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COMPARATIVE EFFICACY OF DIFFERENT THERAPEUTIC AGENTS IN EXPERIMENTALLY INDUCED LEISHMANIASIS IN HAMSTER

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ABSTRACT

Two hundred sand flies were collected from the D. G. Khan and Rajanpur areas of Pakistan from May 2007 to June 2008. The species of sand flies identified were *Phlebotomus. papatasi; P. orientalis; P. major.* Seventy eight out of one hundred twenty flies (65%) dissected, carried the infective leptomonad forms naturally *(leishmania promastigotes)* in their salivary glands and fore-guts. Seven groups (A, B, C, D, E, F and G) of hamsters were experimentally infected. Out of 80 animals 78 (88%) developed characteristic lesions of cutaneous leishmaniasis at the site of subcutaneous injection. The efficacy of therapeutic agents in Group A treated with Sodium Stibogluconate solution was 90 percent, in group B Meglumine Antimonate solution was 100 percent, in group C treated with Clotrimazole 20 percent while in group D treated with Miconazole (20%) the efficacy was 100 percent . Similarly crude extracts of Garlic (*Allium sativa*) was applied on lesions to group-E; thrice a day which cured the lesions within 60 days; Onion (*Allium cepa*) (T-6) were applied on the lesions of group-F; TDS; which cured the lesions within 72 days and Harmal extracts (T-7) were applied on lesions to group-G; TDS; this cured the lesions within 50 days, respectively.

Keywords: *Leishmania, phlebotomus*, hamster, herbal agents.

INTRODUCTION

Leishmaniasis causes lesions on the face and extremities with severe stigmatization amongst affected individuals, particularly children and women. The U.S. Center for Disease Control (CDC) estimated that leishmaniasis occurs as either a disfiguring skin or fatal (if untreated) liver and spleen disease. Skin leishmaniasis may develop into a mucosal affliction of the nose and mouth. Zijlstra *et al.* (1995) reported that in India skin of patients showed nodular lesions. Generally dogs are infected and the vectors pick infection and spread it to humans. Barriga (1997) reported that in dogs most frequent manifestations of leishmaniasis were intermittent fever, anemia, episodes of diarrhea, glomerulonephritis and polyarthritis and antimonial compounds were not very effective and reoccurrences were frequent. Cupolillo *et al.* (2003) reported that the lesions of cutaneous leishmaniasis may heal spontaneously within weeks and depended upon cell mediated immunity and production of gamma interferon. The dog is the principal domestic reservoir of *L. infantum* for human infection (Ashford *et al.,* 1996; Mujtaba and Khalid, 1998; Avila *et al.,* 1990). *L.tropica* has also been isolated from patients with visceral leishmaniasis in India and Israel. According to Lainson (1982) *L. donovani*, *L.infantum* and *L. chagasi* are considered subspecies of a principal species or species complex called *L.donovani-sensu lato*. They can be distinguished easily by serological, enzymatic and by molecular techniques (Momen *et al.,* 1993). This vector transmitted protozoan infection belongs to the most important human diseases world wide (Hervas *et al.,* 1996**).** Drug resistance is reported in virtually all endemic areas, 3/4th of annual occurrences are skin related. Visceral leishmaniasis is considered by far the most severe form of leishmaniasis and is often fatal if left untreated. Of the 500,000 new cases reported annually worldwide, some 90% occur in five developing countries namely Bangladesh, Brazil, India, Nepal and Sudan (Desjeux, 2004). Ashford *et al.* (1998) reported that pentavalent antimony compounds were the drugs of choice for treatment of cutaneous leishmaniasis but for simpler localized lesions local therapy with these compounds was simple, economical and cost effective than systemic therapy. Other drugs like Pentamidine, Amphotricin-B and oral Ketoconazole were useful in resistant cases. The effective control of visceral leishmaniasis could be achieved by application of insecticides during times when vectors were most accessible and through improved nutrition of children (Dhiman and Sen, 1991). Rab *et al.* (1986) reported that garlic (*Allium spp*.) had antibacterial, antifungal and antiprotozoal properties. The principal ingredient was a sulphur compound Diallylthiosulfonate or Allicin, which was produced when garlic cloves are crushed. Allicin was unstable and breaks down into aqueous solvents in 24-36 hours. The main objective of this work was to find out the efficacy of different therapeutic agents in experimentally induced leishmanasis in hamsters.

