reactive substances (before incubation) was subtracted from the optical density of subsequent samples (ΔD_{532}), kinetic curves were plotted, and the duration of the lag phase of induction was calculated [3,5]. This duration was used for evaluation of the effect of the test preparation on the intensity of free-radical processes and AO tissue potential in membrane structures of native tissues.

RESULTS

A typical experiment (Fig. 1) showed that addition of ascorbic acid, α-tocopherol, or β-carotene alone to the

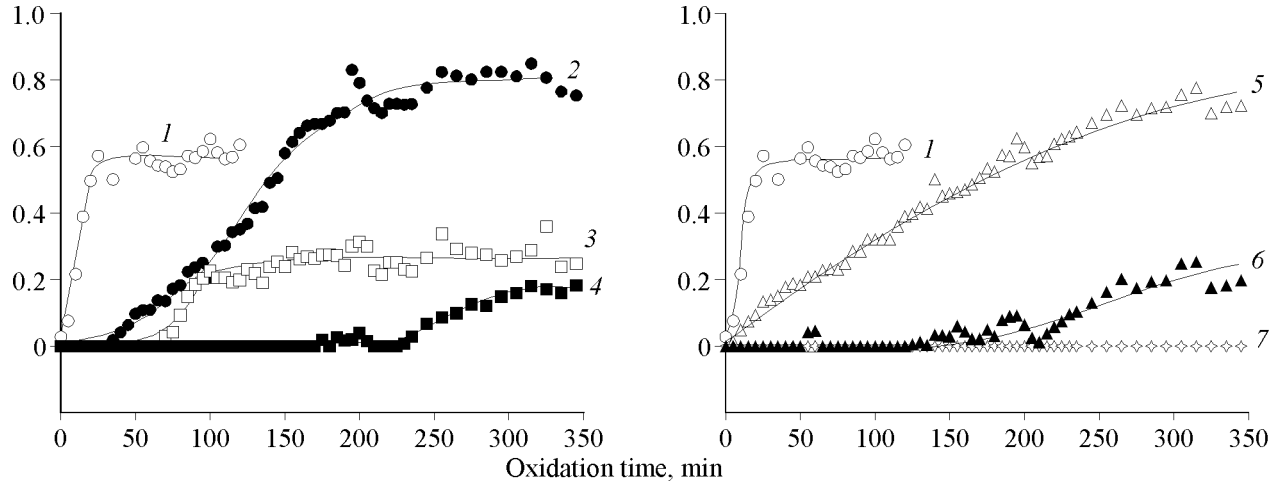

Fig. 1. Effect of antioxidant vitamins, components of Triovit preparation, on kinetics of Cu^{2+}-induced free-radical oxidation of human blood plasma LDL. The control curve shows LDL oxidation without vitamins. Ordinates: lipid peroxide accumulation, ΔD_{233}. *1*) control; *2*) vitamin C; *3*) vitamin E; *4*) vitamins C and E; *5*) β-carotene; *6*) β-carotene and vitamin E; *7*) β-carotene, vitamin C, and vitamin E.

