PHYSIOLOGICAL ACTIVITY OF FAR EAST MARINE ANIMALS SUPERCRITICAL FLUID EXTRACTS

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ABSTRACT

Supercritical carbon dioxide extraction of two far east marine animals was held using ethanol as a cosolvent. Different types of biotest were carried using obtained extracts. The results revealed significant biological activity of SC-CO_2 extracts of those animals which differs significantly from the activity of the extracts gained from the same raw material using traditional water-ethanol extraction. *Halocynthia aurantium* extract possesses the ability to decelerate embryonic stem cell cultures growth and to cause exogastrulation in sea urchin pluteuses. *Scaphechinus mirabilis* extract has a great toxic effect on stem cell cultures leading to their total degradation.

INTRODUCTION

The variety of marine animals is immensely rich. Due to a special environment they live in metabolism of those animals differs strongly from the ones of terrestrial animals. Biological tasks their organisms have to solve oblige them to create powerful biochemical defence systems. Huge amount of biochemical compounds produced by marine animals could be used for medical purposes because of their unique therapeutic properties [1,2]. For instance, native New Zealand tribes used to consume a lot of green-lipped mussels and this food seemed to be responsible for the fact that those people never suffered from inflammatory diseases. Nowadays stabilized green-lipped mussel oil extract is widely used an anti-inflammatory remedy all over the world [3]. A problem of bioactive chemicals recovery from marine animals is of great importance due to a global trend of natural health care products ideology.

The Sea of Japan and other far eastern seas is the richest Russian marine region. Almost all groups of valuable marine organisms live there, including marine mammals, fishes, invertebrates and algae.

Supercritical fluid extraction (SFE) is well known as a powerful and environmentally safe tool for bioactive substances recovery from natural raw materials. It is widely used nowadays for extraction of lipids, essential oils, waxes, steroids, carotenoids and a whole series of other biochemical compounds from numerous plants tissues [4]. On the other hand SFE from animal tissues, especially marine ones, is much less developed. It might be the case according to several reasons, such as: (1) less raw material availability, (2) less active compounds solubility in neat supercritical carbon dioxide and, as a consequence, a need for co-solvent use. The most popular marine objects for SFE are different types of algae and microalgae as a raw material for carotenoids production [4]. Another widespread field of SFE in marine products is extraction of lipid materials from fish, mussels etc [4]. To date, little

data is available on supercritical fluid extraction of bioactive compound from non oil-rich tissues of marine animal.

The aim of this work was to perform SC-CO_2 extraction of two Far East marine animals, ascidia *Halocynthia aurantium* (Ascidiacea, Tunicata, Chordata,) and sea urchin sand dollar *Scaphechinus mirabilis* (Echinoidea, Echinodermata) and to test their biological activity.

MATERIALS AND METHODS

Materials and reactives.

Halocynthia aurantium and *Scaphechinus mirabilis* specimens were collected in the Peter the Great buy of the Sea of Japan at depths of 5 to 20 m. Ethanol used was HPLC grade ("Himmed Synthesis", Russia). CO_2 was of 99.99 % purity (Linde Gas, Germany). Mitomycin C and leukemia inhibition factor were purchased from Sigma, USA with degree of purity 99.98%. All other chemicals were purchased from Fluka and had HPLC degree of purity. Characterized bovine fetal serum was purchased from "HyClone", USA. Commercially available drug Histochrome was used as a purified water-ethanol extract for stem cells biotest. Commercially available drug Haurantin was used as water-ethanol extract of *Halocynthia aurantium* tunic which is prepared from dry ascidia tunic with 70 % ethanol [5].

SFE.

