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Pharmacological Activity of Echinochrome A Alone and in the Biologically Active Additive Timarin

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Abstract—Pharmacological activity of echinochrome A (EchA) alone and in the biologically active additives (BAA) Timarin, administered per os has been investigated in volunteers. Blood hematological, immunological, and biochemical parameters were investigated before and after administration of the substances used. EchA decreased serum glutatione (GSH) and increased catalase activity 1 h after treatment. Later (3 h after administration) catalase activity normalized, while GSH exceeded the initial level. Changes in blood lipids suggest decreased risk of atherogenesis. Changes found in blood sex hormone levels indicate that Timarin may influence sex gland functioning. Changes of hematological and immunological parameters have been interpreted as the result of a mild stressor effect of both EchA alone and in the BAA Timarin increasing adaptation reactivity of the body.

Keywords: echinochrome A, 1,4-naphtoquinone, pharmacological activity, mechanism of action

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INTRODUCTION

Natural naphtoquinones are promising sources for various pharmacologically relevant preparations with various activities [1–4]. Various pigments known as spinochromes, which are structurally related to 1,4-naphtoquinones have been isolated from sea urchins [5]. Among spinochromes the most interesting range of pharmacological activities has been found in 7-ethyl-2,3,5,6,8-pentahydroxy-1,4-naphtoquinone earlier named as echinochrome A (EchA). Marked therapeutic activity of EchA in treatment of ophthalmological and cardiovascular diseases attributed to its high antioxidant activity resulted in introduction of this substance into clinical practice under the trade name Histochrome® [6–8].

Results of our recent studies [4] suggest that the wide range of pharmacological activity of EchA and other 1,4-naphtoquinones cannot be attributed only to the direct antioxidant effect. EchA and other 1,4-naphtoquinones can accept 1 or 2 electrones. All mammalian cell plasma membranes contain the constitutive enzyme DT-diaphorase (NQ01, NAD(P)H : quinone oxidoreductase, EC 1.6.5.2) directly involved in transmembrane translocation of electrons for neutralization of protons, which are constantly removed from cells (to prevent acidosis) by the proton pump H^+-ATPase. DT-diaphorase is equally effective in the use of both 1,4-quinones and 1,4-naphtoquinones as substrates [9–11]. This enzymatic reaction not only results in NAD(P)H-dependent two-electron reduction of 1,4-naphthoquinones into unstable 1,4-hydronaphthoquinones but also spontaneous oxidation of hydronaphthoquinones by oxygen within the call plasma membrane. This redox reaction yields initial substrate 1,4-naphtoquinone and a new product, hydrogen peroxide (H_2O_2). In a simplified form this enzymatic reaction may be written as follows:

$$NAD(P)H + H^+ + O_2 \xrightarrow[\text{diaphorase}]{\text{DT–}} NAD(P)^+ + H_2O_2$$

At low concentrations H_2O_2 acts as a biochemical messenger triggering cascades of physiological changes in cells [12–14]. First of all H_2O_2 activates biochemical processes regulated by latent transcription factors known as peroxisome proliferator-activated receptors (PPARs). Members of the family of these receptors together with their ligands and co-activators stimulate expression of genes encoding peroxisomal proteins and enzymes. Cell peroxisomes contain basically all enzymes of the antioxidant defense (catalase, superoxide dismutase, glutathione, thioredoxin, peroxiredoxin, etc.), enzymes involved in plasmalogen synthesis, fatty acid metabolism and many

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others. The PPARs system plays a key role in regulation of metabolism and suppression of inflammatory processes in cells. Drugs acting at PPARs can influence blood lipid profile, prevent development of type 2 diabetes mellitus, stabilize immune system, suppress inflammatory reactions of various etiology, reduce the effect of inducible NO synthase, and prevent the development of cardiovascular diseases [15].

In addition, decreased oxygen level (oxygen consumption for 1,4-hydronaphthoquinone oxidation) causes local hypoxia and a sharp activation of hypoxia inducible factor-1 (HIF-1), the principal controller and regulator of cell functions under these conditions. HIF-1 promotes significant increase in expression of genes encoding various proteins/enzymes involved in glucose metabolism and mitochondrial functioning, which increases intensity of cell functioning [16].

Despite of significant progress achieved in understanding cellular mechanisms of EchA action, subsequent analysis of its pharmacological activity after oral administration to humans is clearly needed to extent EchA application for therapeutic and prophylactic use in various pathologies.

In this clinical study we have investigated the effect the EchA containing biologically active additive (BAA) Timarin on hematological, immunological, and biochemical parameters of volunteers.

