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Curcuminoids from *Curcuma longa*L. reduced intestinal mucositis induced by 5-fluorouracil in mice: Bioadhesive, proliferative, anti-inflammatory and antioxidant effects

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ABSTRACT

Introduction: Intestinal mucositis is a frequent limiting factor in anticancer therapy and there is currently no broadly effective treatment targeted to cure this side effect.
Objective: This study aimed to evaluate the effects of a mucoadhesive formulation containing curcuminoids (MFC) from *Curcuma longa* L. on the pathogenesis of 5-fluorouracil (5-FU)-induced intestinal mucositis.
Methods: Three intraperitoneal 5-FU injections (200 mg/kg) were used to induce intestinal mucositis in adult Swiss male mice. Treatment was provided orally (MFC 3.75, 7.5 and 15 mg/kg), thirty minutes before 5-FU injections, daily until euthanasia. Duodenal samples were collected to perform morphometric and histopathological analysis, to investigate the expression of Ki-67, p53, Bax and Bcl-2 by immunohistochemistry, to evaluate neutrophil activity myeloperoxidase (MPO)-mediated and oxidative stress by malondialdehyde (MDA) determination. Mice body weight was assessed as well.
Results: As expected, 5-FU induced a significant weight loss (~17%, $P < 0.001$), shortening in villi height (~55.4%) and crypts depth (~47%), and increased (~64%) the histological severity score when compared to other groups ($P < 0.05$). These pathological changes were markedly alleviated by the three MFC treatment doses ($P < 0.05$), in special with the dose MFC 15 mg/kg. This dose also stimulated cell proliferation by ~90% in the epithelial cells lining from villi and crypts ($P < 0.05$), reduced MPO levels and MDA formation by 60% and 44%, respectively ($P < 0.05$).
Conclusions: Our data suggest the therapeutic potential of the formulation for treating intestinal mucositis in mice. Supplementary studies are underway searching for the elucidation of mechanisms involved in the protective effects of MFC in order to make this formulation a clinical tool for mucositis treatment.

1. Introduction

The clinical term mucositis describes a syndrome characterized by ulceration in the entire gastrointestinal tract mucosa and associated symptoms [1]. The frequency of mucositis cases varies and is influenced by factors such as patient's diagnosis, age, health status, and the type, dose and drug administration frequency [2].

It is very difficult to thoroughly survey the occurrence of oral and, in particular, intestinal mucositis induced by chemotherapy and/or radiotherapy in humans. This is due to the fact that it is not a locally mediated process but rather, a systemic one, with concomitant oral lesions, emesis, anorexia, adynamia, abdominal pain and, in particular, diarrhea [3]. 5-Fluorouracil, methotrexate and other antimetabolite agents are the most established causes of chemotherapy-induced mucositis. These agents attack tumor cells and constantly dividing normal cell types, which result in

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destruction of the basal cells and damage to connective tissue in the gastrointestinal tract [1,2,4].

Even though scientists have been studying mucositis for over thirty years [5], there is still no truly effective treatment targeted to cure this side effect [2,6]. Current palliative treatments for intestinal mucositis include anti-inflammatories, systemic antibiotics, antidiarrheal medication and nonspecific painkillers [4,6,7]. While no approach has yet standard gold proven efficacy, numerous agents are in used. Compounds such as growth factors (palifermin), free radical scavengers (amifostine), probiotics (*Lactobacillus* spp.), zinc sulphate, antioxidants (n-acetyl cysteine, L-glutamine), benzydamine hydrochloride and coating agents (CAM2028, Gelclair, MuGard) have been tested with proved efficacy [4–9].

Herbal products, particularly those with anti-inflammatory and antioxidant activities such as acteoside [10], iberogast [11],*Chimonanthus nitens* var. *salicifolius* [12], *Bidens pilosa* L. [13] and *Rhodiola algida* [14] are promising for the treatment of the mucositis. *Curcuma longa* L. (also known as turmeric), belongs to the ginger family (Zingiberaceae) and is traditionally used in cooking for flavoring purposes [15]. The anti-inflammatory, antioxidant and antitumor properties of *C. longa* L. constituents spurred the investigation of the molecular mechanisms associated with these biological activities [15,16]. This is a chemically diverse herb and up until 2011, approximately 235 chemical compounds, mainly phenolic and terpenoid, have been identified [17].

It has been shown that formulations containing mucoadhesive polymers, such as poloxamer 407, have the potential to treat mucositis [4,6,13]. Mucoadhesive systems interact with mucin that covers mucosa, thereby promoting prolonged and localized contact between the pharmaceutical dosage form and absorptive tissue [18].

Given the above, a mucoadhesive liquid formulation based on poloxamer 407 and curcuminoids from *C. longa* L. was developed to improve curcuminoids solubility and to promote intimate contact between the herbal active ingredients and gastrointestinal mucosa. This formulation is hypothesized to be effective in treating mice bearing 5-FU-induced intestinal mucositis.