Extraction with supercritical carbon dioxide was held using Thar Instruments, Inc**.** apparatus SFE-1000. *Scaphechinus mirabilis* bodies were used as is, *Halocynthia aurantium* bodies were dissected in order to retrieve tunica. Only tunica was used in the extraction experiments. A specimen of marine animal was roughly fractioned with a grinding combine, freeze-dried in a Virtis freeze dryer (SP Scientific, USA) at -20°C within 24 hours and then milled electrical milling system LMT-1 (Laborkomplekt, Russia). Residual humidity wasn't measured. A fraction with particle size between 0.5 and 3 mm was collected for SFE experiments. 160 g of milled substance was put into a 200 ml extraction vessel (bulk density ~1 g/ml). Extraction was conducted in a flow regime under the following conditions: P = 300 bar, T = 40 °C, flowrate = 30 g/min, extraction time = 120 min. 10% of ethanol as a modifier was used in the composition of eluent. After the extraction the samples collected were degassed overnight from residual CO_2 solved in ethanol, evaporated with a rotary evaporator (Heidolph, Germany) and weighted.

Sea urchin biotest.

The test object was sea urchin *Strongylocentrotus intermedius*. Sea urchins from the Sea of Japan were kept in the Aquarium Laboratory of the Institute of Marine Biology of the Far East Branch of Russian Academy of Sciences. Sexually mature specimens of approximately the same size at the stage of gonad fluidity were selected. The experiments were performed with eggs, spermatozoa, embryos and zygotes during fertilization. A suspension of moving spermatozoa was added to mature eggs, and then water-ethanol or SCF extract of *Halocynthia aurantium* tunic was applied. The experiments were performed with zygotes during fertilization (200–250 eggs) and larvae (50–100 larvae in each experiment): early and intermediate blastulae, early, intermediate, and late gastrulae and pluteuses. Gametes, embryos, and larvae were transferred to Petri dishes filled with seawater at a temperature of 19 to 20 °C. Embryo cultures with a fertilization percentage of ~98 % were used. In different experiments, the seawater was doped with 0.1–0.4 mg/ml of the test substances. The reference samples were unaffected embryos and larvae at the corresponding stage of development. The

experiments were conducted in three repeatabilities. The experimental procedure was the same for all the development stages studied. Before biotesting, the extracts were evaporated in a vacuum evaporator.

Stem cell test.

In this work R1 line mice were used which were a gift from Dr. A. McLaren, WTCR Institute of Cancer and Developmental Biology, Cambridge, UK. Embryonic stem cells (ESC) of mice were cultivated in a DMEM medium containing 1 mM of L-glutamin, 0.1 mM of nonessential aminoacids, 0.1 mM of β-mercaptoethanol and 15% of characterized bovine fetal serum. Undifferentiated ESC were held in a feeder of primary mice embryonic fibroblasts inactivated by 100 μg/ml mitomycin C or were cultivated in a medium with 10 ng/ml leukemia inhibition factor, LIF [6].

«Hanging droplet» method was used for standard embryonic bodies production. Droplets of ESC suspension were put onto a Petri dish cup (500 cells in a droplet). After a certain amount of time generated embryonic body were retrieved from the droplets and put into new dishes for suspension cultivation for the next 3-5 days [7,8]. In order to register effects of supercritical extracts on embryonic bodies generation the cultivation medium contained certain amounts of extracts addition. Two series of experiments were held with dry extracts concentration of 100 μg/ml and 1000 μg/ml. Stock solutions of extracts in a DMEM medium were prepared straight before the experiment. Cultivation medium exchange was performed daily.

The experiments of the first series were carried out for 120 hours (5 days), the second one – for 72 hours (3 days). All the embryonic bodies have been photographed during the experiment, size estimation and alkaline phosphatase activity measurements were held at the last day of the experiment in each series. An estimation of adhesive properties of embryonic bodies to a non-adhesive surface of cultural plastics (suspension cells cultivation plane tables) was performed on a third day of experiment. A number of cells attached to the surface was counted and an adhesiveness parameter was calculated for each group of cells.

Analysis of photographic images of stem cells cultures was implemented via automatical image analysis program. Statistical analysis was held using pair Student's criterion. Hypothesis reliability on marine animals extracts influence was estimated via ANOVA variation analysis method.

