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Effects of glucocorticoids in *Leishmania major* infection

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Abstract
Leishmania parasites activate NF-κB which induces Th2 expression and inactivates Th1 genes thus subverting the host defense response and promotes the survival and development of the parasite in macrophages. Macrophages were treated artificially with glucocorticoids and incubated with *Leishmania* promastigotes. Interleukin 1 β, Tumor necrosis factor- α and inhibitory nitric oxide synthase gene levels were measured using real time PCR and parasite development monitored *in vitro*. Tumor necrosis factor- α and nitric oxide synthase genes were down-regulated and Interleukin 1 β upregulated in macrophages treated with dexamethasone and hydrocortisone drugs when compared to those treated with lipopolysaccharide and untreated. Dexamethasone treated macrophages had significantly low number of amastigotes compared to hydrocortisone and lipopolysaccharide (p=0.0006). Dexamethasone showed high reduction of infection rates in macrophages as compared to hydrocortisone and lipopolysaccharide treated macrophages, however not significant (p=0.054). With further clinical studies in humans, dexamethasone may be used in the control of leishmaniasis.

Keywords: Glucocorticoids, NF-kB, infection rates, amastigotes, macrophages

1. Introduction
The leishmaniases are diseases caused by obligate intracellular, kinetoplastid protozoa of the genus *Leishmania* (Trypanosomatidae) [1]. Leishmaniases are endemic in 88 countries of the world in which 350 million people who are considered at risk of infection every year approximately 2 million new cases of leishmaniasis are reported[2].

Proven therapies against human leishmaniasis include pentavalent antimonials (sodium stibogluconate and meglumine antimoniate), amphotericin B, pentamidine, miltefosine and paromomycin [3, 4]. These drugs are unsatisfactory because of their limited efficacy, frequent side effects and increasing drug resistance, therefore new, safer and more efficacious drugs are urgently required [5]. Moreover, there is no effective commercially available vaccine against leishmaniasis [6] which posses the danger of over-reliance in the use of anti-protozoan drugs for the management of the disease. Ideally for a successful control strategy, several approaches targeting different aspects of the parasite life cycle should be combined.

Leishmania parasites reside and multiply in their mammalian hosts within macrophages. Macrophages are one of the first lines of defense in the response against pathogenic infection. Infection of a macrophage leads to induction of numerous cellular genes, several of which encode cytokines that stimulate an inflammatory response and resistance to pathogens. To escape the host immune defense and to survive, *Leishmania* parasites have developed different strategies and inhibit several macrophage functions, including phagocytosis, nitric oxide generation, interleukin-12 (IL-12) production, and major histocompatibility complex class II expression [7].

Leishmania invasion induces the activation of the nuclear factor kappa B (NF-κB) transcription factor, which initiates innate immune responses. The NF-κB-Rel family is composed of five different members, NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. These proteins are present in resting cells as inactive complexes sequestered in the cytoplasm by tight binding to the inhibitory protein IκB [8].

Glucocorticoids (dexamethasone and hydrocortisone) inhibit the activation of NF-κB by preventing the degradation of inhibitory kappa beta (Iκβ), leading to retention of NF-κB in the

Cytosol [9]. *Leishmania* up regulates NF-κB pathway in macrophage cytosol, thus resulting to inhibition of Th1 cytokines therefore excerbarating infection.

Glucocorticoids induces synthesis of inhibitor kappa beta (IKβα) leading to upregulation of IKβα protein and cytoplasmic retention of NF-κB p65 in monocytes [10].

Complete and persistent inhibition of NF-κB has been linked directly to apoptosis, inappropriate immune cell development and delayed cell growth. Therefore, development of modulatory strategies targeting this transcription factor may provide a novel therapeutic tool for the treatment or prevention of leishmaniases. This study aims at treatment of macrophages with selected GC drugs and subsequent measurement of IL-β, TNF-α and iNOS mRNA expression levels and determines parasite development in macrophages.

2. Materials and Methods
2.1 Parasite culture
Metacyclic promastigotes of *L. major* strain (Strain IDU/KE/83 =NLB-144) and *L. donovani* (strain NLB-065) were used. Parasites were maintained as previously described [11]. Briefly, promastigotes were cultured in Schneider's Drosophila medium supplemented with 20% foetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin (100 μg/ml). Stationary-phase promastigotes were obtained from 5 to 7 day-old cultures.

