EFFICACY OF GLUCOCORTICOIDS IN CONTROLLING *LEISHMANIA MAJOR* INFECTING BALB/C MICE

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Abstract
Leishmaniasis is a growing health problem in many parts of the world. Efforts to find new chemotherapeutics for leishmaniasis remain a priority. This study was carried out to determine the effect using glucocorticoid drugs to reduce production of chemokine production in a bid to control Leishmania major infection in BALB/c mice.A total of 48 mice were used. In the therapeutic arm (post-infection), 24 Mice were infected with *L major* parasiteswere treated with dexamethasone (0.69 mg/ml), hydrocortisone (2mg/ml) LPS (10 ng/ml) and PBS for 28 days and lesion development monitored for five weeks. For immunoprophylaxia (pre-infection), 24 other mice were treated with the above drugs and then infected with *L major.* LPS was used as the positive control while PBS was the negative. Serum samples were collected before and after infection for cytokine analysis for MIP 1α, MCP 1 and IFNγ using ELISA. Parasite quantification was done by calculating the LDU. Lesion measurement was done by use of a vernier caliper.Lesion sizes after infection of BALB/c mice were similar in all the experimental groups till the onset of therapeutic treatments ($P > 0.05$). At 0.5 months post-treatment, significant differences ($P < 0.05$) were discerned in the lesion sizes of the BALB/c mice in the control groups. However, hydrocortisone and dexamethasone caused substantial elimination of the parasites from the lesions and significantly reduced parasite burden in spleen compared to the controls at the end of the experiment. Generally, hydrocortisone gave better results as compared to dexamethasone. Both hydrocortisone and dexamethasone resulted in substantial clearance of parasitemia from both the lesions on footpads and spleens of infected BALB/c mice. They also led to significantly reduced levels of MCP 1 and MIP-1α and high levels of IFN γ. These results show that glucocorticoids substantially reduce Parasitemia in *Leishmania* infected mice by decreasing production of MCP 1 and MIP-1α chemokines while increasing IFN γ levels. In this regard, a further investigation into the modes of action of the glucocorticoids and probably their efficacy against other Leishmania strains should be explored further.

Key words: MIP-1α, MCP-1, dexamethasone (dexa), hydrocortisone (hydro), Glucocorticoids (GCs) Leishmania infection

1.0 Introduction

Leishmaniasis is a zoonotic disease of major public health importance. At least two million new cases are reported each year, 367 million people at risk and 88 countries most of the tropical are affected WHO (2007); Hotez and Kamath (2009). It is caused by a protozoan parasite of the genus Leishmania and is transmitted to the mammalian host by bites of their vectors of phlebotomine species (Banuls *et al*., 2007).

The disease is manifested through clinical symptoms ranging from skin lesions of cutaneous leishmaniasis to fatal visceral leishmaniasis affecting mainly the liver and the spleen, thus making the disease an important health problem in the world (Chappuis, *et al*., 2007). The Leishmania parasite completes its life cycle in the human host and vector with two main stages of development being amastigote and promastigote (Awasthi *et al*., 2004). They infective stage is metacyclic promastigotes in the sand fly vector, which after entering the human host invades macrophages and differentiate into amastigotes (Assafa *et al*., 2006). The actively replicating stage is the promastigote.

Diseaseoutcome often depends on the particular *Leishmania* sp.and strain causing the disease and the host's immune response. It has been suggested that the host's early innate immune response is critical for parasite containmentandfortheresolutionofdisease.MCP-1 is a potent chemo-attractant for monocytes/macrophages and lymphocytes. It has also been shown to be involved in the regulation of Th1/Th2 lymphocyte differentiation, enhancing Th2 development by increasing IL-4 production and inhibiting IL-12 production.

Control of leishmaniasis depends on early diagnosis and treatment in infected human hosts and reservoirs, dogs (Croft and Coombs 2003). Treatment has for a long time relied mainly on pentavalent antimonials, amphotericin B and pentamidine, which are toxic and difficult to administer because of their long term treatment, high cost and increased drug resistance (Croft *et al.,* 2002, melby 2002, Thakur *et al.,* 2004, mishra *et al*., 2007). Important to note also is that antimonials are very toxic with frequent, sometimes life-threatening, adverse side effects, including cardiac arrhythmia and acute pancreatitis (Croft *et al,.* 2006). This makes the search for cheaper, less toxic drugs with minimal side effects a matter of utmost urgency in leishmaniasis research, considering there is no vaccine for the disease yet.