Histochemical analysis of alkaline phosphatase activity in non-differentiated pluripotent cells was perform according to [6]. Cells were fixed with 3 % solution of paraform in a phosphate buffer within 15-20 minutes. After rinsing with phosphate buffer samples were incubated in a solution containing 10 ml 0.02 M tris-HCl buffer (pH 8.6), 1 mg naphtole AS-BI phosphate and 5 mg of Fast Red-TR dyeing agent within 60 minutes at 37 °C.

RESULTS

SFE.

Both *Halocynthia aurantium* tunica and *Scaphechinus mirabilis* are poorly extracted by supercritical carbon dioxide. Mass yield for *Halocynthia aurantium* in chosen conditions never exceeded 0.5 % from the dry substance, for *Scaphechinus mirabilis* – 2.0 %. *Halocynthia aurantium* extract is a yellow-orange paste, *Scaphechinus mirabilis* is a bright orange extremely viscous liquid (apparently due to the traces of water in a bulk). Both products possess a typical marine smell.

Sea urchin biotest.

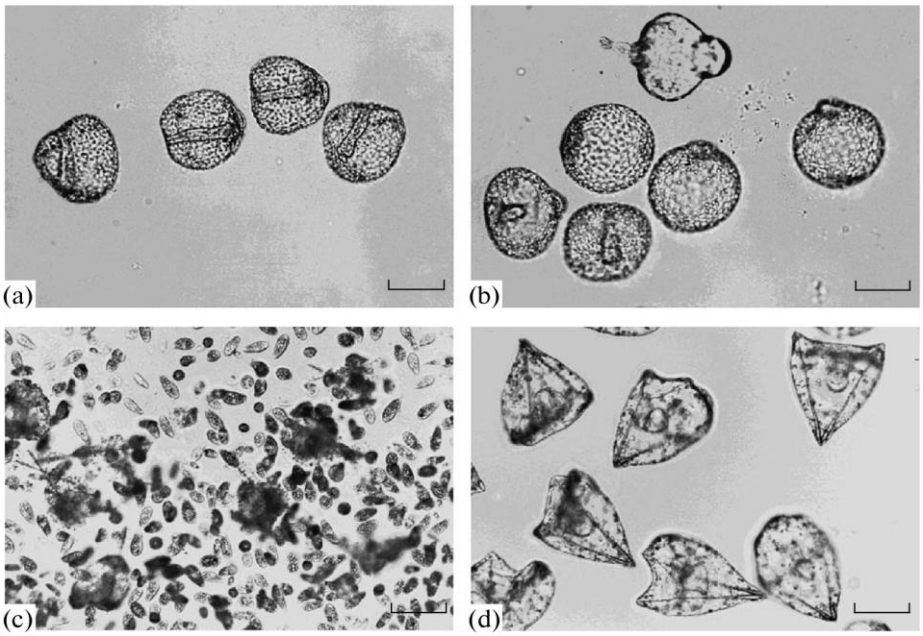

Figure 1. Embryos and larvae of sea urchin (50 μm scale): (a) reference embryos of sea urchin 24 h after fertilization (normal gastrula) (b) test embryos 24 h after fertilization in the presence of the *H. aurantium* supercritical extract (gastrulation disrupted, exogastrulae are formed); (c) reference embryos 73 h after fertilization (numerous infusoria appeared in the cultural media, lysing sea urchin embryos); and (d) sea urchin larvae at the stage of early pluteus, 73 h after fertilization (*H. aurantium* supercritical extract was added to the embryos 24 h after fertilization, at the stage of late gastrula; normal development takes place in the complete absence of infusoria in the cultural medium).

