Comparison of treatment regimens of kala-azar based on culture & sensitivity of amastigotes to sodium antimony gluconate

C.P. Thakur, Shabnam Thakur, S. Narayan* & Arun Sinha**

Balaji Utthan Sansthan, *Rajendra Memorial Research Institute of Medical Sciences & **Department of Statistics, Patna University, Patna, India

Received March 12, 2007

Background & objectives: Present treatment strategies for kala-azar (visceral leishmaniasis, VL) include use of first line drug sodium antimony gluconate (SAG) to all patients but a large number of patients do not get relief with this drug. If a patient does not respond to a full course of SAG, a second or third line drug is given. We undertook this study to test whether an improved outcome can be achieved by employing a strategy of treatment based on culture and sensitivity of amastigotes to SAG compared with conventional empirical treatment.

Methods: In a double-blind, randomized, controlled trial done in Balaji Utthan Sansthan, Patna, of the 181 patients screened, 140 were finally randomly allocated to two groups A and B; group A patients were treated with SAG if their amastigotes were sensitive to SAG, and all patients in group B were treated with SAG to start with. Primary outcome measured was as no relapse within 6 months of follow up after cure and other outcomes measured were period of stay of patients in hospital, expenditure involved in the treatment, and infectivity periods of two groups, two-third of treatment period and whole of untreated period were taken as infectivity period. SAG was used at a dosage of 20 mg/kg given deep intramuscular injections in buttock for 28 days, amphotericin B (AMB) given at a dose of 1 mg/kg body wt daily for 20 days as a slow intravenous infusion in 5 per cent dextrose.

Results: Of the 70 patients in group A, 29 patients whose amastigotes were sensitive to SAG were treated with SAG, 2 patients were withdrawn due to drug toxicity; and 2 relapsed within 6 months of follow up and ultimate cure occurred in 25 (86.2%) patients only. Of the 70 patients in group B treated with SAG, 5 (7.1%) patients withdrew due to drug toxicity, 35 patients (50%) did not respond to treatment, 5 (7.1%) relapsed during 6 months of follow up and thus only 25 patients (35.7%) were ultimately cured. The difference between the two groups was significant (P<0.001). No patient died during treatment due to any toxicity because of early withdrawl of patients from treatment apprehending toxicity. Patients whose amastigotes were resistant to SAG, withdrawn from the study due to SAG toxicity, relapsed after cure with SAG, and who did not respond to SAG in both the groups were treated with AMB and all were cured. Groups B and A patients spent 3065 and 2340 days respectively in hospital, group B 1.3 times more than group A. The likely period of spread of parasites in society was 1965 days in group B and 1644 days in group A, group B 1.4 times more than group A. The total expenditure on treatment in groups B and A was $ 65,575 and $ 50,590 respectively; group B patient had to spend 1.3 times more than group A.

Interpretation & conclusions: A new strategy for treatment of kala-azar based on culture and sensitivity of amastigotes improved the cure rate, saved expenditure on the patient’s treatment, patients had to stay for shorter periods in hospital and reduced the chance of spread of SAG resistant disease in society. Till the government opts for better drugs, the treatment based on culture and sensitivity of the parasites to SAG may be a better method.

Key words Culture - sensitivity of amastigotes - sodium antimony gluconate - treatment
The treatment strategies for kala-azar, (visceral leishmaniasis, VL) in Bihar, India, have been using sodium antimony gluconate (SAG) as a first line drug since 1950s. The drug is administered for 28 days now. If no response to this drug is seen, then only the second line drugs are given. Primary unresponsiveness has been defined as no clinical or parasitological improvement during or after the first course of treatment with pentavalent antimonials. No doubt, the dose and duration of treatment of VL with SAG have been increased from 6 ml intramuscular daily (approximately 10 mg/kg body wt) for 6-10 days in 1950s through 1970s to 20 mg/kg body wt for 4 wk over the years. Increase in dose and duration of treatment with SAG did not decrease the increasing unresponsiveness to the drug which has increased from 30 per cent in 1977 to 85 per cent in 1990s in one study from Bihar. The unresponsiveness to SAG was found widespread in Bihar.