2.2 Drug preparation
The glucocorticoids was dissolved in 15% RPMI1640 and used at a final concentration of 10-100μM. LPS (5mg/ml obtained from invivogen) was diluted with 15% RPMI1640 to a final concentration of 100 μM.

2.3 Macrophage harvesting
BALB/C mice was bred and housed in KEMRI animal house facility. Experimental animals will be housed in designated animal holding facilities. Six mice will be randomly selected for macrophage harvest for each treatment. Each individual cage will be labeled using identification cards that will bear; Name of investigator, species of animal, gender of animal, type of experiment and length of study.

Six to eight week old female BALB/C mice was injected with 2% starch solution, using 18 needle gauge, into the peritoneal cavity for macrophage stimulation. After 48 hrs the mice will be placed in the anaesthetizing chamber with chloroform fumes. The body surface was disinfected with 70% ethanol and torn dorso-ventrally to expose the peritoneum. 10 ml of sterile cold phosphate buffered saline (PBS) was injected again using 18 needle gauge into the peritoneum. Peritoneal macrophages were harvested by withdrawing the PBS. The macrophages were washed through centrifugation at 2 rpm for 10 minutes and pellet obtained was re-suspended in RPMI 1640 culture medium. The macrophages were adsorbed in 24 well plates for 4 hrs at 37ºC in 5% CO_2. Non-adherent macrophages were washed with cold PBS and incubated overnight in RPMI 1640 culture medium. The mice were handled and disposed according to KEMRI animal care and use and guidelines. After macrophage harvesting, the mice was disposed in a red bag labeled with biohazard symbol and transported for incineration.

2.4 Treatment of macrophages with drugs
Macrophages were incubated with the drugs for 8 hrs and 12 hrs. Untreated macrophages were used as negative controls and those treated with LPS was used as positive controls. Each treatment (dexamethasone, hydrocortisone, LPS) was serial diluted four times to obtain four supernants (500μl) from each

2.5 Determination of Nitric oxide production
Nitric Oxide release in macrophages culture was measured using the Greiss reaction for nitrites [12]. Then 100 ml of the supernants were collected 8 hours after treating cultured macrophages with glucocorticoid drugs, LPS and untreated one. The assays were done in duplicate wells in the 96-well micro-titre plates. 60 ml of Greiss reagent A (1% Sulphonilmide in 1.2 M HCL) were added followed by 60ml of Greiss reagent B (0.35 N [1-naphthy] ethlenediamine). The plates were read at 540nm in the enzyme linked immunosorbent assay reader. Sodium nitrite in RPMI was used to construct a standard curve for each plate reading.

2.6 Messenger RNA extraction, cDNA synthesis and quantitative realtime PCR Assay
Messenger RNA extraction from supernatants and cDNA synthesis were done according to qiagen QIAmp RNA min kit, manufacturer's instruction. Every gene run on quantitative realtime PCR was run with a standard. DNA Master SYBR Green I mix (containing *Taq* DNA polymerase, dNTP, $MgCl_2$, and SYBR Green I dye (qiagen manufacturers) was used. Each reaction (20 μl) contained 8 μl of the respective cDNA dilution, 0.4 μl of the respective forward and reverse primers, 1.2 μl of RNAse free water and 10 μlof SYBR Green I mix. The amplification program consists of initial activation, denaturation, annealing and extension (Appendix 1). A negative control without cDNA template was run with every assay to assess the overall specificity.

2.7 Determination of infection rates and multiplication in macrophages
Adherent macrophages (treated and non-treated) were infected with *L. major* promastigotes at a parasite/macrophage ratio of 6:1, incubated at 37ºC in 5% CO_2 for 4 hours, free promastigotes removed by extensive washing with PBS, and the cultures incubated in RPMI 1640 medium for 24 h. After 5 days the monolayers were washed with PBS at 37ºC, fixed in methanol and stained with 10% Giemsa solution. The number of amastigotes was determined by counting at least 100 macrophages in duplicate cultures, and results expressed as infection rate (IR) and multiplication index (MI) , IR = No. of infected macrophages in 100 macrophages

$$MI = \frac{(\text{No. of amastigotes in experimental culture per 100 macrophages}) \times 100}{\text{No. of amastigotes in control culture per 100 macrophages}).}$$

The infection rate was used in the calculation of the association index. The AIs were be determined by multiplying the percentage of infected macrophages by the number of parasites per infected cell. Association indices were the number of parasites that actually infected the macrophages.

