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Amelioration of endotoxin-induced uveitis treated with the sea urchin pigment echinochrome in rats

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Purpose: Echinochrome is a pigment present in the shells and spines of sea urchins. It has been reported to have several biologic protective effects, including in experimental models of myocardial ischemia/reperfusion injury, for which the proposed mechanisms are scavenging reactive oxygen species (ROS) and chelating iron. Endotoxin-induced uveitis (EIU) is an animal model of acute anterior segment intraocular inflammation that is induced by the injection of lipopolysaccharide (LPS). In this study, the therapeutic effect of echinochrome was examined in uveitis using the EIU model.

Methods: EIU was induced in Lewis rats via 200 µg subcutaneous injections of LPS from *Escherichia coli*. Echinochrome was administered intravenously in 10, 1, or 0.1 mg/kg doses suspended in PBS (controls were injected with PBS only). Twenty-four hours after LPS injection, the number of infiltrating cells and the protein concentration in aqueous humor were determined. Aqueous tumor necrosis factor α (TNF-α) concentration was quantified with enzyme-linked immunosorbent assay, eyes were stained with nuclear factor (NF) κB antibodies, and ROS production was determined by dihydroethidium staining in fresh frozen samples.

Results: The number of inflammatory aqueous cells and protein levels were lower in the groups treated with 10 and 1 mg/kg of echinochrome than in the untreated LPS group ($p < 0.01$). Treatment with 10 and 1 mg/kg of echinochrome significantly reduced TNF-α concentrations in aqueous humor ($p < 0.01$). The numbers of NFκB-positive cells and ROS signals were also reduced by echinochrome administration ($p < 0.05$).

Conclusions: Echinochrome ameliorated intraocular inflammation caused by EIU by reducing ROS production, thereby also decreasing the expression of NFκB and TNF-α. As a natural pigment, echinochrome may therefore be a promising candidate for the safe treatment of intraocular inflammation. The use of sea urchin shells and spines in health foods and medical products is thus both economically and environmentally meaningful.

Echinochrome, or 7(2)-ethyl-2,3,5,6,8-pentahydroxy-1,4-naphthoquinone, is one of several spinochromes that occur as pigments in the shells of sea urchins. Part of the chemical structure of echinochrome is appropriate for free-radical scavenging, as it includes 2,3,7-hydroxyl groups and contains a 1,4-naphthoquinone ring, similar to vitamin K. Unlike vitamin K, echinochrome is water soluble and does not affect blood coagulation [1,2]. Naturally derived echinochrome contains several isomers with different location of methyl group with varied proportions, with two most abundant are presented (Figure 1). However, it is considered that these isomers have a similar antioxidant effect, since this is expected to arise from their multiple hydroxyl groups. Echinochrome expression in larval and adult sea urchin tissues

is related to seawater quality [3]: It increases when water quality decreases or contains harmful agents [4]. In adult sea urchins, spinochromes—including echinochrome—are mainly found in the shell and spines of the animal. Sea urchin spinochromes form part of the daily diet of sea otters [5], and lifelong oral consumption of these pigments leads to the pink or light-purple staining of the teeth and bones observed in sea otters [6].

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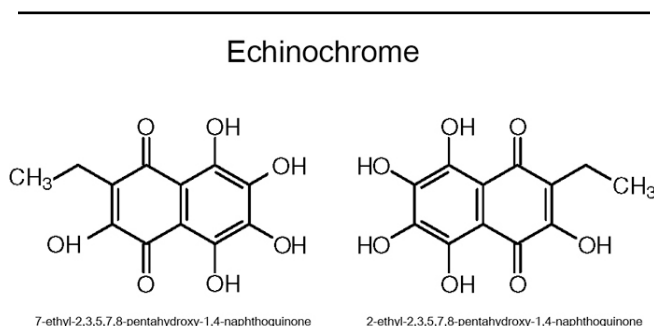


Figure 1. The chemical structure of echinochrome is shown. Echinochrome has two main isomers.

Echinochrome has been reported to have several biologic effects [7,8]. In animal experimental models of myocardial ischemia/reperfusion injury, echinochrome exhibits a considerable protective effect and reduces infarct size by up to 45% [9,10].

