PHARMACOLOGICAL SCREENING OF MUCUS PRODUCED BY THE PACAMÃ FISH, Lophiosilurus alexandri

SUMMARY

Recently, fish byproducts have been appraised as a source of structurally diverse bioactive compounds. In this context, fish mucus has revealed a myriad of pharmacological activities. This study describes a new bioactive molecule release system composed of protein nanoparticles from pacamã (Lophiosilurus alexandri) mucus, an endemic fish to the São Francisco River (Brazil). The process of obtaining and applying these nanoparticles, composed of proteins present in the pacamã mucus, as biocompatible carriers of bioactive molecules is also addressed herein. Therefore, the external mucus of the fish was collected, lyophilized and hydrated in deionized water containing the template molecule. After pH adjustment to 7.5, followed by sonication and centrifugation, the formed nanoparticles were collected in the supernatant. Additionally, the mucus and the particles were characterized, and pharmacological effects were evaluated regarding their antifungal, antibacterial, anticancer, anti or pro-inflammatory and antinoceptive proprieties. NP-Mucus did not exhibit antibacterial activity against Pseudomonas aeruginosa or Staphylococcus aureus. However, it showed potential effects against Candida albicans with a minimum inhibitory concentration (MIC) of 1 µg/mL, and significant pro-inflammatory role, reflected by cellular recruitment activity and healing effects. Therefore, future studies are now needed in order to identify specific compounds in NP-mucus responsible for the observed effects in order to provide new pharmacological and therapeutic strategies.

KEY-WORDS: Nanoparticles. Antifungal. Anticancer. Pro-inflammatory. Fish mucus.

RESUMO

Recentemente, os subprodutos de peixes foram avaliados como uma fonte de compostos bioativos estruturalmente diversos. Nesse contexto, o muco dos peixes revelou uma miríade de atividades farmacológicas. Este estudo descreve um novo sistema de liberação de moléculas bioativas compostas por nanopartículas de proteínas do muco do pacamã (Lophiosilurus alexandri), um peixe do Rio São Francisco (Brasil). O processo de obtenção e aplicação dessas nanopartículas, compostas por proteínas presentes no muco do pacamã, como carreadores biocompatíveis de moléculas bioativas também são abordados neste artigo. Para tanto, o muco externo dos peixes foi coletado, lyophilizado e hidratado em água deionizada contendo a molécula template. Após ajuste do pH para 7.5, seguido de sonicação e centrifugação, as nanopartículas formadas foram coletadas no sobrenadante. Adicionalmente, foram caracterizados o muco e as partículas, sendo avaliados os efeitos farmacológicos quanto às propriedades antifúngicas, antibacterianas, anticancerígenas, anti ou pró-inflamatórias e antinoceptivas. NP-Muco não exibiu atividade antibacteriana contra Pseudomonas aeruginosa ou Staphylococcus aureus. No entanto, apresentou efeitos potenciais contra Candida albicans com concentração inibitória mínima (MIC) de 1 µg/mL, além de importante papel pró-inflamatório, refletido pela atividade de recrutamento celular e efeitos cicatrizantes. Portanto, estudos são necessários para identificar compostos no NP-muco responsáveis pelos efeitos observados, a fim de fornecer novas estratégias farmacológicas e terapêuticas.

PALAVRAS-CHAVE: Nanopartículas. Antifúngico. Anticâncer. Pró-inflamatório. Muco de peixe.

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INTRODUCTION

Fish produce substances that serve as important physical barriers that protect their host against invasion of foreign agents (Guardiola et al., 2017). A complex viscous secretion called mucus covers the epithelial cells (Van der Marel et al., 2010), while its functional properties depend on the ability to form a gel over the epithelial surface (Bragadeeswaran and Thangaraj, 2011). Besides being part of the host defense and osmoregulation system (Jakowska, 1963; Rosen & Cornford, 1971; Brinchnann, 2016), the mucous layer is a key part of fish immunity and contains innate immune components such as lysozyme, proteases, immunoglobulin, lectin, proteolytic enzyme complement, carbonic anhydrase, crinotoxins, calmodulin, C-reactive protein, and peptides (Alexander & Ingram 1992; Subramanian et al., 2007; Whyte, 2007), as well as ceruloplasmin and peroxidase (Guardiola et al., 2017).

