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Omega-3 fatty acids are able to modulate the painful symptoms associated to cyclophosphamide-induced-hemorrhagic cystitis in mice

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Abstract

This study investigated the effects of the long-term dietary fish oil supplementation or the acute administration of the omega-3 fatty acid docosahexaenoic acid (DHA) in the mouse hemorrhagic cystitis (HC) induced by the anticancer drug cyclophosphamide (CYP). HC was induced in mice by a single CYP injection (300 mg/kg ip). Animals received four different diets containing 10% and 20% of corn or fish oil, during 21 days. Separated groups received DHA by ip (1 μmol/kg) or intrathecal (i.t.; 10 μg/site) routes, 1 h or 15 min before CYP. The behavioral tests (spontaneous nociception and mechanical allodynia) were carried out from 1 h to 6 h following CYP injection. Bladder inflammatory changes, blood cell counts and serum cytokines were evaluated after euthanasia (at 6 h). Immunohistochemistry analysis was performed for assessing spinal astrocyte and microglia activation or GPR40/FFAR1 expression. Either fish oil supplementation or DHA treatment (ip and i.t.) markedly prevented visceral pain, without affecting CYP-evoked bladder inflammatory changes. Moreover, systemic DHA significantly prevented the neutrophilia/lymphopenia caused by CYP, whereas this fatty acid did not significantly affect serum cytokines. DHA also modulated the spinal astrocyte activation and the GPR40/FFAR1 expression. The supplementation with fish oil enriched in omega-3 fatty acids or parenteral DHA might be interesting nutritional approaches for cancer patients under chemotherapy schemes with CYP.

Keywords: Hemorrhagic cystitis; Cyclophosphamide; Omega-3; Mice; GPR40; DHA

1. Introduction

The beneficial prophylactic effects of fish-oil-derived omega-3 fatty acids, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been extensively demonstrated in metabolic disorders and cardiovascular diseases [1–3]. As a consequence, the dietary supplementation with omega-3 fatty acids for the general adult population has been widely recommended during the last decade [4]. Compelling evidence also indicates that omega-3 fatty acids might be useful for cancer patients, even by enhancing the antitumor effects of chemotherapy agents or by preventing the related side effects [1,5,6]. Furthermore, it has been shown that dietary daily intake of supplements enriched with EPA/DHA prevents cancer-induced cachexia and/or sarcopenia, providing body weight maintenance in affected individuals [3]. For instance, Murphy *et al.*[7] demonstrated that supplementation with 2.2-g fish oil per day prevented the losses of body weight and muscle mass in patients with nonsmall cell lung cancer under chemotherapy. In addition, Finocchiaro *et al.*[8] presented similar results in a multicenter study conducted with the same cancer type, revealing a reduction of inflammatory and oxidative parameters in patients receiving omega-3 fatty acids. Interestingly, it was demonstrated that DHA-rich diet displayed protective effects on the body weight loss in rats treated with the chemotherapy drug doxorubicin [9].

Hemorrhagic cystitis (HC) is an adverse effect of chemotherapy or radiotherapy on the pubic region. The most common symptoms are dysuria, frequency, nocturia, urgency, intense suprapubic pain and gross hematuria [10,11]. Cyclophosphamide (CYP) is an alkylating agent employed in chemotherapy schemes for a series of different types of cancer, such as non-Hodgkin's lymphoma, leukemia and breast cancer, among other solid tumors [12]. Despite the potential antitumor effects of CYP, its use is highly associated to the occurrence of HC due to the generation of the urotoxic metabolite acrolein, affecting 2–40% of the treated patients. Preventive approaches, including intense bladder irrigation and the coadministration of sodium-2-mercaptoethane sulfonate (Mesna), have been used,

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although these strategies are not totally effective in clinics, especially after long-term exposure to high doses of CYP [13]. Therefore, it is reasonable to propose that omega-3 rich diets could be useful in preventing CYP-induced HC. In fact, it was previously demonstrated that repeated treatment with EPA (100–300 mg/kg) was able to reduce CYP-elicited genotoxicity and oxidative stress in mice, although the inflammatory or painful urological alterations have not been assessed in this study [14]. Notably, high levels of omega-3 fatty acids have been positively correlated with disease remission rates in patients with non-Hodgkin's lymphoma that had been treated with different chemotherapy agents, including CYP [15].

In light of literature data, the present study investigated to what extent the long-term supplementation with marine omega-3 fatty acids or the acute treatment with DHA might prevent the collateral effects associated with HC induced by CYP in mice, aiming to assess the mechanisms related to the protective effects of omega-3 in this *in vivo* experimental model.

2. Methods

2.1. Animals

Male Swiss mice (25–30 g; total number of 200 mice) obtained from the Universidade Federal de Pelotas were used throughout the study. The animals were housed in groups of five per cage and maintained in controlled temperature (22±2°C) and humidity (60–70%), under a 12-h light–dark cycle, with food and water *ad libitum*. Experiments were conducted in accordance with current guidelines for the care of laboratory animals, ethical guidelines for the investigation of experimental pain in conscious animals and the ARRIVE Guidelines Checklist [16,17]. All the experimental procedures were approved by the Animal Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul (RS) (Protocol Number: CEUA 12/00303). The experiments were performed between 8:00 and 12:00 a.m. to minimize the potential circadian variations in the behavioral responses. The number of animals and the intensity of noxious stimuli were the minimum necessary to demonstrate the consistent effects of the treatments.

