Short communication

Hexadecyl-phosphorylcholine ointment for treatment of cutaneous leishmaniasis: an animal trial

J. Iqbal,[1] I. Bukhari,[1] M. Jamshid,[3] S. Bashir,[1] M. Masoom Yasinzai[2] and M. Anwar [4]

مرهم هيكساديسيل فسفوريل كولين في معالجة داء الليشمانيات الجلدي: تجربة على الحيوانات

جاويد إقبال، عشرت بخاري، محمد جمشيد، ساجد بشير، معصوم ياسينازي، محمد أنور

الخلاصة: تقارن هذه الدراسة المشهَّدة بالدواء الغفل تأثير مرهم هيكساديسيل فسفوريل كولين بتركيز 6٪ ممزوجاً مع كلوريد البنزونيوم بالمرهم الغفل في معالجة داء الليشمانيات في 60 من حيوانات القداد الذهبية، إذ عولج بالمرهم الدوائي أربعون من هذه الحيوانات في حين طبّق مرهم غُفُل مرتين يومياً على العشرين حيواناً الآخرين. وبعد المعالجة لوحظ تقلُّص ملحوظ في مساحة وحجم الآفات في الحيوانات المعالجة بالمرهم الدوائي مقارنةً بالحيوانات التي طبّق عليها المرهم الغُفل؛ و لم تُلاحَظ الطفيليات الليشمانية في اللطاخات المأخوذة من 35 حيواناً من بين الحيوانات الأربعين التي عولجت بالدواء و لم يحدث أيضاً أيُّ نُكْس خلال فترة ملاحظة استمرت 120 يوماً.

ABSTRACT A placebo-controlled trial compared 6% hexadecyl-phosphorylcholine (HePC) and 12% benzethonium chloride ointment with placebo ointment for treatment of cutaneous leishmaniasis. Cutaneous lesions were experimentally induced by inoculation with leishmania promastigotes in 60 golden hamsters. Forty (40) animals were treated with drug and 20 with placebo ointment applied twice daily for 15 days. After treatment, all lesions were significantly reduced in size in the treatment group compared with the placebo ointment. No parasites were detected in smears from 35/40 of the drug-treated lesions and no relapses occurred over 120 days of observation.

La pommade d'héxadécylphosphocholine pour le traitement de la leishmaniose cutanée : essai sur l'animal

RÉSUMÉ Un essai contrôlé contre placebo a comparé une pommade contenant 6 % d'héxadécylphosphocholine et 12 % de chlorure de benzéthonium avec une pommade placebo pour le traitement de la leishmaniose cutanée. Les lésions cutanées ont été induites expérimentalement par inoculation de promastigotes de leishmania sur 60 hamsters dorés. Quarante (40) animaux ont été traités avec le médicament et 20 avec la pommade placebo appliquée deux fois par jour pendant 15 jours. Après le traitement, toutes les lésions ont significativement diminué en taille dans le groupe du traitement par rapport à la pommade placebo. Aucun parasite n'a été détecté dans les frottis de 35 des 40 lésions traitées par le médicament et aucune rechute ne s'est produite pendant 120 jours d'observation.

[1]Department of Pharmacy; [2]Institute of Biochemistry; [4]Department of Chemistry, University of Balochistan, Quetta, Pakistan.

[3]Faculty of Pharmacy, University of the Punjab, Lahore, Pakistan (Correspondence to J. Iqbal: drjaveidiqbal@hotmail.com).

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Introduction

The principal causative agents of leishmaniasis in Asia, Middle East, Africa and parts of Europe are *Leishmania donovani*, *L. major*, *L. tropica* and *L. infantum* [*1*]. Currently, the disease appears to be on the rise in Pakistan. Both cutaneous and visceral leishmaniasis are prevalent. The cutaneous form of the disease is seen throughout the country, being highly endemic in Baluchistan, whereas the visceral form is prevalent in the northern part of the country [*2*]. The cutaneous form occasionally becomes epidemic.