In the latter years, interest in mucus has increased greatly among researchers as recent studies have shown that the mucus that covers the body of fish contains molecules with activity of pharmacological interest, such as anticancer, haemolytic (Balasubramanian et al., 2016), anti-inflammatory (Ramos et al., 2012), bactericidal (Krishna, 2017; Guardiola et al., 2017) and antibacterial activity (Nagashima et al., 2001). However, there is no information regarding the pharmacological effects of the mucus produced by the carnivorous freshwater fish Lophiosilurus alexandri, also known as pacamã. This is the largest species of the pseudopimelodidae family, being able to reach 500 mm in length (Britski et al., 1986) and between 4 to 8 kg in body mass (Sato et al., 2006; Salaro et al., 2015). Endemic to the San Francisco River (Shibata, 2003), one of the longest rivers in Brazil spanning about 2,756 km (Guimarães, Landau & Barros, 2011), this ecosystem is home to a rich and diversified, however poorly studied, ichthyofauna (Barbosa et al., 2017) regarding the occurrence of biologically active compounds. Therefore, this study sorted and characterized nanostructured systems produced from fish mucus, and assessed the mucus for a range of pharmacological activities, thus revealing great resource for drug discovery.

MATERIAL AND METHODS

2.1. Mucus collection

The epidermal mucus from catfish pacamã (Lophiosilurus alexandri) was collected as described by Ross et al. (2000) with slight modifications. The skin of the lateral back region of the fish body was gently scraped with a spatula and the mucus was transferred to a falcon plastic bottle and conditioned at -80 °C and subsequent freeze-drying. The fish samples (n=15) used in this study came from breeders that were collected in São Francisco River and were then adapted to captive conditions in the Laboratory of Aquaculture (Laqua) of the Federal University of Minas Gerais (UFMG). The fish were kept in adequate tanks of oxygen and food until their identification and subsequent extraction of mucus. All the methodological data from mucus production were registered in two patents, BR 10 2018 073304 4 and BR 13 2020 016849 4. We characterized the measures of size, polydispersity index and zeta potential. The nanoparticle tracking analysis was used to provide size and concentration distribution. The surface morphology of the nanoparticles was analyzed by Atomic Force Microscopy (Charlie-Silva et al., 2019).

2.2. In vitro assays

2.2.1. Antimicrobial Assay

To determine the antimicrobial activity, an assay was performed through the microtiter broth dilution method, as described by Bulet et al. (1993) and Conceição et al. (2011) with modifications. The samples were tested against Pseudomonas aeruginosa (ATCC 15442), Staphylococcus aureus (ATCC 25923) and Candida albicans (ATCC 10231). Pre-inoculum of the strains were prepared in BHI broth (Kasvi, Curitiba, PR, Brazil) and standardized to 10^7 cells per ml through spectrophotometry by diluting the inoculum in saline solution. The mucus and peptide fractions were dissolved in Ultrapure water and applied to the wells with inoculum, with the final volume of 150 μL (20μL from the sample diluted in Ultrapure water and 130 μL from the inoculum in BHI broth) in each well. The positive control prepared in this assay consisted of 130 μL of inoculum with addition of 20 μL of Streptomycin (15μg/mL being the initial concentration). After 18 hours of incubation at 37°C, the microorganisms growth inhibition was determined in the spectrophotometer through the absorbance at 590nm.

2.3. In vivo assays

2.3.1. Animals

The experiments were conducted on Wistar rats (160–200 g, male, age ≥2 months) from CEBIO-UFMG. The animals were housed in a temperature-controlled room (23 ± 1 °C) on an automatic 12 h light/dark cycle (06:00–18:00 h). All of the tests were conducted during the light phase (08:00–17:00 h). Food and water were freely available until the beginning of the experiments. All of the animal procedures and protocols were approved by the ethics committee of Minas Gerais Federal University (CEUA-UFMG, 336/2017).

