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N.P. MISHCHENKO, E.A. VASILEVA, S.A. FEDOREYEV,
O.A. LEBED'KO, B.Ya. RYZHAVSKII, M.S. KUZNETSOVA

Antioxidant composition of echinochrome, ascorbic acid and αtocopherol for treating inflammatory processes in lungs

A composition of echinochrome, ascorbic acid and α-tocopherol acetate (5:5:1), which exhibits a high antioxidant effect, can be used in the therapy of the inflammatory process in the lungs. This composition exhibits a pronounced synergistic anti-inflammatory effect, decreasing as compared with echinochrome and a complex of ascorbic acid– α-tocopherol (5:1), perivascular and peribronchial edema, lymphoid infiltration and alveolar expansion in rat lungs caused by the administration of lipopolysaccharide.

Key words: antioxidants, echinocrome, inflammatory process, lungs, rats.

INTRODUCTION

Diseases of the respiratory system are a serious medical and social problem, which is determined by their significance in the level of morbidity, disability and mortality. Currently there has been a steady increase in the number of patients with inflammatory lung diseases that are difficult to treat and have a continuously recurrent nature of the course. Therefore, the active search for pharmacological agents for inflammatory processes in the lung continues.

In patients with chronic inflammatory diseases of the lungs in the stage of remission, pronounced changes in the biogenesis of reactive oxygen species (ROS) occur at different levels of systemic organization. The disturbance of the oxidative metabolism of granulocytes, the development of oxidative stress at the membrane-cellular, organ, organism levels have been revealed [2, 5]. It is known that ROS activate redox-sensitive factors of transcription and stress kinase, regulate cellular and humoral immunogenesis, are triggers of inflammatory processes [7]. Therefore, the violation of redox regulation, of course, plays an important role in the course of the bronchopulmonary inflammatory process. The above indicates the need to include in the anti-inflammatory pharmacotherapy of these patients antioxidant agents that affect the production / detoxification of the ROS and restore the redox balance.

It was shown that intramuscular injection of the preparation "Histochrome 0.02%" (isotonic solution of di- and trisodium salt of echinochrome 0.2 mg/ml) in the therapy of children with chronic inflammatory lung disease increases antioxidant protection of the organism, corrects

* MISHCHENKO Natalia Petrovna – PhD, Leading Researcher; VASILEVA Elena Andreevna – Junior Researcher; FEDOREYEV Sergei Alexandrovich – DSc, Head of Laboratory (G.B. Elyakov Pacific Institute of Bioorganic Chemistry, FEB RAS, Vladivostok, Russia); LEBED'KO Olga Antonovna – DSc, Director of the Khabarovsk branch of federal state budgetary institution «Far Eastern Scientific Centre of Physiology and Pathology of Respiration» Research Institute of Maternity and Childhood, Khabarovsk); RYZHAVSKII Boris Yakovlevich– DSc, Professor, Head of Department of Biology and Histology of the Far Eastern State Medical University, Khabarovsk; KUZNETSOVA Maria Stanislavovna – Graduate Student (Far Eastern State Medical University, Khabarovsk). *E-mail: mischenkonp@mail.ru

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violations free radical and immune status, reduces the number of relapses of the disease, while reducing the severity of exacerbation and shortening the duration of hospitalization [3, 4].

It is shown that at the early stage of postnatal ontogeny, oral administration of echinochrome eliminates the structural-metabolic disturbances caused by lipopolysaccharide or bleomycin, and positively affects the antioxidant status in rat lungs [6].

The development of new drugs for the treatment of inflammatory processes in the lungs is a priority in therapeutic pulmonological practice and relevant for the expansion of the arsenal of oral antioxidant drugs.

MATERIALS AND METHODS

Echinochrome – substance (pharmaceutical pure, manufactured by PIBOC FEB RAS), ascorbic acid (99.8%, pharmaceutical, AppliChem, Germany) and α-tocopherol (≥96%, Ph. Eur. Carl Roth, Germany),lipopolysaccharide *E. coli* 026:B6 (Sigma).

