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Evaluation of acute and subchronic toxicity of a non-anticoagulant, but antithrombotic algal heterofucan from the *Spatoglossum schröederi* in Wistar rats

Jailma Almeida-Lima,[1] Nednaldo Dantas-Santos,[1] Dayanne L. Gomes,[1] Sara L. Cordeiro,[1] Diego A. Sabry,[1] Leandro S. Costa,[1,4] Maria de L. Freitas,[2] Naisandra B. Silva,[2] Carlos E. B. Moura,[2] Telma M. A. M. Lemos,[3] Edda L. Leite,[1] Hugo A. O. Rocha*[*,1]

[1]Departamento de Bioquímica, Universidade Federal do Rio Grande do Norte, Brazil,
[2]Departamento de Morfologia, Universidade Federal do Rio Grande do Norte, Brazil,
[3]Departamento de Análises Clínicas e Toxicológicas, Universidade Federal do Rio Grande do Norte, Brazil,
[4]Instituto Federal de Educação, Ciência e Tecnologia do Rio Grande do Norte, Campus Santa Cruz, Brazil.

Abstract: Fucan is a term used to denominate a family of sulfated polysaccharides rich in L-fucose. The brown alga *Spatoglossum schröederi*, Dictyotaceae, synthesizes three heterofucans named A, B, and C. Fucan A is a non-anticoagulant heterofucan which possesses potent antithrombotic (*in vivo*) and antiproliferative (*in vitro*) activities. However, its toxicity *in vivo* has not been determined. The present study examined the acute and subchronic toxicity of the fucan A in Wistar rats after subcutaneous administration. After that, the animals were killed and examined. The results showed in the acute study that fucan A did not cause general adverse effects and mortality in the concentrations 0, 20, 100, 1000, and 2000 µg/g body weight per rat for seven days. Regarding the subchronic study, the data showed that the fucan A did not cause any change in hematological and biochemistry parameters, as well as in the morphology, and in the size of the rat's organs analyzed at a concentration of 20 µg/g body weight per rat during a 62-day period. In conclusion, this study indicates this heterofucan is a compound with potential pharmacological value that has no toxicity *in vivo.*

Introduction

The general sulfated polysaccharides of brown seaweeds are called fucans, which comprise families of polydisperse molecules based on sulfated L-fucose; heterofucans are also called fucoidans (Rocha et al., 2005a; Bilan et al., 2008). Fucans from algae express important pharmacological activities such as anticoagulant, antioxidant, antiproliferative, antitumoral, anticomplementary, anti-inflammatory, antiviral, antipeptic, and antiadhesive activities (Rocha et al., 2005b; Cumanshi et al., 2007; Li et al., 2008; Costa et al., 2010). In spite of several studies on the biological activities of fucans, detailed studies on the toxicology of fucans from different sources are limited. Few studies have shown that some algal fucans are not toxic compounds (Li et al., 2005; Gideon & Rengasamy, 2008; Zaragozá et al., 2008; Chung et al., 2010). However, each new fucan purified from a marine alga is a new compound with unique structures and, consequently, with potential pharmacological and toxicological properties.

The brown alga *Spatoglossum schröederi*, Dictyotaceae, synthesizes three heterofucans (Rocha et al., 2005a), the polysaccharide - namely fucan A - is the most abundant heterofucan produced by this alga (Leite et al., 1998). This compound is practically devoid of anticoagulant activity *in vitro* and hemorrhagic activity *in vivo*, but when fucan A was injected endovenously 24 h before the ligature of the venae cavea, it showed a dose-dependent antithrombotic effect, reaching saturation around 20 µg/g of rat weight. In addition, this effect was also time-dependent, reaching saturation around 16 h after fucan administration. Besides, regardless of administration pathway, fucan A displayed antithrombotic action, the exception was the oral pathway (Barroso et al., 2008). These data suggest that it may be an ideal antithrombotic agent *in vivo*. Earlier,

we showed fucan A as an antiproliverative compound against several tumor cell lines which did show genotoxic and mutagenic effects *in vitro* (Almeida-Lima et al., 2010). However, information about fucan A toxicity *in vivo* is not available compared with that of other fucans. On the basis of these considerations, the purpose of the present study was to investigate the toxic effects of fucan A extracted from *S. schröederi*.