Leishmania virulence has been linked to the modulation of chemokine expression by macrophages and reports have indicated that *Leishmania* parasites induce expression of both MIP 1α and MCP 1 (Liu 2002, Teixeira 2005).Some progress toward understanding how Leishmania suppresses macrophage inflammatory responses has been made, however, the extent to which Leishmania modulates macrophage cytokine responses and the underlying molecular mechanisms involved are yet to be known. It is also not known how inhibition of production of MIP-1α and MCP 1 chemokines affects the parasite and disease progression and its influence if any on lesion development in mice. This study was aimed at evaluating the effect of glucocorticoids on lesion development, parasite infectivity and multiplication indices. The effect of GCs on the production of MIP-1α and MCP-1 chemokines and IFN γ in BALB/c mice was also determined.

2.0 Material and Methods

2.1 Leishmania Parasites

Metacyclic promastigotes of *L. major* (IDUB/KE/83= NLB-144 strain) were used. This strain has been maintained by cryopreservation and *in vitro* culture, and periodic passage in BALB/c mice at Kenya Medical Research Institute (KEMRI). Parasites were grown at 26^{o}C in Schneider's Drosophila medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS).

Mice: Six to eight week old female BALB/c mice were used in this study. They were strictly maintained under the rules and regulations of the animal Care and Use Committee-KEMRI (ACUC-KEMRI, Kenya) in specific pathogen-free conditions with laminal air flow. 28 mice were inoculated intramuscularly with 2 x 10^6 stationary phase promastigotes of *L. major* on the left hind footpad (LHFD). All animal experiments were approved by Kenya Medical Research Institute (KEMRI) Committee. Lesion sizes were estimated weekly for 5 weeks post-infection. Infected control mice were not treated. To compare the effect of the drug, the difference between the infected left hind footpad and the uninfected right hind footpads was taken on a weekly basis according to the method of Nolan and Farrell (1877). Briefly, the lesion size was measured in two dimensions (D and d) at right angles to each other with a caliper gauge, and the lesion size(s) determined by the following formulation: S = (D x d) / 2. The mice were anesthetized with sodium thiopental (50 μg/g of body weight) and sacrificed at the end of the experiment and their spleens removed for parasite load determination.

2.2 Drug preparation and Administration

The glucocorticoids, hydrocortisone and dexamethasone were dissolved in PBS and used at different concentrations depending on their cytotoxicity (data not shown). Dexa was more toxic and therefore used at a concentration of 0.67 mg/ml while dexaethasone was used at 2 mg/ml. LPS (5mg/ml obtained from Invitrogen^{TM}) was diluted with PBS to a final concentration of 10 ng/ml. After skin lesions were confirmed (one and half months after inoculation), the infected mice were randomly assigned to four groups of six mice each. The drugs were subcutaneously injected into the mice daily for fifteen days. For experimental handling, the animals were grouped as follows: group A, received a dose each of dexa; group B, received a dose each of hydro; group C received LPS being the positive control while group D received PBS being the negative control group. PBS was the diluent for the drugs and hence was used as negative control. To measure the effects of GCs on production of MCP 1 and MIP 1α, there were two sampling points being before and after infection with L.*major* promastigotes.

2.3 Promastigote Cultivation

Leishmaniamajor (strain IDUB/KE/83=NLB144) which was originally isolated in 1983 from a female *P. duboscqi* collected near Marigat, Baringo district ,Rift Valley province in Kenya, was used (Beach *et al.,*1984).This strain had

since been maintained by cryopreservation in *in vitro* cultures in liquid nitrogen, with periodic passage in BALB/c mice at KEMRI. Stationary phase promastigote cultures were used in this study. The romastigoteformsof*L.major*wereculturedincompleteSchneider'smediumpreparedwith20%heatinactivatedfetalcalfserum (FCS) and filtered in sterile conditions. They were diluted to1x10 cell permlofmedium.

2.4 Chemokine Analyses by Sandwich ELISA

The level of mouse MIP 1αand MCP 1 in the sera of GC treated mice was measured by use of mouse CCL3/MIP-1α and CCL2/JE/MCP-1ELISA kits (R&D Systems), in accordance with the manufacturer's instructions. The minimum detectable dose of mouse MIP-1α is less than 1.5 pg/mLwhile that of MCP 1 is less than 2 pg/mL.

