GAMMA RADIATION AFFECTS THE ANTI-*Leishmania* ACTIVITY OF *Bothrops moojeni* VENOM AND CORRELATES WITH L-AMINO ACID OXIDASE ACTIVITY

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ABSTRACT

Leishmania causes human disfiguring skin disease in endemic areas of Amazon and North Eastern Brazil. Those parasites present a remarkable resistance to most treatments, except those using toxic antimonial salts. We detected a specific anti-*Leishmania* activity in snake venoms, using an *in vitro* promastigote assay. In this report, we analyzed the activity of *Bothrops moojeni* venom against *L.amazonensis*, using whole venom or fractions of L-amino acid oxidase (L-AO). Crude venom of *B.moojeni*, was fractionated by molecular exclusion chromatography. Activity against promastigotes was detected by respiratory oxidative conversion of MTT in a colorimetric assay and L-AO activity was detected by a colorimetric assay with peroxidase and OPD as revealing reagents. Crude venom was irradiated with 500, 1000, and 2000 Gy in a ^{60}Co gamma radiation source. The venom had an anti-*Leishmania* activity of 33pg/promastigote and the active fraction migrates as 100–150 kDa, close to the size described for L-AOs, and also presented L-AO activity. The radiation reduces both the L-AO and anti-*Leishmania* activity in a dose dependent effect. Those data suggests the anti-*Leishmania* activity in this venom is closely related to the L-amino acid oxidase activity and also that radiation could be used as a tool to detect specific activities reduction in water solutions, similarly to observed in dry preparations.

Key words: *Bothrops moojeni*, *Leishmania*, L-amino acid oxidase, therapy, radiation

I. INTRODUCTION

Leishmaniasis is a widespread cutaneous disease found in Latin America specially in Amazon and NorthEastern Brazil. It is caused by several species of sand fly transmitted protozoa of the order Kinetoplastida[1]. The parasite lives as an intracellular amastigote in macrophages of mammalian hosts, mainly in the cold areas of the skin, or as free living promastigotes in the gut of sand fly vectors[2].

Despite of intense research in immunology and the disfiguring effect of the disease in some patients the treatment is based in early described toxic drugs, such as sodium stibogluconate, with few and also toxic alternatives, amphotericin B or pentamidine. Treatment failures and drug resistance have been recently increasing, which precludes the development of new drugs [3].

Snake venoms are complex mixture of enzymes and biological active peptides, with many pharmacological activities[4]. Growth inhibition of *Trypanosomatideae* by snake venoms, assayed in *in vitro* systems, have been described[5]. .L-amino acid oxidase (L-AO) is an enzime found in most snake venoms that catalyzes the oxidative deamination of L-amino acids, producing hydrogen peroxide and ammonia[6]. Those by-products could interact with the parasites, resulting in oxidative death.

Gamma radiation has been described as a useful tool in the detoxification of snake venoms, [7].

In this work, we tested *Bothrops moojeni* venom against *Leishmania* promastigote in an in vitro assay, associated to molecular exclusion fractionation, using gamma radiation of crude venom as a tool for molecular weight dependent detoxification.

II. MATERIAL AND METHODS

Crude *B.moojeni* venom was kindly furnished by Prof.Dr. J.R.Rogero, IPEN/CNEN –SP, as dry preparation, obtained from Instituto Butantan. All reagents used in the experiments were from pro-analysis quality, and the solutions prepared with Milli Q high purity water. *Leishmania L. amazonensis* was kindly furnished by Prof.Dr. J.J.Shaw, and maintained as stabilates in liquid nitrogen.

Crude venom of *B. moojeni* was weighted and dissolved in saline solution. After dissolution, the cloudy was cleared by centrifugation at 13500 rpm for 3 minutes and the protein concentration adjusted for 2mg.mL^{-1}, after protein determination[8].

Samples were irradiated with 500-1000-2000 Gy in a ^{60}Co gamma radiation source (GAMMACELL). After irradiation, the venom was maintained at 4°C.

Crude venom (20mg), was submitted at a molecular exclusion chromatography (Superdex 200) previously equilibrated with sodium acetate buffer 50mM, pH 5.0 in a flow rate of 0,5mL/min.

Crude and irradiated samples, were added to 96 well microplate, in triplicate, at a start concentration of 20µg/mL, in a serial dilution up to 0,312µg/mL.

Leishmania (Leishmania) amazonensis promastigotes were cultured in RPMI 1640 medium w/o phenol red, with 10% fetal calf serum at 25°C. Promastigotes were added to the plate at 3x10^6/mL, resulting in a final volume of 100µL/well. The plate was incubated at 25°C for 16 hours.

The MTT method was used to detect the viability of the *Leishmania amazonensis* promastigotes after incubation with the venom[9]. A modification was introduce, to the original method, because of the large amount of proteins found in snake venoms, that caused an interference of reading at 570nm [10]. Briefly, we added 20µL/well of a 5mg/mL MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and incubated the plate for 4 hours at 25°C. After this, 100µL/well of SDS-HCl 10%-1mM solution was used and the plate incubated for 18 hours at the same conditions described before. The Optical Density (O.D.) was read at 570nm in a MULTISKAN MS reader. Adequate assays with increasing promastigotes concentration showed a good correlation (r^2>90%) between O.D. and parasites counts in automatic haemocytometer.