Materials and Methods

Algal material

The brown seaweed *Spatoglossum schröederi*, Dictyotaceae, (voucher specimen number 1976) was collected on the seashore of Natal, RN, Brazil. Immediately after collection, the alga was dried at 50 ºC under ventilation and grounded in a blender. The seaweed was then treated with acetone to eliminate lipids and pigments.

Extraction of fucans

Using a methodology which combined proteolysis and sequential acetone precipitation we obtained seven polysaccharides fractions from the brown seaweed *S. schröederi* (Barroso et al. 2008). After electrophoresis, the fraction precipitated with 0.6 volumes of acetone showed fucan A as a major compound. The polysaccharides from 0.6 vol. acetone fraction were further purified by ion exchange chromatography. Fraction eluted with 1.0 M NaCl which contains fucan A as described by Leite el al. (1998) was then subjected to Sephadex G-75 chromatography which revealed the presence of a single component. Fractions 65-82 eluted from the column were pooled, concentrated, and subjected to electrophoresis in agarose gel.

Animals

Male Wistar rats (250-300 g) were housed at a temperature of 22±3 °C under a 12 h light/dark cycle and given a standard pelleted diet and water *ad libitum* during the experimental period. The protocol for these experiments was approved by the Committee of Ethics in Research of the Hospital Universitário Onofre Lopes (HUOL, UFRN) under approval number 082/07.

Acute toxicity study

The rats were divided into groups of six. Fucan A was dissolved in saline and administered subcutaneously at doses of 0, 20, 100, 1000, and 2000 µg/g body weight per rat (500 µL). The control group received the same volume of sterile saline. Toxicity symptoms and mortality were observed. After 7-days the animals from each group were weighed, anesthetized with 10 mg/kg xylazine and 90 mg/kg ketamine *i.m.*, and bled by cardiac puncture and autopsied to analyze the biochemical, hematological, and histopathological alterations induced by fucan A.

Subchronic toxicity study

Twelve healthy rats were selected for the test and equally distributed into two groups (six rats per group). Only the concentration of (20 µg/g body weight per rat, 500 µL) was used for the subchronic toxicity study because this concentration had greater antithrombotic activity in another study (Barroso et al., 2008) and 500 µL of saline solution (0.09%), for both treated and control group, respectively, were administered daily by subcutaneous route to each rat for 62 consecutive days. The control animals received equal volumes of saline via the same administration route of the treated.

Body weight and organ weight gain

Individual body weights were recorded once weekly during the study period. Mean body weight gains were calculated for each group during the testing interval (days 1-62). Animals were also weighed immediately after euthanasia for calculation of weight of the organs.

Necropsy and histopathology

At necropsy, all the animals were killed by anesthetic with xylazine and ketamine. The principal organs (kidneys, spleen, liver, testicle, prostate, heart, brain and lungs) were carefully examined macroscopically and then weighed. Histological examinations (Gideon & Rengasamy, 2008) were performed on the preserved organs and tissues of the animals from the control group and treated with fucan A. The organs were fixed, trimmed, processed, embedded in paraffin, sectioned (5 µm diameter), placed on glass microscope slides, and stained with hematoxylin and eosin.

Clinical observations

All animals were observed three times daily for mortality in studies, acute toxicity, and sub-chronic toxicity. Cage-side observations were made daily during the study and all abnormal findings were recorded. All observations included, but were not limited to: changes in skin, eyes, and mucous membranes, occurrence of secretions and excretions, and autonomic activity (*e.g.*,

lacrimation, pilo-erection, unusual respiratory pattern). Aberrant behavior (*e.g.,* self-mutilation, walking backwards) were also recorded.

