

## Full Length Research Paper

# Efficacy of glucocorticoids in controlling leishmania major infecting Balb/c mice

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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 of 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* parasites. Six were treated with dexamethasone (0.69 mg/ml), another six hydrocortisone (2 mg/ml), six lipopolysaccharide (LPS) (10 ng/ml) and six phosphate-buffered saline (PBS) for 28 days and lesion development monitored for five weeks. For immunoprophylaxis (pre-infection), 24 other mice were treated with the same drugs and then infected with *L. major*. LPS was used as the positive control while PBS was the negative. Serum samples were collected for cytokine analysis for MIP 1 $\alpha$ , MCP 1 and IFN $\gamma$  using enzyme linked immunosorbent assay (ELISA). Parasite quantification was done by calculating the leishmania donovan unit (LDU). Lesion measurement was done by use of a vernier caliper. Lesion sizes after infection of BALB/c mice were similar in all the chemotherapeutic 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. 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-1 $\alpha$  and high levels of IFN  $\gamma$ . We show that glucocorticoids substantially reduce parasitemia in *Leishmania* infected mice by decreasing production of MCP 1 and MIP-1 $\alpha$  chemokines while increasing IFN  $\gamma$  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-1 $\alpha$ , MCP-1, dexamethasone, hydrocortisone, glucocorticoids (GCs), *Leishmania*.

## INTRODUCTION

Leishmaniasis is a zoonotic disease of major public health importance. At least two million new cases are

reported each year, 367 million people are at risk and 88 countries, mostly tropical countries, are affected (World

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Health Organization (WHO), 2007; Hotez and Kamath, 2009; Grimaldi and Tesh, 2012). It is a neglected disease caused by a protozoan parasite of the genus *Leishmania* and is transmitted to the mammalian host by bites of their vectors of *Phlebotomine* species (Yamey and Torreele, 2000; 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). The infective stage is metacyclic promastigote in the sandfly vector, which after entering the human host invades macrophages and differentiates into amastigotes (Assafa et al., 2006). The actively replicating stage is the promastigote.

Disease outcome often depends on the particular *Leishmania* sp. or 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 containment and for the resolution of disease. 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; Croft et al., 2006; Melby, 2002; Thakur et al., 2004; mishra et al., 2007). 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.

In *Leishmania major* infections, cutaneous lesions develop at sites of insect bites. Most of the time, the lesions heal spontaneously within weeks or even months. The infection may involve visceralization in susceptible animal models BALB/c strain of mice but is highly uncommon in man though draining lymph nodes may be involved. Following self-healing, there results immunity. *L. major*, inoculated in the skin of BALB/c mice, produces large ulcers which do not resolve due to the susceptibility of this particular strain. The parasite metastasizes has uncontrolled growth and eventually leads to death. In most strains of experimental mice, *L. major* infection causes no or only transient pathological changes, whereas some strains develop systemic visceral disease with splenomegaly, hepatomegaly, anemia, hypergammaglobulinemia and skin lesions (Phillips, 1993; Reithinger et al., 2007).

Both MCP-1 and MIP 1 $\alpha$  are potent chemo-attractants 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. *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 1 $\alpha$  and MCP 1 (Gregory et al., 2008; Teixeira et al., 2005). Silencing of CCR5, a receptor for MIP-1 $\alpha$ , has been reported to cause low parasite entry in *L. donovani* infected macrophages (Bhattacharyya et al., 2008). Some progress towards 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 unclear how inhibition of production of MIP-1 $\alpha$  and MCP-1 chemokines affects the parasite and disease progression and its influence if any on lesion development in mice.

NF- $\kappa$ B (nuclear factor kappa beta) is a critical transcriptional factor which regulates the expression of many gene encoding proteins. Activation of the NF- $\kappa$ B pathway is a strategy that protects the host cells from apoptosis, allowing pathogens to survive, replicate and disseminate (Guizani-Tabbane et al., 2004). Dexamethasone and Hydrocortisone inhibit the activation of NF- $\kappa$ B by preventing the degradation of I $\kappa$ B, hence retention of NF- $\kappa$ B in the cytosol (Kopp and Ghosh, 1994). *Leishmania* infection upregulates the NF- $\kappa$ B pathway, resulting in increase of MIP-1 $\alpha$  and MCP-1 chemokines and thus induction of macrophage chemotaxis therefore aggravating the infection though the inflammatory pathway is shut off (Gregory et al., 2008).