MATERIALS AND METHODS

The biologically active additive to food (BAA) Timarin is an alcohol extract from sea urchins; it contains natural antioxidants (echinochrome, ascorbic acid), a wide range of natural mineral compounds and organic substances. Timarin was patented in Russia (RU patent no 2340216) [17] and approved by the Russian Federal Service for Supervision of Consumer Rights Protection and Human Welfare no. 77.99.03.935.B.000138.06.04 (14.06.2004).

A polyhydroxynaphthoquinone echinochrome A (EchA) was isolated from the flat sea urchin *Scaphechinus mirabilis* by researchers of the Laboratory of Biotechnology of the Pacific Institute of Bioorganic Chemistry (Far Eastern Branch, Russian Academy of Sciences) as described in [5].

The study was performed on 30 healthy volunteers (16 females and 14 males, aged 54 ± 8 and 52 ± 10 years, respectively). Timarin (daily dose expressed as 1 mg EchA) was administered per os in 100 mL of distilled water once a day for 20 days.

In a separate experiment volunteers received a single dose administration of 50 mg EchA in 100 mL distilled water and rapid changes in reduced glutathione (GSH) and glucose (Glc) were investigated.

During pharmacological studies blood was taken into vacutainers with EDTA, while biochemical studies were carried out using serum. Initial levels of hematological, immunological, and biochemical parameters were evaluated in blood samples taken one day before the beginning of Timarin administration.

Hematological studies were performed using a Cell Dyn 3700 hematological analyzer (Abbot, USA). The study of the blood lipid profile included determination of total cholesterol content, triglycerides, cholesterol of HDL, LDL, and VLDL, calculation of the atherogenicity coefficient. Total cholesterol and cholesterol of LDL and HDL in serum were analyzed using a Cobas INTEGRA 400 PLUS biochemical analyzer (ROCHE, Switzerland); the atherogenicity coefficient (AC) was calculated by the following formula: AC (arbitrary units) = (Total cholesterol – HDL cholesterol)/HDL cholesterol. Blood glucose concentrations were evaluated using an One Touch Ultra glucometer (Germany). Triglycerides, and also aspartate amino transferase (AST) and alanine aminotransferase (ALT) activity were determined in serum using a diagnostic kit Novogluc-K.M. (Vector-Best, Russia). The antioxidant defense state was evaluated by the level of lipid peroxidation (LPO) products determined as described in [18].

Immunological parameters were evaluated in the whole blood (with EDTA) using a flow cytofluorimeter BD FaksCalibur and BD diagnostic test-systems (USA). Immunophenotyping of lymphocytes was performed using the following CD markers: CD3, 4, 8, 16, 19, 25, 95 and HLA-DR.

Steroid sex hormones (estradiol, testosterone) were determined by the method of competitive ELISA using a plate photometer (EL808iu (BioTek, USA) following the protocol to the Nova Tek kit (Germany).

Levels of reduced and oxidized glutathione were determined spectrofluorimetrically as described [19]; catalase activity was evaluated by a decrease of H_2O_2 [20].

Statistical differences were evaluated using Student-Fisher test by means of the SPP 11/0 program. Differences were considered as statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Studying effects of EchA on blood biochemical parameters we have found that 1 h after a single dose administration of this substance there was a decrease in blood glutatione (GSH) and an increase in the activity of such important antioxidant enzyme as catalase. Later (3 h after EchA administration) catalase activity normalized, while GSH exceeded the initial level (Fig. 1). These results may suggest that appearance of H_2O_2 in blood and tissues of volunteers due to EchA reaction with cell DT diaphorase influences the state of their antioxidant system. Since tissue H_2O_2 content is strictly controlled by the enzymes of the

antioxidant defense catalase and glutathione-dependent peroxidase (GPx) its utilization is associated with consumption of reduced glutathione (GSH)