Hematological and clinical biochemistry

Hematological examination was performed using an automatic hematology analyzer (Horiba ABX Micros 60) to measure the following parameters: hematocrit, hemoglobin, erythrocytes, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and leukocyte and platelet count. Clinical biochemicals parameters were analyzed as described early (Messias et al., 2010). The parameters measured with an automated biochemical analyzer (BIOPLUS 2000) were total protein (TP), albumin (Alb), total bilirubin (T. Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamil transferase (GGT), total cholesterol (TC), urea, creatinine (Cr), and glucose.

Statistical analysis

The values were expressed as mean±standard error (S.E.). The statistical analysis of data was by Mann-Whitney test (comparing the treated groups to control) using a 5% level of significance. The statistical program used was GraphPad Prism software version 3.05.

Results

Acute toxicity study

No animals died during the experimental period, and fucan A did not produce toxic signs in any animals. In addition, the findings of gross necropsy performed at study termination did not reveal any signs of toxicity (data not shown). Under the conditions of this acute test, the LD50 value for fucan A was greater than 2000 µg/g body weight.

Subchronic toxicity study

Body and organ weights

No toxicologically significant changes were observed in the biochemical or hematological parameters (Table 1). Post-mortem examination did not reveal pathologic changes in organs and tissues that were related to administration of the test compound. No correlation between the dose level of fucan A and alterations in organ weights were observed; mean body weight of rats was measured weekly. The relative body weights and organ weights are shown in Figure 1 and 2, respectively. Fucan A treatment did not alter body weight in either of the groups treated and no alterations were revealed in any of the organs examined by gross necropsy.

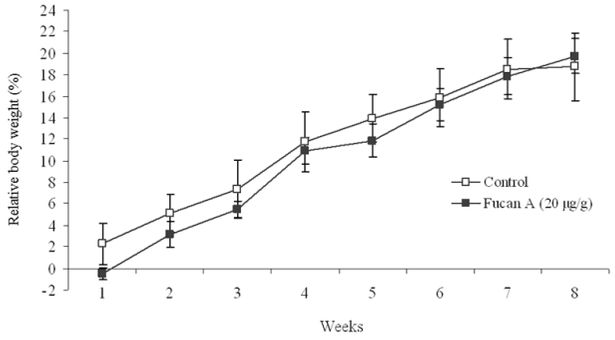

Figure 1. Effect of fucan A administered by subcutaneous injection for 62 days in sub-chronic toxicity study on relative body weight (g/100g body weight) in male Wistar rats. (n=6, per group), data expressed as mean±SE.

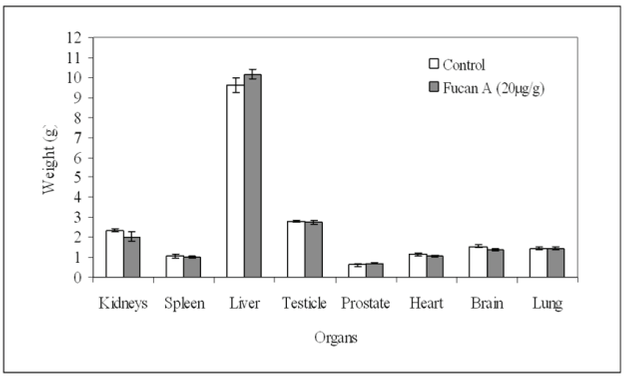

Figure 2. Organ weights in Wistar rats treated with fucan A for 62 days in sub-chronic toxicity study. (n=6, per group), data expressed as mean±SE.

Clinical observations

The results of individual clinical examinations in both the treated and control groups of the male Wistar rats indicate that fucan A was well tolerated during the treatment period (62-days), with no mortality or signs of morbidity observed. In addition, observations were made daily during the study and no differences between treated rats and controls in changes in skin, eyes, and mucous membranes, occurrence of secretions and excretions, and autonomic activity, as well as in behavioral changes.