2.3.2. Evaluation of the anti-inflammatory activity

The anti-inflammatory properties from the NP-Mucus from pacamã (L. alexandri) were evaluated using a carrageenan-induced peritonitis model in rats. For this, 24 male wistar rats were randomly divided into four groups (n = 6 each): 1- Control (C) (Unstimulated), 2- Carrageenan 200 μg (CG), 3-Carrageenan 200 μg + DMSO 10% in PBS (vehicle) and 4- Carrageenan 200 μg + DMSO 10% in PBS (vehicle) + NP-Mucus (2% mucus). The agents tested were injected intraperitoneally 30 min prior to the intrathoracic injection (i.t.) of carrageenan (200 μg/100gL) or its vehicle (PBS/100μL). Four hours after the induction of peritonitis, all rats were killed in a CO2 (carbon dioxide) chamber. Cells from the...
peritoneal cavity were harvested by injection of 4 mL of PBS and total cell counts were performed in a modified Neubauer chamber using Turk stain. The results were presented as the number of cells per cavity.

2.3.3. Nociceptive activity

Hyperalgesia was induced by subcutaneous injection of carrageenan (Sigma USA; 200 μg per paw) into the plantar surface of the hind paw. Hyperalgesia was measured according to the paw pressure test described by Randall and Sellito (1957). An analgesimeter was used (Ugo-Basile, Italy) with a cone-shaped paw-presser with a rounded tip, which applies a linearly increasing force to the hind paw. The weight in grams (g) required to elicit the nociceptive response of paw flexion was determined as the nociceptive threshold. A cutoff value of 300 g was used to reduce the possibility of damage to the paws. The nociceptive threshold was measured in the right paw, determined as the average of three consecutive trials, and expressed in grams (g). The threshold was recorded before the carrageenan-injection and three hours after this procedure. One group (n=4) received DMSO 10% in PBS (vehicle) and the second group (n=4) received DMSO 10% in PBS (vehicle) + NP-Mucus (2% mucus). Measurements were taken every ten minutes. The results were analyzed using the Graph Pad Prism 5.0 and expressed as mean ± SEM. Statistically significant differences among groups were calculated by the application of analysis of variance (ANOVA) followed by the Bonferroni post-test, with the level of significance set at P<0.05.

2.3.4. Wound healing rat model

Ten rats were anesthetized with ketamine hydrochloride and xylazine by intramuscular injection (IM). After shaving the hair on the back of each rat, the skin was asepticised with iodine three times followed by 70% alcohol to remove the iodine, to realize one incision with area of 1 cm², located on the dorsal line of each animal. Postoperative animals were housed in properly disinfected cages to prevent infection or further damage to the wounds after recovering from anesthesia. After surgery, the rats were randomly divided into two groups with 5 animals each. The control group (DMSO 10% solution) was administered. The nanoparticle treatment group (Nanoparticle-Mucus) was administered 100 microliters. Wound areas were recorded on days 1, 2, 3, 4, 5, 6 and 7 post-wounding using a digital caliper. All rats from each group were euthanized with underdosage of anesthetics on day 7 post-wounding. Histological sections of the skin (5 μm, stained with H&E) were examined by light microscopy (Zeiss Axiophot microscope, Oberkochen, Germany), and a blinded experienced pathologist performed histopathologic analyses.

RESULTS

In the present study, NP-Mucus did not exhibit antibacterial activity against *Pseudomonas aeruginosa* (Gram-negative) or *Staphylococcus aureus* (Gram-positive). However, it showed potential effects against *Candida albicans* with a minimum inhibitory concentration (MIC) of 1 µg.

All carrageenan-induced animals showed increased recruitment of leukocytes in the peritoneal cavity. The highest recruitment was observed with the NP-Mucus treatments (p <0.05; Figure 1). Regarding the differential cell count, granulocytes, lymphocytes and monocytes were observed in all treatments, post-stimulus the granulocytes showed increase and no difference was observed between treatments (p <0.05). Rats with stimulation and treated with NP-Mucus presented higher percentages of granulocytes and lymphocytes in the inflammatory focus. When analyzing the granulocytes count, a significant increase (p <0.05) was observed in all treatments after carrageenan induction.