Tested preparations:

ECH –echinochrome –reference preparation;

ASC+TOC –compositionofantioxidantswithoutechinochrome: ascorbic acid 3,0 gandαtocopherolacetate 0,6 g, excipients (MKC, aerosilиmagnesiumstearate) to 10 g–reference preparation;

ECH+ASC+TOC– compositionofantioxidants: echinochrome 30 g, ascorbic acid30 g, αtocopherolacetate6 g, MKC 28 g, Aerosil 5 gandmagnesiumstearate 1 g.

Determination of echinochrome in compositions. *The test sample*. Sample of the composition in 0.1000 g (accuracy 0.0005 g)is placed in a 100 ml volumetric flask and brought to the mark with ethanol (solution 1). 5 ml of solution 1 are added to a 100 ml volumetric flask and brought to the mark with acidified ethanol (1 ml of 1N hydrochloric acid per 100 ml of ethanol). *A standard sample* of 0.0200 g of Echinochrome-standard sample, weighed with an accuracy of 0.0005 g, is placed in a 50 ml volumetric flask and the volume is adjusted to the mark with ethyl alcohol(solution 2). 5 ml of solution 2 are added to a 100 ml volumetric flask and brought to the mark with acidified ethanol. The optical density of the test solution and the standard sample solution is measured on a spectrophotometer at **λ** 468 nm in a cuvette with a layer thickness of 10 mm using an acidified ethanol as the reference solution.

Determination of ascorbic acid in the compositions. A sample of the composition of 0.300 g (accuracy 0.0005 g)is placed in a 100 ml volumetric flask, dissolved in 10 ml of bidistilled water and 10 ml of hydrochloric acid 2%, and shaken for 10 minutes. The volume of the obtained solution is made up with bidistilled water to a mark, mixed and filtered. The first 10 ml of the filtrate are discarded. 10 ml are taken from the resulting solution and placed in a conical flask with a capacity of 100 ml, and 1 ml of hydrochloric acid, 0.5 ml of potassium iodide 1%, 2 ml of starch 0.5%, water bidistilled to a total volume of 20 ml are added. The resulting solution is titrated with 0.00167 M (0.01N) potassium iodate solution until a persistent light blue staining appears. The quantitative content of ascorbic acid in the composition is determined from the calculation of 1 ml of potassium iodate 0.00167 M (0.01 N), which goes for titration, corresponds to 0.008824 g of ascorbic acid.

Determination of α-tocopherol acetate. *Test solution:* Weigh a composition of 0.500 g, weighed to the nearest 0.0005 g, in a 25 ml volumetric flask.A sample of the composition of 0.500 g (accuracy 0.0005 g) is placed in a 25 ml volumetric flask and is added 15 ml of hexane, kept at room temperature for 2 hours and then volume in a flask with hexane is brought to the mark. *Standard sample:* 0.0250 g of alpha-tocopherol acetate is placed in a 25 ml volumetric flask, 15 ml of hexane is added, it is kept until completely dissolved and the volume is adjusted to the mark with hexane. The solutions obtained are chromatographed at least three times. High-performance liquid chromatograph “Agilent 1100” with column Hypersil ODS C18, grain size 5 μm, length 250 mm diameter 4 mm, eluent methanol 80%, acetonitrile 20%,andλ 292 nm were used.

