Leishmaniasis refers to a spectrum of diseases that have diverse clinical manifestations caused by the protozoan flagellates of the genus *Leishmania*. Visceral leishmaniasis (VL), caused by the intracellular parasite *Leishmania donovani*, *L. chagasi* and *L. infantum* is characterized by defective cell-mediated immunity (CMI) and is usually fatal if not treated properly (Handman, 2001). Visceral leishmaniasis affects approximately half a million new patients every year in many countries of the tropics and subtropics (Desjeux, 2004). In India, VL is widespread especially in the states of Bihar, Eastern Uttar Pradesh, West Bengal, Assam, Sikkim and to lesser extent in Tamil Nadu and Orissa. Maximum people affected with this disease are from the economically weaker sections of the society. The drugs used for the treatment are effective against the parasite but they cause immense toxicity and come with baggage of a multitude of side effects and frequent failure due to drug resistance. Growing limitations in available chemotherapeutic strategies due to emerging resistant strains and lack of an effective vaccine strategy against visceral leishmaniasis deepens the crisis. Due to these problems with the existing chemotherapy, there is a need to discover new and more effective antileishmanial compounds having negligible side effects.

Cisplatin (CP) is a first-generation platinum-containing drug, used in the treatment of various solid tumors. We have for the first time characterized the *in vivo* effect of cisplatin in murine experimental visceral leishmaniasis but at higher doses it is nephrotoxic. Considering the above findings, the present study was designed to evaluate the protective efficacy of the drug in combination with various antioxidants to reduce or prevent cisplatin-induced nephrotoxicity. The immunological, hematological, biochemical, molecular and histological changes induced by CP and antioxidants in uninfected and *L. donovani* infected BALB/c mice were investigated and compared with sodium stibogluconate (SSG).
Antileishmanial potential of CP (5 mg and 2.5 mg/kg b.wt.), CP+ antioxidants and SSG treatment on the course of infection in L. donovani infected BALB/c mice

To assess the antileishmanial effect of cisplatin and its combination with different antioxidants, various groups of animals were treated and sacrificed on 1, 15 and 30 post treatment days. The liver of mice of each group was removed and weighed. Impression smears were made and the parasite load was assessed in terms of Leishman Donovan Units.

Animals treated with cisplatin at both dosages (5 and 2.5 mg/kg b.wt.) significantly (p<0.001) reduced the parasite load as compared to infected controls. Cisplatin at the dosage of 5 mg/kg b.wt. showed significantly (p<0.001) lesser parasite burden as compared to those treated with 2.5 mg/kg b.wt. of cisplatin. When different antioxidants were used along with cisplatin, a significant reduction in parasite load was observed as compared to the infected controls and the decrease was found to be comparable to cisplatin treated animals. The results were also comparable with the group of infected animals treated with SSG (positive control) where reduction in parasite load was reported on 1, 15 and 30 p.t.d.

Effect of CP (5 mg and 2.5 mg/kg b.wt.), CP+ antioxidants and SSG treatment on the humoral immune responses

Humoral immune responses in different group of animals were evaluated by measuring total specific IgG, IgG1 and IgG2a by indirect ELISA from serum samples collected on 1, 15 and 30 treatment post days/post infection days.

The IgG levels were found to be highest in the infected controls as compared to the cisplatin treated animals. With increase in post treatment days, the IgG antibody response in infected mice treated with cisplatin (5 mg/kg b.wt. and 2.5 mg/kg b.wt.) was observed to be lesser as compared to infected controls and the maximum antibody response was produced in infected controls. The IgG levels in the infected animals treated with 5 mg/kg b.wt. of cisplatin were found to be lower than those treated with 2.5 mg/kg b.wt. of the drug. When antioxidants
were supplemented along with cisplatin, decrease in antibody titre was observed as compared to the infected controls. When cisplatin treated animals were compared with SSG treated animals then the decrease in antibody titre was found to be comparable.

The IgG1 which is an indicator of Th2 immune responses, was found to be maximum in infected controls. The decreased IgG1 levels were found in cisplatin treated animals as compared to infected controls. When antioxidants were given along with cisplatin, decrease in antibody titre was observed as compared to the infected controls. When cisplatin treated animals were compared with SSG treated animals then the decrease in antibody titre was found to be comparable.