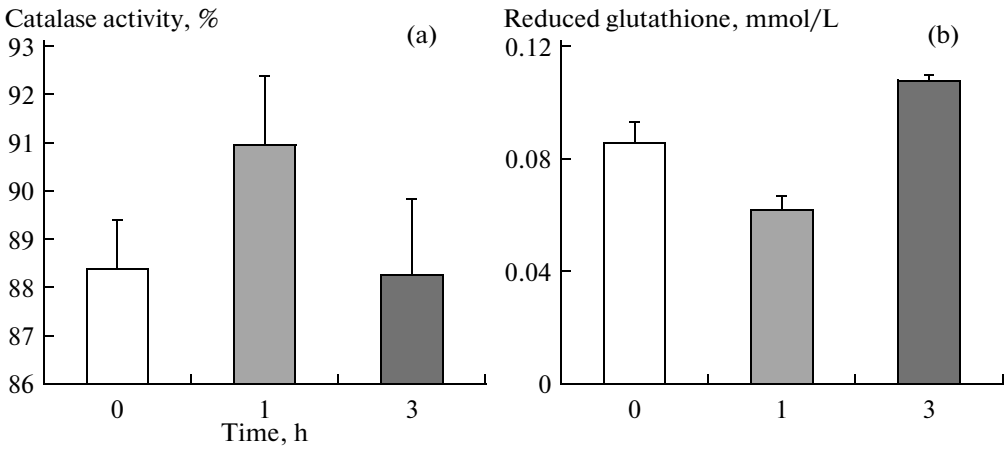

Fig. 1. Catalase activity (a) and reduced glutathione (b) in blood of volunteers before (0), 1, and 3 h after EchA administration. Data represent mean ± SEM of three independent experiments. Abscissa: time, h. Ordinate: (a) catalase activity, %; (b) reduced glutathione content, mmol/L.

in cells (GSH is the main cytosolic antioxidant of cells). Our data on blood GSH content and its rapid recovery from oxidized glutathione (GSSG) suggest that EchA may potentially influence carbohydrate metabolism in the body (as GSSG reduction by glutathione reductase requires NAD(P)H supply, which is generated from the hexose monophosphate pathway activated by glucose transported to the cell).

In a series of experiments we have investigated content of glucose, insulin and C-peptide formed during activation of pro-insulin in vesicles of pancreatic β-cells of healthy volunteers before and after Timarin administration. In vesicles of these cells mature insulin does exist as a zinc-containing hexamer up to its secretion. We have investigated the effect of Timarin on blood insulin, C-peptide, glycated hemoglobin (Hbal) and glucose. Healthy volunteers (in terms of type 1 or type 2 diabetes mellitus), which had normal blood glucose (3.1—6.4 mmol/L) received the daily dose of EchA of 1 mg (as Timarin) in the morning (30 min before meal) for 20 days. Table summarizes results of this experiment.

Table shows that the daily treatment with 1 mg EchA (as Timarin) for 20 days decreased blood insulin, C-peptide, glucose, and Hbal; this suggests marked stimulation of carbohydrate metabolism. Results on the effect of Timarin on glucose content and metabolism well correspond to experimental data on high protector activity of EchA in alloxan diabetes [1, 3, 4].

Taking into consideration the historic trend of the use of EchA-based preparations with emphasis on prophylaxis and treatment of cardiovascular diseases it was especially interesting to analyze the effect of Timarin on blood lipid parameters and predictors of the risk of atherogenesis. Figure 2 shows positive changes in most parameters characterizing this risk. Healthy volunteers treated with Timarin were characterized by decreased levels of blood triglycerides, total cholesterol, LDL cholesterol and VLDL cholesterol and also by decreased value of the calculated AC. These changes in blood lipid profiles were the same in males and females. However, in males the blood lipid profile is characterized by some features, which determine higher levels of AC than in females ($p < 0.05$). Thus, the group of male volunteers has a higher risk for atherosclerosis (and characteristic consequences typical for this pathological process). However, in males the course of Timarin therapy resulted in a more pronounced decrease of this parameter than in females, although the absolute values of AC in males remained somewhat higher than in females. The latter suggests prolonged (or repeated) treatment of males with Timarin in order to obtain more pronounced results.

The effect of Timarin administration for 20 days on the levels of insulin, C-peptide, glycated hemoglobin (Hbal) and glucose in blood of volunteers

Parameters	Insulin (μU/mL)		C-peptide (ng/mL)		Hbal (%)		Glucose (mmol/L)	
Physiological norm	3.00–17.00		1.10–4.40		4.00–6.00		3.10–6.40	
Mean value	Before	After	Before	After	Before	After	Before	After
	12.4	10.13	3.92	2.93	4.03	3.98	5.53	5.25
Mean decrease	18.8 ± 1.5		25.2 ± 2.1		1.24 ± 0.2		5.0 ± 0.4	

