Cisplatin along with herbal drug treatment reduces the percentage of regulatory T cells and decreased the severity of experimental visceral leishmaniasis

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Abstract  Background: Visceral leishmaniasis is the most alarming and devastating amongst the various forms of leishmaniasis. It is caused by Leishmania donovani, an obligate intracellular parasite of macrophages that survives through immunosuppression. Absence of T regulatory cells provides complete clearance of the parasite. A few immunoprophylactics have been sought to battle instinctive leishmaniasis, with fluctuating achievement. Our previous studies have shown that treatment of L. donovani infected mice with cisplatin along with herbal drugs resulted in decreased parasite load with heightened delayed type hypersensitivity responses (DTH), increased levels of IgG2a, IFN-γ, IL-2, CD4+ cells, NK 1.1 cells over that of IgG1, IL-4, 1L-10, CD8+ and CD19 in infected mice.

Methods: Along the above lines, the present study further evaluated the percentage of CD4+ CD25+ FoxP3+ T regulatory cells and ultra structural changes in kidney, liver and spleen. Cisplatin (5 mg/kg b.wt. daily for 5 days, i.p.) along with Tinospora cordifolia (100 mg/kg b.wt. daily for 15 days, p.o.) or Withania somnifera (350 mg/kg b.wt. daily for 15 days, p.o.) or Asparagus racemosus (650 mg/kg b.wt. daily for 15 days, p.o.) was administered to L. donovani infected BALB/c and after 30 days post treatment mice were sacrificed.

Results: The findings uncover a significant reduction in parasite load coupled with decreased percentage of Treg cells and no pathological changes at ultra structural level.

Conclusion: In this manner, results acquired recommend that the decrease in percentage of T reg cells may further help the antileishmanial remedial impact of cisplatin alongside natural medications.

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Introduction

Visceral leishmaniasis (VL) is a life-threatening systemic disease caused by the parasite *L. donovani* and is positioned by World Health Organization as the second most paramount disease after malaria. It is a significant reason of morbidity and mortality in East Africa and the Indian subcontinent. 90 percent of worldwide VL cases occur in Bangladesh, India (mainly northeastern region) Nepal, Sudan, Ethiopia and Brazil. In Indian subcontinent, the disease has been reported from 109 districts (45 in Bangladesh, 52 in India and 12 in Nepal) with yearly occurrence of 136,500, 270,000 and 12,600 cases in Bangladesh, India and Nepal respectively. Treatment options for VL are limited. Pentavalent antimonials have viably served as the therapeutic mainstay against leishmaniasis, yet reports of large-scale resistance in India have required assessment of alternative therapeutic modalities. These include amphotericin B together with its liposomal formulation, paromomycin and sitamaquine, however, each have their own limitations of affordability and toxicity and require parenteral administration. Even the orally effective antileishmanial, miltefosine, is associated with gastrointestinal disturbances and teratogenicity.

The disease is portrayed by depressed cell-mediated immunity (CMI) and agents which directly stimulate the macrophage to kill intracellular amastigotes through enhanced release of nitric oxide (NO) and/or induce the basic T-helper type 1 (Th1)-cell anti-leishmania immune response would provide a rationale for treatment in visceral infection. Several studies have already been reported emphasizing the benefits of combination of antileishmanial drugs with immunostimulants.

Cisplatin is a highly efficient anti-neoplastic drug commonly used as a first-line therapy for treatment of various solid tumors. The anticancer effect of cisplatin is mediated by apoptosis and DNA-crosslinks with subsequent cytotoxic lesions in malignant cells. However, its clinical use is associated with dose and duration-dependent nephrotoxic side effect. Generation of reactive oxygen species (ROS), impaired glutathione metabolism, alterations in the mitochondrial antioxidant enzymes and increase in lipid peroxidation are the most plausible mechanisms of cisplatin induced toxicity. Medicinal plants and natural herbal products have potential antioxidant activity and are therefore often administered along with chemotherapeutic agents to provide better protection against their toxic side effects.