2.5 Determination of Parasite Burdens

The assessment of the amastigote burden was carried out by blinded microscopic enumeration with Giemsa-stained splenic impressions. The total amastigote burdens were calculated as Leishman Donovan units (LDU, number of amastigotes per 1,000 nucleated cells x organ weight [in grams] x 2 x 105), according to the formula of Bradley and Kirkley (1977). The percent efficacy was calculated as [1- (mean amastigote load in treated mice/mean amastigote load in 9 NTC)] x 100. An LDU reduction of at least 80% was adopted as the minimal criterion for drug efficacy.

2.6 Statistical Analysis

All experiments were carried out in triplicate. The mean and standard deviation of at least three experiments were determined. ANOVA was used to compare the difference between means and all significantly different treatments were separated using Duncan's Multiple Range Test (DMRT). P values of 0.05 or less were considered significant.

3.0 Results

3.1 Effect of GC Therapy on L. Major Lesion Development

BALB/c mice infected on the LHFD with *L. major* promastigotes showed skin lesions as a single nodule with or without ulceration one and half months after inoculation. The lesions developed to full size in most of the infected mice. Efficacy of GC treatments on the development of lesion sizes in BALB/c mice is presented in figure 1. Lesion sizes after infection of the BALB/c mice were similar in all the treatment till the onset of therapeutic treatments. Treatment was started after one and half months of infection. At 0.5 months post-treatment, significant differences were discerned in the lesion sizes of the BALB/c mice. The untreated controls for infection of Balb/c mice with *L. major* (PBS) displayed the highest increase in lesion size after infection as was lesion in *L. major* treated with LPS. However, the low rates of increase in lesion was recorded in dexa, which was significantly ($P < 0.05$) higher than lesion size in Balb/c mice treated with hydro.

3.2 Effects of GC therapy on spleen parasite Burdens (Determination of LDU)

Splenic impression enumeration of amastigotes yielded no significant differences in the body weights of Balb/c mice among the various treatments ($P > 0.05$). Weight of the spleen, spleno-somatic index, number of parasites and LDU followed similar trends where hydro had the lowest value followed by values of dexa while PBS has the highest values (Table 1).

3.3 Effects of GCs on Production of MIP 1α, in BALB/c Mice

There was a significant difference between the concentrations of MIP-1α before *L. major* infection among the various ranges of concentration tested ($F = 32.222$, $df = 12$, $P = 0.0000$). However, hydro appeared to elicit the higher reduction in production of MIP-1α compared to dexa. The efficacious ranges of hydro ranged from 0.25 mg/l to 0.8 mg/l while similar efficacious range of dexa ranged from 0.4 mg/l to 0.9 mg/l. Dexamethasone resulted in 80% reduction in the concentration of MIP-1α compared to 92.6% reduction in the concentration in hydro Figure 2). There was a significant difference between the concentrations of MIP-1α after *L. major* infection among the various ranges of concentration tested ($F = 16.785$, $df = 12$, $P = 0.0001$). Again, hydro elicited a greater reduction in production of MIP-1α as compared to dexa. The efficacious ranges of hydro were from 0.16 mg/l to 0.85 mg/l while the efficacious range of dexa ranged from 0.45 mg/l to 1.0 mg/l. Dexa resulted in 80.5% reduction in the concentration of MIP-1α compared to 95.2% reduction in the concentration in hydrocortisone (Figure 3).

3.4 Effects of GCs on Production of MCP 1 in BALB/c Mice

The results on efficacy of the two GCs on the concentration of MCP in macrophage of Balb/c mice before infection with *L. major* promastigote indicates a significant difference between the concentrations of MCP before *L. major* infection among the various ranges of concentration tested (F = 29.542, df = 12, p = 0.0000). The efficacious ranges of hydro ranged from 0.08 mg/l to 0.72 mg/ml while similar efficacious range of dexa ranged from 0.17 mg/l to 0.67 mg/ml. Dexa also resulted in 80% reduction in the concentration of MCP while hydro resulted in 92.4% reduction in the concentration in MCP (Figure 4). After infection, there is recorded a significant difference between the concentrations of MCP after infection with *L. major* promastigotes among the various ranges of concentration tested (F = 56.115.542, df = 12, p = 0.0000) with efficacious ranges of hydro ranging from 0.08 mg/l to 0.67 mg/ml while those of dexa ranged from 0.17 mg/l to 0.67 mg/ml. Dexa also resulted in 80% reduction in the concentration of MCP while hydro resulted in 92.4% reduction in the concentration in MCP (Figure 5).