2. Material and methods

2.1. Mucoadhesive formulation containing curcuminoids (MFC)

MFC was prepared by mixing polyethylene glycol 400 and propilenglycol (1:1, v/v) in a heated reactor (65–70 °C). Then, 9.0% (w/v) poloxamer 407 (Basf, Ludwigshafen, Germany) was added to the mixture under mechanical stirring until complete dispersion. After that, curcuminoids from *C. longa* L. extract (>95% curcuminoid content, Gamma Comércio Importação & Exportação LTDA, São Paulo, Brazil) was added to the organic phase and pH was adjusted by using 0.1 M citric acid (pH 6.5). The final concentration of curcuminoids was 25 mg/mL. An aqueous fraction was prepared by dispersing 6% (w/v) of poloxamer 407 in purified water, under constant stirring. After dispersion, Soluplus® (Basf, Ludwigshafen, Germany), sodium metabisulphite and sodium bisulphite were added under stirring. The aqueous fraction was then heated up to 65 °C and poured into organic fraction. The mixture was stirred for 30 min. MFC was then bottled in ambar flask and stored at room temperature until use. A blank mucoadhesive formulation (control) was prepared as described above, but without adding curcuminoids extract.

2.2. Mice

Swiss (8–10-week-old) male albino mice provided by our Institute's Central Bioterium were kept under controlled temperature (25 ± 2 °C) and light conditions (12h-light–dark cycle), with free access to water and standard chow pellets (Presence nutrição animal, São Paulo, Brazil). Mice were acclimatized for a week before beginning the experiments. All animal treatment and surgical procedures were carried out in accordance with the norms of the Brazilian College of Animal Experimentation (COBEA) and approved by the local Research Ethics Committee (Federal University of Goiás 036/2012).

2.3. Experimental design

Considering that the 5-FU regime was able to induce mucositis homogeneously in the mice, as well as ethical aspects in the use of animals, three independent essays were performed with mice randomly allocated into five experimental groups (Fig. 1) of 5 animals each. Groups were as follow: negative control (blank mucoadhesive formulation 0.6 mL/animal, via gavage, from day 1–6); positive control (5-FU 200 mg/kg, i.p., days 4–6); and treated (MFC 3.75 mg/kg; 7.5 mg/kg and 15 mg/kg, via gavage, from day 1–6). 5-FU (Sigma–Aldrich, St. Louis, USA) was diluted in sterile water and injected intraperitoneally (i.p.), daily, for 3 consecutive (days 4, 5 and 6) days into mice to induce mucositis. During the induction of mucositis, MFC treatment was given thirty minutes before 5-FU injections, daily throughout the trial to the respective groups via gavage. On the 7th day of the experiment, animals were anesthetized by 10 mg/kg of xylazine and 100 mg/kg of ketamine hydrochloride administered i.p. and euthanized by cervical dislocation [19]. Duodenal samples, 10 cm after pyloric sphincter were removed to perform the assays.

2.4. Body weight assessment

Mice were daily weighed throughout the entire experimental period (7 days). The values were expressed as weight variation (%) in relation to the weight at the beginning of the experimental period [13].

2.5. Morphometric and histopathological analysis

Duodenal samples were fixed in 10% phosphate buffered formalin, embedded in paraffin, sectioned at 5 μm thickness (Leica RM 2155, Heidelberg, Germany) and stained by hematoxylin and eosin (H&E). For intestinal morphometric examination, villus height (estimated from top of the villus to crypt-villus junction) and crypt depth (defined as adjacent intussusception) were determined using the AxioVision 40 software (Carl Zeiss, Jena, Germany) and a light microscope (Axio Scope A1Carl Zeiss, Jena, Germany) at 20X and 40X objectives. In this triple-blinded trial, ten villi and crypts from each animal duodenal slide were measured and averaged for each group.

Histopathological analysis evaluated the severity of intestinal tissue damage caused by 5-FU using ten different histological parameters, which consisted in villi and crypts size reduction, villi and crypts disruptions and abscess formation, muscularis thickening, epithelium and muscular layer integrity, inflammatory cells infiltration, vacuolization and mucosal edema. For each score, a value of 0–3 was given and combined for an overall score [10,13].

2.6. Immunohistochemistry for Ki-67, p53, Bax and Bcl-2

Duodenal samples were embedded in paraffin, sectioned at 3 μm thickness (Leica RM 2155, Heidelberg, Germany) and mounted on silane-coated slides. The slides were then deparaffinized, rehydrated in graded ethanol and washed with tris-buffered saline (TBS) (pH 7.4). This was followed by incubation in a citric acid buffer solution (pH 6.0) at 96 °C for 20 min.