Determination of antioxidant activity. Stock solutions of echinochrome, ascorbic acid and α-tocopherol are prepared at a concentration of 10 mg/ml in ethanol. Binary and ternaryantioxidant compositions are obtained by mixing the volumes of stock solutions in the indicated proportions, then 10 μl of each solution is placed in glass vial, 300 μl of linetol are added and the reaction vessels are placed in a thermostat (37 °C). The concentration of antioxidant in linetol in all cases was 0.05 mg/ml or 0.005%. Twice a day, the mass (accuracy 0.0005 g)of the reaction mixtures pre-cooled to room temperature is measured, when the mass increases by about 10 mg, the reaction is stopped. The period of inhibition of the oxidation of linetol ($\Delta\tau$) is calculated as the difference in the times over which the weight of linetol was increased by 10 mg in experiments with and without additive antioxidants by the formula $\Delta\tau = \tau - \tau_0$, where τ is the time of initiation of oxidation of linetol in the presence of an antioxidant (h); τ_0 is the time of initiation of oxidation of linetol without the addition of an antioxidant (h) [9].

Determination of anti-inflammatory action. The experiments were performed on Wistar rats at the age of 1 month. To model the inflammatory process, animals were injected intraperitoneally withLPSat a dose of 2.5 mg/kg (groups 2-5). The rats were administered three times in a dose of 10 mg/kg immediately before the administration of LPS, and 24 and 48 hours after the administration of LPS, aqueous solutions of ECH (group 3), ASK+TOK (group 4) and ECH+ASK+TOK (group 5). To the control group of animals (group 2) water was introduced through the probe in an equivalent volume with solutions of the test preparations. The group 1 was intact animals.

Euthanasia of rats was performed 3 days after the administration of LPS. The lungs of the animals were fixed in a Carnoy liquid, the paraffin sections with a thickness of 7 μm were stained with hematoxylin and eosin. Using the eyepiece micrometer, the maximum dimensions of the alveoli were determined. The processing of these data was carried out in the program Statistica.

RESULTS AND DISCUSSION

An increase in the effectiveness of therapy of the inflammatory process in the lungs with the use of a composition of antioxidants can be provided by a complex of multifaceted and diverse properties inherent in each of the components and is due to fundamentally different mechanisms of their antioxidant activity.

Echinochrome, unlike the main endogenous antioxidants, simultaneously blocks a number of links of free radical reactions. It acts as an interceptor of the ROS, neutralizes lipoperoxide radicals, chelates metal ions, inhibits lipid peroxidation, and regulates the cellular redox potential.

The water-soluble antioxidant vitamin C (ascorbic acid) is an essential co-factor of prolyl hydroxylases inhibiting the transcription factor of HIF-1 (hypoxia-inducible factor 1). Vitamin C-mediated inhibition of transcription of HIF-1-reactive genes is one of the main mechanisms governing the course of infectious-inflammatory processes [8].

In turn, the fat-soluble antioxidant vitamin E (**α**-tocopherol), participating in signal transduction processes, interacts with protein kinase C and inhibits its activity. It is with this mechanism that the manifestation of anti-inflammatory effects of **α**-tocopherol in the lungs is associated [1].

Echinochrome and α-tocopherol are practically insoluble in water, which limits their bioavailability when administered orally. To improve the bioavailability of the complex preparation, we performed experiments on the selection of auxiliary substances for the application of α-tocopherol acetate on them, with the condition of obtaining a mixture having sufficient flowability and sliding and suitable for encapsulation. Auxiliary substances such as kaolin, casein, magnesium carbonate basic, Aerosil, magnesium stearate to obtain bulk microcapsules of α-tocopherol acetate were investigate. As a result, auxiliary substances were selected, such as methylcarboxycellulose (MCC) (4.7 g per 1 g of α-tocopherol acetate), Aerosil (5 g) and magnesium stearate (1 g). Aerosil (sliding auxiliary) and magnesium stearate (lubricating auxiliaries) were added in accordance with the norms - 5% and 1% of the total mass, respectively, and the

composition of the pharmaceutical composition (wt%) is proposed: echinochrome – 30, ascorbic acid – 30, α-tocopherol acetate – 6, MCC – up to 28, Aerosil – up to 5, magnesium stearate – up to 1. This antioxidant composition is a homogeneous, fine crystalline powder of dark red-brown color, suitable for tableting or capsulation.