This study was aimed at evaluating the effect of glucocorticoids on lesion development, parasite infectivity and multiplication indices. The effect of glucocorticoids (GCs) on the production of MIP-1 $\alpha$  and MCP-1 chemokines and IFN  $\gamma$  in BALB/c mice was also determined.

## MATERIALS AND METHODS

### *Leishmania* parasites

Metacyclic promastigotes of *L. major* (IDUB/KE/83= NLB-144 strain) were used. This strain has been maintained by cryopreservation, *in vitro* culture and periodic passage in BALB/c mice at Kenya Medical Research Institute (KEMRI). Parasites were grown at 26°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 weighing 20  $\pm$  2 g 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 laminar air flow. Twenty eight mice were inoculated intramuscularly with 2  $\times$  10<sup>6</sup> 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 infected left hind footpad was compared against the uninfected right hind footpads on a weekly basis according to the method of Nolan and Farrell (1987). 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:  $S = (D \times d) / 2$ . 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.

### 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). Dexamethasone was more toxic and therefore used at a concentration of 0.67 mg/ml while dexamethasone was used at 2 mg/ml. Lipopolysaccharide (LPS) (5 mg/ml obtained from Invitrogen™) was diluted with phosphate-buffered saline (PBS) to a final concentration of 10 ng/ml. Five weeks after inoculation (day 29), skin lesions were confirmed and the infected mice were randomly assigned to four groups of six mice each. The mice were administered with the drugs intraperitoneally for twenty eight days. For experimental handling, the animals were grouped as follows: group A received a dose each of dexamethasone; group B received a dose each of hydrocortisone; group C received LPS which served as 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.

### Promastigote cultivation

*Leishmania major* (strain IDUB/KE/83 = NLB 144) 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 promastigote forms of *L. major* were cultured in complete Schneider's medium prepared with 20% heat inactivated fetal calf serum (FCS) and filtered in sterile conditions. They were diluted to  $1 \times 10^6$  cell per ml of medium.

### Chemokine analyses by sandwich ELISA

The level of mouse MIP-1α and MCP-1 in the sera of GC treated mice was measured by use of mouse CCL3/MIP-1α and CCL2/JE/MCP-1 ELISA 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/ml, while that of MCP 1 is less than 2 pg/ml.

### 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  $\times$  organ weight [in grams]  $\times 2 \times 10^5$ ), according to the formula of Bradley and Kirkley (1977). The percent efficacy was calculated as  $[1 - (\text{mean amastigote load in treated mice} / \text{mean amastigote load in 9 NTC})] \times 100$ . An LDU reduction of at least 80% was adopted as the minimal criterion for drug efficacy.

### Statistical analysis

All experiments were carried out in triplicates. The mean and standard deviation of at least three experiments were determined. Analysis of variance (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.

## RESULTS

### Effect of GC therapy on *L. major* lesion development

BALB/c mice infected on the left hind footpad 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 five weeks of infection. After 15 days post-treatment, significant differences were discerned in the lesion sizes of the BALB/c mice. The untreated controls as well as the LPS treated mice displayed the highest increase in lesion size. However, low rates of increase in lesion was recorded in dexamethasone, which was significantly ( $P < 0.05$ ) higher than the lesion size in mice treated with hydrocortisone.

### Effects of GC therapy on spleen parasite burdens (determination of LDU)

Splenic impression enumeration of amastigotes yielded no significant differences in the body weights among the treated mice ( $P > 0.05$ ). Weight of the spleen, spleno-somatic index, number of parasites and LDU followed similar trends where hydrocortisone<sup>(a)</sup> had the lowest value followed by values of dexamethasone<sup>(b)</sup>, while PBS, the negative control group<sup>(d)</sup> had the highest values. For weight of spleen, spleno-somatic index and number of parasites, significant differences were observed in all the four treatment groups (Table 1).

