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Leishmania major: *In vitro* and *in vivo* anti-leishmanial effect of cantharidin

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ABSTRACT

Cantharidin is a natural poisonous compound secreted by male blister beetles. The effect of different doses of cantharidin on *Leishmania major* (MRHO/IR/75/ER) were investigated both *in vitro* (promastigote and amastigote viability) and in experimentally-infected BALB/c mice (skin lesions) using ointment or soluble cantharidin. In this study, cantharidin with concentrations of 0.5, 1, 2, 5, 10, 20 and 50 μg/ml inhibited the growth of *L. major* promastigotes after 24 h and the resultant inhibition levels were 39.22%, 41.95%, 49.88%, 54.78%, 58.01%, 68.30% and 80.04%, respectively. After 72 h, the mean number of amastigotes per macrophage in the culture using 2 μg/ml of cantharidin, (the 50% inhibitory concentration dose (IC_{50})), was 1.2 while in the control group it was 2.7. In order to perform the inflammatory blister technique, 500 μg of cantharidin were solved in 25 μl of DMSO to show the formation of the blister which leads to treatment of cutaneous leishmaniasis. Using the blister technique, the small lesions (<5 mm) healed after one session. Two weeks of topical treatment with 0.1% cantharidin ointment was an effective method for treating cutaneous leishmaniasis in infected BALB/c mice.

1. Introduction

Leishmaniasis is a major public health problem in many, mostly developing, countries. Chemotherapy is currently the best way for treatment of leishmaniasis; however, these are moderately toxic, and have risks of recurrence and unacceptable side effects. Photodynamic therapy has been introduced as a therapeutic option with rapid localized destruction of lesions without affecting the adjacent normal tissues. Furthermore, in contrast to systemic treatments, photodynamic therapy has no risk of toxicity; but it is nonetheless an expensive approach (Ghaffarifar et al., 2006).

The terpenoid, cantharidin (2,6-dimethyl-4,10-dioxatricyclodecane-3,5-dione) is a natural poisonous compound secreted by the male blister beetles of *Meloidae* and *Oedemeridae* families. All body fluids of blister beetles have cantharidin and which, in its pure form and at room temperature is an odorless and colorless solid (Carrel et al., 1993; Dettner, 1997; Dettner et al., 2003). Cantharidin has been used for treatment of warts and molluscum contagiosum by topical application (Moed et al., 2001; Cathcart, 2009).

Like other natural toxins, cantharidin is a strong inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) (Graziano et al., 1988; Li et al., 1993). When cantharidin is applied to the skin, it causes acantholysis and blister formation (Pierard and Pierard, 1988; Yell et al., 2006).

The aim of this study is to assess cantharidin in the treatment of cutaneous leishmaniasis using an ointment preparation and the inflammatory blister technique.

2. Materials and methods

2.1. Leishmania culture and host animals

L. major (MRHO/IR/75/ER) was cultured in RPMI 1640 (Gibco) with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) for preparation of promastigotes.

Female BALB/c mice aged 8–10 weeks were used as the source of peritoneal macrophages and also for intradermal (i.d.) inoculation at the base of the tail with 2×10^6 promastigotes of *L. major* (strain MHRO/IR/75/ER). The lesions appeared at the fourth week after inoculation. All mice were followed up for 22 weeks.

2.2. In vitro assay

2.2.1. Preparation of drug solution

Cantharidin (Sigma–Aldrich Laboratories, St. Louis, MO) was initially dissolved in dimethylsulfoxide (DMSO) (Sigma–Aldrich Laboratories, St. Louis, MO), at concentration of 20 mg/ml and was then stored at 4 °C until use.

2.2.2. Promastigote assay

Promastigote assays were carried out using a direct counting assay, previously described based on growth inhibition (Callahan

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et al., 1996). The effects of cantharidin were evaluated in 24-well microtitre plates (Nunclon, Denmark). The promastigotes were seeded at an initial concentration equivalent to that of early log phase (2×10^5 promastigotes/ml) and they were then allowed to multiply for 72 h in the following media: (I) only in the medium (RPMI with 10% FBS) as control group; (II) in the medium with DMSO (another control group); (III) in the presence of different concentrations of cantharidin (0.5, 1, 2, 5, 10, 20 and 50 μg/ml) without renewing the medium or drug. The parasites were counted daily for 3 days in a Neubauer chamber with a light microscope, and the results were compared with controls. Each assay was performed in triplicate and whole series of experiments was repeated twice. The drug IC_{50} was determined using a plotted graph (Callahan et al., 1996) and used for further studies.

2.2.3. Amastigotes/macrophage assay

Drug susceptibility of the amastigotes in the BALB/c mice macrophages was determined using a modification of the method by Chang (1980). Briefly, peritoneal macrophages were collected and infected *in vitro* with promastigotes in RPMI medium with 10% FBS, at a concentration of 10^6 cells and 10^7 promastigotes per ml. The cultures were incubated for 24 h at 37 °C in 5% CO_2 to allow promastigotes to be phagocytized by macrophages as well as to permit the adhesion of macrophages to the plate surface. The number of parasites were measured in 100 macrophages; then 2×10^5 macrophages/well were cultured in 24-well plates with RPMI and 10% FBS. Cantharidin at its IC_{50} concentration was added to the wells. The plates were incubated in 37 °C and 5% CO_2 incubator and number of amastigotes per macrophage were counted at 24, 48 and 72 h after adding cantharidin to the culture.