Hematological and clinical biochemistry

The data from the hematological and serum biochemical examinations are summarized in Table 1. Although not statistically significant (p>0.05) increases

in glucose (25%), urea (30%), AST (22.45%) and TC (21.26%) were sporadic at the dose (20 µg/g body weight/day); however, the differences did not exceed the maximum of physiological and historical control data ranges (140-261 mg/dL; 12.6-33 mg/dL; 54-298 U/L; 26-82.4 mg/dL, for glucose, urea, AST, and TC, respectively). In relation to the hematological parameters, similar data were observed for the values of the control group; the effects were considered to be of no toxicological significance (p>0.05).

Table 1. Biochemical and hematological parameters of Wistar rats treated with fucan A from *S. schröederi* for 62 days.

Parameters	Control	Fucan A (20 µg/g)
Glucose (mg/dL)	193.83±17.41	243.83±11.54
Urea (mg/dL)	39.33±3.77	51.33±3.53
Creatinine (mg/dL)	0.75±0.10	0.81±0.12
AST (U/L)	123.17±6.88	150.83±12.81
ALT (U/L)	82.33±6.92	86.67±8.07
GGT (U/L)	2.50±0.76	2.00±0.37
TP (g/L)	6.10±0.39	5.27±0.20
Albumin (g/L)	2.22±0.21	2.12±0.05
Globulin (g/L)	3.88±0.36	3.02±0.16
TC (mg/L)	57.17±6.05	69.33±1.26
TB (mg/L)	1.73±0.17	1.87±0.17
Hematocrit (%)	42.08±1.45	36.90±1.77
Hemoglobin (g/dL)	13.95±0.43	12.60±0.61
Erythrocytes (10^6/mm^3)	5.92±0.06	6.07±0.21
MCV (fL)	50.17±1.47	50.83±0.31
MCH (pg)	16.57±0.66	16.32±1.67
MCHC (g/dL)	33.13±0.40	35.42±0.44
Leukocytes (10^3/mm^3)	7.38±0.72	4.92±1.10
Platelets (10^3/mm^3)	418.67±24.74	456.33±65.37

Data are presented as mean±SE of six animals per group. Abbreviations: AST, aspartate aminotransferate; ALT, alanine aminotransferase; GGT, γ-glutamil transferase; TP, total protein; TC, total cholesterol; TB, total bilirubin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration (p>0.05).

Necropsy and histopathology

The organs including heart, liver, spleen, lung, kidney, brain, testicle, and prostate were carefully examined. No noticeable pathological changes were observed by the naked eye. No histopathological changes were observed in all organs of control rats, as well as, those of rats treated with fucan A (20 µg/g body weight/ rats), including liver and kidneys (Figure 3).

Discussion

Polysaccharides, which represent a very interesting class of macromolecules that are widespread in nature, have recently attracted more attention in the biochemical and medical areas due to their several biological activities (Cumashi et al., 2007; Li et al., 2008). In fact, seaweeds are a great source of sulfated polysaccharides expressing anticoagulant, cytotoxic, antitumor, antimetastic activities (Alekseyenko et al., 2007; Li et al., 2008).

Although polysaccharides from several sources are safely exhibiting no toxic effect even at high concentration of 300 to 4000 µg/g of body weight (Gideon & Rengasamy et al., 2008), although some polysaccharides can be toxic at low concentration, such as the sulfated galactan (25 µg/g of body weight) from the red alga *Champia feldmani,* which was administered in Wistar rats (Lins et al., 2009). Histopatology and morphology changes were observed both in the liver and kidneys of the treated animals (Lins et al., 2009). In addition, alginic acid (another acid polysaccharide synthesized by brown algae) from *Sargassum vulgare* induced necrosis of renal tubule epithelium in experimental animals, but these effects were reversible and related to direct vascular effects (De Paula Alves Sousa et al., 2008).