Certain generic preparations of SAG have been blamed for increasing inefficacy and toxicity. We have always used the best generic preparation of SAG (Albert David (India) Pvt. Ltd., Kolkata) which was found comparable to the brand drug pentostam (Glaxo-Welcome, England). Even with this best generic drug we have found high percentage of unresponsiveness and fatal toxicity with SAG. Therefore, we attempted to develop a better treatment strategy for kala-azar patients based on culture and sensitivity of amastigotes of Leishmania donovani to SAG.

Promastigotes' culture was not found useful for routine use and therefore it was not done. In the doses we used SAG in man, the drug used in corresponding dose was found ineffective on promastigotes in culture. Also promastigotes are not found in humans. We conducted a randomized double blind trial of the new therapeutic strategy and compared it with classical one with respect to efficacy, expenditure incurred on treatment, duration of stay of patients in hospital, and chance of spread of the disease in society.

Material & Methods

The study was conducted between January 2004 to April 2005 and the protocol was cleared by the Ethics Committee of Balaji Utthan Sansthan, Patna. Written informed consent was taken from each patient or the guardian of the patients below 18 yr of age. Consecutive patients with kala-azar coming to Balaji Utthan Sansthan, Patna, confirmed by the finding of Leishmania amastigotes (Fig. 1) in a smear of bone marrow or spleen stained with Giemsa (Nice Chemicals Pvt. Ltd., Cochin) were considered for inclusion in the study. Patients were excluded if they had a haemoglobin concentration below 50g/dl, had complications such as pneumonia, jaundice, tuberculosis, renal and cardiac diseases, or had received treatment with antimony or amphotericin B (AMB) or any treatment for kala-azar or refused to be included in the study. Patients with parasitological infections with hookworm, roundworm and Entamoeba histolytica were not excluded and these infections were treated concurrently. E. dispar is nonpathogenic and its infection was treated with appropriable drug as used for E. histolytica. We needed a sample size of at least 60 based on past experience and assuming a rate of drop out and spontaneous cure of zero and a difference in the rate of cure between the standard treatment and the new treatment of 20 per cent.

Of the 181 patients screened, only 140 met the inclusion and exclusion criteria and gave written consent. These were divided randomly in two groups with 70 patients, each matched for age and sex; 55 men and 15 women were included in each group (Fig. 2). As there was some difficulties in culturing grade-1 amastigotes, patients with grade-1 amastigotes were excluded from both the groups to maintain parity.

The clinical state of each patient was thoroughly assessed before the start of treatment. The duration of illness was noted. The spleen was measured in the anterior axillary line from costal margin to its tip and the liver was measured in the mid-clavicular line from the costal margin to its margin. Patient’s weight was noted. Total and differential white cell counts,
haemoglobin, (Merck’s automatic blood cell counter, Medonic CA/530, Melet Schloesing Laboratories, France), serum creatinine, serum alanine transaminase and aspartate transaminase activities (Transasia Bio medical Ltd, Daman, India ERBA Smart Lab autoanalyzer Transasia), serum electrolytes, (Flame photometer, India), prothrombin time (PT), (SM Diagnostics India, Fibran 20 coagulation Analyzer), were measured, urine was analysed and chest radiography and electrocardiography performed in each patient. Samples of bone marrow or spleen were aspirated and of the bone marrow aspirate (BMA) was used for Giemsa staining, and for macrophage culture. For amastigotes culture only BMA was used as it contained enough macrophages with parasites. For culture study for assessment of drug effect number of infected macrophage must reach 1x10^6. We had some difficulty in reaching that level with grade-1 parasites, and are working on it to improve culture of grade-1 parasites.

