PART III/III: DEVELOPMENT AND EVALUATION OF CHITOSAN ENCAGED LIPOSOMES FOR IMMUNOMODULATION TO INVESTIGATE SYNERGISTIC EFFICACY FOR THE CONDITIONS OF LEISHMANIASIS
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5.1 INTRODUCTION

Visceral leishmaniasis (VL) treatment is challenged worldwide due to escalating prevalence of resistance to antimonials and therefore development of affordable alternative drugs is urgently advocated. Moreover, a major obstacle in developing new drugs for such neglected diseases is attributed to their little prospect of financial return (Murray 2001; Murray, Brooks et al. 2003). In last few decades, some new drugs as well as formulations of conventional antileishmanial drugs has been developed. Out of these, Amphotericin B (AmB) emerged as a powerful molecule against sensitive as well as resistant leishmaniasis, which has been used as a second line treatment for VL. Though AmB has been used in conventional dosage form since last few decades but nowadays its newer formulations is being dominantly prescribed in comparison to other agents. Furthermore, unresponsiveness and relapses rarely occurs. Several AmB formulations are available in markets in which liposomal AmB (AmBisome®) occupies major share of market and proved to be effective drug delivery system in terms of enhanced efficacy and minimization of side effects. Other commercial amphotericin B - lipid formulations have also been manufactured, namely an AmB lipid complex (Abelcet®) and an AmB colloidal dispersion (Amphocil™) but their use against VL has not been as extensive as AmBisome® and they too, are costly (Murray 2004).

Approaches to improved treatment include: (i) efficacy trials of single dose AmBisome treatment for VL, with 90 per cent cure rate reported to date,(Sundar, Chakravarty et al. 2010) and (ii) the use of cost effective liposomal formulations (Croft and Coombs 2003). To meet this expectation, alternative AmB formulations have been developed. For example, arabinogalactan derivatives, (Louzir, Melby et al. 1998) nanoparticles, (Arora, Melby et al. 1995; Melby, Valencia-Pacheco et al. 1996) and other lipid formulations, (Melby, Andrade-Narvaez et al. 1994) or chemical derivatives, (Melby, Darnell et al. 1993) have proved efficacy in experimental models (Ibrahim, Gebremariam et al. 2010). The act of heating AmB to form superaggregates reduces in vivo toxicity without loss of efficacy and warrants further investigation as a cheap alternative to the lipid formulations (Henderson, Leon et al. 2009; Laniado-
Laborin and Cabrales-Vargas 2009). It has already been evaluated for the treatment of canine VL (Sasakawa, Yamamoto et al. 2008).

Alternative formulations which can act synergistically i.e., immunomodulatory with chemotherapeutic potential could be the new strategy to enhance potency (Saldarriaga, Perez et al. 2006; Saldarriaga, Travi et al. 2006). Doxorubicin, oxaliplatin and 5-fluorouracil combined with interleukin-21 (IL-21) has shown synergistic antitumor effects (Skak, Søndergaard et al. 2009). Recently we have proved enhanced efficacy of chitosan decorated lipid nanoparticles and alginate coated liposomes for enhanced synergistic action against cancer and leishmaniasis respectively (Singodia, Khare et al. 2011; Singodia, Talegaonkar et al. 2011). Recently some of the scientists have shown interest in using novel AmB formulations and reported efficacy in the conditions of cutaneous leishmania (CL) in immuno-compromised (Choi and Lerner 2002) and pediatric patients (Pace, Williams et al. 2011). As self-limiting syndrome is associated with CL, there has been limited efforts made in exploring AmB for this disease (Alvar, Croft et al. 2006). Various scientists have recently tried to prove that immuno-intervention with antimonials and AmB can be a better treatment for visceral leishmaniasis (Murray, Brooks et al. 2003; Banerjee, De et al. 2008; Murray 2008) but toxicity remains a major concern. Developing novel formulations using biocompatible polymers having properties of immuno-stimulation might be an interesting area for better management of leishmaniasis.