The proposed protective mechanisms of echinochrome are reactive oxygen species (ROS) scavenging and iron chelation [11]. In the Russian Federation, echinochrome is approved as an experimental treatment for acute infarction in humans as the commercially available drug Hystochrome® (Pacific Institute of Bioorganic Chemistry [PIBOC], Vladivostok, Russia) [12,13] and the dietary supplement Timarin® (PIBOC) [14].

Endotoxin-induced uveitis (EIU) is an animal model of acute anterior segment intraocular inflammation that is induced by the injection of an endotoxin, the lipopolysaccharide (LPS) component of the Gram-negative bacterial cell wall [15]. It has been reported that increased ROS production is one of the factors that triggers inflammation, leading to activation of nuclear factor (NF) κ B; this is one of the most ubiquitous transcription factors, and it has been suggested that it has a key role in inflammatory reactions [16,17]. The inflammatory feedback loop progresses with cellular infiltration and protein leakage into the anterior chamber of the eye, which reaches a maximum 24 h after LPS injection [18]. Elevated expression of cytokines and chemokines has been observed to be concomitant with maximum EIU [18,19]. Therefore, reducing ROS expression in the eye may be beneficial in limiting inflammatory response. The purpose of this study was to examine the therapeutic effects of echinochrome in a rat EIU model.

METHODS

Animals and reagents: Echinochrome was obtained from PIBOC. The substance was dissolved in PBS (NaCl 8 g, (Na₂HPO₄)12H₂O 2.9 g, KCl 0.2 g, KH₂PO₄ 0.2 g, H₂O 1 l; pH 7.4) immediately before use.

Eight-week-old male Lewis rats (180–220 g) were used. All procedures involving animals were performed in accordance with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. EIU was induced by subcutaneous injection with 200 μ g LPS from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO) that had been diluted in 300 μ l PBS. At the same time, the animals were injected intravenously with 10, 1, or 0.1 mg/kg of echinochrome diluted in 1.0 ml PBS. Control EIU rats were intravenously administered 1.0 ml PBS (no echinochrome). Euthanasia was performed by intravenous injection of 100 mg/kg of sodium pentobarbital (Sigma, Tokyo,

Japan). All experiments were performed in triplicate, with four animals in each group.

Number of infiltrating cells, concentrations of protein, and tumor necrosis factor α in aqueous humor: Twenty-four hours after LPS injection, the rats were euthanatized, and the aqueous humor was collected immediately from both eyes via an anterior chamber puncture (25–50 μ l/rat) using a 30-gauge needle (Terumo Corporation, Tokyo, Japan), under an Olympus SZ61 microscope (Olympus, Tokyo, Japan). To count the cells, the aqueous humor samples were suspended in an equal amount of Türk stain solution (Merck, Darmstadt, Germany). The cells were counted with a hemocytometer under an Olympus CKX31 microscope (Olympus), and the number of cells was obtained by averaging the results of four fields from each sample. The total protein concentration in the aqueous humor samples was measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). The aqueous humor samples were stored in ice water until testing, and cell counts and total protein concentrations were measured on the day of sample collection.

TNF- α levels in the aqueous humor were assessed with a commercially available rat TNF- α enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Each assay was performed in triplicate.

Immunohistochemical studies for nuclear factor κ B: NF κ B translocation precedes the development of systemic and ocular inflammation. Based on the preliminary results, the rats were deeply anesthetized by intravenous injection of sodium pentobarbital (50 mg/kg) 3 h after LPS injection, and the eyes were fixed by an intracardiac perfusion of Superfix 100 solution (Kurabo, Osaka, Japan). The eyes were then enucleated and immersed in the same fixative for 12 h, and then embedded in paraffin. Next, 5 μ m sagittal sections were cut near the optic nerve head.

Sections were dewaxed with xylene and rehydrated with ethanol. Antigens were retrieved by heating sections in citrate buffer solution in a microwave oven. To evaluate the NF κ B-positive cells in the iris/ciliary body (ICB), a 1:100 dilution of NF κ B p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was applied to the sections for 12 h, after which they were washed with PBS. Following this, a 1:1,000 dilution of a secondary goat-anti-rabbit antibody dye conjugate (Invitrogen, Carlsbad, CA), which produces red fluorescence, was applied. Because NF κ B is widely present in intercellular tissue, to detect its expression in cell nuclei, section slides were stained with a 1:1,000 dilution of YO-PRO-1 (Invitrogen), which produces a green signal. YO-PRO-1 is a sensitive detector of membrane permeabilization, and a