MATERIALS AND METHODS

Collection of flies and culture of promastigotes: Two hundred sand flies were collected from D.G.Khan and Rajanpur areas. The flies were dissected in accordance to the procedure adopted by Crampton *et al.* (1997). The promastigotes of genus *Leishmania* were collected from salivary glands and fore-gut of flies. None of the dissected flies carried the infective stages in their mid-guts, hind-guts and reproductive systems. The promastigotes were grown in Iscove's commercial medium (Sigma) containing 10% foetal calf serum, 2 mM. Glutamine and Gentamycin 25 µg/ml. The culture was set up in glass culture flasks containing 50 ml. of medium and with starting density of 5×10^5 promastigotes per ml. Flasks were incubated at 37ºC for four to five days. After 4-5 days the promastigote density was 2-4×10^7 cells/ml. (optimal density). Once the culture had achieved optimal parasite density, the promastigotes were collected by centrifugation and were washed three times with PBS in order to remove traces of medium and serum. After the last wash the promastigotes were aliquoted in Eppendorf tubes (100 µl. of packed promastigotes / tube) and stored at −20ºC until use. Stored promastigotes were suspended in 1 ml. PBS and sonicated for 2 minutes in 10 second bursts as done by Rahim *et al.* (1998). The sonicated antigen was either used to immunize hamsters or for further experimentation. Counting of promastigotes was made in a Neubauer haemocytometer.

Experimental infection in hamsters: A group of eighty hamsters was constituted and the infectious stages of protozoa found in the salivary glands and fore-guts of flies were injected (0.2 ml) subcutaneously into two or three previously shaven backs of hamsters diluted in PBS. The development of lesions produced were studied and confirmed by making smears of biopsy material on 3rd, 5th, 7th, 9th, 11th, 13th, 15th and 19th day post inoculation. (Crampton *et al.*, 1997).

Efficacy of different anti-leishmanial agents: For this experiment seven groups of hamsters (A, B, C, D, E, F and G) were constituted out of previous experiment having ten animals each with confirm lesions of cutaneous leishmaniasis. Group-A was administered intralesional injection of Sodium Stibogluconate sol. 10% (T-1) and group-B was administered intralesional injection of Meglumine Antimonate sol. 10% (T-2); OD, respectively, after cleaning the wound with luke warm double distilled water, at dose rate of 0.5 ml. in and around the lesion (Wilson *et al.*, 2005).

Group-C was applied Clotrimazole 20% (T-3) and group-D was applied Miconazole 20% (T-4) in cream base. Both were applied to the lesions TDS, respectively, after cleaning the wound with luke warm double distilled water. Similarly crude extracts of Garlic (Allium sativa) (T-5) were applied to lesions of group-E; Onion (Allium cepa) (T-6) were applied on the lesions of group-F and Harmal extracts (T-7) were applied on lesions to group-G; thrice a day (TDS); respectively. The crude extracts were obtained after grinding the herbs (100 grams each) in a pestle and mortar and adding 100 ml. of double distilled H_2O. Extract was filtered through filter paper. Fresh extract was prepared for daily use and kept in a refrigerator. The extract was applied after cleaning the wound with luke warm double distilled H_2O. (Louzir *et al.*, 1998).The efficacy of each drug was examined. Recovery rate was recorded for different treatments and χ^2 test was applied for significance ($P<0.05$).

RESULTS

Identification of sand flies: The species of sand flies identified were *Phlebotmus. papatas, P. oriental and P. major.*

Histopathology: Upon experimental transmission growth of *Leishmania* in the tissues of skin lead to hypertrophy of stratum corneum with hypertrophy and proliferation of the papilla. There was infiltration of plasma cells, lymphoid cells and mononuclear leukocytes. Large mononuclear cells containing many parasites were also observed. The lesion appeared as a reddish papule on the 3rd day and developed a covering of dry scales by the 5th day. The lesion became moist from the crust on the 7th day; if the crust was removed a shallow ulcer was seen. The ulcer gradually enlarged and by 19th day, had sharp-cut, raised edges surrounded by an indurate area. Secondary bacterial infection of the sore was observed after 19th day. Sores healed spontaneously in untreated animals after four to six months, leaving a depigmented scar. Out of 80 animals 78 developed characteristic lesions of cutaneous leishmaniasis at the site of subcutaneous injection (88%). The hamsters which didn't develop any lesions showed no hypersensitivity reaction.

Efficacy of anti-leishmanial agents: Group-A was administered intralesional injection of Sodium Stibogluconate Sol. 10% (T-1); Once a day (OD); which cured the lesions in nine out of ten animals within 7 days of treatment (90%); group-B was administered intralesional injection of Meglumine Antimonate sol. 10% (T-2); OD; which cured the lesion in all of the ten animals within 12 days of treatment (100%). The lesions healed leaving minor scar after both of the treatments.