3.5 Effects of GCs on Production of IFN γ in BALB/c Mice

There were significant changes in IFN-γ levels in serum of Balb/c mice infected with *L. major* among the two treatments, hydro and dexa (F = 8.745, df = 7, P = 0.0001). There were increased IFN-γ levels from the start of the experiments in the *L. major* infected Balb/c mice albeit the hydro treated mice produced larger quantities of IFN-γ (Figure 6).

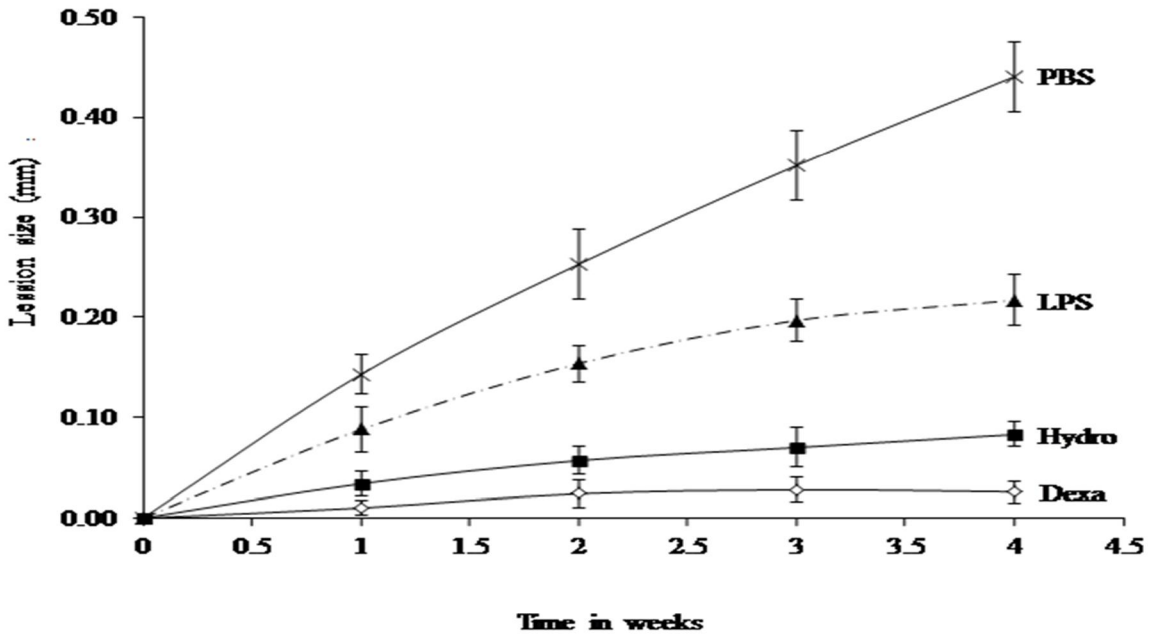

Figure 1: Lesion size in Balb/c mice subjected to different drug treatments

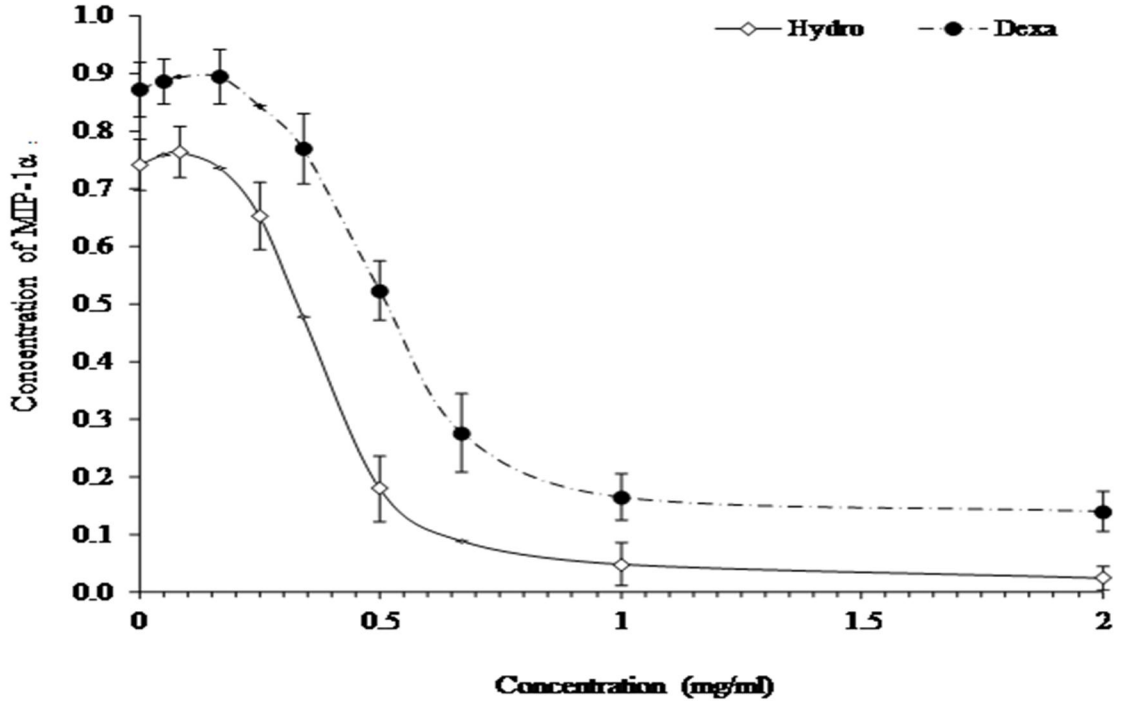

Figure 2: Concentration of MIP-1α in serum of Balb/c mice before infection with L. major promastigotes

Table 1: Body weight, weight of spleen, spleeno-somatic index, number of parasites and LDU in Balb/c mice following treatment with hydro, dexa, LPS and PBS

Treatment	Body weight	Weight of spleen	Spleeno-somatic index	No of parasites	LDU
Dexa	23.75 ± 0.48	0.14 ± 0.012[b]	0.61 ± 0.06[b]	71.75 ± 2.98[b]	2.05 ± 0.41[b]
Hydro	23.52 ± 0.65	0.13 ± 0.006[a]	0.55 ± 0.02[a]	28.33 ± 2.38[a]	0.74 ± 0.14[a]
LPS	23.50 ± 0.35	0.25 ± 0.05[c]	1.08 ± 0.08[c]	92.40 ± 12.25[c]	4.76 ± 1.21[c]
PBS	23.52 ± 0.35	0.30 ± 0.03[d]	1.31 ± 0.13[d]	196.50 ± 6.50[d]	11.95 ± 2.64[d]
ANOVA					
F-value	0.987	45.895	65.125	76.125	74.122
P-value	0.762	0.0000	0.0000	0.0000	0.0000