This type of biotesting was performed only for the *Halocynthia aurantium* extracts. Two types of extracts were compared: supercritical fluid extract and water-ethanol extract from *Halocynthia aurantium* tunica. When water-ethanol one were added to Petri dishes containing spermatozoids, eggs experiencing fertilization, dividing embryos, embryos at the stages of early and intermediate gastrula, and larvae at the stage of early pluteus, no visible distinctions from the reference were observed: spermatozoids remained mobile, normal fertilization percentage (98 %) was in place, fission occurred without deviations, and normal gastrula and early pluteuses were formed. Only upon addition of the water-ethanol extract, a small decrease in the fractionation rate was observed, which disappeared at the stage of late blastula. When the same samples (spermia, embryos, and larvae) were treated with supercritical extracts from *Halocynthia aurantium* tunica also no deviations from the reference samples were found. However, if a supercritical extract was added at the stage when gametes fuse together, the development was disrupted. The formation of a fertilization membrane and fission occurred as usual, but the process of gastrulation was perturbed. Instead of the formation of a normal archenteron through the bulging of the blastomeres of the vegetative pole of the blastula, as it occurred in the reference sample (Fig. 1a), exogastrulae were formed (Fig. 1b). During exogastrulation, the blastomeres of the vegetative pole do not invaginate into the blastocoel, but, on the contrary, bulge outward. Later, such exogastrulae do not develop, but remain alive and mobile for several days. They can somewhat increase in

size compared to the reference late blastulae and become more transparent because of a depletion of yolk granules. Embryos and larvae were cultivated in seawater from the aquarium of the Institute of Marine Biology of the Far East Branch of the Russian Academy of Sciences, which, in turn, was supplied from the Amur Gulf. This water contains a large number of bacteria and unicellular eukaryotes, which intensely replicate in Petri dishes with sea urchin embryos, especially infusoria. After four or five days, became so numerous that they cause lysis of sea urchin embryos and larvae (Fig. 1c). A similar picture was observed when the culture was treated with the water-ethanol extract. Note, however, that addition of the SC-extract to a medium with embryos after gastrulation suppressed the propargation of infusoria, allowing larvae to develop in a clean medium with a number of unicellular eukaryotes too small to interfere (Fig. 1d).

Stem cell test.

Two series of experiment were conducted in this block of tests. Concentration of supercritical extracts of both animals of 100 μg/ml was taken in series 1, 1000 μg/ml – in series 2. Cultivated cells in each series were divided into 4:

- control group
- treated with histochrome
- treated with *Halocynthia aurantium* supercritical extract
- treated with *Scaphechinus mirabilis* supercritical extract

The first experimental series showed no abnormalities in stem cells cultures development *in vitro*. The presence of pluripotent cells with high alkaline phosphatase activity as long as differentiated cells on the embryonic bodies surface not expressing alkaline phosphatase correspond to a normal spatio-temporal pattern of stem cells differentiation (Fig. 2). No statistically reliable distinction in embryonic bodies sizes of cultures administrated with Histochrome drug and supercritical extract of *Scaphechinus mirabilis* were detected. On the other hand, statistically reliable embryonic cells growth inhibition up to 10% was registered in the cultured treated with *Halocynthia aurantium* SC-extract.

Adhesion ability of embryonic bodies investigation showed no differences in adhesiveness of control group, third and forth series. Adhesion coefficient kept in an interval of 28-30%. The second group surprisingly showed 0%. Histochrome administration caused loss of cells ability to attach to the surface. The only bioactive compound included into the composition of this drug, the only other components are sodium chloride, sodium bicarbonate and water. The observed result is quite unexpected and needs further examination.

Series 2 of experiments (1000 μg/ml) revealed a significant toxic effect of *Scaphechinus mirabilis* supercritical extract. Intensive cell destruction followed by full degradation occurred in the forth experimental group within 24 hours. Partial cells destruction is already visually detectable after several hours from the start. Third group of cells revealed a statistically reliable embryonic bodies growth decrease by 32 % in comparison to control group cells after 72 hours of cultivation. No morphological distinctions were detected in this group. Adhesiveness test result appeared to be quite similar to the ones of series 1. Total lack of adhesion was detected in group 2, and adhesion ability decrease by 30 % was registered in group 3.

