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Original Article

Leishmanicidal Activity of Films Containing Paromomycin and Gentamicin Sulfate both In Vitro and In Vivo

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ABSTRACT

Background: Based on the efficacy of paromomycin ointment and recent ongoing clinical trials of combination of paromomycin and gentamicin, a new physical form of films of the paromomycin and gentamicin was prepared and anti-*Leishmania* activities of the prepared films were assessed in vitro and in vivo.

Methods: Paromomycin 15% and gentamicin 0.5% was incorporated in a film using ethyl cellulose and HPMC (Hydroxyl Propyl Methyl Cellulose). In order to assess the drug release and anti-*Leishmania* activities of the preparation, a clone *L. major* parasite was established using a set of modified NNN medium without overlay liquid layer. Therapeutic effects of the films were evaluated using Balb/c mice model. The mice were inoculated with 2×10^6 *L. major* promastigotes (MRHO/IR/75/ER) and then when the lesions developed the mice were randomly divided in 3 groups, 10 mice per group, and treated with either perpetrated films or placebo for 28 days or left untreated.

Results: Growth inhibition of cloned promastigotes showed that the films have enough releasing capacity and in vivo system, the films containing paromomycin and gentamicin was able to reduce the lesion size and induced complete cure in 80% of the mice but relapse was seen in 60% of the cured mice and overall 50% cure rate was seen during 20 weeks period of the study.

Conclusion: It seems that the prepared films might be further used in human clinical trials.

Keywords: Cutaneous leishmaniasis, Paromomycin, Gentamicin, Drug Film

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Introduction

Currently 350 million individuals in more than 88 countries are at risk of leishmaniasis. Recently due to increase in CL incidence rate and co-infection of leishmaniasis with HIV, more attention is demanded to the neglected disease. Approximately 90% of all CL cases are reported from eight countries including Iran (1). However using antimony needs multiple injections, accompanies with side effects and the efficacy of antimony is very low especially in CL lesions induced by *Leishmania tropica* and resistant to antimony is reported (2-4). Aminoglycoside antibiotics such as paromomycin showed to be effective against *Leishmania* parasites and promising results generated in phase 3 clinical trials (5-8). Topical therapy against CL if available is ideal since such topical therapy dramatically improves the patients' compliance. Currently topical formulation of paromomycin and gentamicin is in phase 3 clinical trials (9) (Personal communication with Alan Magill and Max Grogl).

In this study, a physical form of film containing paromomycin 15% and gentamicin 0.5% was prepared. The anti *Leishmania* activities of the films were assessed in vitro and in vivo.

Material and Methods

Parasite

Leishmania major strain (MRHO/IR/75/ER) used in this experiment is the same strain which was used for leishmanization and preparation of experimental *Leishmania* vaccine (10,11).

Drug films

The films were prepared at School of Pharmacy, Isfahan University of Medical Sciences using paromomycin 15% and gentamicin 0.5% incorporated into ethyl cellulose and Hydroxyl Propyl Methyl cellulose (HPMC). The films contain of backing layer, drug reservoir, rate controlling membrane, and adhesive layer. Direct compression and solvent casting methods are used to make films containing paromomycin and gentamicin with permeation enhancer in polymeric matrices. Physical properties of the prepared films such as flexibility, stability, thickness, and apparent uniformity were checked. Releasing study was done by putting the films on *Bacillus subtilis* culture and growth inhibition zone was assessed and compared with standard curve of discs containing the same amount of paromomycin.

In vitro experiments

Leishmania promastigotes were harvested at stationary phase using a set of modified NNN medium without overlaid liquid layer. The plates were sealed with parafilm and incubated at 26 ± 1°C for 7-10 days, the surface of the plates were checked for the growth of the promastigotes. Three types of culture plates were prepared triplicity as follow; paromomycin and gentamicin incorporated into ethyl cellulose and HPMC film plates, placebo film plates and control media with no film. The same number of promastigotes was cultured in the abovementioned three types of plates. After 4-5 days, the plates were checked for the growth of the promastigotes by direct microscopy and the number of motile and non-motile promastigotes was determined. Viability of promastigotes was also evaluated using trypan blue.

In vivo experiments

Female 6 -8 weeks old Balb/c mice were purchased from Pasteur Institute of Iran. The mice were inoculated at the ramp of the tail with 2×10^6 *L. major* promastigotes harvested

at stationary phase. The lesion was developed in the inoculation site after 2-3 weeks.
Thirty mice with ulcerated lesion were selected and randomly divided in to 3 groups (10 per group), the mice were treated with either films containing paromomycin 15% and gentamicin 0.5% or placebo, and one group was left untreated. The films were changed every 4 days for 28 days; each mouse received 7 films. At the time of changing the film, the size of ulcer in each mouse was measured using vernier scale. The mice were followed up for up to 4 months.
ANOVA test was used to analyze the data and p<0.05% was considered significant.

Results

In vitro experiments

No growth of *Leishmania* was seen in a 30 mm radius of the drug films. On the other hand, growth of *Leishmania* promastigotes was seen in placebo and control plates, wet mount smears proved the presence of alive, and motile promastigotes in placebo and control plates. Among 1,000 promastigotes was counted, 971±3.6 (97%), in placebo plates and 980±5 (98%) in control plates showed to be alive, but in the experimental plates 101±6 (10%) promastigotes showed to be alive (Table 1).