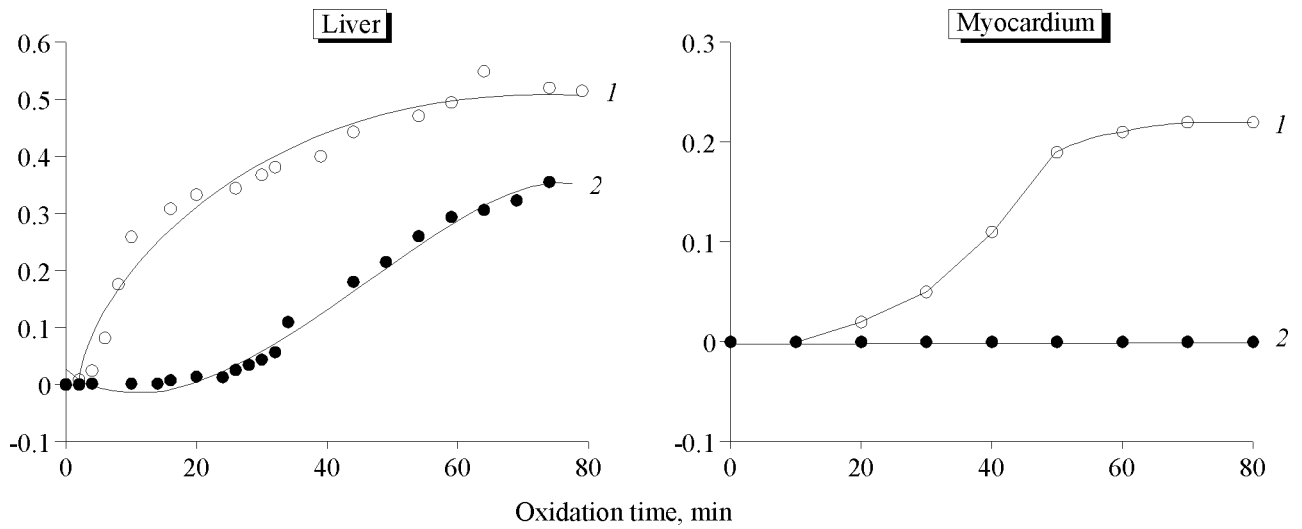

Fig. 2. Ascorbate-dependent free-radical oxidation of unsaturated membrane lipids in liver and myocardium homogenates of control Wistar rats (*1*) and the rats treated *per os* with Triovit for 30 days (*2*). Ordinates: MDA accumulation, ΔD_{535}.

incubation medium in the above concentrations pronouncedly suppressed LDL oxidation. Combinations of α-tocopherol with ascorbic acid or with β-carotene inhibited oxidation of LDL far more efficiently compared to individual components. When all examined AO-vitamins were applied simultaneously (ascorbic acid+α-tocopherol+β-carotene), LDL oxidation was completely suppressed. Therefore, AO-vitamins used in proportions corresponding to Triovit preparation efficiently suppressed free-radical oxidation of unsaturated phospholipids in LDL *in vitro*. The maximum effect was observed when total complex of Triovit vitamins was administered. Ascorbic acid (HO—Asc—OH) participates in regeneration of tocopheroxyl radical (α-TO$^\bullet$) formed during interaction of α-tocopherol (α-TOH) with unsaturated phospholipid radicals in LDL ($LO_2^\bullet$ and LO$^\bullet$) according to the following reactions [4]:

$$LO_2^\bullet + \alpha\text{-TOH} \rightarrow LOOH + \alpha\text{-TO}^\bullet,$$
$$\text{or } LO^\bullet + \alpha\text{-TOH} \rightarrow LOH + \alpha\text{-TO}^\bullet;$$
$$\alpha\text{-TO}^\bullet + \text{HO-Asc-OH} \rightarrow \alpha\text{-TOH} + \text{HO-Asc-O}^\bullet,$$
$$\alpha\text{-TO}^\bullet + \text{HO-Asc-O} \rightarrow \alpha\text{-TOH} + \text{O=Asc=O}.$$

In a similar way, α-tocopherol inhibits free-radical oxidation of β-carotene, a polyunsaturated lipid. The mechanism of antiradical effect of β-carotene is based on the formation of low-active radicals in isoprenoid chain during interaction with active lipid radicals and/or regeneration of α-TO$^\bullet$ radicals [4]. We found previously that β-carotene can exhibit both AO- and pro-oxidant effects *in vivo* depending on its intracellular concentration [6]. Similar concentration-dependent inversion of *in vivo* AO-effect is characteristic of other natural AO agents (α-tocopherol and ascorbic acid) and even of synthetic AO preparations [6]. Evidently, only strictly determined proportions of AO-vitamins in a complex preparation provides the maximum AO effect during free-radical oxidation of LDL. The data presented in Fig. 1 suggest that the proportions of AO components in Triovit preparation are optimal.

The results of *in vivo* study of AO efficiency of Triovit preparation are shown in Fig. 2. Thirty days after daily administration of Triovit to experimental rats, the free-radical processes were drastically inhibited in native membrane structures in the liver and almost completely suppressed in myocardial membranes. These data attest to efficiency of examined preparation and its pronounced trophicity to the myocardium. Therefore, our study proved the protective action of Triovit preparation both in model system and in the whole organism, which substantiates the clinical research for the use of Triovit.

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