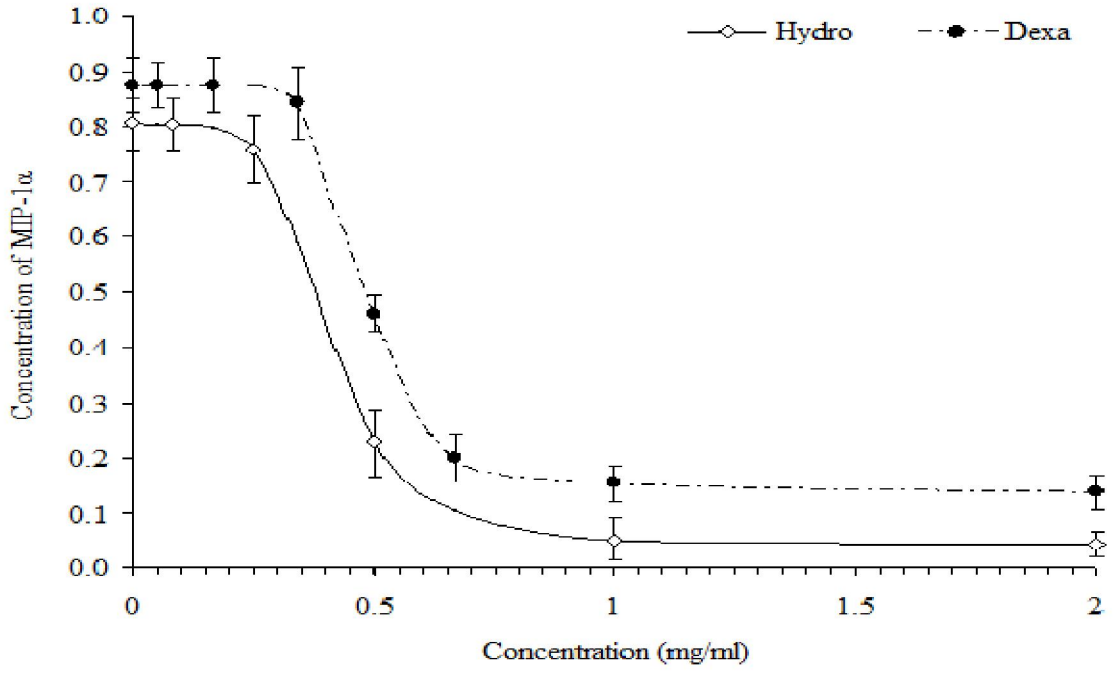

Figure 3: Concentration of MIP-1α in serum of Balb/c mice after infection with L. major promastigotes

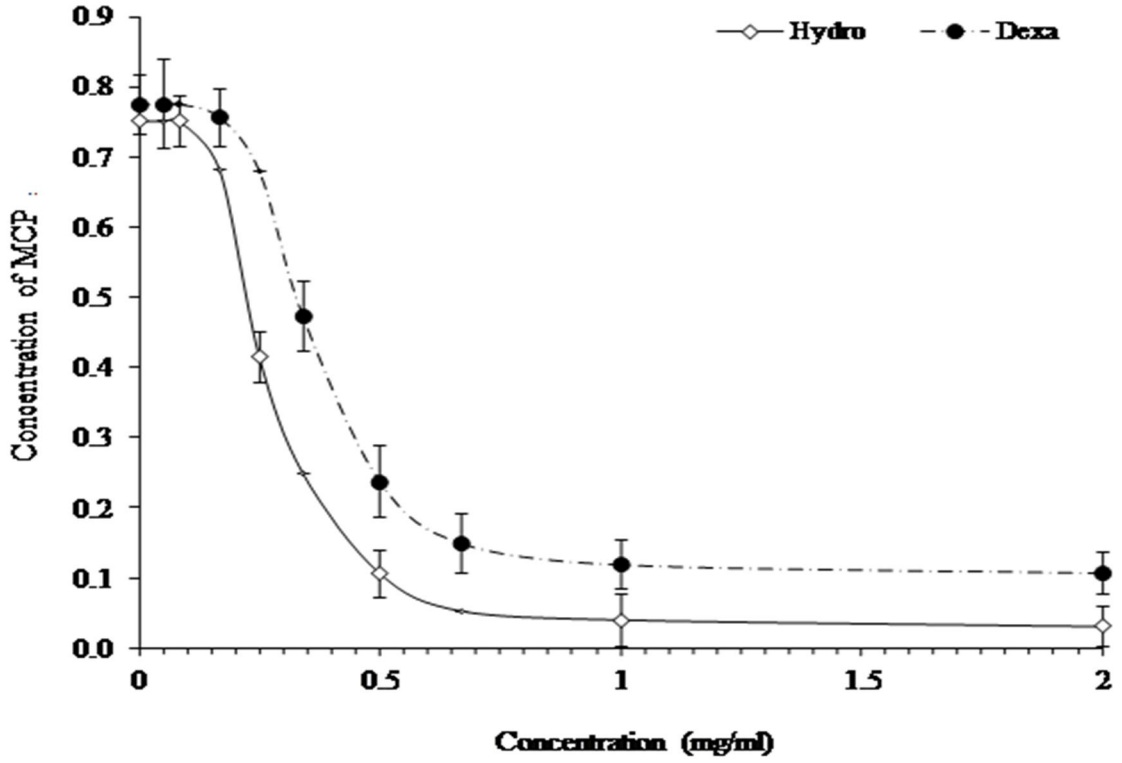

Figure 4: Concentration of MCP in serum of Balb/c mice before infection with L. major promastigotes