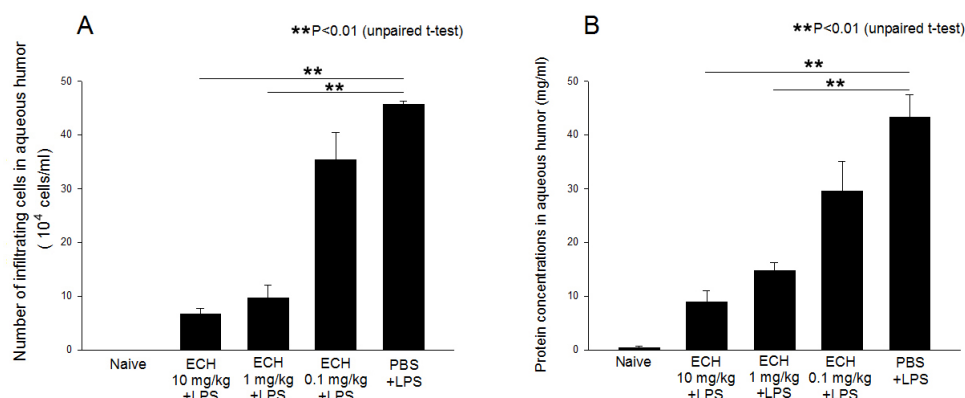


Figure 2. Effects of echinochrome (10, 1, and 0.1 mg/kg) on **A**: cellular infiltration and **B**: protein concentration in aqueous humor collected 24 h after lipopolysaccharide (LPS) treatment are shown. Data are presented as mean $\pm$ standard error of the mean (SEM; n=4). **Groups

treated with echinochrome 10 and 1 mg/kg have significantly less infiltrating cells and protein concentrations than untreated endotoxin-induced uveitis (EIU) controls ($p<0.01$).

normally cell-impermeant, monomeric, cyanine dye with a strong binding affinity to nucleic acids. NF κ B-positive nuclei (yellow) were quantified in merged images of the two ICB areas of the eyes, as we reported previously [20]. In brief, the results of two areas of each eye were averaged for each sample and in each group and then compared with the total number of stained nuclei in ICB areas to obtain percentage of NF κ B colocalized cells. Unless stated otherwise, slide imaging and evaluations were performed under a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) using software bundled with the apparatus.

Detection of reactive oxygen species: The eyes were dissected from the rats 24 h after LPS injection and frozen fresh in FSC22 frozen section compound (Leica Microsystems, Wetzlar, Germany) with liquid nitrogen. Dihydroethidium (DHE, Sigma), an oxidative fluorescent dye, was used for immunohistochemical detection of cytosolic superoxide anion (O_2^-). ROS production was determined by conversion of DHE to ethidium bromide. Square areas of ICB microphotographs (1.44 megapixels in size) were selected, and mean gray values of the DHE signal were evaluated with ImageJ software, as we reported recently [21]. Two whole ICB areas were evaluated in each eye and then four eyes were examined in each group.

Statistical analysis: All values are expressed as the mean $\pm$ standard error of the mean (SEM) for the respective groups. Statistical significance was evaluated by an unpaired t test. P values less than 0.05 were considered statistically significant.

RESULTS

Effect on cellular infiltration and protein concentration in aqueous humor: Severe uveitis was found in the anterior segments of the rats with EIU 24 h after LPS administration.

In the untreated LPS group, the number of inflammatory cells was $288.0 \pm 25.0 \times 10^4$ cells/ml. The aqueous infiltrating cell numbers of the rats treated with 10, 1, and 0.1 mg/kg of echinochrome were $11.5 \pm 0.9 \times 10^4$, $19.8 \pm 2.2 \times 10^4$, and $308.0 \pm 16.0 \times 10^4$ cells/ml, respectively. The mean numbers of aqueous inflammatory cells were significantly reduced by treatment with 10 ($p<0.01$) and 1 ($p<0.05$) mg/kg of echinochrome (Figure 2A). No infiltrating cells were detected in the aqueous humor of the naïve rats.

A high protein level (43.3 ± 4.2 mg/ml) was detected in the aqueous humor of the untreated EIU rats. The aqueous protein levels of the rats injected with 10, 1, and 0.1 mg/kg of echinochrome were 8.9 ± 2.2 , 14.8 ± 1.5 , and 29.5 ± 5.5 mg/ml, respectively. Aqueous protein concentration was only 0.48 ± 9.2 mg/ml in the naïve rats.