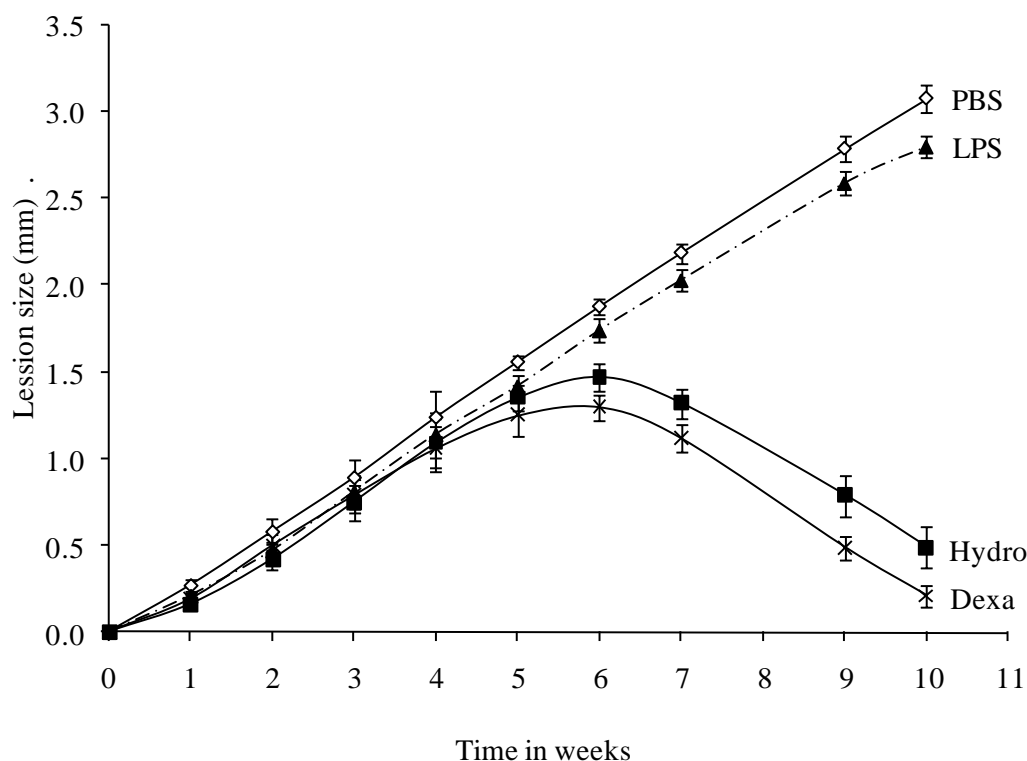
### Effects of GCs on production of MIP-1α, in BALB/c mice

For treatment before *L. major* infection, there was a significant difference at all concentration levels while for

**Table 1.** Body weight, weight of spleen, spleno-somatic index, number of parasites and LDU in Balb/c mice following treatment with hydrocortisone, dexamethasone, LPS and PBS.

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

Means with the same letters as superscripts are not significantly different ( $P > 0.05$ ) based on ANOVA followed by Duncans multiple range test (DMRT).

**Figure 1.** Lesion development in Balb/c mice subjected to different drug treatments.

treatment after infection, there was no significant difference until a concentration of 0.4 mg/l. The significant difference recorded ( $F = 32.222$ ,  $df = 12$ ,  $P = 0.0000$ ) was statistically significant. However, hydrocortisone appeared to elicit the higher reduction in production of MIP-1 $\alpha$  compared to dexamethasone. The efficacious ranges of hydrocortisone ranged from 0.25 to 0.8 mg/l while similar efficacious range of dexamethasone ranged from 0.4 to 0.9 mg/l. Dexamethasone resulted in 80% reduction in the

concentration of MIP-1 $\alpha$  compared to 92.6% reduction in the concentration for hydrocortisone (Figure 2). There was a significant difference between the concentrations of MIP-1 $\alpha$  after *L. major* infection among the various ranges of concentrations tested ( $F = 16.785$ ,  $df = 12$ ,  $P = 0.0001$ ). Again, hydrocortisone elicited a greater reduction in the MIP-1 $\alpha$  level as compared to dexamethasone. The efficacious ranges of hydrocortisone were from 0.16 to 0.85 mg/l while the efficacious range of dexamethasone ranged from 0.45 to