Treatment of animals with cisplatin (5 mg/kg b.wt. and 2.5 mg/kg b.wt.) increased the IgG2a antibody titre which is an indicator of Th1 immune response. Treatment of animals with cisplatin led to a sudden increase in the IgG2a levels on 1 p.t.d. and further decreased on 30 p.t.d. but was still higher than infected controls. The increase was more pronounced in infected animals treated with cisplatin at a dosage of 5 mg/kg b.wt. as compared to infected animals treated with cisplatin at a dosage of 2.5 mg/kg b.wt. When antioxidants were supplemented along with cisplatin, the IgG2a antibody titre was found to be still higher than the infected controls. When cisplatin treated animals were compared with SSG treated animals then the increase in antibody titres was found to be comparable.

**Effect of CP (5 mg and 2.5 mg/kg b.wt.), CP+antioxidants and SSG treatment on cell mediated immune responses**

**DTH responses**

A profound delayed type hypersensitivity response was induced by cisplatin treated *L. donovani* infected animals, suggesting the generation of cell-mediated immune responses. The percentage increase in footpad thickness in the infected animals treated with 5 mg/kg b.wt. of cisplatin was found to be higher than those treated with 2.5 mg/kg b.wt. of the drug. In animals where antioxidants were given along with cisplatin, the DTH responses increased from 1 to 30 p.t.d.
When cisplatin treated animals were compared with SSG treated animals then the increase was found to be comparable.

**Cytokine responses**

The immune responses augmented in different groups of animals were analysed by quantifying the cytokines (IFN-γ, IL-2, IL-10 and IL-4) produced by spleen cells on different post infection/treatment days. Higher concentrations of IFN-γ and IL-2 indicate protective Th1 type of immune response whereas higher concentration of IL-10 and IL-4 are indicators of protective Th2 type of immune response.

IFN-γ and IL-2 levels were found to be higher in infected animals treated with cisplatin (5 mg/kg b.wt. and 2.5 mg/kg b.wt.) as compared to infected and normal controls. Treatment of animals with cisplatin led to a sudden increase in the IFN-γ and IL-2 levels on day 1 post treatment and further decreased on 30 p.t.d. but was still higher than infected controls. Increase in IFN-γ and IL-2 levels has been observed in animals treated with cisplatin at both the dosages even when the antioxidant combinations were given. The maximum production of IFN-γ and IL-2 was observed in splenocytes of animals treated with cisplatin (5 mg/kg bwt. and 2.5 mg/kg bwt.)+vitC+vitE+silibinin as compared to animals treated with cisplatin (5 mg/kg bwt. and 2.5 mg/kg bwt.)+vitC+vitE and cisplatin (5 mg/kg bwt. and 2.5 mg/kg bwt.)+silibinin treated animals. When the cisplatin treated animals were compared with SSG treated animals then the increase in IFN-γ and IL-2 levels were found to be comparable to SSG treated animals on different post treatment days.

Th2-regulated cytokines IL-4 and IL-10 levels were minimum in animals treated with cisplatin as compared to infected controls. Spleen cells from infected controls, however, produced much more IL-4 than the cisplatin treated groups. This effect of cisplatin in down-regulating IL-4 and IL-10 production was seen in almost all the animals treated along with different antioxidants on different post treatment days. The levels of IL-10 and IL-4 produced by splenocytes of cisplatin treated mice were comparable to that induced by SSG treatment.
Assessment of toxicity induced by CP at higher doses (5 mg and 2.5 mg/kg b.wt.) and its prevention by supplementing antioxidants along with cisplatin in different group of animals and their comparison with controls

Hematological investigations

The induction of hematological responses by the drug was studied by estimating hemoglobin (Hb), total leucocyte count (TLC) and differential leucocyte count (DLC) in different groups of animals on 1, 15 and 30 post treatment days/post infection days in all treated animals and controls.

Hemoglobin estimation

A decrease in hemoglobin levels were observed in infected and uninfected cisplatin (5 mg/kg b.wt. and 2.5 mg/kg b.wt.) treated animals as compared to normal controls. When antioxidants were given along with the drug in infected animals, the hemoglobin levels were found to be in normal range of 8–10 g/dl.

Total Leucocyte Count (TLC)

Leucopenia was observed in infected and uninfected cisplatin (5 mg/kg b.wt. and 2.5 mg/kg b.wt.) treated animals while leucocytosis was observed in infected untreated animals. TLC was found to be in normal range of 7000-12000/mm^3 when antioxidants were supplemented along with cisplatin. When compared with SSG, the results were found to be comparable and the values were within the normal range.

Differential Leucocyte Count (DLC)

Neutrophils

Maximum percentage of neutrophils was observed in infected controls and normal range was observed in cisplatin treated animals and in animals treated with cisplatin along with various antioxidants. The difference in treated and infected controls was found to be significant (p<0.001) and results were found to be equivalent to SSG treated animals where normal range was observed.