2.8 Statistical analyses
Data were presented as mean and standard error mean or standard deviation. Comparison between multiple groups was performed by analysis of variance (ANOVA) and when significant, comparisons between two groups were performed by student's t-test. All analyses were carried out at 5% level of significance.

3 Results

3.1 Expression levels of IL-1β, TNF-α and iNOS mRNA in macrophages treated with Dexamethasone, hydrocortisone and LPS drugs

Total RNA extracted from peritoneal cells of mice was reverse transcribed. Complementary DNA samples were amplified using real-time quantitative polymerase chain reaction and SYBR Green detection. Expression of IL-1β, TNF-α, iNOS in macrophage cells (Figures 3, 4, 5 respectively) was compared between GC treated cells and LPS treated and untreated cells. Macrophage cells were treated with serial dilution of dexamethasone and Hydrocortisone drugs (100 μM) for 8 hrs. Values are mean ± SEM from three independent experiments performed in triplicate. These results suggest that dexamethasone upregulates IL-1β but downregulates TNF-α and iNOS in macrophages. Hydrocortisone down regulates IL-1β, TNF-α and iNOS in macrophages, however LPS treated macrophages had upregulated expression of IL-1β, TNF-α and iNOS.

3.2 Infection rates

Infection rates of macrophages treated with the three drugs were not significant (Figure 4). However the infection rates was found to be lower in macrophages treated with dexamethasone as compared to those treated with hydrocortisone and LPS.

3.3 *Leishmania* amastigote multiplication index

The number of parasite multiplication in the macrophages decreases as the concentration of each drug increases but there is a steady parasite load in the negative control as the concentration is increased. Hydrocortisone had a high number of parasite load as compared to the LPS and dexamethasone treated macrophages (Figure 5). In this study LPS was used as a positive control and have shown the same multiplication index of the parasite with dexamethasone treated macrophages, this study suggests that the dexamethasone is more effective in clearing parasite as compared to hydrocortisone.

3.4 Measurement of nitric oxide (NO) stimulation

The macrophages were incubated with the drugs at 37°C in 5% CO_2 incubator and 100μl of the supernatant tested for NO production using nitrite test method.

The nitric oxide production in supernatants of macrophage culture treated with test drugs and controls were determined using a representative standard curve for samples at concentrations between 0 and 1000 μg/ml. No significant NO levels (p>0.05) were produced compared to the standards (Figure 6). None of the samples had optical density readings of more than 0.5 indicating that less NO was produced compared to negative controls that produced similar levels.

4 Discussion

Macrophages are proposed primary host cells for *Leishmania* but the role of these cells have not been well characterized neither in disease prevention nor in progression independent of T cell. The effector functions of macrophages for *Leishmania* have always been described in a T-dependent manner. Parasite modulates its host in terms of signalling or antigen presentation for its own benefit and induces factors that provide disease progressive environment and prime T cells for Th2 differentiation.

It is also possible that parasites starts modulating the macrophages at the time of entry and later on modulated parasitized macrophages interact with T cells and may stimulate disease inducing factors from T cells that help in disease progression and parasite survival in susceptible host.

Chemotherapeutic cure of leishmaniasis is largely dependent upon the development of an effective immune response that activates macrophages to produce toxic nitrogen and oxygen intermediates to kill the amastigotes. This process is suppressed by the infection itself which down regulates the requisite signaling between macrophage and T cells. In this study, treatment of macrophages with glucocorticoids, upregulated the genes responsible for inducible nitric oxide synthase enzyme production, thus high levels of NO. In comparison with infection rates and amastigote growth in the macrophages (Figures 4 and 5), this study suggest that hydrocortisone and dexamethasone may be used in control of *leishmania* infection. Inhibition of NO production renders macrophages unable to restrict *L. major* replication *in vitro* [13, 14] but from the study dexamethasone and hydrocortisone down regulates iNOS enzyme thus implying nitric oxide may not be one of the mechanism used to kill *Leishmania* parasite, which is also evident in low NO production by macrophage stimulated by same drugs. iNOS catalyzes the synthesis of nitric oxide (NO) from arginine, a potent microbicidal agent that leads to killing of intracellular parasites and other microbes p [15,16,17]. In other studies iNOS has been shown to be indispensable for the innate natural killer cell response to *Leishmania major* [18]. INOS-dependent parasite control has also been observed in the livers of *Leishmania donovani*-infected mice [19]. More recently, a number of studies revealed that the production of NO is induced in human monocytes and/or macrophages *in vitro* when stimuli other than Interferon-γ (IFN-γ) and lipopolysaccharide are applied, for example, type I interferon or interleukin 4 (IL-4) plus anti-CD23 [20,21].