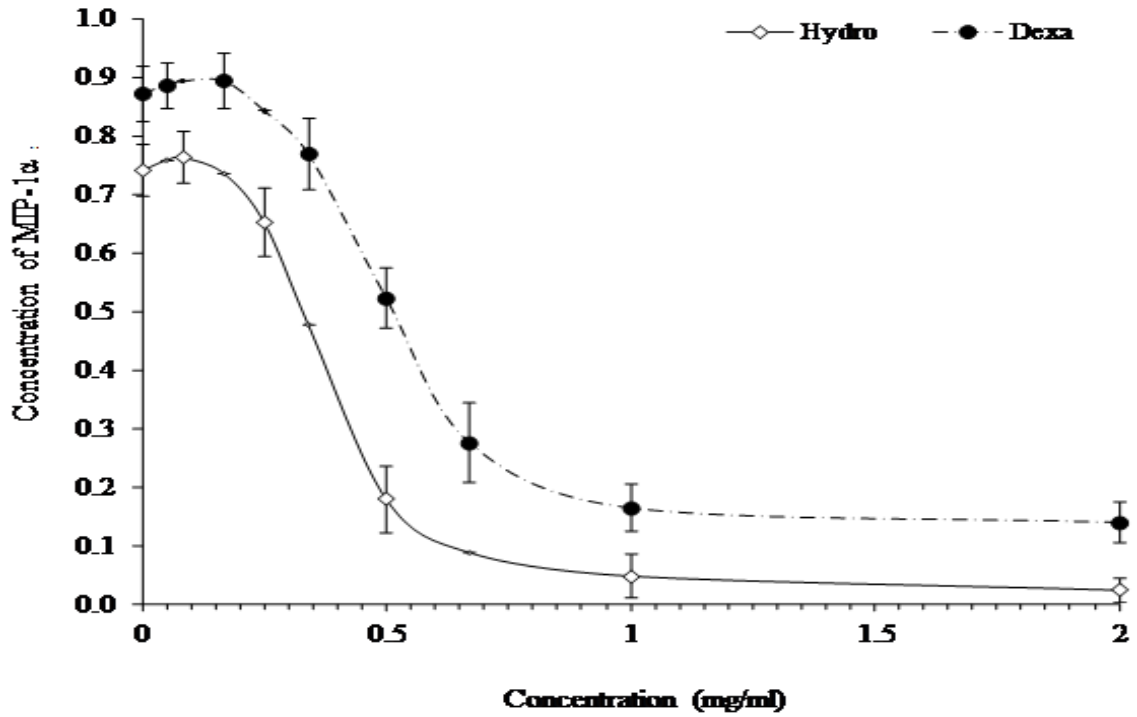


Figure 2. Concentration of MIP-1α in serum of Balb/c mice before infection with *L. major* promastigotes.