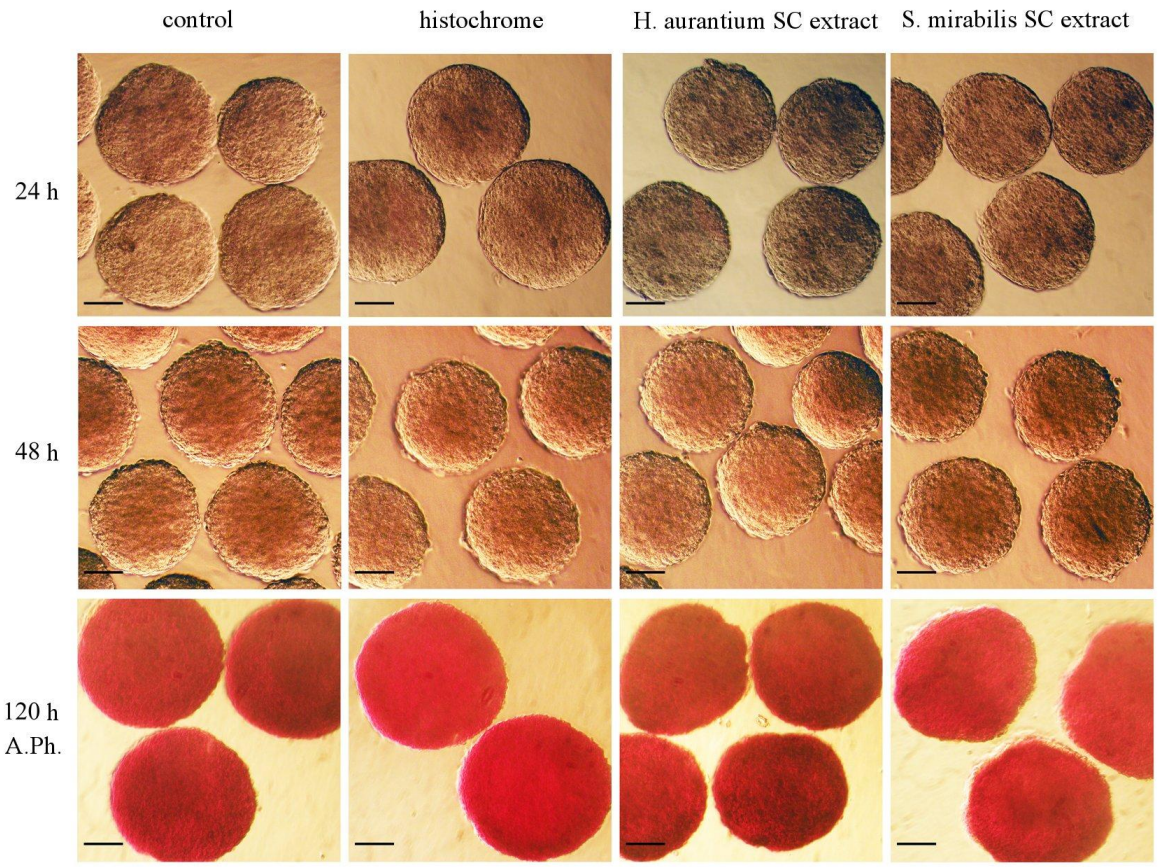

Figure 2. Morphology of embryonic bodies and alkaline phosphatase activity in series 1 experiments (C=100 μg/ml).