This study also found out that the activity of dexamethasone and hydrocortisone against *leishmania* parasites is not through the stimulation of macrophages in the production of nitric oxide since the OD levels of the test drugs was the same as those of negative control (RPMI 1640). The NO production levels of the macrophages treated with dexamethasone and hydrocortisone were the same as those of RPMI 1640 and LPS treated macrophages. NO undergoes oxidative degradation in aqueous solution to form nitrite (NO_2^-) and nitrate (NO_3^-) [22]. NO_2^- can be measured by a simple colorimetric assay known as the Greiss reaction. A number of reports have shown that *in vitro* cytotoxicity against the intracellular form of leishmanias is mediated by NO [23, 24, 25]. Macrophages have been identified as the iNOS-producing cells within the skin lesions, and regions with high level of iNOS contain few or no parasites [26]. In other studies mutant mice with disruption of the iNOS gene have shown failure of macrophage to produce NO after stimulation with IFN-γ plus LPS and likewise failed to control *L. major* infection *in vivo* [27].

Tumor necrosis factor-alpha synergizes with IFN-γ in the induction of iNOS and NO production by macrophages *in vitro* [28]. Thus downregulation of TNF-α may lead to the subsequent down regulation of iNOS enzyme which is evident in this study. The Th1 cytokines (TNF-α and IL-1β) mediates the elimination of *Leishmania* pathogens. *Leishmania* suppresses the expression of genes for the Th1 and upregulates genes for Th2 (IL-4, IL-5 and IL-10) cytokines. And this study found out that the levels of TNF-α were down regulated and in

both dexamethasone and hydrocortisone treated macrophages whereas IL-1β cytokine were upregulated in dexamethasone treated macrophages. This study therefore, suggests the protectiveness of the drugs against *leishmania* infection is through and IL-1β cytokines. The *in vivo* concentration of NO can vary from basal levels produced by epithelial cells (< 2 nM) to that of an activated macrophage (> 1 μM) [29]. At lower concentrations, NO has anti-inflammatory properties and modulate T cell functions, whereas high concentrations of NO results in bacterial killing, T cell dysfunction as well as tissue injury [30]. In macrophages, iNOS mediated NO production is enhanced by IFN-γ, TNF-α, bacterial LPS, IL-1ß, IL-6, and IL-17, whereas transforming growth factor beta (TGF-ß), IL-4, IL-10, IL-11, and IL-13 suppress the induction of iNOS in macrophages [31,32]. Serine/threonine phosphatase- protein phosphatase 2 and dual-specificity phosphatase/ MKP1 have been found to regulate p38 activity, whereas MKP3 preferentially controls ERK1/2. These interventions have shown to result in the upregulation of IL-10 and decreased TNF-α and/or NO production and this study suggest the same [33] (Figure 3).

Experimental data suggested the role of *Leishmania* specific CD4+ T cells to passively transfer the resistance or exacerbation of disease in immunodeficient or sublethally irradiated naïve hosts, correlating with their production of Th1 or Th2 cytokines [34, 35, 36, 37] and this study correlates with previous studies.

The cytokine interleukin 1 beta is a potent mediator in response to infection and injury [38]. It is produced mainly by blood monocytes, but also by macrophages, dendritic cells and a variety of other cells in the body [39, 40] significant elevations of plasma IL-1β have been detected in healthy humans injected with LPS [41]. This also found out that when macrophages are stimulated with LPS they upregulate expression of IL-1β (Figure 1).