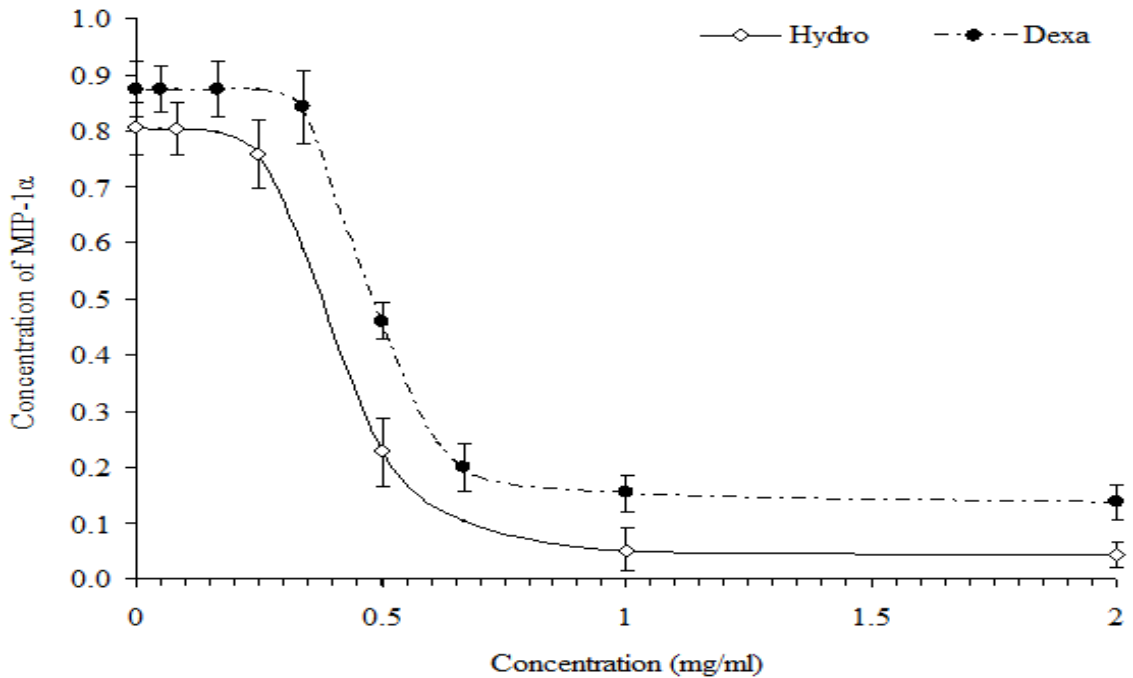


Figure 3. Concentration of MIP-1α in serum of Balb/c mice after infection with *L. major* promastigotes.

1.0 mg/l. Dexamethasone resulted in 80.5% reduction in the concentration of MIP-1α compared to 95.2%

reduction in the concentration in hydrocortisone (Figure 3).

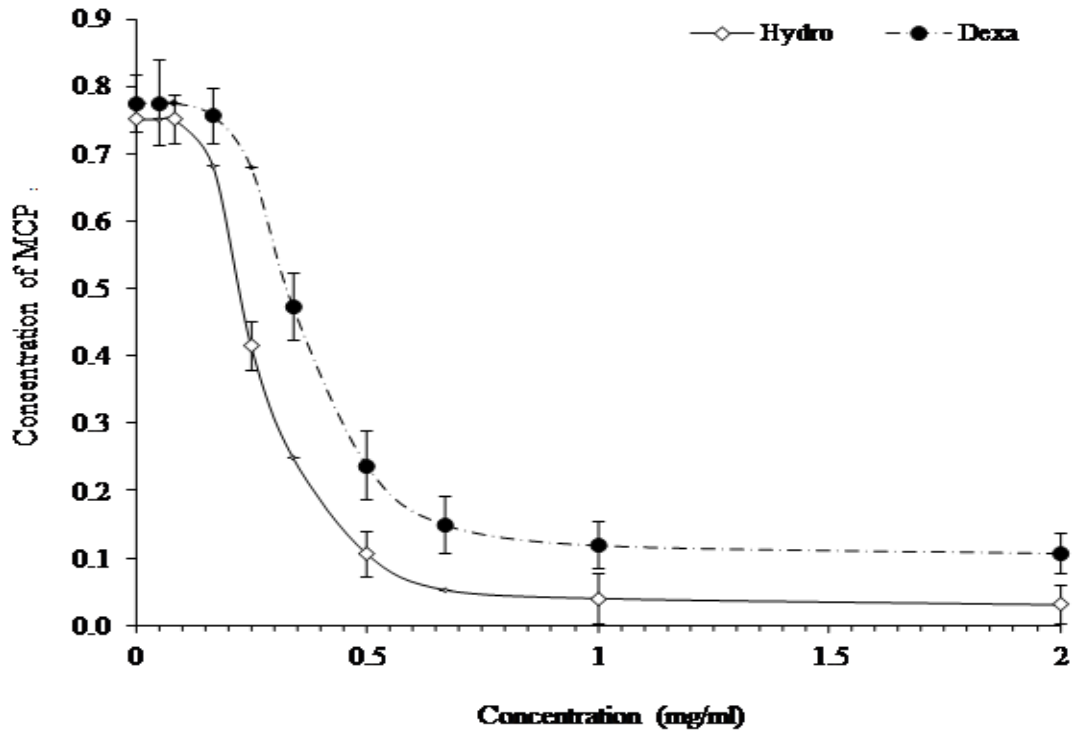


Figure 4. Concentration of MCP in serum of Balb/c mice before infection with *L. major* promastigotes.