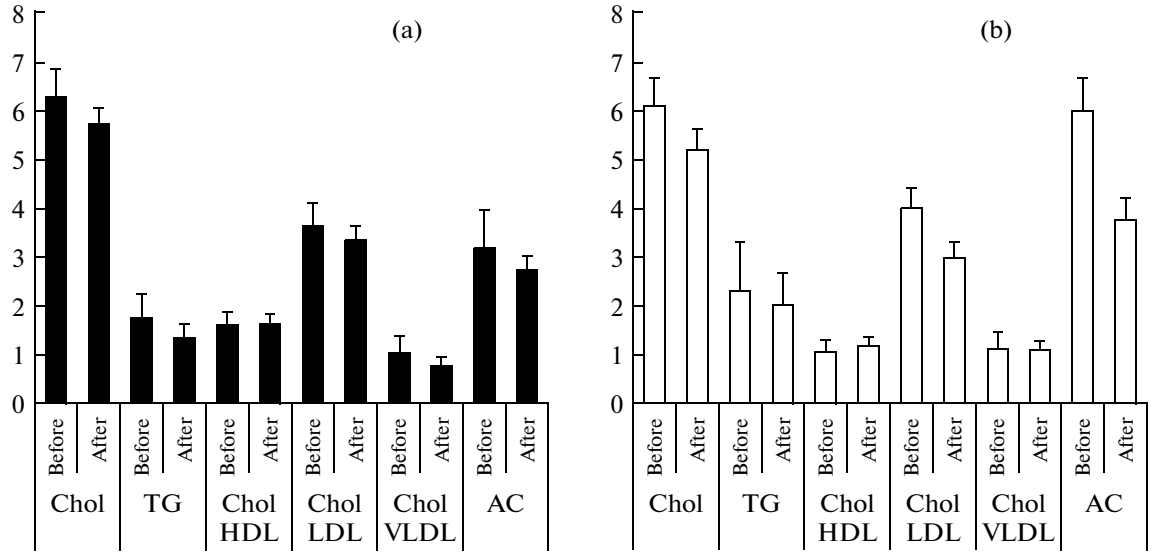

Fig. 2. Parameters of lipid metabolism of volunteers receiving Timarin 1 mg per os daily for 20 days. (a) females, (b) males.
Abscissa: Chol—cholesterol; TG—triglycerides, HDL Chol—cholesterol of high density lipoproteins; LDL chol—cholesterol of low density lipoproteins; VLDL chol—cholesterol of very low density lipoproteins; AC—atherogenicity coefficient. Parameters before and after course of Timarin administration.
Ordinate: Cholesterol (Chol) and triglycerides (TG) are expressed in mmol/L, AC is expressed in calculated units.

Under conditions of oral administration for 20 days Timarin insignificantly influenced hematological and immunological parameters. The detected trends suggest that it exhibits mild modulating effects on the blood and immune system; these effects may be generally characterized as activation of immune system. It is reasonable to suggest that these changes are determined by a mild stressor effect of Timarin followed by the development of adaptation processes in the blood and immune system.

It was interesting to analyze gender differences in the reaction to Timarin administration. After the course of Timarin administration we found similar trends to the decrease of estradiol in female and male volunteers. This decrease was more pronounced in female volunteers, where an almost twofold decrease was observed (from 20.1 ± 1.1 to 11,7 ± 0.6 pmol/L before and after the course of Timarin administration, respectively; $p < 0.05$). In male volunteers the change of the estradiol level induced by Timarin administration (32.7 ± 2.8 versus 24.8 ± 0.7 pmol/L; $p < 0.05$) was lower than in females. There was an insignificant trend to the decrease in blood testosterone in both females (from 0.32 ± 0.04 to 0.28 ± 0.04 ng/mL) and males (from 5.64 ± 0.7 to 5.29 ± 0.45 ng/mL), which did not reach the level of statistical significance ($p > 0.05$). It is known that the ratio of sex hormones is a more informative parameter than analysis of individual values. The estradiol : testosterone ratio decreased after treatment with Timarin from 62.53 to 41.42 in females and from 5.8 to 4.7 in males ($p < 0.05$). It should be noted that all these parameters are within the age norm and the trend to the increase of blood testosterone in males should be interpreted as a positive effect. In the context of analysis of mechanisms underlying Timarin action these data suggest that the course of Timarin administration enhances physiological capacities of the body: together with the hypothalamo-pituitary adrenal system, sex glands are one of highly sensitive organs reflecting general changes in the body in response to stressor effects.

For analysis of the effect of Timarin administration on the immune system we have investigated population and subpopulation of blood leukocytes by means of evaluation of membrane CD differentiation antigens, which help to evaluate the level of the immune system activation (Fig. 3). Analysis revealed that Timarin administration caused a tendency to the increase of the immunoregulatory index and the increase in natural killer cells (NK-cells) in both groups and opposite changes in the content of T- and B-lymphocytes. The group of female volunteers was characterized by a small (and statistically insignificant) negative dynamics, while the group of male volunteers was characterized by some increase in this parameter. These data suggest that the course of Timarin administration resulted in a mild modulating effect on the immune system. For detalization of this effect we have investigated relative content of blood cells expression the differentiation CD antigens, the markers of functional state of cells. These included interleukin 2 receptors (CD25), FAS/APO-1 receptors (CD95, apoptosis markers) and antigens of the major histocompatibility complex (MHC) HLA-DR.