TNF-α (Cachectin) is a protein produced mainly by macrophages, with a wide range of biological activities and may be important in inflammatory processes. In parasitic infection, administration of recombinant human TNF released from intraperitoneal osmotic pumps could effectively suppress the *plasmodium chabaudi adami* infection in CBA mice [42]. In experimental *Trypanosome cruzi* infections treatment of macrophages with recombinant TNF-plus lipopolysaccharide resulted in a significant reduction in the number of intracellular organisims compared with mock-treated macrophages [43]. TNF-α is host protective in experimental cutaneous leishmaniasis. However in this study TNF-α was downregulated in macrophages treated with the drugs which suggest that TNF-α is may have not been involved in protecting against *leishmania* infection (Figure 2).
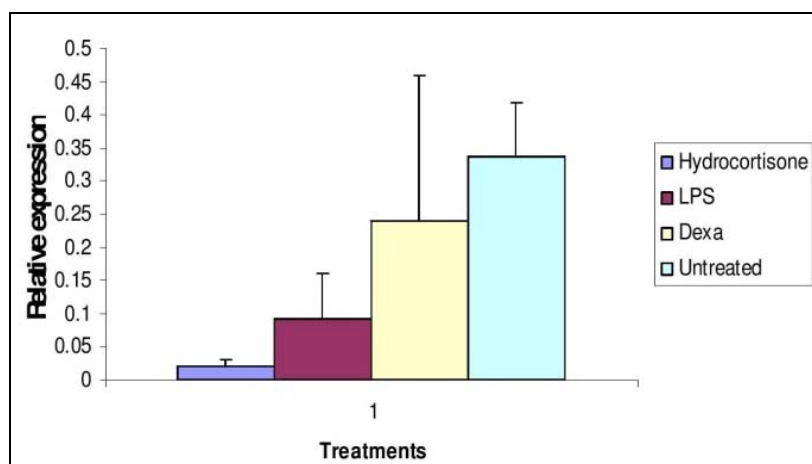

Fig 1: IL-1β mRNA levels in macrophage cells treated with Dexamethasone, hydrocortisone and LPS. IL-1-β was down regulated in Hydrocortisone and LPS treated macrophages as compared to untreated macrophages. However Dexamethasone treated macrophages had upregulated IL-1-β expression as compared to LPS treated macrophages.
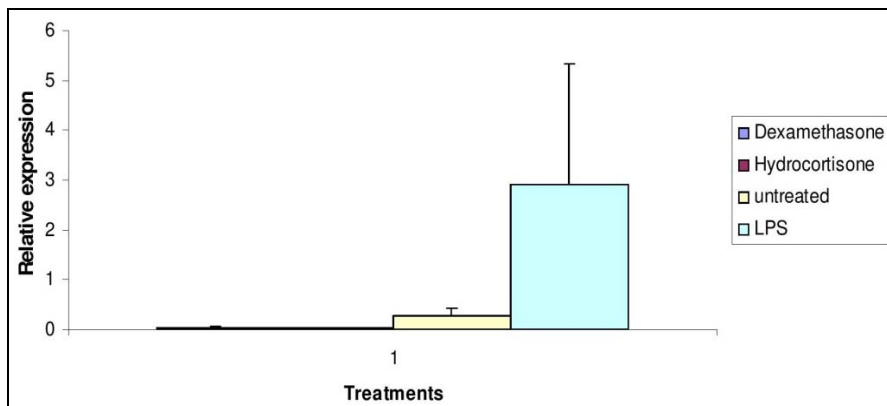

Fig 2: Cytokine TNF-α mRNA levels in macrophage cells treated with dexamethasone, hydrocortisone and LPS. TNF-α gene down regulated in Dexamethasone, hydrocortisone treated macrophages compared to LPS treated macrophages.
This data is complimented with the microscopy results that showed significant decrease in the infection rates and growth of amastigotes in the macrophages after treatment with the glucocorticoids.
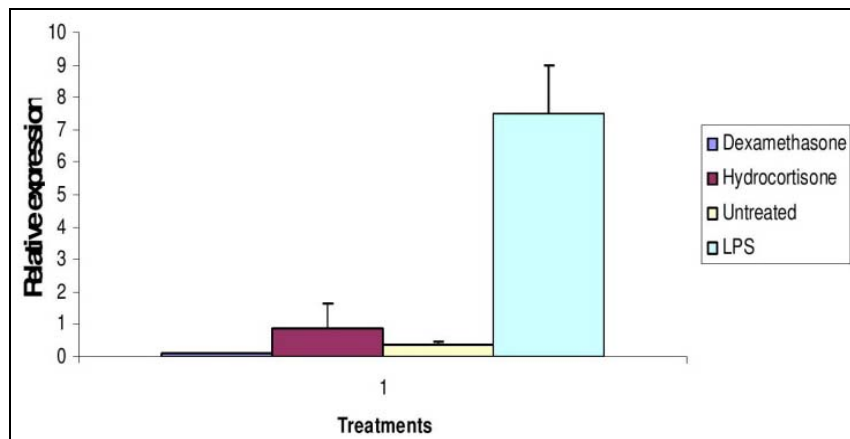
Fig 3: iNOS gene was downregulation in dexamethasone, hydrocortisone treated macrophage compared to LPS treated macrophages.
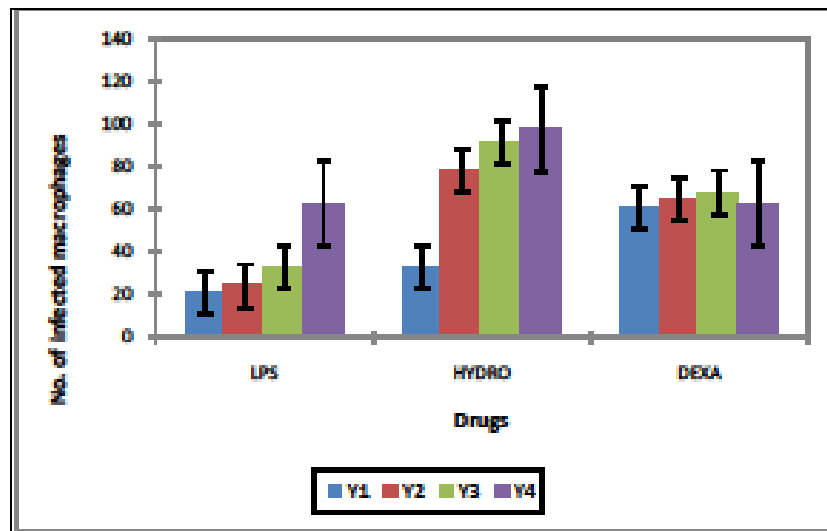