Cisplatin has been found to have antileishmanial activity in vitro at a concentration of 0.25–64 μM. It’s in vivo antileishmanial activity has also been reported from our laboratory. The treatment of *L. donovani* infected BALB/c mice with cisplatin resulted in decreased parasite load and it was 81.76% on 21 post treatment day (p.t.d.). However, treatment with cisplatin even at low doses generated nephrotoxicity and hepatotoxicity.

Thus, keeping in mind, the immunosuppression caused during visceral leishmaniasis infection and the side effects of the drug cisplatin, we have previously used immunomodulatory and protective herbal drugs *T. cordifolia*, *W. somnifera* and *A. racemosus* along with cisplatin against visceral leishmaniasis. Our results showed decrease in parasite load with enhanced generation of DTH responses, increased levels of Th1 cytokines (IL-2 and IFN-γ), IgG2a, CD4+ T cells and NK 1.1 cells.

Evidence from experimental murine models of infection suggests that natural Treg cells promote survival of *Leishmania* parasites and reactivation of disease. Because immunopathology is thought to play a major role in the pathogenesis of active VL and Treg cells are considered important in the regulatory mechanisms of immunity in VL and there are not any reports regarding the percentage of Treg cells prior and after the treatment, thus we herein, extend our studies to explore the percentages of Treg cells amid infection and after the drug treatment. Additionally, pathological changes have furthermore been observed at the ultra structural level.

Methods

Parasite

*L. donovani* (MHOM/IN/80/Dd8) promastigotes were obtained from the London School of Tropical Hygiene and Medicine, London. The promastigote culture was maintained in vitro at 22 ± 1 °C in modified Novy, McNeal and Nicolle’s (NNN) medium by serial subcultures after every 48–72 h.

Animals

Inbred BALB/c mice were purchased from the Institute of Microbial Technology, Chandigarh, India and those at 6–8 week of age were used for the experiment. All mice were maintained at controlled temperature and humidity, with a 12 h light-dark cycle, and sterile food and water ad libitum. Experiments were carried out according to the guidelines of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA). The ethical clearance for conducting various experiments mentioned in the study on BALB/c mice was taken from Institutional Animal Ethics Committee (IAEC) of the Panjab University, Chandigarh (Approval No. 10/33/CAH). This work was approved by institutional animal ethics Committee.

**In vivo infection of mice**

The parasites in the logarithmic phase of growth were used for antigen preparation. The culture was pooled and centrifuged for 15 min at 2500 rpm. The supernatant was discarded and the pellet (promastigotes) was washed thrice in PBS. Finally, after last wash, supernatant was discarded and to the remaining pellet, 1 ml of PBS was added. The promastigotes were then counted in the Neubauer’s chamber. For this promastigote suspension was diluted in 10% buffered formalin. Promastigotes were then adjusted to a concentration of 10⁷ parasites/promastigotes/ml. Mice were then injected 0.1 ml of this suspension containing 10⁷ parasites, intracardially.
Experimental design

For each experimental group, inbred BALB/c mice were used. Nine such groups were selected, A, B, C, D, E, F, G, H and I. Healthy control Group A) received PBS only, infected control Group B) was infected with L. donovani and kept for 30 days for the progressive development of the disease, Group C) consisted of infected mice treated with cisplatin (Sigma–Aldrich, St. Louis, MO, USA) at the dose of 5 mg/kg b.wt./day daily for 5 days, i.p., Groups D, E and F) consisted of infected mice treated with cisplatin (5 mg/kg b.wt./day daily for 5 days, i.p.) along with herbal drugs (Himalaya Drugs Company, Bangalore, India) T. cordifolia (100 mg/kg b.wt./day), W. somnifera (350 mg/kg b.wt./day) and A. racemosus (650 mg/kg b.wt./day) respectively for 15 days orally (five days along with cisplatin and then alone for ten days), groups G, H and I) consisted of infected mice treated with only herbal drugs, T. cordifolia (100 mg/kg b.wt./day), W. somnifera (350 mg/kg b.wt./day) and A. racemosus (650 mg/kg b.wt./day) alone daily for 15 days. Mice from each group were sacrificed and 30 post infection/post treatment days for the assessment of parasite load, percentage of Treg cells and ultrastructural changes in kidney, liver and spleen.