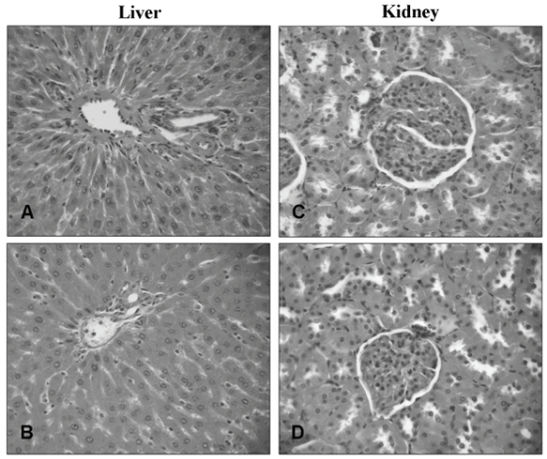

Figure 3. Effect of fucan A on the sub-chronic toxicity study on histological morphology of rat liver (A and B) and kidney (C and D) shown by hematoxylin and eosin staining (x 400): (A and C) group control and (B and D) Fucan A (20 µg/g).

An earlier study from our group with fucan A extracted from the seaweed brown *S. schroederi* suggests that fucan A presents no toxicity in several *in vitro* tests (Almeida-Lima et al., 2010). In the present studies, the acute and subchronic toxicity of the fucan A administered to Wistar rats was investigated.

In this study, no acute mortality of Wistar rats was observed, indicating that the LD50 of fucan A is more than 2000 µg/g. In addition, the subchronic toxicity of fucan A (20 µg/g for 62-days) also resulted in no mortality, nor in changes in body and organ weights (Figure 1 and 2).

Only the concentration of 20 µg/g was used for the subchronic toxicity study because this concentration had greater antithrombotic activity in another study (Barroso et al., 2008). In regard to the hematological analysis, no significant differences were observed for most of the parameters between the control group and the group treated with fucan A. As to biochemical parameters, the increase in glucose, urea, and AST could be interpreted as treatment-related. However, this seems to have no toxicological significance because the change was small and within the control ranges in contemporary subchronic toxicity studies performed with fucans (Li et al., 2005; Gideon & Rengasamy et al., 2008; Kim et al., 2010).

Through histopathological analyses, this study also evaluated the integrity of the all organs in Wistar rats subjected to fucan A treatment. In addition, we did not observe modifications of liver and kidney tissues. In addition, there were no alterations in enzymatic activity of ALT and GGT and in the levels of creatinine and total protein of the peripheral blood from treated rats, suggesting a normal function of the liver and kidneys (Figure 3 and Table 1).

Several articles have recommended primary anticoagulant/antithrombotic compounds for all cancer patients admitted to the hospital for surgical or medical reasons (Lee, 2010). Previously, we showed fucan A to be an antithrombotic compound that exhibits a tumor cell proliferation-inhibition effect against pancreatic carcinoma, prostatic cancer epithelial, promyelocytic leukemia, and cervical adenocarcinoma cells. In addition, this polymer did not show genotoxic and mutagenic effects *in vitro* (Almeida-Lima et al., 2010). Here, this study demonstrates that fucan A showed no toxicity even at high doses after subcutaneous administration in rats for seven days. Furthermore, fucan A was not toxic when administered for 62 days, not causing biochemical, hematological and histopathological alterations. These data led us to propose fucan A as a promising new and safe antithrombotic and antitumoral drug. Moreover, our findings indicate that further investigation is needed to evaluate fucan A activity in tumor progression *in vivo*.

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***Correspondence**

Hugo A. O. Rocha
Departamento de Bioquímica, Universidade Federal do Rio Grande do Norte
Av. Senador Salgado Filho, 3000, Lagoa Nova, 59078-900 Natal-RN, Brazil
hugo@cb.ufrn.br
Tel.: +55 84 3215 3416
Fax: +55 84 3211 9208