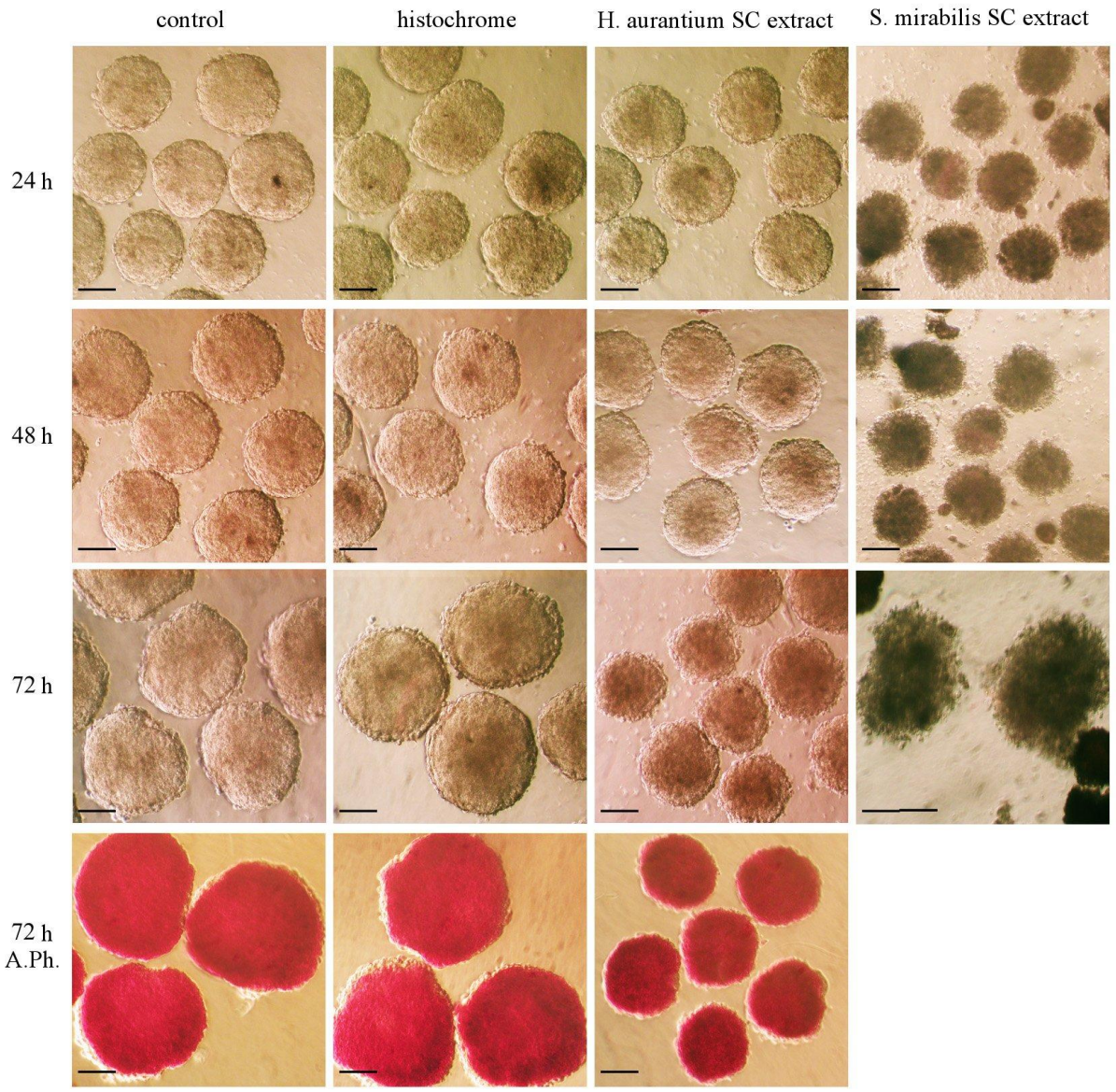

Figure 3. Morphology of embryonic bodies and alkaline phosphatase activity in series 2 experiments (C=1000 μg/ml).

CONCLUSION

Supercritical carbon dioxide is a poor solvent for bioactive compounds from chosen marine animals tissues which makes cosolvents usage inevitable. Although mass yields of SFE of both tested marine animals was rather disappointing they tend to possess strikingly different and significantly more powerful biological effect in comparison to extracts from the same raw material gained by traditional water-ethanol technique. *Halocynthia aurantium* SC-CO_2 extract appears to be toxic to infusoria, cause exogastrulation in *Strongylocentrotus intermedius* embryos and possess the ability to slow down the growth rate in stem cells cultures. *Scaphechinus mirabilis* supercritical extract turned out to be extremely toxic to the stem cells culture at concenctrations of 1000 μg/ml but cause no influence in ten times smaller concentration. Certain biological mechanisms of such behaviour of two examined supercritical extracts are not clearly understood at the moment and require further detailed investigation.

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