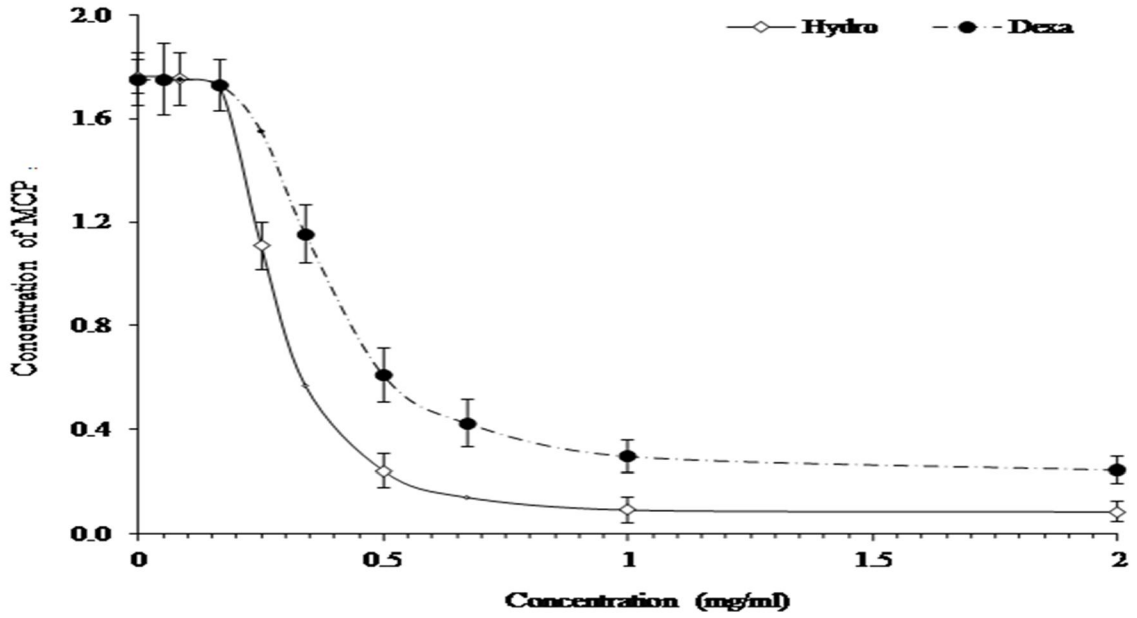

Figure 5: Concentration of MCP in serum of Balb/c mice after infection by L. major promastigotes

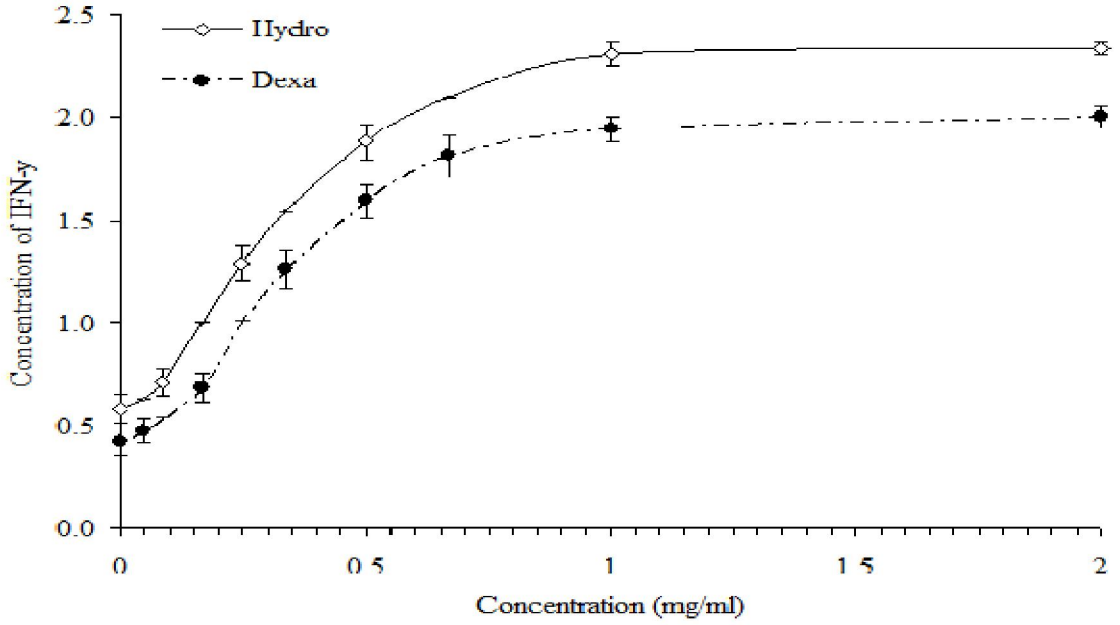

Figure 6: serum IFN-γ levels of Balb/c mice infected with L. major and treated with GC drugs