Figure 3 shows statistically significant increase ($p < 0.05$) of lymphocytes expressing HLA-DR antigens

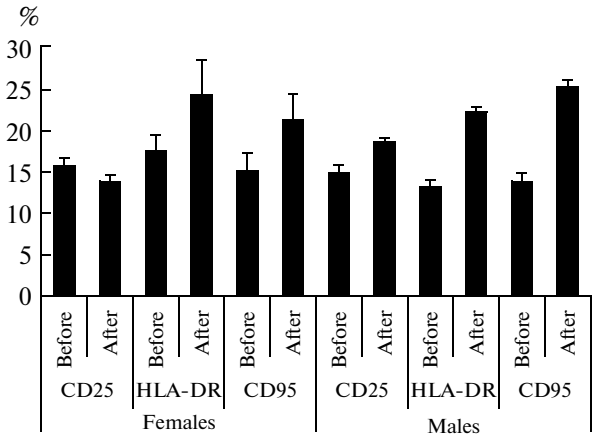

Fig. 3. The content of lymphocytes expressing CD markers of cell activation and apoptosis in volunteers treated with the daily dose of Timarin for 20 days.

Abscissa: CD25—lymphocytes expressing a low affinity α chain of IL-2 receptor; HLA-DR—lymhocytes expressing MHC HLA-DR antigens; CD95—lymphocytes expressing a membrane marker of apoptosis. Parameters are shown before and after the course of Timarin administration. Ordinate: percent of total lymphocyte content.

and apoptosis markers both in male and female blood samples. However, gender differences were found in the content of lymphocytes expressing CD25. In the group of female volunteers there was a statistically insignificant trend to the decrease in this parameter, while in the group of male volunteers there was a statistically significant increase ($p < 0.05$) in lymphocytes expressing IL-2 receptor. However, in all cases the parameters studied were within the physiological norm. Analyzing the observed changes it is relevant to suggest that treatment with Timarin enhances functional capacities of the immune system. The increase in HLA-DR expression reflects increased capacity of immune competent cells to intercellular cooperation and increases effectiveness of antigen processing and presentation, while increased levels of expression of the apoptosis marker suggests stimulation of processes of cell renewal in the body.

CONCLUSIONS

In general, results of the present study reflect the development of adaptation processes activated in the human body after mild stressor effect of hydrogen peroxide; its additional amounts are synthesized in response to the effect of EchA, the main acting component of Timarin. This results in enhanced functional activity of various systems of the body. The ability to perceive and to adapt to stress signals is the key factor determining survival and viability of various cell systems of the human body. Reactive oxygen species (ROS) may be involved in various signaling processes; for example, H_2O_2 mediates distant signals triggered by such stressor signals as skin damage, thermal injuries induced by both high and low temperatures. At the molecular level hydrogen peroxides mediates systemic signals with very important cellular pathways, controlled by various factors: nuclear factor kappa B (NF-κB), which is involved in stress response and inflammation; nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which is involved in cell protection against oxidative stress; peroxisome proliferator-activated receptor- γ?(PPAR-γ), a regulator of cell metabolism; heat shock factors (HSFs), regulators of synthesis of heat shock proteins [9]. This determines the multiple protective effect of EchA, the components of the BAA Timarin, on the human body.

Thus, based on results of the present study we have come to the following conclusions:

1. The course of oral administration of Timarin exhibits a mild modulating effect on various systems of the body and enhances their functional activities.

2. Timarin influences blood lipid parameters and decreases risk of atherosclerosis and diseases associated with this process.

3. Timarin demonstrates modulating (preferentially activating) effects on the blood system thus determining higher reactivity and optimization of adaptation processes.

4. Timarin exhibits the immunostimulating effect realized via the increase in the lymphocyte subpopulation possessing properties of natural killer cells and also via increased expression of membrane markers of immune competent cells (IL-2 receptors and HLA-DR antigens).

5. The course of oral administration of Timarin increases processes of cell renewal realized via apoptotic mechanisms.

6. Monotherapy with Timarin is characterized by some insignificant gender differences and (in some cases) opposite reaction to this BAA.

7. Taking into consideration a wide range of biological effect Timarin may be applicable in clinical practice for various purpose; however, particular cases of its application and doses require subsequent investigations.

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Translated by A.Medvedev

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