Assessment of infection

Six mice from each group were sacrificed after 30 post infection and post treatment days. Liver parasite burdens were determined from Giemsa-stained multiple impression smears, expressed as Leishman-Donovan Units (LDU) as per the method of Bradley and Kirkley (1977). Cure can be defined as elimination of parasites to negligible levels.

Flow cytometry

For the immunophenotyping, antibodies viz. CD4 (FITC conjugated), CD25 (APC conjugated) and FOXP3 (PE conjugated), permeabilization buffer and cytofix were purchased from BD Biosciences.

Single cell suspensions from peripheral blood were prepared from all the groups of mice after 30 post infection days (p.i.d.)/post treatment days (p.t.d.) and enriched for lymphocytes using Ficoll-Hypaque density gradient centrifugation. For the determination of percentage of Treg cells, lymphocytes were then stained with antibodies CD4 and CD25 and were kept in dark for 30 min at 4 °C. Cells were then washed with wash buffer at 1500 rpm for 5 min. After that supernatant was discarded and to the pellet 60 μl of cytofix was added and it was kept for half hour in dark at 4 °C. Fixative was then removed and the pellet was again washed with wash buffer. Supernatant was discarded and to the pellet 400 μl of permeabilization buffer was added and the cells were kept for 10 min. Foxp3 was then added and it was then kept again for half hour in dark at 4 °C. As regulatory T cells may express the α-chain of IL-2R, we had used the CD4+ CD25+ T reg cell specific marker Foxp3. The pellet was then again washed in wash buffer and finally resuspended in the same to make final volume of 350–400 μl. Samples were then analyzed on FACSCalibur (B.D) after collecting 10,000 events.

Transmission electron microscopy

Kidney, liver and spleen samples were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 45 min at 26 °C, the fixative was discarded and it was replaced with 0.1 M cacodylate buffer and the samples were stored at 4 °C until being processed further.

Statistical analysis

All data comparisons were tested for significance by using one-way ANOVA. Results were expressed as mean ± S.D. of one of three independent experiments.

Results

Parasite load

Infected mice upon treatment with cisplatin demonstrated a significant (p < 0.0001) decline in parasite load in terms of LDU as compared with infected control. A percentage reduction of 96.7% was observed. Similarly in the groups of infected mice treated with cisplatin along with T. cordifolia, W. somnifera and A. racemosus, the LDU has been found to be decreased which translated into a parasite removal of 97.1%, 97.2% and 97.2% respectively. Moreover, treatment of infected mice with T. cordifolia, W. somnifera and A. racemosus alone resulted in decreased parasite load (Fig. 1).

![Parasite Load in different groups of BALB/c mice. P value: Infected control vs infected + cisplatin treated/ infected + cisplatin + T. cordifolia treated/infected + cisplatin + W. somnifera treated/infected + cisplatin + A. racemosus treated/infected + T. cordifolia treated/infected + W. somnifera treated/infected + A. racemosus treated *p < 0.0001.](image-url)
Percentage of Treg cells CD4+CD25+ in various groups of BALB/c mice

On flow cytometry, as compared with normal control, the percentage of CD4+CD25+ Treg was significantly higher in L. donovani infected BALB/c mice at 30 day post infection. However, percentage of Treg cells was found to be decreased significantly in the group of infected mice treated with cisplatin alone at the dose of 5 mg/kg b.wt. daily for five days, i.p., as compared to infected control. A significant reduction in the percentage of Treg cells was also observed in the group of infected mice treated with cisplatin in combination with herbal drugs T. cordifolia (100 mg/kg b.wt), W. somnifera (350 mg/kg b.wt.) and A. racemosus (650 mg/kg b.wt.) daily for 15 days, orally when compared with infected controls. A significant reduction in

Fig 2. Percentage of Treg cells in normal, infected and infected+cisplatin treated mice. a) Gating of lymphocytes in normal control b) dot plot showing percentage of Treg cells in normal control c) expression of Foxp3 d) gating of lymphocytes in infected control e) dot plot showing percentage of Treg cells in infected control f) expression of Foxp3 g) gating of lymphocytes in infected+cisplatin treated mice h) dot plot showing percentage of Treg cells i) expression of Foxp3.
the percentage of Treg cells has also been observed in the
groups of infected mice treated with herbal drugs alone T.
cordifolia (100 mg/kg b.wt), W. somnifera (350 mg/kg
b.wt.) and A. racemosus (650 mg/kg b.wt.) respectively
daily for 15 days, orally as contrast to the infected control
(Figs. 2–4).