A fast screening procedure for LAO activity was elaborated by Venom Group of Radiobiology -IPEN. We use a LAO screening medium, a solution with RPMI 1640 w/o phenol red as substrate, with horseradish peroxidase (30IU) and OPD (ortho phenylene diamine) at100µg/mL.

The whole/crude venom, irradiated and the fractions from chromatography, were added to a 96 well microplate (100µL/well), in duplicate, containing 100µL/well of LAO screening medium. The plate was incubated for 30 minutes, at room temperature and in a dark place, and read at 405nm in a MULTISKAN MS reader.

III.RESULTS

Detection of Anti-*Leishmania* Activity

The crude and irradiated venom in different doses, were compared to the capability of inhibition of promastigotes, resulting in a decrease of the activity in high doses of irradiation (Fig.1).

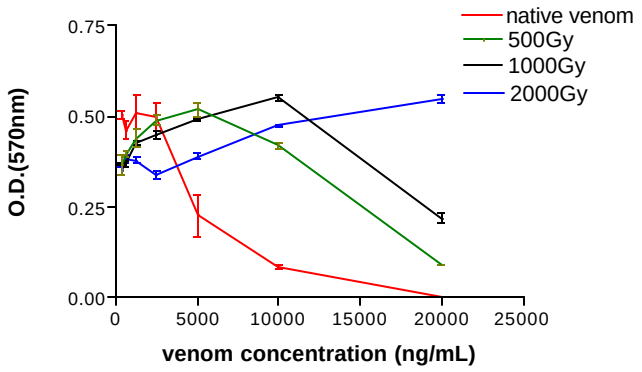

Fig.1-Inhibition of *Bothrops moojeni* venom after treated by gamma radiation (GAMMACELL) at 500-2000Gy doses.

The fractions from the molecular exclusion chromatography, showed the active peak in the region of 100-150kDa, corroborating the results described in literature for LAOs.

Using the samples from chromatography, we detected L-aminoacid oxidase activity in the same peak responsible for the inhibition of promastigotes.(Fig.2).

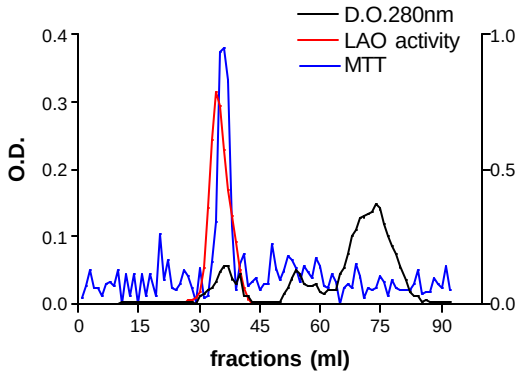

Fig.2 Screening of L-aminoacid oxidase and the anti-*Leishmania* activity for *Bothrops moojeni* crude venom Molecular exclusion chromatography (Superdex 200 –XK 1.6/70cm) previously equilibrated with sodium acetate buffer 50mM pH5,0. The flow rate was 0,5mL/min and the eluted fractions 1ml/tube.

Decreasing of LAO Activity After Irradiation

Crude venom was assayed after irradiation in order to verify the decreasing of LAO activity. This data was presented in Fig.3, showing an effect-dose dependency.

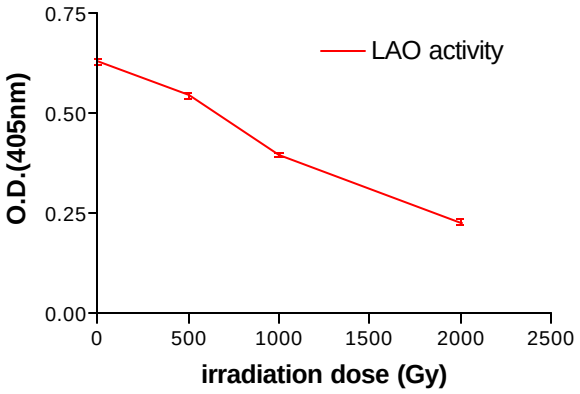

Fig.3 Dose-dependency inhibition of LAO activity of *Bothrops moojeni* venom after irradiation with 500 to 2000Gy.

DISCUSSION

Our data clearly shown a effect of *B.moojeni* venom on promastigotes of *L.L.amazonensis*, in an *in vitro* assay. This effect was similar to those already described by others authors, using *Cerastes cerastes* venom, a Viperidae snake from Old World. Those authors were unable to define which activity of the venom was effective in producing those effect [5]. Our data on purification and L-AO activity suggest that this effect is probably derived of this activity. Free radicals are capable to induce the death of parasites, as detected in similar systems with the use of other free radicals inducing enzymes added to the medium, like xanthine oxidase in Plasmodium systems[11]. This effect induces an higher levels of peroxides, overwhelming the scavenger systems of those parasites[12].

The use of gamma radiation also clearly demonstrates a inhibition of the anti-parasitic effect in the crude venom, at low doses, confirming the proteic origin of this action, and also suggest an high molecular weight of this protein, by its low radioresistance. L-AO had a molecular weight of 100-150 kDa, usually affected at those doses. This approach has been used in dry preparations, with the same purpose, but its use in solutions has some criticism yet unresolved.[13]

The purification steps for L-AO and its activity on parasites suggests that the use of purified proteins from snakes venoms could be an interesting model for developing alterantive drugs in therapy of leishmaniasis.

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