4.0 Discussion

The *L. major*, the aetiological agent of Cutaneous leishmaniasis in humans skin. However, in the BALB/c mice, itproduces visceral infection in BALB/c mice in addition to the local lesion at the point of inoculation which made it a suitable model for this study. Spontaneous healing cannot be achieved in the mice and therefore, the healing of the lesions was due to the GC therapy administered only. To better evaluate if the drugs effectively reduced the parasites burden in the spleen after treatment, varied concentration efficacies for the GCs were determined. In the spleen, the non-treated controls had the highest LDU. Significantly ($P < 0.05$) higher parasite load in the spleen occurred in dexa as compared to hydro.

These results indicate that hydro and dexa are promising drugs for development as immunoprophylactic antileishmanial agents. PBS was used as diluent in the drug preparations. LPS was the positive control because endotoxin or its purified derivative LPS is a key activator of the innate immune system, stimulating mononuclear phagocytes to synthesize an array of cytokines and chemokine that recruit inflammatory cells to the involved tissue as well as activating immune and inflammatory responses. Hydro was the most effective in reducing both the lesion sizes and splenic parasite load; followed by dexa. These drugs were used in a dose daily injected in mice previously infected with *L. major*. The significant decrease in the lesion sizes in hydro is an indicator of antileishmanial properties of hydro and it is interesting to note that the administration of hydro significantly ($P < 0.05$) reduced footpad size and concomitantly inhibited parasite growth.

Reported was a remarkable long-term *in vivo* activity hydro, 2mg/ml and dexa 0.67mg/ml administered subcutaneously against *L. major* infection in BALB/c mice. The most frequently used drug in treatment of leishmaniasis is pentostam and, for completeness, since a direct extrapolation of the drug doses from mice to humans is unreliable, it is important to note that in *Leishmania* therapy the recommended therapeutic dose in humans has been established to be 20 mg/kg of body weight/day for 20 days. The short-term efficacy of the test compounds against a *Leishmania* infection which evolved for 1.5 months prior to drug administration was demonstrated. This phase of murine cutaneous leishmaniasis caused by *L. major* corresponds to the end of the acute phase of the disease and was characterized in this study as demonstrated by Lapara (2010) Figure 3.

The amastigote loads were significantly lower in the spleens of hydro as compared to dexa-treated mice than in those of controls at all-time points tested. Moreover, the progression of the splenomegally and the increase of the splenic load were found to be significantly lower in treated mice than in controls, demonstrating that parasite suppression and the inhibition of growth persistent. Although a direct measure of the parasite killing (1-[amastigote load at the end of the treatment/amastigote load before the start of the treatment]) is not provided by data in this study, induction of effective anti-leishmanial activities evident. In leishmaniasis, the spleen is a major site of multiplication in the natural infection in susceptible hosts. In BALB/c mice, the splenic parasite burden is initially quite low, but it increases steadily for at least 3 months, and does not decline spontaneously without treatment (Hoffman *et al,.* 2009). This is why the splenic efficacy of the drugs is emphasized, since until recently splenectomy has been performed as the last recourse for cases of antimony resistant leishmaniasis. The efficacy of the drugs in the spleen is compatible with the available data on its tissue distribution as demonstrated by Hoffman (2009). The prolonged effect on parasite growth suggests a low catabolism and/or slow clearance, resulting in a long biological half-life for the drugs.

The results of this study indicate that hydrocortisone was effective in both healing the lesion and eliminating the parasites from the spleen at a concentration of 2 mg/ml as opposed to the negative control, PBS. Complete parasite elimination was not obtained but Hydro still gave better result in parasite elimination as compared to dexa. In 1993, Marion and others reported that higher efficacy may be correlated with molecular structural features of compounds under study. This is the first time antileishmanial activity in GCs has been reported with emphasis on cortisol (hydrocortisone) and dexamethasone.

5.0 Conclusion

We report that hydro resulted in partial clearance of parasitemia from both the lesions on footpads and spleens of *L. major*-infected BALB/c mice. Based on these results and existing studies reporting on safe doses and side effects of GCs, and administration by the subcutaneous route, use of GCs is a promising approach for immunoprophylaxis of *L. major* infection.

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