In vivo experiments

Lesions appeared at 2-3 weeks after inoculation at the base of the tails. The lesions size in group of mice which received the films was significantly (P<0.05%) smaller than the control groups (Fig. 1). In control groups receiving either placebo or no treatment the lesion size continue to progress throughout the 20 weeks duration of the study (Fig. 1). Mean diameter of lesion size in the experimental group was 0.4±0.04 mm, in placebo group was 5.04±0.20 mm and in none treated group was 6.42±0.44 mm. At the end of the treatment course 80% of the animals were cured in the experimental group and the mean diameter of the lesions showed to be significantly (P<0.05) decreased in the 20% of the mice with the lesion (1 mm).

Table 1: In vitro effect of drug and placebo film on *L. major* growth

Plate	Live parasite X±SD	%	Dead parasite X±SD	%	Total count
Plate with drug film	101±6	10	899±6	90	1000
Plate with placebo	971±3.6	97	29±6	3	1000
Plate without drug film	980±5	98	20±5	2	1000

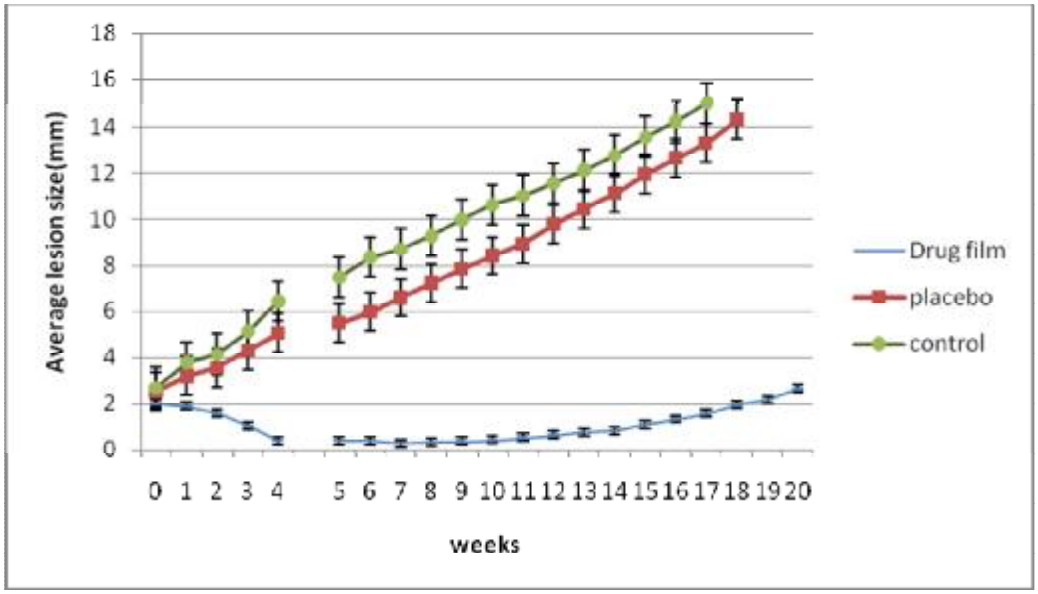

Fig. 1: Ulcer size in Balb/c mice infected with *L. major*, animals were treated with drug films or placebo or left untreated for 28 days (4 weeks)

Discussion

Although leishmaniasis is a major health problem in some of the endemic areas(12) , no vaccine is available against any form of leishmaniasis (10-13). Available chemotherapy against CL is limited and daily injections is difficult to tolerate by the patients and justifies search for more practical formulation of chemotherapy and topical therapy is highly acceptable if available (14-16).Therapeutic activities of paromomycin against CL is tested in several clinical trials, although the results are not consistent but the overall showed that at least paromomycin is effective against some *Leishmania* strains (5,7,15,17,18). Combination of paromomycin and gentamicin was showed to cure *L. major* and *L. mexicana* in infected Balb/c mice (19).

The same formulation called WR279396 was checked in phase 2 trials and showed to be safe with acceptable cure rate and currently is in phase 3 clinical trial in Tunesia and Columbian male army (9, 20) (Personal communication with Alan Magill and Max Grogl). In the current study, a new form of film containing of paromomycin and gentamicin was prepared and tested in vitro against *L. major* growth and in vivo against *L. major* infection in Balb/c mice.

Paromomycin is effective against *Leishmania* infection and gentamicin is a wide spectrum antibiotic that has synergic effects in combination of paromomycin (17-19). In this study, drug films were shown to be effective against in vitro promastigote growth with inhibition rate of 98% within 5 days. The in vivo results indicated that the new physical form of combination of paromomycin and gentamicin was able to significantly (P<0.05) reduce the lesion size in Balb/c mice. Drug films were able to induce lesion cure in 80% of the mice at 28 days after the treatment initiation. However relapses characterized by the reappearance

of the lesion, was observed at week 9 to 11 in cured mice which declined the cure rate from 80% to 50% in the experimental group, considering the fact that Balb/c mice are highly susceptible to *L. major* infection and this strain of mice is unable to control the infection even with therapy (6).

In conclusion, the results suggested that topical films might be useful for the treatment of cutaneous leishmaniasis. The advantages of using film form of the drug as follows:

Continuous contact of the drug with the lesions, which promote cure rate, also gradual release of the drug provides parasiticidal effects of the drug continuously. Low efficacy of different forms of paromomycin like lotion or ointment in some studies might be due to a short contact period of the drug with the lesions or removal of the drug by the patients' daily activities, in case of using films containing the drug guarantees continues contact of the drug with the lesion. Finally, Human trials are needed to prove the possible efficacy of the preparation.

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