#### 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 mice before infection with *L. major* promastigote indicated a significant difference between the concentrations of MCP before *L. major* infection from a concentration of 0.4 mg/l among the various ranges of concentration tested ( $F = 29.542$ ,  $df = 12$ ,  $P = 0.0000$ ). The efficacious ranges of hydrocortisone ranged from 0.081 to 0.72 mg/ml while similar efficacious range of dexamethasone ranged from 0.17 to 0.67 mg/ml. Dexamethasone also resulted in 80% reduction in the concentration of MCP while hydrocortisone resulted in 92.4% reduction in the concentration in MCP (Figure 4). Post infection, a significant difference in the concentrations of MCP was recorded among the various ranges of concentrations tested ( $F = 56.115.542$ ,  $df = 12$ ,  $P = 0.0000$ ) with efficacious ranges of hydrocortisone ranging from 0.08 to 0.67 mg/ml while those of dexamethasone ranged from 0.17 to 0.67 mg/ml. Dexamethasone also resulted in 80% reduction in the concentration of MCP while hydrocortisone resulted in 92.4% reduction in the concentration of MCP (Figure 5).

#### Effects of GCs on production of IFN $\gamma$ in BALB/c mice

There were significant changes in IFN- $\gamma$  levels in serum of

infected mice treated with hydrocortisone or dexamethasone ( $F = 8.745$ ,  $df = 7$ ,  $P = 0.0001$ ). IFN- $\gamma$  levels increased from the start of the experiments in both the treatment groups albeit the hydrocortisone treated mice produced larger quantities of IFN- $\gamma$  (Figure 6).

#### DISCUSSION

This study was aimed at determining the effects of glucocorticoids on *L. major* infection in Balb/c mice. The two glucocorticoids used in this study were hydrocortisone and dexamethasone. *L. major*, the aetiological agent of cutaneous leishmaniasis in humans, is a parasite of the skin. However, in the BALB/c mice, it produces visceral infection 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 infected 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, treatment efficacies of various concentrations of 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 dexamethasone as compared to hydrocortisone. These results indicate that hydrocortisone and dexamethasone are promising drugs for development as immunoprophylactic antileishmanial agents. PBS was used as diluent in the drug preparations.

