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Photoprotective effect of coumarin and 3-hydroxycoumarin in sea urchin gametes and embryonic cells

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ABSTRACT

Ultraviolet radiation B (UVB) represents 5% of all solar UV radiation and chronic exposure can induce harmful biological responses, including skin cancer. Prospection of new drugs with photoprotective properties and less toxic effects is constant and natural products have been the main options in this field. Coumarins are a group of natural phenolic compounds that shows several pharmacological activities. The aim of present work was to investigate the effect of coumarin and six derivatives in sea urchin gametes and zygotes exposed to UVB. Embryonic development assay was used to monitor UVB embryotoxicity. Firstly, we demonstrated that coumarin inhibited first embryonic cell division from 5 μM (EC_{50} = 52.9 μM) and its derivatives showed an embryotoxic effect ten times higher. Then, gametes or zygotes were treated with coumarin compounds before or after UVB exposure (UVB doses ranged from 0.056 to 0.9 kJ m^{-2}). Pretreatment of gametes or zygotes with coumarin or 3-hydroxycoumarin (1 μM, both) decreased UVB embryotoxic effect. Protective effect of the compounds was observed only when cells were treated previous to UVB exposure. Coumarin derivatives 4-hydroxycoumarin, 6-hydroxycoumarin, 7-hydroxycoumarin, 6,7-dihydroxycoumarin and 6-methoxy-7-hydroxycoumarin did not exhibit photoprotective activity. Our data provides evidences that coumarin and 3-hydroxycoumarin can be a promising class of photoprotective drugs.

1. Introduction

Ultraviolet (UV) radiation environmental levels have increased in the three last decades and a high incidence is expected until half of this century [1]. Chronic exposure to UV radiation, especially UVB, can induce harmful biological responses, including erythema, edema, burning, hyperplasia, immunosuppression and premature aging. These changes are directly or indirectly related to skin diseases, including cancer [2].

UVB represents approximately 5% of all solar UV radiation [3] and promotes cell damage by photochemical (direct effects) or photodynamic reactions (indirect effects). Photochemical effects are induced when proteins or nucleic acids are photochemically degraded, resulting in partial or total loss of their biological functions. Photodynamics reactions produce reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydroxyl radical (**.**OH) and hydrogen peroxide (H_2O_2). These species react with lipids, proteins and DNA, inactivating its biological functions [4].

In the last decades, there was an increasing incidence of diseases related to UVB [1]. This fact provides a great concern in the search for substances to be used in the prevention of damage caused by excessive UVB exposure. The prospection of new drugs with photoactive properties without toxic effects is constant and natural products are the main options in this field [3]. Furthermore, synthetic analogs of natural products with improved potency and safety have been synthetized and natural products are often used as starting points for drug discovery [5,6].

Coumarins are a group of natural phenolic compounds found in plants [7], bacteria [8] and fungi [9]. Nearby, 3400 coumarins were isolated from natural resources and others were synthesized from the basic molecular structure (1,2-benzopiron) [10,11]. The simplest compound of this class is coumarin, which is a lactone derived from acid-hydroxy-cinnamic. Coumarin is a fragrant organic chemical compound and has been used as an aroma enhancer in pipe tobaccos and certain alcoholic drinks, although it is banned as a flavorant food additive, due to concerns regarding its

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hepatotoxicity in animal models [12]. These compounds are used in pharmaceutical industry – in combination with heparin or troxerutine hydroxyethyl – to varicose veins treatment and were used as chemotherapeutic against lung, kidney and prostate carcinoma [13]. Diverse pharmacological activities are attributed to coumarins, such as antiviral and antibacterial [14], anti-inflammatory [15,16], antioxidant [17,18], anticancer [19], antiparasitic [20], and vasodilator [21]. Kaidbey and Kligman [22] showed a photosensitization activity of coumarin and several of its derivatives. Nevertheless, photoprotection activity of coumarin derivatives has not been investigated.

Hydroxycoumarins are an important class of coumarins derivatives that exhibits significant roles in the prospection of pharmacological active compounds [23]. Some hydroxycoumarins have been used in pharmaceutical formulations, such as 4-hydroxycoumarin, a known vitamin K antagonist clinically used as anticoagulant [24]; and 7-hydroxycoumarin, an antioxidant and photoprotective compound used as a sunscreen component [25,26]. However, the pharmacological proprieties of certain hydroxycoumarins (*e.g.*, 3-hydroxycoumarin) have not been investigated. Despite having a potential antioxidant activity due to oxygen atoms in the ortho-position, 3-hydroxycoumarin is an understudied compound [27].

The sea urchin embryonic development is a suitable experimental model for the study of the biological effects of UV radiation. Since 1908 echinoderms development has been widely used in scientific works [28]. This model has been used in the investigation of pharmacological activities of many compounds [29–31] as well as widely used to elucidate the effects of UV radiation [32,33]. Sequencing of the sea urchin *Strongylocentrotus purpuratus* genome [34] and a detailed description of the developmental transcriptome [35] led to the identification of genes implicated in a variety of biological processes, and highlighted the importance of such organisms to biological science. Genome investigations additionally provided a platform for predicting and analyzing proteins that are involved in responses to stresses, such as UV radiation [36].