Transmission electron microscopy

During observation, kidney micrographs of normal mice
showed intact glomerular basal membrane and podocytes
in renal corpuscles also appeared intact. However, in
cisplatin administered group of infected BALB/c mice there

![Fig 3. Percentage of Treg cells in cisplatin along with herbal drugs treated infected mice. a) Gating of lymphocytes in infected+cisplatin+T. cordifolia treated mice b) dot plot showing percentage of Treg cells c) expression of Foxp3 d) gating of lymphocytes in infected+cisplatin+W. somnifera treated mice e) dot plot showing percentage of Treg cells f) expression of Foxp3 g) gating of lymphocytes in infected+cisplatin+A. racemosus treated mice h) dot plot showing percentage of Treg cells i) expression of Foxp3]
was a prominent mitochondrial degeneration in proximal convoluted tubular cells. These changes were found to be reduced greatly in the groups of infected mice treated with cisplatin along with herbal drugs. In the cisplatin treated infected group of mice paramount cytoplasmic changes appeared in the micrographs of liver, space of Disse was found to be dilated. However, these changes were found to be significantly depressed in the groups of infected mice treated with cisplatin along with herbal drugs. The spleen sections of cisplatin treated infected mice showed heterochromatin condensation in the nucleus of plasma cells. However, after the treatment of infected mice with cisplatin in combination with herbal drugs, normal architecture of spleen was observed (Figs. 5–7).

Discussion

In our previous studies, treatment of *L. donovani* infected BALB/c mice with cisplatin alone and in combination with

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**Fig 4.** Percentage of Treg cells in infected mice treated with herbal drugs. a) Gating of lymphocytes in infected + *T. cordifolia* treated mice b) dot plot showing percentage of Treg cells c) expression of Foxp3 d) gating of lymphocytes in infected + *W. somnifera* treated mice e) dot plot showing percentage of Treg cells f) expression of Foxp3 g) gating of lymphocytes in infected + *A. racemosus* treated mice h) dot plot showing percentage of Treg cells i) expression of Foxp3
herbal drugs led to a significant reduction in parasite load with restoration of Th1 type of immune responses.\textsuperscript{19,20a,b} Therefore, herein, we now extend the immunomodulatory efficacy of cisplatin alone and in combination with herbal drugs through the evaluation of parasite load along with percentage of Treg cells in experimentally induced visceral leishmaniasis infection in BALB/c mice, thus meriting the use of these drugs against VL infection. For the target parasite (\textit{L. donovani}), an appropriate laboratory host is very important to carrying on research for the evaluation of antileishmanial action of newer compounds.\textsuperscript{21} The BALB/c model fulfills the required eligibility as the chronic infection bears resemblance to human VL.\textsuperscript{22}

The parasite load was assessed in all the groups of mice on 30 post infection and post treatment days in liver as LDU.\textsuperscript{23} The treatment of \textit{L. donovani} infected BALB/c mice with cisplatin, decreased the parasite load and previous studies have also demonstrated that \textit{in vitro} antileishmanial activity of cisplatin has also been reported at a concentration of 0.25--0.64 \textmu M.\textsuperscript{17}

A further decrease in parasite load was observed in the infected mice treated with cisplatin in combination with

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\textbf{Fig 5.} Transmission electron micrograph of kidney. a) normal control b) infected control c) infected + cisplatin treated d) infected+ cisplatin+\textit{T. cordifolia} e) infected+ cisplatin+\textit{W. somnifera} f) infected+ cisplatin+\textit{A. racemosus}. Abbreviations: M-Mitochondria, DM-degenerating mitochondria, SER-smooth endoplasmic reticulum, RER-rough endoplasmic reticulum.
herbal drug *T. cordifolia* at the dose of 100 mg/kg b.wt. daily for 15 days, orally and the reason that may be responsible for the reduction of parasite burden in infected mice treated with cisplatin in combination with *T. cordifolia* could be the higher berberine concentration in *T. cordifolia*.\(^24\)