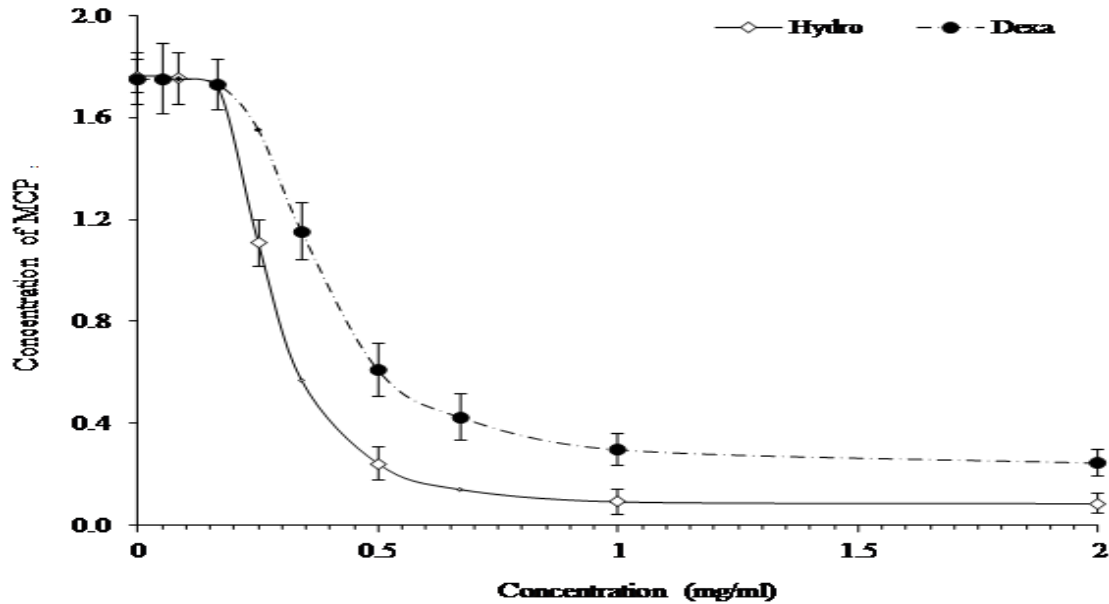


Figure 5. Concentration of MCP in serum of Balb/c mice after infection by *L. major* promastigotes.

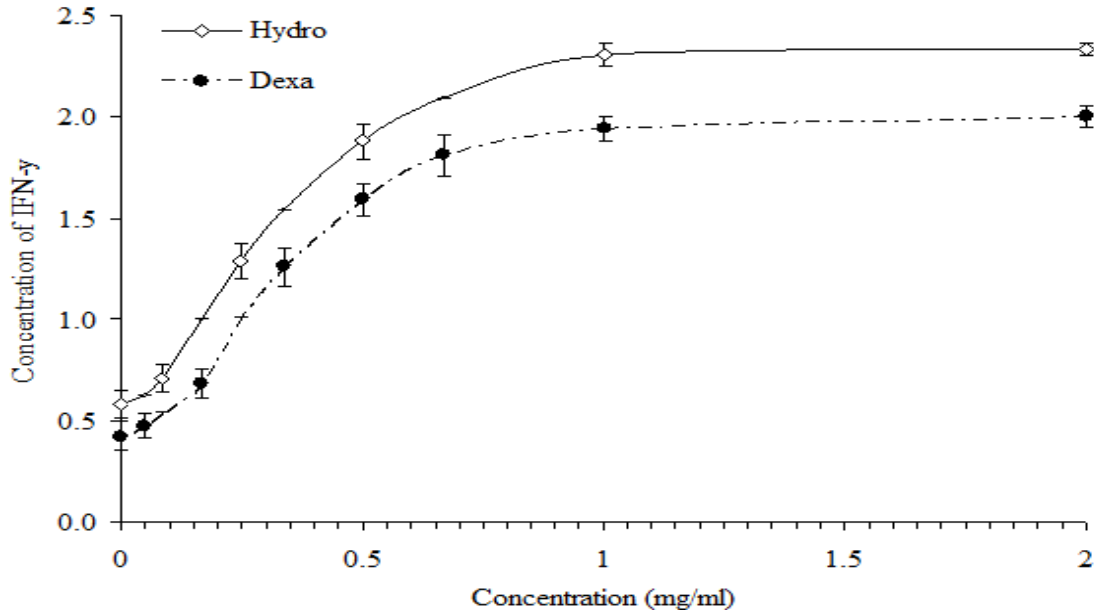
LPS was the positive control because endotoxin or its purified derivative is a key activator of the innate immune system. It acts to stimulate mononuclear phagocytes to synthesize an array of cytokines and chemokines (MIP-1 $\alpha$  and MCP-1) that recruit inflammatory cells to the involved tissue as well as activating immune and inflammatory responses. Hydrocortisone was most effective in reducing both the lesion sizes and splenic parasite load; followed by dexamethasone. These drugs were administered daily to mice previously infected with *L. major*. The significant decrease in the lesion sizes in hydrocortisone is an indicator of antileishmanial properties of hydrocortisone and it is interesting to note that the administration of hydrocortisone significantly ( $P < 0.05$ ) reduced footpad size and concomitantly inhibited parasite growth.