Protein concentrations in the rats treated with 10 mg/kg ($p<0.01$) and 1 mg/kg ($p<0.01$) of echinochrome were significantly lower than that of the untreated LPS group (Figure 2B). The decrease in aqueous cellular infiltration and protein levels indicates a dose-dependent response to echinochrome (Figure 2).

Effect of echinochrome on tumor necrosis factor α in aqueous humor: The aqueous humor of the untreated EIU rats showed considerable production of TNF- α , with a concentration of 457.0 ± 29.0 pg/ml. The aqueous TNF- α levels of the rats treated with 10, 1, and 0.1 mg/kg of echinochrome were 225.7 ± 9.4 , 276.0 ± 12.0 , and 405.4 ± 26.0 pg/ml, respectively. Treatment with 10 ($p<0.05$) and 1 mg/kg ($p<0.01$) of echinochrome significantly reduced the concentrations of TNF- α in the aqueous humor (Figure 3). No TNF- α was detected in the aqueous humor of the naïve controls (below the 15 pg/ml sensitivity threshold of the enzyme-linked immunosorbent assay kit).

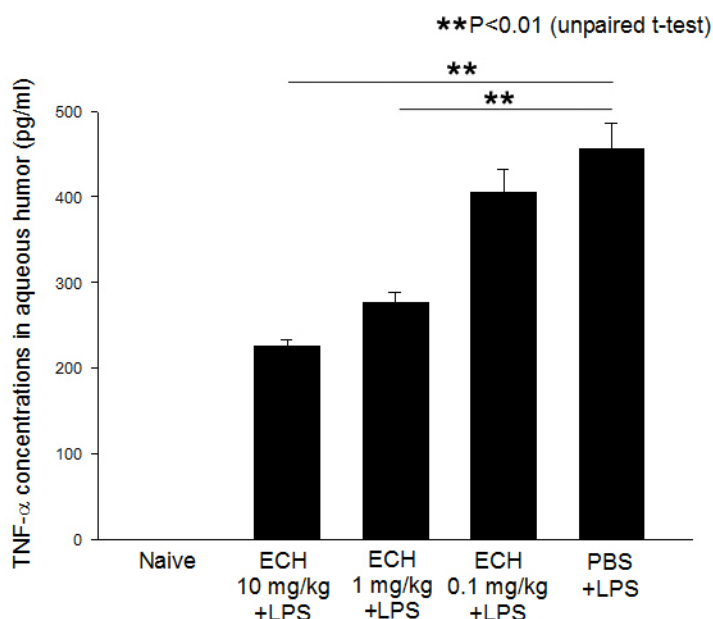


Figure 3. Effects of echinochrome (10, 1, and 0.1 mg/kg) on tumor necrosis factor- α (TNF- α) in aqueous humor collected 24 h after lipopolysaccharide treatment are shown. Data are presented as mean $\pm$ standard error of the mean (SEM; n=4). ** TNF- α concentration is significantly lower in echinochrome (10 and 1 mg/kg) treated groups than untreated endotoxin-induced uveitis (EIU) rats ($p<0.01$).