Regimens of treatment, assessment and follow up:
Patients in group A received SAG (Albert David, India) according to culture and sensitivity report of amastigotes of patient. The drug was given intramuscularly at buttocks at a dose of 20 mg/kg body wt for 28 days. The patients whose amastigotes were unresponsive to SAG were given amphotericin B as intravenous infusion at a dose of 1mg/kg body wt daily for 20 days, slowly in more than 2 h, without any incremental dose. The drug (available as dry powder), was dissolved in 10 ml of sterile water and diluted in 500 ml of 5 per cent dextrose and infused slowly through a scalp vein canula. Patients were properly hydrated and serum electrolytes deficiency, if any, was corrected before start of AMB. Serum creatinine was estimated weekly, if the creatinine level increased >3 mg/100 ml, the drug was discontinued and restarted only when the creatinine level became normal. Those patients who did not respond to SAG, those who were withdrawn from SAG therapy due to toxicity, and those who relapsed after SAG therapy were treated with AMB. AMB was started only when ECG changes caused by the use of SAG normalized with a minimum of 10 days of rest. A weekly watch on serum creatinine and serum electrolytes was kept for those patients treated with AMB.

The criteria for withdrawal from the study were fixed before the start of the trial. If the condition of patients started to deteriorate or they experienced any serious side effect of the drug, they were withdrawn from the trial. The response of treatment was noted for each patients. For calculation of expenditure on each patient in the two group, 30 days of stay at hospital, (one day before start of treatment and one day after treatment for doing pretreatment and post treatment investigations were added) and 22 days for AMB treatment (one day before and one day after for investigations and 20 days for treatment) were taken. On an average, 20 $ a day was calculated for stay in the hospital, 30 $ for a course of SAG and 50 $ for a course of AMB treatment, 10$ per patient for culture and sensitivity.

Infectivity period: Based on our earlier study we found that maximum number of nuclear remnants were present in the blood during two third of treatment period, even at the end of treatment with 20 days of AMB some remnants still were there. On the average we took two third of treatment period as infectivity period and whole of untreated period.

Apparent cure was indicated by the return of temperature to normal, decrease in the size of spleen, and an improvement in general condition. Parasitological cure was indicated by the absence of parasites in the smear taken after treatment. Ultimate cure was defined as clinical and parasitological cure with no relapse during six months of follow up.

The patients were followed up every month for six months. A card or messenger was sent if a patient did not turn up on the correct day. On each occasion patients were examined and routine investigations done.
At the final follow up examination, a sample of bone marrow/spleen was aspirated and examined for parasites.

**Infected macrophage culture and sensitivity:** Cultures of Leishmania-infected macrophages were set up by inoculating the leucocytes from subsamples of the bone marrow aspirates, (collected pre-treatment) into complete culture medium [RPMI 1640 medium (Hi-media, Mumbai, India)] with 20 per cent of heat-inactivated foetal calf serum, (HI FCS; Biological Industries, Beit Haemek, Israel) and 25 µg gentamycin, 50U penicillin and 50 µg streptomycin/ml (Nicholas Piramal India Ltd. Kalipore, Ahmedabad). To separate out the leucocytes each subsample of aspirate was mixed with three volumes of Ficoll Hypaque® (Pharmacia, Uppsala, Sweden) and centrifuged (Remi International Ltd., Mumbai) at (120 x g x 20 min) at 4°C after each wash. The resultant buffy coat of leucocyte was gently collected with a Pasteur pipette (Micro cell) and then washed three times with plain RPMI- 1640 medium and centrifuged (100 x g x 10 min) after each wash. The final pellet was re-suspended in the complete medium to give approximately 2 x 10^5 cells/ml. After the number of macrophages/ml was determined in a haemocytometer the suspension was plated out onto sterile glass cover slips in four-wells, tissue culture plate (Nunc, Roskilde Denmark). After overnight incubation in a humidified, 5 per cent CO₂ incubator at 35 ± 1°C, the non- adhering cells were removed with three gentle washes in RPMI 1640. The coverslips with their adherent macrophages were then covered again with complete medium with 20 and 1 µg of SAG, AMB and per ml respectively and no drug/ml before incubation for a further 72 h. The coverslips were then carefully removed so that the macrophages adhering to them could be fixed in 10 per cent neutral formalin and stained with Giemsa or haematoxylin and eosin (H&E). A hundred macrophages on each cover slip were then examined under the microscope, so that percentage of infected macrophages, the mean number of amastigotes/infected macrophage and total number of amastigotes in each culture (with or without drug) could be estimated. The cells left adhering to the tissue culture plate were gently washed with Schneider’s Drosophila medium sigma containing 20 per cent HI-FCS covered with the same medium and incubated at 24±1°C, to check that any surviving amastigotes were viable and converted into promastigotes when cultured under these conditions.