![Figure 1 - Effects of NP-Mucus from pacamã Lophiosilurus alexandri on total leukocytes recruitment in response to carrageenan injection into the peritoneal cavity of rats. The results are expressed as the mean ± SEM of 6 animals in each group. Equal letters reflect there are no differences between treatments (p< 0.05, ANOVA +Bonferroni’s test).](image)

Regarding the nociceptive activity, intraplantar administration of the epidermal mucus from *Lophiosilurus alexandri* at doses of 100 µg/paw in rats, at the third hour after injection of Cg (200 µg), induced a significant hyperalgesic effect which lasted for almost 90 minutes (Figure 2).
Regarding the healing assays, for all groups, the wound healing rates increased with time. However, a significant increase in wound-healing activity in NP-Mucus was observed when compared with the control group. At 7 days after wounding, the healing effects were good in the NP-Mucus, and their average wound healing rates were higher than the healing rate of control group (p < 0.5).

Histological sections from all groups did not demonstrate differences between treatments. After 7 days, all the fragment of hairy skin presented focally extensive areas of loss of the epithelium with exposure of the dermis (ulcer) (Figure 3). There was an intense amount of cellular debris, fibrin, neutrophilic inflammatory infiltrate, hemorrhage and few bacterial colonies (crust) over the area of ulceration. Some keratinocytes adjacent to the ulcer had discrete cytoplasmic vacuolization (discrete hydropic degeneration). In the superficial and deep dermis underlying the ulcer, there was intense fibroplasia and neovascularization (granulation tissue). Fibroblasts were predominantly organized parallel to the epidermis. The new blood vessels were arranged perpendicular to the epidermis and lined by reactive endothelium. Within the granulation tissue, there was a discrete multifocal neutrophilic inflammatory infiltrate. In the deep dermis, in the peripheral region of the lesion, there was a lymphohistiocytic inflammatory infiltrate with some multinucleated giant cells surrounding elongated and refringent (foreign body) material. The same material was also found in the cytoplasm of some multinucleated giant cells.

Figure 3 - Wound healing rat model. (A) mean values (n = 5) followed by the same letter do not differ by the Tukey test (P < 0.05). The variance analysis is represented by capital letters to compare the different treatments within each experimental period, lowercase letters to compare the evolution of each treatment in the different experimental periods. The histological images show that there were no differences in the histological lesion patterns on treatment and control groups. (B) Scarring reaction – Granulation tissue (asterisks) and dermis with discrete neutrophil inflammatory infiltrates at the margin of the lesion (arrow). H&E-staining.
DISCUSSION

In this study, we reported for the first time that an aquatic animal was studied with the aim of producing nanoparticles that can serve as carriers of bioactive molecules for human and veterinary health. The use of an epidermal mucus from a freshwater fish (*Lophiosilurus alexandri*) indicated a significant pro-healing activity. It is a biotechnological innovation that epidermal fish mucus can be applied, as adjuvant treatment, to wounds to help heal. In addition, the mucus has an important role as a stimulator to increase leukocyte migration. Furthermore, the antimicrobial property of epidermal mucus against infectious pathogens has been demonstrated.

Many reports indicate that fish skin can be used in the treatment of burns in Brazil. A biological dressing of leather fish skin of the species *Pseudoplatystoma coruscans*, popularly known as pintado, surubim-caparari, brutelo, caparari or urchin, has been described (Costa et al. 2019). The skins are treated and stored under refrigeration, until the moment of use with the objective of providing a biological dressing to be applied on extensive uninfected mammalian wounds for temporary lining or occlusion, reducing the risk of external contamination and promoting pain relief, accelerating the healing process.

In the present study, we identified the potential antimicrobial activity against *Candida albicans* of the NP-Mucus of *Lophiosilurus alexandri*. This NP-Mucus has already been found to have a minimum inhibitory concentration (MIC) of 1µg/mL. A recent study by Conceição et al. (2012) also showed antimicrobial activity against *Candida* sp of stingray mucus. Other reports have expanded the antibacterial spectrum found in fish skin mucus (Nagashima et al. 2001; Hellio et al. 2002). In addition, antimicrobial activity against infectious pathogens has also been demonstrated in epidermal mucus from rainbow trout (*Oncorhynchus mykiss*) (Austin & McIntosh, 1988).