Similarly, significant inhibition in the parasite load was also noticed when cisplatin was administered in combination with *W. somnifera* in infected mice. The killing of parasites with *W. somnifera* owes to the presence of withaferin A (steroidal lactone) and withanolide A.\(^25\)

The combination of *A. racemosus* with cisplatin also resulted in comparable clearance of *L. donovani* parasites from the liver of infected BALB/c mice. This may be due to the involvement of racemoside A, which is an efficacious antileishmanial molecule. This compound showed IC\(_{50}\) values of 1.31 μg/ml against promastigotes of *L. donovani*.\(^26\) It can be concluded that maximum parasite clearance was achieved in the groups of mice which included cisplatin in their treatment regime as cisplatin has direct role in killing of parasites and the possible mechanism of parasite killing has been reported to arrest the S and G2 stages of the life cycle of promastigotes and axenic amastigotes. However, complete clearance of the parasites cannot be achieved as elimination of the parasites later depends upon the development and persistence of Th1 responses.\(^27\) Also, apart from the leishmanicidal activities of drugs, Th1 mediated protection plays an important role.\(^28\)

Treg cells are the key regulators of immune responses, express CD4, Fox P3, and CD25 surface markers, and regulate the activation, proliferation, and effector function.

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Fig 6. Transmission electron micrograph of liver. a) normal control b) infected control c) infected + cisplatin treated d) infected + cisplatin + *T. cordifolia* e) infected + cisplatin + *W. somnifera* f) infected + cisplatin + *A. racemosus*; Abbreviations: SD-Space of Disse
of activated conventional T cells in several immunological conditions such as immunopathology, autoimmune diseases, transplantation, tumor immunity and infectious diseases.

In experimental models of leishmaniasis, Treg cells seem to play a role in the maintenance of chronic infections, with persistence of pathogens, consequently enabling the disease reactivation. The role of Treg cells in the disease leishmaniasis has been already explored as Foxp3+CD4+ cells were found to be the major source of elevated IL-10 mRNA in spleen of VL patient. Foxp3+ T cells are an important source of IL-10 in human VL and that these cells suppress effector T cell activation. Therefore, current study was done to evaluate the increase or decrease in the percentage of Treg cells.

In accordance with the above reports our results also showed that percentage of Treg cells were found to be increased in L. donovani infected BALB/c mice as compared to normal controls. However, administration of cisplatin to L. donovani infected BALB/c mice decreased the percentage of Treg cells as compared to infected control. Cisplatin is thought to stimulate the immune response by activating macrophages.

Fig 7. Transmission electron micrograph of spleen. a) normal control b) infected control c) infected + cisplatin treated d) infected + cisplatin + T. cordifolia e) infected + cisplatin + W. somnifera f) infected + cisplatin + A. racemosus. Abbreviations: H-Heterochromatin, EU-Euchromatin, HC-Heterochromatin, N-Nucleus.
and alternative immune cells. \(^{32}\) Cisplatin induced macrophage activation leads to the synthesis and release of nitric oxide (critical in parasite killing), TNF-\(\alpha\) and IL-1.\(^ {13}\) Authors have also reported the immunomodulatory role of cisplatin pretreatment in a mouse model with colon adenocarcinoma. They observed that pretreatment with cisplatin enhanced the anti-tumor activity of cytokine induced killer (CIK) cells, which share functional properties with both NK cells and T cells\(^ {34,35}\) by providing CD3\(^ +\) T lymphocytes into the tumor mass. Moreover, cisplatin also reduced the percentage of intratumoral and splenic Treg cells.\(^ {36}\)