For each patient providing the macrophages the effect of different drugs was calculated as the percentage reduction in the mean number of amastigotes/macrophage in the culture with the drug compared with that seen in the corresponding drug free culture. The mean reduction for each drug was then calculated. Ten to 40 per cent of macrophages were infected with a mean of 25 per cent and 3 to 10 parasites were found in each macrophage. Clearance of all parasites at 20 µg per ml of SAG was taken as efficacy of SAG.

**Statistical analysis:** Mann-Whitney test, t test and Boxplots method were used to compare the two groups, chi square and Fisher Exact test were used to determine the significance between the outcome of treatment group.

**Results**

Patients in two groups had intermittent fever, splenohepatomegaly, anaemia and leucopenia and all patients were parasitologically positive (Table). The statistical analysis of the characteristics of the patients of groups A and B was done with respect to their age, spleen size, liver size, leucocyte counts, haemoglobin levels, platelet counts, serum aspartate aminotransferase, serum alanine aminotransferase, and serum creatinine levels, and parasitological scores. First, a graphical approach was adopted, which is based on Boxplots method (Fig. 3). The widely used technique showed the median levels of these characteristics as well as pointed out the outliers (unusual values) in the two groups. There was no outlier in the two groups. There was no difference between various parameter in both the groups (Table).

Only 10-40 macrophages were infected with amastigotes, mean being 25 per cent. The number of parasites varied between 3 to 10 per macrophage, average being 7 parasites per macrophage. There was no association between number of parasites and their sensitivity to SAG.

Only 29 (42.8%) patients in group A whose amastigotes were sensitive to SAG, were treated with...
SAG. Amastigotes of all 70 patients were sensitive to AMB; 2 patients were withdrawn from the SAG treatment as they developed myocardial toxicity. Two patients cured with SAG relapsed during 6 months of follow up. Thus only 25 patients (86.2%) were ultimately cured with SAG in group A. Two patients who developed myocardial toxicity, were given 10 days of rest before start of AMB treatment. Forty five patients of group A, comprising of 41 patients with amastigotes not sensitive to SAG, 2 patients who were withdrawn from study due to SAG toxicity and 2 patients who relapsed after cure were treated with AMB and were cured.

In group B, SAG was started in all 70 patients; 5 patients (7.1%) developed toxicity and were withdrawn, 5 patients relapsed within 6 months of follow up, only 25 patients (35.7%) were ultimately cured, the difference between the two groups was significant (Test of equality of proportion). From group A, 25 of 29 patients (86.6%) got cured while the same number of 70 patients (35.71%) of group B could be successfully treated. The test of the equality of the two proportions rejected the null hypothesis ($P<0.001$). This, in turn, indicated the significantly higher proportion of the cured patients under the approach adopted for the treatment of the disease.

Only 45 patients of this group (5 patients who developed toxicity, 5 patients who relapsed and 35 patients who did not respond to SAG) were treated with AMB and were cured. Ninety patients out of 140 were treated with AMB. Adverse events encountered in two groups of patients, one treated with SAG and another with AMB were pain and swelling at the site of injection, thrombophlebitis at infusion site, shivering and rigor during infusion, fever, rise in serum creatinine, ECG changes, and relapses. Early withdrawal of patients did not allow any patient to develop fatal toxicity.