The cytotoxic effect of cantharidin was assessed on the mouse macrophages. The test was carried out in 24-well microtitre plates. A suspension of 5×10^5 macrophages was added to each well, and subsequently incubated in a 37 °C and 5% CO_2 incubator for 60 h without renewal of the medium. The IC_{50} value was afterwards calculated (Bodley et al., 1995).

2.3. In vivo assay

2.3.1. Ointment preparation and application

An ointment was prepared with 1 mg/ml (0.1%) cantharidin in standard ointment base (Eucerin). The ointment was topically applied to tail lesions in the mice. Treatment was performed once a day for 2 weeks. Lesion size was measured with vernier calipers on two different diameters. The number of mice in treatment group was eight, and in each control group (treated and non-treated with Eucerin) was four.

2.3.2. Inflammatory blister technique

In order to evaluate the blister formation caused by soluble cantharidin, the lesion was cleaned with 70% alcohol and allowed to dry. A blank paper disc, 6.4 mm in diameter was placed on a 2×2 cm^2 of sterilized vaseline gauze and then 25 μl of 0.1% cantharidin solution was applied to the disc (Fig. 1) (Chang, 1980).

The disc with cantharidin was placed on the cleaned lesion and fixed with tensoplast tape. After 24 h, direct smears and samples for culture in NNN media were obtained from the lesions. The number of mice in experimental group was eight, and in each control groups (treated and non-treated with DMSO) was four.

2.4. Statistical analysis

Results of each experiment were summarized with the mean and standard deviation (SD) values. The percentage of killed promastigotes was obtained in comparison to control group. Mean lesion size was compared by Mann Whitney test (*P*-values of <0.05 were considered significant).

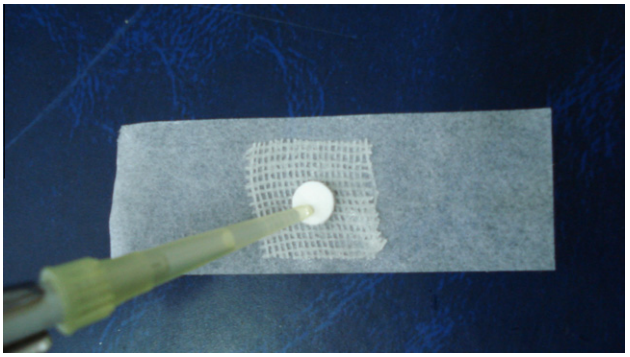

Fig. 1. The 25 μl of 0.1% cantharidin solution was added to blank filter paper disc with a 6.4 mm diameter, on a 2×2 square of vaseline gauze.

3. Results

3.1. The effect of cantharidin on promastigotes in vitro

Growth inhibition of promastigotes was evaluated at seven concentrations of cantharidin (0.5, 1, 2, 5, 10, 20 and 50 μg/ml) for 24, 48 and 72 h (Table 1). The resultant IC_{50} was determined to be 2 ± 0.28 μg/ml.

3.2. The effect of cantharidin on amastigotes and macrophages in vitro

Prior to adding cantharidin, the mean number of amastigotes/macrophage was 1.8. After adding cantharidin, the mean number of amastigotes/macrophage after 24, 48 and 72 h were 1.6, 1.4 and 1.2, while the values in control group were 2.1, 2.4 and 2.7, respectively. With regard to cytotoxicity of cantharidin to macrophages, the IC_{50} was determined to be 7.7 ± 2.6 μg/ml.

3.3. In vivo assay

3.3.1. Effect of topical treatment with cantharidin ointment

Cantharidin ointment prepared in Eucerin cured the lesion in all the mice. The mean and SD values of lesion diameters at the onset of treatment and at the end of the first and second weeks of treatment were 4.48 ± 0.23 mm, 2.38 ± 0.26 mm and 0, respectively (Figs. 2–4). The follow up showed relapse of lesions for two mice at the eighth and ninth weeks after beginning of treatment. On the other hand, all the mice in control group died between the 14th to the 20th week after inoculation and there was no significant difference between the group treated with Eucerin and the other control group.

Table 1
Percentage of growth inhibition of promastigotes following exposure to different concentration of cantharidin after 24, 48 and 72 h.

Cantharidin concentrations (μg/ml)	Percentage of killed promastigotes following exposure to the drugs		
	24 h	48 h	72 h
50	80.04	83.98	90.52
20	68.30	73.51	82.54
10	58.01	64.03	78.22
5	54.78	59.04	72.42
2	49.88	56.02	69.40
1	41.95	46.01	51.97
0.5	39.22	45.64	50.31