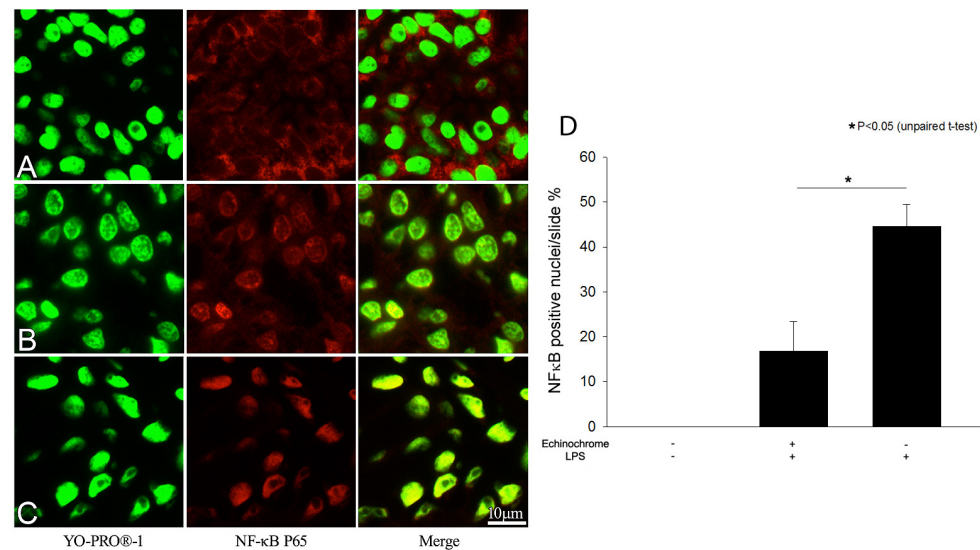
Immunohistochemistry of nuclear factor κ B p65 translocation in the iris/ciliary body: Activated NF κ B p65 immunoreactivity was examined 3 h after the LPS injections were administered. In the naïve controls, NF κ B was stained only in the cytoplasm in its inactivated form, with no nuclear translocation detected (Figure 4A). A few activated NF κ B-positive cells were observed in the ICB of the rats treated with echinochrome at 10 mg/kg (Figure 4B); in contrast, NF κ B p65 was strongly expressed in the nuclei of the untreated EIU rats (Figure 4C). Active NF κ B-positive nuclei were counted to obtain a quantitative measure of NF κ B activity in the ICB (Figure 4D). Most of the stained cells were ICB cells. LPS injection resulted in a significant increase in the number of active NF κ B-positive cells in the ICB after 3 h ($44.5\pm5.0\%$). In the group treated with 10 mg/kg echinochrome, the number of active NF κ B-positive cells decreased significantly (16.7 ± 6.6 ; $p<0.05$). In the naïve control group, no active NF κ B-translocated nuclei were detected in the ICB (Figure 4D).

Detection of reactive oxygen species: Confocal microscopic images of ROS production were examined in the ICB tissues. All of the images were made in parallel at identical exposure settings (Figure 5). Only a background ROS signal was detected in the ICB of the naïve rats (Figure 5A). ROS production was lower in the echinochrome-treated EIU rats (Figure 5B) than in the untreated EIU rats, in which a strong ROS signal was detected (Figure 5C). The color of the corneal epithelium on the DHE-stained slides was transformed into grayscale; then, gray values were evaluated by Image J

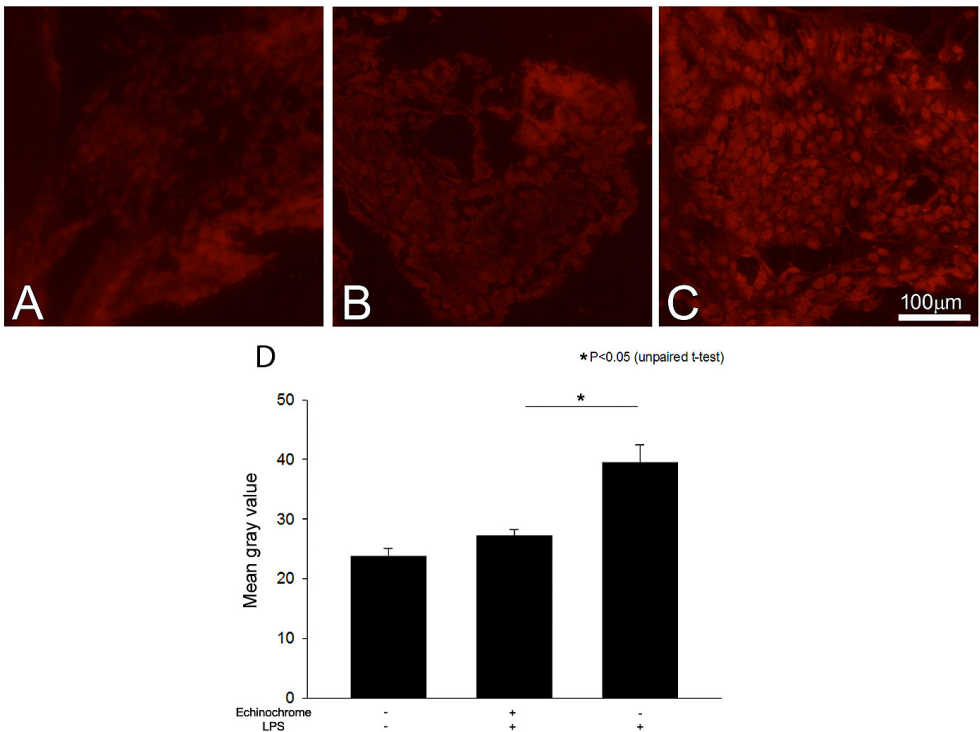
software and summarized (Figure 5D). The mean gray value in the ICB of the naïve rats was 23.8 ± 1.4 . The mean gray value of the EIU rats treated with 10 mg/kg of echinochrome (27.2 ± 1.0) was significantly lower ($p<0.05$) than that of the untreated EIU rats (39.4 ± 3.0).