The decrease in the percentage of Treg cells in the group of infected mice treated with cisplatin in combination with \(T. \) cordifolia, is most probably due to the immunostimulating properties of \(T. \) cordifolia. An \(\alpha\)-d-glucan \([(RR1) (immunostimulant)]\), isolated from \(T. \) cordifolia has been found to elucidate the synthesis of IL-1\(\beta\), IL-6, IL-12, IL-12 p40, IL-18, TNF-\(\alpha\) and monocyte chemo attractant protein (MCP)-1, while it did not induce the production of IL-2, IL-4, IL-10, IFN-\(\gamma\) and TNF-\(\beta\).\(^ {22}\)

The results of the present study also indicated the beneficial immunomodulatory effect of cisplatin along with \(W. \) somnifera in the treatment of experimental visceral leishmaniasis. It has been found that combined treatment of infected mice with cisplatin along with \(W. \) somnifera also resulted in decreased percentage of Treg cells as compared to infected control. The obtained results are in agreement with the previous studies which indicate towards the immunomodulatory potential of \(W. \) somnifera. The aqueous root extract of \(W. \) somnifera stimulated the cell mediated immunity, IgM, IgG, enhanced production of CD3\(^ +\), CD4\(^ +\), CD8\(^ +\), CD19, IFN-\(\gamma\), IL-2 and IgG2\(\alpha\) in the SRBC immunized BALB/c mice. Similarly, our results also demonstrated that \(W. \) somnifera along with cisplatin successfully generated protective immune responses against \(L. \) donovani infection in BALB/c mice as depicted by the increased levels of IFN-\(\gamma\), IL-2, CD4\(^ +\) and natural killer cells with decreased CD8\(^ +\) and CD19 cells.\(^ {38}\)

Previous studies have demonstrated that immunity can be enhanced by \(A. \) racemosus through T-cells. Steroidal sapogenins and steroidal saponins (shatavaroside A and shatavaroside B) are major secondary metabolites present in \(A. \) racemosus that might be attributed to its immunomodulatory effects.\(^ {39}\) In the present study treatment of \(L. \) donovani infected BALB/c mice with cisplatin along with \(A. \) racemosus reduced the percentage of Treg cells as compared to infected control. As regards to ultrastructural studies, in cisplatin administered group of infected mice, kidney micrographs showed mitochondrial degeneration in proximal convoluted tubular cells, in the micrographs of liver, space of Disse was found to be dilated and spleen sections showed heterochromatin condensation in the nucleus of plasma cells. However, after the treatment of infected mice with cisplatin in combination with herbal drugs all the above changes have been found to be attenuated. Our results are in accordance with the previous findings where cisplatin administration at the dose of 7.5 mg/kg, i.p. in adult male rats resulted in hemorrhage, glomerular atrophy, inflammatory cell infiltration and tubular necrosis in kidneys. Moreover, at ultrastructural level, the renal corpuscles of cisplatin treated rats showed wide capsular space, fused foot processes, dilated congested capillary loops, irregular capillary basement membrane and mesangial cell hyperplasia with excessive deposited mesangial matrix. Whereas, in the aged garlic extract pre-treated rats, normal podocytes, uniform thickness of the capillary basal lamina and a few dilated congested capillary loops were observed.\(^ {40}\) The protection being provided by the herbal drugs at the ultrastructural level is due the presence of various active constituents in the drugs.\(^ {41-43}\) \(W. \) somnifera offers protection due to the presence of active components viz. alkaloids, withanoloids and flavonoids by influencing the levels of lipid peroxidation products, free radical scavenging and antioxidant property.\(^ {41}\) \(A. \) racemosus has been found to ameliorate the damage caused to tissue by inhibiting the production and scavenging of free radical through the induction of antioxidant enzymes and improving non-enzymatic thiol antioxidant GSH.\(^ {42}\) The biologically active phytoconstituents of \(T. \) cordifolia such as flavonoids and alkaloids may be responsible for its protective activity.\(^ {13}\)

Thus, taken, together, these findings suggest that cisplatin in combination with herbal drugs may play a role in attenuating the immunosuppression caused during VL infection by reducing the percentage of Treg cells. Therefore, it is possible to speculate that cisplatin in combination with herbal drug treatment could be used as anti-leishmanial therapy in future.

**Conflict of interest**

The authors declare no conflict of interest.

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**References**

Visceral leishmaniasis, Treg cells & ultrastructure