Lymphocytes

Lymphocytosis was observed in CP treated animals as compared to infected and normal controls. When cisplatin treated animals were compared with
SSG treated animals, the percentage of lymphocytes was found to be increased. The percentage of lymphocytes was also found to be significantly (p<0.001) increased on different post treatment days when SSG treated animals were compared to infected controls.

**Basophils, Eosinophils and Monocytes**

The percentage of basophils, eosinophils and monocytes were found to be in normal range of less than 1.7%, less than 6% and in the range of 9-15% in all the groups of animals.

**Biochemical investigations**

The induction of biochemical responses by the drug was studied by evaluating liver and kidney function tests in sera samples of different groups of animals. All the assays were performed on 1, 15 and 30 post treatment days/post infection days in all treated and control animals.

**Liver function tests**

Quantitative estimation of SGOT and SGPT activity revealed maximum activity in infected and uninfected animals treated with cisplatin as compared to infected and normal controls. The enzyme activity in mice treated with 5 mg/kg b.wt. of cisplatin was found to be maximum in comparison to those treated with 2.5 mg/kg b.wt. of the drug. Enzyme activity showed a sharp decline from 1 to 30 p.t.d. when antioxidants were supplemented along with the cisplatin and normal range was attained on all post treatment days. The increase in SGOT and SGPT activity was observed in SSG treated animals on 1 p.t.d. and with increase in subsequent post treatment days normal range was observed.

The alkaline phosphatase and acid phosphatase activity was found to be in the normal range of 4–11 KA units and 0 to 0.6 U/L respectively in all groups of animals on different post treatment days.

The activity of lactate dehydrogenase was found to be maximum in animals treated with cisplatin followed by the infected and normal controls. When antioxidants were supplemented along with cisplatin, reduced levels of LDH were
Summary and Conclusions

found on different post treatment days and normal range of 114-240 IU/L was observed.

**Kidney function tests**

Treatment of infected and uninfected animals with cisplatin (5 mg/kg bwt and 2.5 mg/kg bwt) led to a sudden increase in the concentration of blood urea, BUN, uric acid and creatinine. The increase was more pronounced in animals treated with cisplatin at the dosage of 5 mg/kg b.wt. as compared 2.5 mg/kg b.wt. To reduce the nephrotoxicity induced by cisplatin, antioxidants (vitamin C, vitamin E and silibinin) were supplemented along with cisplatin. The levels of serum urea, BUN, uric acid and creatinine were found to be within the normal range of 10-45 mg/dl, 5-21 mg/dl, 3-6.7 mg/dl and 0.85-1.35 mg/dl respectively in animals treated with cisplatin along with antioxidants. The results were quite comparable to the SSG treated mice where normal levels were found on different post treatment days.

A significant decrease was found in electrolyte levels when infected animals were treated with cisplatin which leads to hyponatremia, hypomagnesemia, hypocalcemia, hypokalemia, hypochloremia and hypophosphatemia. The decrease in electrolytes was more pronounced in animals treated with 5 mg/kg b.wt. of cisplatin in comparison to those treated with 2.5 mg/kg b.wt. of cisplatin. When the antioxidants were supplemented to reduce the nephrotoxicity, the normal electrolyte levels were attained and the serum sodium, potassium, phosphorus, chloride, calcium and magnesium concentration was found to be within the range of 135 to 155 mmol/l, 3.6 to 5.5 mmol/l, 2.5-5 mg/dl, 98-109 mMols/l, 8.7 to 10.5 mg/dl and 1.3 to 2.5 mg/dl respectively.

**Mortality rate**

The death rate was maximum in animals treated with cisplatin (5 mg/kg b.wt. and 2.5 mg/kg b.wt.). The death rate increased from 30-85% and 25-75% in animals treated with cisplatin at the dosage of 5 mg/kg bwt. and 2.5 mg/kg bwt. on different post treatment days. When antioxidants were given along with the drug in infected animals, the death rate was found to be 0%. The results were found to
be comparable to SSG treated animals where death rate was found to be 0% on 30 post treatment day.

**Histopathological studies**

The histopathological studies of different organs of all the groups of animals was done with the light microscope and photography was done using Phase contrast microscope (Nikon) fitted with a digital camera (ProRes, Jenoptik-Germany).