DISCUSSION

In this study, we examined the therapeutic effects of the antioxidant compound echinochrome on EIU, an animal model for acute ocular inflammation. Echinochrome is a type of pigment present in sea urchin shells and spines. Small-scale research studies of its biologic effects have been conducted locally in the Russian Far East, which is rich in northern marine resources. However, because many of the studies were reported in Russian, the data from the basic research regarding echinochrome are quite limited outside the area. In the present study, a single intravenous administration of echinochrome significantly ameliorated EIU. Inflammatory cell infiltration and protein levels in the aqueous humor of EIU rats treated with echinochrome were significantly lower than those of untreated EIU rats. Proinflammatory cytokines and ROS activate NF κ B, which is a key transcription factor that regulates various inflammatory processes [22]. NF κ B is found in the cytoplasm and is bound to inhibitor of kappa B alpha (I κ B α) and I κ B β . When cells are stimulated, NF κ B dissociates from I κ B and migrates into the nucleus, where it binds to specific sequences in the promoter regions of the target genes [23]. The activation of NF κ B promotes the expression of several genes, including TNF- α , which mediate



C: untreated EIU rats. **D:** Quantitative analysis of NFκB-positive cells in the iris/ciliary body (ICB). Data are shown as mean±standard error of mean (SEM; n=4). *Amount of NFκB nuclear colocalization was significantly lower in echinochrome treated group than untreated EIU controls (p<0.05).



as mean ± standard error of the mean (SEM; n=4). *Mean gray value of echinochrome treated eyes was significantly lower than untreated EIU controls (p<0.05).

inflammation and active immune responses. These cytokines sometimes activate—and on other occasions are activated by—NFκB, thereby inducing a positive feedback loop in inflammatory response [24].

The present study shows that NFκB p65 nuclear translocation in the ICB was significantly suppressed by the single administration of echinochrome. Immunohistochemical findings indicate that NFκB inhibition by echinochrome is achieved via significant reduction of ROS production in ocular tissue, which supports previous data obtained in vivo [7] and in vitro [11,25]. However, this kind of study has some limitations, including the following: (1) the number of test subjects was limited, (2) EIU is an acute model so duration of the study was not long enough to fully access all biological effect of the echinochrome administration, (3) evaluation at different time points were lacking during the course of the experiments. Further studies are needed in the near future.

Several other substances, including resveratrol and astaxanthin, have been reported to have ameliorative effects on EIU by suppressing ROS production [26,27]. However, most of these antioxidant substances are poorly soluble in water, and therefore can only be applied orally or subcutaneously; this reduces the amount of substances that effectively reaches the ocular tissue and delays the onset of the drug's effect. Echinochrome is water soluble and can be administered intravenously, thereby reaching the eyes within seconds after administration. Echinochrome has already been approved as an experimental treatment for acute infarction in humans, and it has been used as a human dietary supplement in Russia with no reported serious side effects [14]. This supports the findings of the present study. We also checked the systemic side effects of echinochrome injection, and no serious effects were observed in rats at concentrations of up to 25 mg/kg (undiluted solution), as long as the substance was administered slowly (data not shown). Echinochrome can be chemically synthesized [28], but the shells and spines of several sea urchin species provide a cheaper source of spinochromes [29-31]. In Japan and other countries, these parts of sea urchins are usually disposed as industrial trash in food-processing factories after gonads are harvested [32]. The use of sea urchin shells and spines (echinochrome) in health foods and medical products is meaningful both economically and environmentally. As such, the naturally derived pigment echinochrome appears to be a promising candidate as an agent for the safe treatment of ocular inflammatory disorders.

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