It has been shown experimentally that the active components of the composition completely pass within 20 minutes into a solution of hydrochloric acid simulating gastric juice (pH 1.2). It was determined that the active components of the composition remained unchanged for 12 months at room temperature, hence the composition was stable.

A comparative study of the antioxidant activity of echinochrome, ascorbic acid and α-tocopherol and their mixtures in vitro on the peroxide oxidation model of linetol was carried out. Table 1 shows the inhibition of linetol oxidation in the presence of echinochrome, ascorbic acid, α-tocopherol, and mixtures thereof in different ratios.

Table 1

Antioxidant activity of drugs on the autotoxidation model of linetol

Antioxidants and their compositions	The period of inhibition of linetol autoxidation, h	The effect of the mixture with respect to the effect of ECH
ECH	100 ± 5	-
ASK	24 ± 3	-
TOC	125 ± 7	-
ECH+ASK (1:1)	69 ± 4	No effect
ECH+TOC (1:1)	201 ± 8*	Synergy
ASK+TOC (2:1)	195 ± 7*	Synergy
ECH+ASK+TOC (5:5:1)	223 ± 10**	Synergy
Control - Linetol	24 ± 2	

*Notes: Echinochrome (ECH), ascorbic acid (ASC), α-tocopherol asetate (TOC) and their compositions (concentration of compounds - 0.05 mg/ml). * - statistically significant differences between the parameters of echinochrome and antioxidant compositions (p≤0.05), ** - statistically significant differences between the values of the ternary mixture and the double mixtures of antioxidants (p≤0.05).*

Table 1 shows that the most effective antioxidant in this experiment was α-tocopherol (Δτ 125 h). Echinochrome was less effective (Δτ 100 h), ascorbic acid showed no antioxidant effect in this model. The low efficiency of ascorbic acid in this model is due to its high ability for autooxidation in linetol solution. It is known that in experiments *in vitro* ascorbic acid has no antioxidant activity in the absence of α-tocopherol, which was demonstrated by our experiment. The best result for the protection of linetol from oxidation was shown by a mixture of three components ECH+ASK+TOC, in which the antioxidants had a synergistic effect (Δτ 223 h).

Since the antioxidant composition was developed for oral use, we introduced α-tocopherol acetate and, after the experimental selection, the ratio of the active components of the mixture echinochrome, ascorbic acid and α-tocopherol acetate was found to be 5:5:1.

The anti-inflammatory properties of the composition of antioxidants ECH+ASK+TOC *in vivo* were studied. Introduction LPS intraperitoneally at a dose of 2.5 mg/kg caused severe morphological changes in the lungs of rats (group 2), which were slightly suppressed by comparison drugs (groups 3 and 4). Perivascular and peribronchial edema, combined with migration to the perivascular space of leukocytes, are important markers of interstitial pneumonia. They were clearly expressed in groups 2-4 (found in all animals) and are much less common in group 5 (11.1%). Lymphoid lung infiltration is also a sign of inflammation, it was observed in all experimental animals in groups 2-4. Dimensions of emphysematous dilated alveoli in group 5 (72 ± 3.5 μm) were smaller than group 2 (85 ± 4.4 μm), which may be due to a lesser degree of alteration of alveoli, interstitial lung tissue in animals that received the ECH+ASK+TOC.

CONCLUSION

Thus, the ECH+ASK+TOC (5:5:1 weight ratio) antioxidant composition exhibits a pronounced synergistic anti-inflammatory effect, decreasing in a greater degree compared to echinochrome and the ascorbic acid– α-tocopherol acetate (5:1) complex, perivascular and peribronchial edema, lymphoid infiltration and expansion of the alveoli in the lungs caused by the introduction of LPS.The ECH+ASK+TOC (5:5:1) antioxidant composition exhibits good bioavailability, is completely soluble in the stomach and is suitable for the preparation of oral medicaments in the form of tablets and capsules.

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