**Kidney**

The kidneys of infected animals showed focal interstitial nephritis. Cisplatin treatment at both doses induced mild vascular and inflammatory changes with signs of vascular congestion, exhibited contracted glomerulus and unknown distinct bodies of the size of monocytes were also seen. Tubular lumen filled with cellular debris and elevated tissue in interstitium indicative of necrotic cell, pyknotic nucleus, brush border epithelial damage of tubules and focal interstitial nephritis was also observed after cisplatin treatment. In contrast, animals treated with SSG did not show any of the above changes in kidney sections, however swelling, desquamation and congestion in dilated glomeruli was observed in infected plus SSG treated animals. Moreover, the damage caused by cisplatin was ameliorated after the supplementation of antioxidants showing a marked reduction in the extent of tubular damage and normal morphology of kidney tissue was observed.

**Liver**

Hematoxylin/eosin stained tranverse sections of liver of infected and drug treated animals showed focal reaction changes in liver and mild kupffer cell hyperplasia was also observed. The accumulation of red blood cells around the draining pathways of the central vein after cisplatin treatment was observed. After the supplementation of antioxidants, the focal reaction changes were reversed and liver attained a normal structure.

**Spleen**

In infected controls, the spleen shows the reactive enlargement of follicles. Brown pigment (haemozoin) within the cells has been observed indicating
intravascular hemolysis may be due to the presence of *Leishmania* in spleen tissue. Both red pulp and white pulp were undistinguished and proliferation of marginal zone was observed. In cisplatin treated animals, spleen shows the expansion of marginal zone and white pulp with occasional focus of acute abscess containing neutrophils. The number of megakaryocytes was increased. The above changes suggest that the spleen is reactive suggesting septicemia. In SSG treated animals, mild expansion of mantle zone around the follicles was observed. There were no signs of toxicity in the spleen in groups of animals treated with cisplatin along with various antioxidants.

**Testes**

Hematoxylin/Eosin stained transverse sections of testes of infected animals showed maturation arrest. In drug treated animals, the testes were normal and mild reduced spermatogenesis was observed. In animals treated with cisplatin along with various antioxidants, the morphological characteristics of testes were comparable to those in control groups.

**Ovary**

Hematoxylin/Eosin stained transverse sections of ovary of infected controls showed many rounded or oval bodies called ovarian or graaffian follicles at various stages of development. Each follicle contains a large ovum surrounded by many layers of follicle cells. In drug treated animals and animals treated with cisplatin along with various antioxidants, the ovary was normal in the oestrus phase with development of corpus luteum.

**DNA fragmentation assay**

The isolated DNA from spleen and kidney tissue was analysed for DNA fragmentation assay. In infected controls, DNA fragmentation was observed in the spleen tissue but no DNA damage was observed in kidney tissues. On the contrary, infected plus cisplatin treated animals showed DNA fragmentation in the form of smear or faint ladder in both the tissues. No DNA damage was observed in animals treated with cisplatin along with various antioxidants.
Detection of parasite antigen in the spleen cells of infected and treated BALB/c mice by PCR

A 792 base pair fragment of gene encoding kinetoplast mini-circle of *L. donovani* was amplified in DNA of spleen cells collected from different group of animals which confirmed the presence of visceral infection. No amplification was observed in DNA sample of normal un-infected mice. The sensitivity and specificity of 100% was achieved by PCR assay.

From these results, following conclusions can be withdrawn:

- A considerable therapeutic efficacy was shown by cisplatin against murine leishmaniasis which was evident from significant reduction in parasite load.
- Significantly heightened DTH responses generated in cisplatin treated animals and in animals treated with various antioxidants suggested the generation of cell mediated immune responses.
- Increased levels of IgG2a, IFN-γ and IL-2 suggested the generation of Th1 type of immune responses in CP and SSG treated animals.
- Decreased levels of IgG, IgG1, IL-10 and IL-4 suggested the abolishment of non-protective Th2 type of immune responses in CP and SSG treated animals.
- Decreased activity of SGOT, SGPT and LDH in animals treated with cisplatin along with various antioxidants as compared to cisplatin treated animals showed the hepatoprotective efficacy of various antioxidants.
- Decreased activity of various kidney function tests in animals treated with cisplatin along with various antioxidants as compared to cisplatin treated animals showed the nephroprotective efficacy of various antioxidants.
- Protective efficacy of various antioxidants was also observed during histopathological studies of different tissues where normal histology of the tissues was observed.
- Animals treated with cisplatin along with various antioxidants also showed protection against DNA damage in spleen and kidney tissue.
Amplification of DNA on last day of experimentation confirmed the presence of parasite antigen in the spleen cells of infected and treated animals.