Antimicrobial activity is most commonly found in specialized peptides, with a number of unusual structures (Rajanbabu and Chen, 2011). Bioassay-guided isolation and characterization showed a new antimicrobial peptide called myxinidine from an acidic extract of the epidermal mucus of hagfish (*Myxine glutinosa L*). These authors suggest that myxinidine may be an alternative therapeutic agent for humans. In this study, we identified and characterized the formation of nanoparticles of 210.8 nm and with Potential Zeta of -13.4 (mV). These characteristics suggest a possible application of this material as a biocompatible coating, besides the production of nanomaterials as nanoparticles (NP). Fish produces substances that serve as the important physical barriers complex viscous secretion called mucus (Van der Marel et al. 2010). The mucus is rich in proteins and 5 fractions were identified (PC1 to PC5). Herein, we suggested the formation of this nanoparticle by these proteins are strongly glycosylated, which gives the mucous layers viscoelastic and rheological properties (Jevtov et al., 2014). The mucin polymer present in the external secretion of fish is responsible for gel formation in the mucosal barrier. Therefore, the cooperation of two protein domains (Von Wildebrand Factor D domain and Proline-Threonine-Serine domain) contributes to the polymerization of mucin (Jevtov et al., 2014). Due to its characteristic pattern of glycosylation, the polypeptide backbone extends above the cell surface forming a protective barrier against pathogens. The distinct interfacial activity of mucin is due to its unique structure of repetition of strongly glycosylated hydrophilic domains, interspersed by domains of hydrophobic proteins, conferring natural surfactant property (Thasneem et al., 2013). Its biological origin mediates favorable reactions showing controlled cellular interaction and compatibility.

Other biological activities have been described in numerous species of fish, such as anti-inflammatory (Ramos et al., 2012), bactericidal activity (Krishna, 2017; Guardiola et al., 2017) and anticancer (Balasubramanian et al., 2016).

Pain measurement and observation of leukocytes recruitment induced by carrageenan are screening tools for the assessment of analgescic or anti-inflammatory properties of natural or synthetic compounds. We evaluated hyperalgesia and the total number of cells at the inflammatory site. We observed pro-inflammatory activity from NP-Mucus epidermal mucus of *Lophiosilurus alexandri*, and increased nociceptive response induced by the mucus. Pain is frequently identified in patients who come into contact with fish mucus and venom (Fenner et al., 1989). Monteiro-dos-Santos et al. (2011) and Conceição et al. (2012) reported increased recruitment of cells in response to mucus injection in the microcirculatory environment. These results can be explained by the tissue injury caused by the mucus such as degranulation of mast cells with a release of cytokines and chemokines with the degradation of the membrane phospholipids and production of arachidonic acid (AA). This acid, when metabolized by cyclooxygenase (COX), originates prostaglandins (PG), which increases vascular permeability (VP) and also causing pain (Luster, 2005).

In addition, we used an experimental model of peritonitis induced by carrageenan to verify leukocyte migration. In this model, a significant increase in leukocyte migration was observed in mucus treated animals, meaning that a significant number of cells are being attracted to the inflammatory site.

Future studies are still needed in order to identify specific compounds in NP-mucus in order to provide new pharmacological strategies for the development of a new therapeutic agent.

CONCLUSION

The mucus and the particles were characterized, and pharmacological effects were evaluated regarding their antifungal, antibacterial, anticancer, anti or pro-inflammatory and antinociceptive proprieties. NP-Mucus did not exhibit antibacterial activity against *Pseudomonas aeruginosa* or *Staphylococcus aureus*. However, it showed potential effects against *Candida albicans* with a minimum inhibitory concentration (MIC) of 1 µg/mL, and significant pro-inflammatory role, reflected by cellular recruitment activity and healing effects.
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