Fig 4: Graph showing infection rates of *Leishmania major* parasites on macrophages treated with drugs.
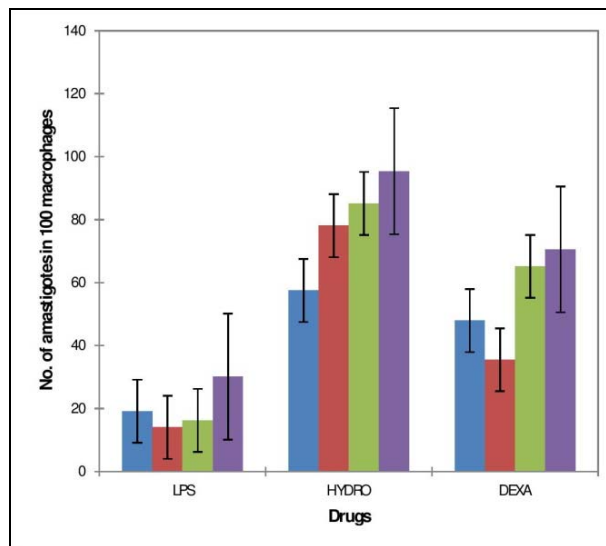

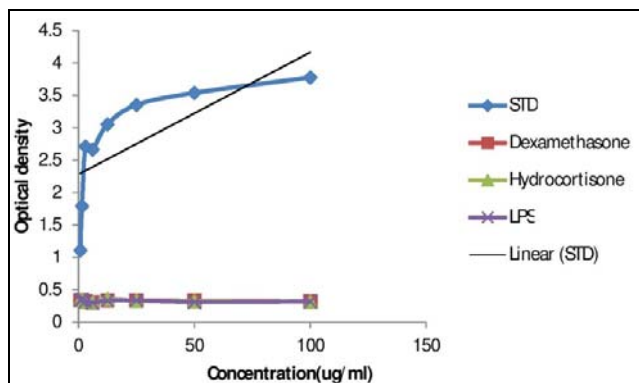**Fig 5:** Parasite growth in macrophages treated with GC and LPS drugs.

Fig 6: Shows the NO production stimulation on macrophages by test drugs, positive control (LPS) and RPMI 1640. LPS, dexamethasone and Hydrocortisone treated macrophages measured very low levels of NO compared to standard nitrite samples used.

5 Conclusion

Hydrocortisone inhibits the traslocation of NF-kB from cytosol into the nucleus as seen in the down regulation of the three genes; IL-1β, TNF-α and iNOS. Dexamethasone inhibits the translocation NF-kB from cytosol into the nucleus as supported by downregulation of TNF-α and iNOS, however IL-1β was upregulated. These data suggest that administration of dexamethasone in inhibition of NF-kB translocation into nucleus may be valuable treatment strategies in leishmaniasis. Dexamethasone showed reduced infection rates and amastigote growth in macrophages than hydrocortisone and RPMI treated macrophages.

6 Acknowledgements

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