Patients in group A had to stay for 2340 days in hospital. A total of 3065 days were spent by patients of group B, 1.3 times more than group A. Group B patients had spent a total of 65575 $, which included hospital and treatment costs. Hospital cost included bed charge, nursing and investigation costs. The cost of culture was taken as 10$ per patient. The expenditure incurred on group B patients was 65575 $ compared to 50590 $ on group A patients, 1.3 times more than group A. Infectivity period of group A was 1644 days compared to 2265 days in group B, 1.3 times more than group A.

**Discussion**

Our findings support to the hypothesis that treatment of kala-azar (VL) with SAG based on culture and sensitivity of amastigotes of patients markedly improves cure rate in a place where incidence of resistance to SAG is high and widespread. In group B where patients were treated empirically i.e., all patients were treated with SAG to start with, cure rate was only 35.7 per cent compared 86.6 per cent cure rate in group A where SAG was used only in those patients whose amastigotes were sensitive to SAG in vitro. Moreover, exposing a patient to a toxic drug to which his amastigotes are not sensitive, appears unethical.

Amphotericin B cured all patients in both the groups who were unresponsive to SAG, relapsed after initial cure with SAG and patients withdrawn from study due to SAG toxicity. In vitro also AMB cleared amastigotes.
from all isolates. AMB was effective in clearing promastigotes from isolates but SAG did not clear promastigotes. Therefore, promastigotes culture was not useful for routine use. Resistance to amphotericin B has been reported in laboratory or in wild parasites.

Therefore, judicious use of AMB is required to minimize emergence of resistance. Due to superior efficacy AMB has gradually replaced SAG as a first line drug in Bihar except in government’s clinics and hospitals. With certain measures taken during AMB therapy we had been able to reduce toxicity of AMB but we have not been able to reduce toxicity of SAG except by early withdrawal of cases from the study, as we adopted this principle in this study. Patients in group A had shorter stay in hospital than patients of group B. The stay of a patient in a hospital is not confined to the patient only but attendant/family members also stay with patient to look after. Therefore stay in hospital dose not cost only expenditure incurred on the patient but also incurred on the two family members. Hospital cost is also much more in group B than in group A because of lengthy process of assessing the unresponsive to SAG. Clinically, it takes 28 days to know that patient is not responding to SAG.

It has been shown in laboratory that exposing the parasites to low doses and given irregularly led to emergence of resistance to SAG. Similarly resistance to AMB can also emerge. Therefore, urgent measures need to be taken to prevent the emergence of resistance.

Patients, being the only reservoir of parasites, if remain untreated for a longer period, chance of spread of the disease is more especially of resistant parasites. Thus the culture and sensitivity of amastigotes and choosing the appropriate drug on that basis could be a better method of treatment.

The only disadvantage of this method is that it requires good and trained technician to do culture and sensitivity of amastigotes and the reagents are expensive. Recently thrombocytopenia and haemorrhages due to SAG have been reported. Two improved versions of amphotericin B, amphotericin B emulsion and AmBisome have come in market. Both have minimal side effects, the former is expensive than plain amphotericin B. If these two drugs are made cheaper, they would be ideal for treatment. Single dose of AmBisome can cure a patient. Paromomycin which has been approved by the regulatory authority and is ready for phase 4 trial, is given for 21 days at the dose of 11 mg/kg body wt intramuscularly. These newer drugs are better, more effective and highly suited for elimination of kala-azar.

In conclusion, the treatment of kala-azar with SAG based on culture and sensitivity of amastigotes was found to be better, saved stay of patient in a hospital and total expenditure on the treatment and the expenditure incurred on their relatives for staying with him/her. The decrease in parasite load started from the first day of treatment and thus minimized the risk of spread of the disease in society. It was recommended that till better drugs are within the reach of patients the present treatment strategy of use of SAG based on culture and sensitivity of amastigotes to SAG may be adopted.

References
proprietary sodium stibogluconate are equivalent; HIV co-infected patients have a poor outcome. Trans R Soc Trop Med Hyg 2001; 95 : 668-72.