In this study, hydrocortisone and dexamethasone significantly ( $P < 0.05$ ) reduced levels of MIP-1 $\alpha$  and MCP-1 chemokines both before and after infection with *L. major* promastigotes. Both MIP-1 $\alpha$  and MCP-1 have been reported to influence the Th1/Th2 paradigm shift responsible for either resistance or susceptibility to leishmaniasis (Alexander and Bryson, 2005; Rolao et al., 2007). Both hydrocortisone and dexamethasone have been reported to have remarkable long-term *in vivo* activity in BALB/c mice at 2 and 0.67 mg/ml, respectively after subcutaneous administration. The anti-parasitic activity of these drugs can be attributed to the reduction in production of MIP-1 $\alpha$  and MCP-1 chemokines which are induced by the parasites to propagate infection as reported by Teixeira et al. (2006).

Pentavalent antimony compounds, mainly sodium stibogluconate (pentostam) and meglumine antimonite

(glucantime) have mainly been used for antileishmanial chemotherapy. Although they are usually highly effective, their disadvantages include parenteral mode of administration thus long duration of therapy normally lasting several weeks, suboptimal effectiveness in some settings, bothersome and frequent though almost always-reversible toxic effects that include fatigue, body aches, electrocardiographic abnormalities, raised aminotransferase levels and chemical pancreatitis (Roberts, 2006; Santos et al., 2008), the most frequently used drug is pentostam. 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 (Pearson and Sousa, 2009; Rey, 2012). The short-term efficacy of glucocorticoids 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 and Kelly (2010).

In infected mice, the splenic parasite burden is initially quite low, but it increases steadily and does not decline spontaneously without treatment unlike the hepatic parasite burden. The spleen is a major site of *Leishmania* multiplication during natural infection in susceptible hosts (Croft et al., 2003; Hoffman et al., 2009). The amastigote loads in this study were significantly lower in the spleens for hydrocortisone as compared to dexamethasone-treated mice than in those of controls at all time points tested. Moreover, the progression of the splenomegaly



**Figure 6.** serum IFN- $\gamma$  levels of Balb/c mice infected with *L. major* and treated with GC drugs.

and the increase of the splenic load were found to be significantly lower in treated mice than in controls, demonstrating parasite suppression and the inhibition of growth. The efficacy of the drugs in clearing infection in the spleen needs to be emphasized because for a long time, splenectomy has been performed as the last resort in 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 Croft (2003).

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 activity is evident. The prolonged effect on parasite growth suggests a low catabolism and/or slow clearance, resulting in a long biological half-life for the drugs. During *L. major* infection in mice, IFN- $\gamma$  plays an essential role in controlling parasite growth and disease progression. It is also reported to activate macrophages to kill promastigotes (Grimm et al., 2011).

The resolution mechanism in leishmaniasis is majorly characterized by induction of specific IFN- $\gamma$  releasing CD4<sup>+</sup> T cells while the failure to cure is associated with elevated levels of IL-4, IL-10 and IL-13 with low IFN- $\gamma$  responses from *Leishmania*-specific CD4<sup>+</sup> T cells (Ajdari et al., 2000; Mansueto et al., 2011). In this study, the glucocorticoids significantly increased the production of IFN- $\gamma$  and the decrease in parasitaemia could be attributed to the high levels of this potent cytokine. These results are supported by the study by Kemp et al. (1999) which reported that a population of antigen-specific T cells co-producing IL-10 and IFN- $\gamma$  which expand in

response to *L. donovani* infection in humans contributed to disease resistance. Literature reports further suggest that both genetic and environmental factors influence the outcome of infection in humans (Karplus et al., 2002; Mohamed et al., 2003).

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. Although complete parasite elimination was not obtained by either of the glucocorticoids, hydrocortisone was more effective than dexamethasone in eliminating parasite load. Marion et al. (1993) reported that higher efficacy may be correlated with molecular structural features of compounds under study. This is the first time anti-leishmanial activity in GCs has been reported with emphasis on cortisol (hydrocortisone) and dexamethasone.

## Conclusion

We report that both hydrocortisone and dexamethasone 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 on safe doses and side effects of GCs, use of GCs is a promising approach for immunoprophylaxis and chemotherapy of *L. major* infection.

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### Conflict of Interest

The authors declare no competing interest.

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