2.2. Drugs

The following drugs were used: CYP (Genuxal — Baxter Oncology GmbH, Halle/Westfalen, Germany) was purchased at Medilar (Porto Alegre, Brasil), being diluted in distilled water. DHA in 1% ethanol was purchased from Cayman Chemicals (Michigan, USA) and was diluted in phosphate-buffered saline (PBS) until the desired concentration. The final ethanol concentration never exceeded 0.1%.

2.3. Diets and treatments

Four different diets were prepared using Nuvilab CR-1 chow, with the addition of corn oil (50% of omega-6 fatty acids) or concentrated fish oil (55% of omega-3 fatty acids); each oil was added at two distinct concentrations, 10% and 20%. The detailed composition of diets is provided in Supplementary Table 1. Dietary scheme started 1 month after birth, lasting 21 days, being HC induced at the 22th day [18]. In a separate series of experiments, the animals received DHA at different schedules of administration. DHA was acutely dosed, 1 h or 15 min before the induction of HC, at 1 μmol/kg (intraperitoneal; ip) or 10 μg/site (intrathecal; i.t.), respectively [19,20]. DHA-treated groups received only regular chow during all the experimental periods.

2.4. Induction of cystitis and nociception assessment

HC was induced by a single ip administration of CYP (300 mg/kg). Immediately after the ip injection of CYP, mice were housed in individual plastic cages, without sawdust bedding, and the spontaneous nociception behavior was measured for 2 min, every 30 min, over a total period of 4 h. The behavioral alterations were scored according to the following scale: 0=normal, 1=piloerection, 2=strong piloerection, 3=labored breathing, 4=licking of the abdomen or 5=stretching and contractions of the abdomen; the activity (walking, grooming and rearing) was recorded in seconds [21].

At the end of the fifth hour, von Frey test was conducted to evaluate the mechanical allodynia in the lower abdominal area. For this experimental set, 6–10 mice/group (supplementation-treated animals) or 8 mice/group (DHA ip and i.t.) were used. Mice were placed individually in clear Plexiglas boxes (9 cm×7 cm×11 cm) on elevated wire mesh platforms to allow access to the abdomen. The withdrawal response frequency was measured after 10 applications (duration of 1 s each) of 0.4 g von Frey hair (VFH) (Stoelting, Chicago, IL), obtaining the percentage of frequency responses. The following reactions were considered as a positive withdrawal response: sharp retraction of the abdomen, immediate licking, scratching at the site of filament application and/or jumping [22].

In separate experimental groups, to evaluate the mechanical allodynia during all the 6-h period after HC induction, the 0.4-g VFH was applied below to the plantar surface of the right hind paw (to assess referred pain) or to the lower abdomen area, as described by Meotti *et al.*[23], with slight modifications. The nociceptive responses were evaluated at different timepoints (1, 2, 4 and 6 h) following CYP injection. The 0.4-g VFH filament application to the hind paw and to the lower abdomen area produces a mean withdrawal frequency of about 10% and 30%, respectively, which are adequate values for the measurement of mechanical hypersensitivity. For this series of experiments, 5–6 mice or 9–11 mice/group (10% and 20% dietary lipid concentration, respectively) or 7–8 mice/group (DHA ip and i.t.) were used. All the behavior assessments were performed by trained experimenters blind to the treatment groups. In all the cases, after 6 h of the CYP injection, the animals were killed by deep inhalation of sevoflurane for further evaluation of inflammatory parameters.

2.5. Determination of bladder inflammatory parameters

This method was based on criteria established by Gray *et al.*[24]. Following euthanasia (6 h after CYP application), all the bladders were dissected free from connecting tissues and transected at the bladder neck. Each bladder was macroscopically evaluated, by an examiner unaware of the treatment groups. The edema formation was categorized as severe (3), moderate (2), mild (1) or absent (0). Edema was considered severe when fluid was seen externally in the walls of the bladder, as well as internally. When edema was confined to the internal mucosa, it was reported as moderate; when it was between normal and moderate, the edema was defined as mild. Bladders were also examined for hemorrhage and categorized into four classes, depending on the presence of intravesical clots (3), mucosal hematomas (2), dilatation of the bladder vessels (1) or normal aspect (0). As an additional measure of bladder edema, the wet weight of each bladder was recorded and expressed as milligrams (mg) per 100 g of animal [24]. For this purpose, animals from the first set of behavioral tests were used.

2.6. Hematological parameters

After euthanasia, a small drop of blood was collected for the smear evaluation, using Giemsa staining [25]. Differential cell counts (neutrophils, eosinophils, basophils, lymphocytes, monocytes and immature cells) were estimated under an ×40 objective, by counting 100 cells [26]. Representative pictures were captured. For this analysis, the animals from the second set of mechanical allodynia experiments were used.