In the absence of a vaccine, drug treatment with pentavalent antimonials is still the first line of treatment for leishmaniasis in Pakistan and worldwide. The major drawback associated with the treatment is that it is painful and costly, relapses occur and above all, resistance develops. During the last 2 decades, many attempts have been made to develop effective new compounds for treating cutaneous leishmaniasis (CL) that would be economical, that could be applied topically to lesions and that would avoid the development of resistance. Many other drugs have been tested for leishmanicidal activity, including allopurional, ketaconazole and dapsone [*3*]. Paromomycine, an aminoglycoside, has been tested as a topical ointment for the possible treatment of CL but more clinical trials are needed against the main parasite causing CL [*4*].

Hexadecyl-phosphorylcholine (HePC), an alkylphosphorylcholine compound, is the most promising of the new class of anti-tumour agent. The compound is an inhibitor of mammalian protein kinase and has been applied topically for the treatment of skin metastasis showing good local tolerability [*5*]. HePC also appears to be useful for CL [*6–8*]. We have shown that this compound has potent anti-leishmanial activity *in vitro* [*6*]. The toxicity data of protein kinase inhibitors in humans is also well established [*5*]. In order to investigate the effectiveness of HePC ointment as a topical anti-leishmanial preparation, we tested its efficacy on experimentally induced lesions in hamsters.

Methods

Preparation of HePC ointment

An ointment containing 6.0% HePC and 12.0% benzethonium chloride was prepared according to the British Pharmacopoeia and British Pharmaceutical Codex methods by fusion techniques. Benzethonium chloride (Sigma, USA) was used as a surfactant and penetration enhancer. HePC was generously gifted by Asta Pharma, Germany. Other chemicals and reagents used were of Analar grade.

Parasite cultures

For primary isolation of parasites for inoculation into experimental animals a drop of aspirate was taken from underneath a cutaneous leishmaniasis lesion infected with *L. tropica* and inoculated onto 1% agar base containing 0.9% saline and 10% rabbit defibrinated blood. This was supplemented with an overlay of Medium 199 + antibiotic + 1% sterile human urine. Parasites were grown to a density of 5×10^7 parasites/mL, spun down, reconstituted in Medium 199 (Gibco, Eggenstein, Germany) at a density of 10 million parasites/20 µL.

Parasites were initially maintained in modified Tobies medium overlaid with Medium 199, 10% heat inactivated fetal bovine serum (HIFBS) and 2% urine at 20 ºC in a cooled incubator. Parasites were subsequently bulk cultivated in monophasic liquid culture Medium 199 supplemented with 10% HIFBS and 2% urine at 20 ºC in a cooled incubator.

Induction of lesions

Sixty (60) golden hamsters (*Mesocricetus auratus*) aged 8 to 25 weeks were obtained from the Institute of Health, Islamabad, Pakistan.

Parasites maintained in Medium 199 were sedimented down at 3000 rpm for 15 min and washed twice with sterile phosphate buffered saline. The washed parasites were resuspended in sterile phosphate buffered saline to a final concentration of 10^7 parasites/mL. The parasite culture was used at a concentration of 5×10^6 cells/mL to inoculate the hamsters on their nose.

Hamsters were monitored for lesion development and the size of lesions was measured daily with vernier callipers until they were full developed (15 days on average).

Treatment

Two preparations were compared as treatments: HePC ointment containing 6.0% HePC with 12% benzethonium chloride in a simple ointment base, and placebo ointment containing simple ointment base only. The hamsters were divided into 6 groups of 10 animals and were kept in different cages: 4 groups received the HePC ointment and 2 groups received placebo ointment. A standard amount of ointment was applied to lesions twice daily at 09.00 and 18.00 h and the lesions left uncovered. At the end of the study all hamsters had received 15 consecutive days of treatment with HePC or placebo ointment.

The size of the lesions was inspected visually every day during treatment and were measured using callipers after the end of treatment at 120 days. The mean lesion size was determined by measuring the lesion diameter at its widest point and then at right angles and taking the average diameter.