Previous studies demonstrated the sensibility of sea urchin gametes and embryos to UVB. Au and colleagues [37] showed that spermatozoa exposure of *Anthocidaris crassispina* inhibited the fertilization. Exposure of eggs also inhibited the fertilization process in *Sphaerechinus granularis* [38]. Similarly, Irradiated fresh fertilized eggs of *Strongylocentrotus droebachiensis* exhibited significant decrease in cell viability, DNA damage and overexpression of cell cycle genes, such as p53 and p21 [39]. Recently, our group demonstrated that spermatozoa, eggs or zygotes of *Echinometra lucunter* were sensitive to UVB relevant doses [40].

Thus, the aim of the present work was to investigate the photoactivity of coumarin derivatives in sea urchin gametes or zygotes exposed to UVB. We demonstrate that coumarin and 3-hydroxycoumarin protected spermatozoa, eggs or zygote against UVB damage and this activity was observed when treatment with both compounds was performed after to irradiation.

2. Materials and methods

2.1. Coumarins and its derivatives

Coumarin (**1**), 3-hydroxycoumarin (**2**), 4-hydroxycoumarin (**3**), 6-hydroxycoumarin (**4**), 7-hydroxycoumarin (**5**), 6,7-dihydroxycoumarin (**6**) and 6-methoxy-7-hydroxycoumarin (**7** – Fig. 1) were purchased from Sigma–Aldrich (St. Louis, MO). Stock solutions were prepared in DMSO and stored at 4 °C.

2.2. Capture and maintenance of sea urchins

Adult *E. lucunter* (Linnaeus, 1758) sea urchins were collected at Atlantic Ocean (João Pessoa/Brazil, 7°07′S, 34°49′O). Specimens were transported to the laboratory in plastic containers with local seawater. Individuals were washed with filtered sea water (FSW – filtered with a 50 μm filter mesh) to remove biological contaminants, and placed in a reservoir with 4 L FSW/animal, under constant oxygen supply for a maximum of 15 days. Animal capture and experimental procedures were authorized by the Instituto Chico Mendes de Conservação da Biodiversidade (authorization code 32105-1).

2.3. Collection of gametes and fertilization

This procedure was adapted from Costa-Lotufo and colleagues [41]. Spawning was induced by an intracoelomic injection of 0.5 M KCl (3 mL). Gametes were collected for a maximum of 15 min after injection. Sperm were collected with glass Pasteur pipette and kept at 4 °C until use; eggs were directly collected in FSW and washed twice in 500 mL of FSW. Egg suspensions were adjusted to 1×10^4 cells/mL in FSW. Dry sperm were diluted to 1:5000 in FSW for fertilization procedure. Fertilization envelope elevation was monitored under light microscopy.

2.4. Embryonic development assays

Embryos (1×10^4 embryos/mL) were incubated in sterile 24 multiwells plates at 26 ± 2 °C and coumarin (**1**) or coumarin derivatives (**2**–**7**) were added 10 min after fertilization at different concentrations and kept until the end of the assay (embryo cultures were not washed). Embryos aliquots were fixed with 2% formaldehyde at 90 min (2-cell embryos/first cleavage stage); 120 min (4-cell embryos/second cleavage stage) or 240 min (morula stage, embryos with at least 16 cells) post-fertilization. Embryonic developmental stages were monitored under light microscopy at 400× magnification. A total of 100 embryos were evaluated for each sample at each time point. Each experiment was independently performed three times in triplicate. Negative controls contained the same amount of DMSO used at the maximum compounds concentration assayed (1% v/v). Under this concentration, DMSO *per se* had no effect on embryo development.

2.5. UVB irradiation

UVB source was two lamps emitting a continuous spectrum between 290 and 315 nm (TL20 W/12RS, Phillips, Amsterdam, Holland). UVB irradiance was 20 W/m^{-2}. UVB radiation was carried out according to Carini and colleagues [42]. UV doses ranged from 0.056 to 0.9 kJ m^{-2}, as measured with Vilber Lourmat dosimeter (model VLX-3W, UV Products Inc.) equipped with wavelength sensor to UVB (312 nm). Samples were irradiated 10 min before – or 10 min after – coumarins derivatives treatment in uncovered 50 mm Petri dishes. Eggs or spermatozoa (gamete irradiation protocol) were treated with UVB doses and fertilized as described above. Freshly fertilized eggs (zygote/embryo irradiation protocol) were treated with UVB doses, 10 min post-fertilization. UVB exposure never exceeded 1 min. Embryonic development was conducted in the absence of white light. Embryos aliquots were fixed with 2% formaldehyde at 90 min (2-cell embryos/first cleavage stage), 120 min (4-cell embryos/second cleavage stage) or 240 min (morula stage) post-fertilization and embryonic developmental stages monitored under light microscopy at 400× magnification. A total of 100 embryos were evaluated for each sample at each time point. Each experiment was independently performed three times in triplicate.

2.6. Statistical analysis

All results are expressed as mean ± standard error of the mean. The statistical significance of differences among groups was