In Group-C after application of Clotrimazole 20 (T-3); thrice a day (TDS). The lesions cured within 21 days and in group-D treated with Miconazole 20% (T-4); TDS; the lesions cured in 36 days. Recovery rate in both therapies was 100%. Healed lesions left minimal scar after both of the treatments. Similarly crude extracts of Garlic (*Allium sativa*) (T-5) was applied on lesions to

group-E; TDS; which cured the lesions within 60 days; Onion (*Allium cepa*) (T-6) were applied on the lesions of group-F; TDS; which cured the lesions within 72 days and Harmal extracts (T-7) were applied on lesions to group-G; TDS; treatments cured the lesions within 50 days. Extracts were applied after cleaning the wound with luke warm double distilled water. However scar formation was seen after healing. (Figure 1).The results indicated that efficacy of hermal extract was highest (87%) followed by Garlic (70%) while lowest efficacy (67%) of Onion was seen. The difference in efficacy of therapeutic agents was statistically significant ($p < 0.05$).

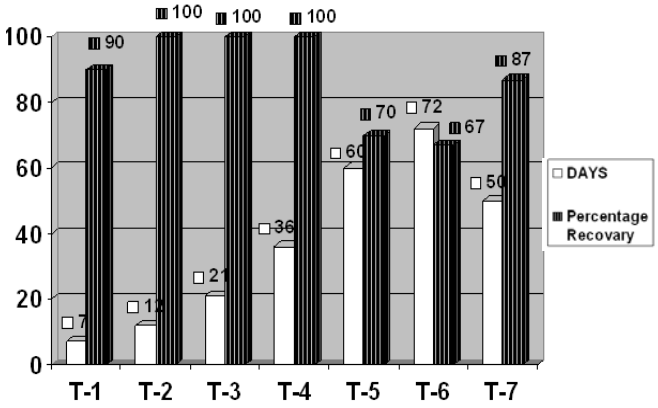

Figure 1: Recovery percentage after therapeutic trails.

DISCUSSION

Leishmaniasis is caused by flagellate protozoa of family Trypanosomatidae; genus *Leishmania*. Life cycle of the parasite is simple. The amastigotes exist within the definitive host and the promastigotes exist in the salivary glands of the vectors (Barral *et al.*, 1991). The vectors in this case are small flies of family Phlebotomidae; genus *Phlebotomus* (*Lutzomyia*). The flies ingest the amastigotes while feeding on infected host's blood, once inside the vector's gut the amastigotes become motile by developing flagellum and become promastigotes which are the infective forms, they travel to the salivary glands from where they are transmitted to a new host during the next blood meal. Once inside the definitive host the promastigotes loose the flagella and enter the macrophages, where they multiply inside the parasitophorous vacuole (Yasinzai and Chang, 1996). Endemic areas of disease in Pakistan were districts of Chitral, Dir, Swat , Gilgit; Mansehra, Skardu, Chilas, Abbottabad, Rawalpindi, Azad Kashmir, Lasbela, Khuzdar, Derabughti, D.G.Khan, Rajanpur, Jacobabad, Larkana, Dadu; Quetta, Qila Abdullah, Pishin and Qila Saifullah. The above mentioned areas are foot hills of mountainous ranges that are present in the North, West and South Western Pakistan, which cover all the four provinces including Azad Kashmir The south-eastern areas of Pakistan are non-endemic as reported by Ayub *et al*.(2003). During the present study the samples of sand flies were collected from D. G. Khan and Rajanpur areas. It was experienced that dogs were seen wandering in bushes and damp places during the day to rest; such places were often found to be the hide-outs of sand-flies.The species of sand flies identified during present study were *Phlebotomus. Papatasi, P. orientali and P. major*. The presence of sand flies and Leishmaniasis cases in both dogs and human population was evident from the results of the present study. Seventy eight out of one hundred twenty sand-flies (65%) dissected, carried the infective leptomonad forms naturally *(leishmania promastigotes)* in their salivary glands and fore-guts belonging to sub-genus *Leishmania*. Upon experimental transmission, it was seen that growth of *Leishmania* in the tissues of skin lead to hypertrophy of stratum corneum with hypertrophy and proliferation of the papilla which coinsides with the findings of Zeledon (1992), Yasinzai and Chang (1996) who adopted topical Immidazole Compounds in rabbits and reported infiltration of plasma cells, lymphoid cells and mononuclear leukocytes alongwith large mononuclear cells containing parasites were also observed by them. It was evident from the results of present study that the use of intralesional injections of pentavalent antimony compounds was an effective method which being safe on one hand (reduced toxicity of antimony comp.) was also cost effective due to minimal use (dosage). Topical use of Immidazole Compounds also gave good results and both of the treatments suppressed the infection on hamsters leaving bare minimal scar. Sharifi *et al.* (1998) also reported traditional herbal extracts known to have anti-leishmanial properties. In present study harmal extracts cured the lesions in 50 days and the recovery percentage was 87% and was quite effective, however scar formation was evident after healing of lesions.

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