Samples were taken from the lesions, stained and examined under the microscope for Leishmania parasites.

Analysis was made using analysis of variance.

Results

In the 4 treatment groups the mean lesion size decreased significantly after treatment with HePC ointment (Table 1). In the 2 control groups, mean lesion sizes also decreased significantly (from 0.6 cm to 0.4 cm) in one group and increased slightly (from 0.5 cm to 0.6 cm) in the other group.

On day 7 of treatment, the lesions of most of the hamsters in the experimental groups were parasitologically negative as assessed by *in vitro* culture. After 7 weeks, the lesions in 35 out of 40 treated rodents were totally cured, while in the placebo groups, still no healing was observed after 120 days of observation. There were no relapses in the experimental groups of animals.

Discussion

The results of topical HePC in cutaneous leishmaniasis were encouraging. HePC ointment significantly reduced the size of cutaneous lesions produced by experimentally induced infection with *L. tropica* in susceptible golden hamsters. Most of the lesions were parasitologically negative after treatment and there were no relapses after 120 days of observation.

The ED50 value of 20 µM for HePC was obtained for *Leishmania* isolates from Baluchistan [*7,8*]. HePC also suppressed the differentiation of amastigotes to promostigotes, even at a concentration as low as 1.0 µM [*9*]. We have found that this

Table 1 Effect of hexadecyl-phosphorylcholine (HePC) ointment on golden hamsters inoculated with promastigotes. HePC and placebo ointment was applied twice daily to experimentally induced lesions for 15 days

Group	Treatment	Days of treatment	Lesion size (cm)			
			Before treatment		After treatment	
			Mean	SD	Mean	SD
1 (n =10)	HePC	15	0.6	0.11	0.0*	0.02
2 (n =10)	HePC	15	1.0	0.27	0.0*	0.003
3 (n =10)	HePC	15	0.7	0.18	0.1*	0.29
4 (n =10)	HePC	15	0.7	0.20	0.2*	0.006
5 (n =10)	Placebo	15	0.6	0.16	0.4*	0.18
6 (n =10)	Placebo	15	0.5	0.32	0.6	0.009

*P > 0.01, comparing lesion sizes before and after treatment.
n = number of hamsters per cage.
SD = standard deviation.

compound shows potent anti-leishmanial activity *in vitro* [*6*]. However, further study of its mechanism of action is needed in the mammalian system as this compound is known to be a protein kinase inhibitor. We tried to find out if the mechanism was the same in the case of leishmania. The leishmanial protein kinase was isolated, partially purified and the effect of HePC studied on the partially purified enzyme. It was observed that HePC effectively inhibited the activity of leishmanial protein kinase [*5*]. The inhibitory effect was stronger than the other known protein kinase inhibitors.

The study needs to be repeated with a larger group of animals before any conclusion about the efficacy of HePC can be reached. Plans are underway to test HePC ointment in human patients.

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Leishnet: web-based database and information system on Leishmania/HIV co-infection

There is an emerging problem related to the increased overlap between leishmaniasis and AIDS. A WHO surveillance network for Leishmania/HIV co-infections already exists and includes 28 member institutions worldwide. The Leishnet database has recently been created on the basis of a standardized case report form used by each member institution to report to WHO/HQ. The problem, initially restricted to southern Europe, has now moved to eastern Africa and Asia.

Objectives:

- to improve the reliability and standardization of collected epidemiological information through frequent updates;
- to make the information accessible, not only to the network members but also to other interested persons through the web using a decentralized database (graphs, maps and tables);
- to improve coordination between the member institutions and between the institutions and WHO;
- to involve each centre more directly in the process of surveillance and to implement remote data entry;
- to evaluate the dynamics of the Leishmania/HIV co-infection worldwide.

Further information can be obtained from leishnet@who.int, or on the home page at http://www.who.int/leishmaniasis/home_leishnet/en/.