Various polymers as chitosan, glycol chitosan and sodium alginates etc. has been proved to be effective in activation of macrophages and releasing various cytokines and cytotoxic agents (Porporatto, Bianco et al. 2003; Yu, Zhao et al. 2004; Clauss and Samuel 2008; Yang and Jones 2009; Singodia, Khare et al. 2011). Low molecular weight chitosan has been proved to be effective as tumoricidal and antiviral agents by releasing various cytokines as nitric oxide (NO), TNF-α, interferon, IL-6, IL-12 etc (Yu, Zhao et al. 2004). This study suggests that reactive NO induced by chitosan is one of the effector molecule involved in macrophages-mediated tumor cytostasis. Higuchi et al (1990) has demonstrated that TNF-α and L-arginine-derived NO act synergistically in macrophage cytotoxicity (Higuchi, Higashi et al. 1990). The leishmaniasis comprise a group of diseases with extensive morbidity and mortality in most developing countries(Vatsyayan and Roy 2007). Bearing in mind the urgent need for new antileishmanial drugs, due to the limitations of the current therapeutics, we have embarked on to extended study to investigate the antileishmanial potencies of
combination therapy with AmB liposomes having surface modified with chitosan as immune-modulator.

Keeping this hypothesis, we have anticipated to develop chitosan decorated AmB liposomes which can be highly effective in control of leishmaniasis with enhanced efficacy. Simultaneous activity of liposomal AmB and released cytokines by activation of macrophages can prove to be effective medium against leishmaniasis. This synergistic action can generate good antileishmanial activity at low concentration of AmB which can further reduce toxic side effects.

5.2 MATERIALS AND METHODS

5.2.1 Material:

AmB was obtained as a gift sample from M/s Dabur Research Foundation, Ghaziabad, India. Mouse TNF-α and IL-12 Elisa kit was purchased from R&D Systems, San Diego, CA, USA. Griess reagent for nitrite estimation, low molecular weight chitosan (CHL), Nile red, MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide), 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, Dulbecco’s Modified Eagle’s Medium (DMEM), RPMI and supplements were purchased from Sigma-Aldrich, St. Louis, MO, USA. Well plates for cytotoxicity and uptake studies were from Greiner Bio One (Frickenhausen, Germany). All other chemicals and solvents were of HPLC or analytical grade.

5.2.2 Preparation of plain and chitosan coated liposomes:

AmB liposomes were prepared by novel ethanol injection method with slight modification as reported previously (Singodia, Khare et al. 2011). Briefly SPC, cholesterol and EPG in molar ratio of 7:3:1 were dissolved in ethanol by heating at 60°C. Separately AmB was dissolved in acidified DMA. These two solutions were mixed in same vial to obtain clear drug lipid premix solution. Then this premix solution was rapidly injected into phosphate buffer which forms liposomes (Plain-Lip) instantaneously. For preparation of chitosan coated liposomes (CHL), a solution consisting of chitosan (0.25, 0.5 and 1% w/v, in 0.5M acetate buffer, pH 4.4) was prepared and liposomes were incubated for 3 hours. The coated liposomes (CHL) were recovered by ultracentrifugation at 60,000 rpm [Beckman Optima MAX
ultracentrifuge (Beckman Coulter, Fullerton, CA, US) and re-suspended in PBS at pH 7.4. Liposomes were stored at 4°C till further characterization.

5.2.3 Coating efficiency of Chitosan
Aliquots of CHL suspensions (0.4 mL) were centrifuged at 12,000 rpm for 5 min in filtration tube (molecular weight cut, 12 kd). The non-adhered chitosan at the bottom of the tube was determined by a colorimetric method described by Muzzarelli with slight modification (Muzzarelli 1998). To prepare the standard curve, a stock solution of chitosan was prepared at a concentration of 5 mg/mL. Different volumes of the stock solution were transferred into volumetric flasks. A solution of the dye was prepared by dissolving the powder (150 mg) in demineralized water (100 ml). Aliquots of the dye solution (5 ml) were made up to 100 ml with 0.1 M glycine hydrochloride buffer. The final concentration of the dye was 0.075 g/liter. The buffer solution was prepared by dissolving glycine (1.87 g) and sodium chloride (1.46 g) and made up to 250 ml with demineralized water. Aliquots (81 ml) of this solution were made up to 100 ml with 0.1 M HCl. The final pH was 3.2. The following procedure was adopted: 15, 30, 45, 60, 80, 100, 150, 200, and 250 µl of chitosan solution were introduced into volumetric flasks, followed by different volumes of buffer, to reach 300 µl. Then, aliquots of dye solution (3 ml) were added to each. The absorbance values were measured at 575 nm using UV-1700 Shimadzu spectrophotometer. Buffer (0.3 ml) and dye (3 ml) solutions were used to prepare the reference solution.

5.2.4 Characterization of surface decorated liposomes
5.2.4.1 Vesicle size and zeta potential:
Particle size and zeta potential of formulations were determined by a Nano-ZS zetasizer (Malvern Instruments, Malvern, UK) at 25°C. The analysis was carried out on both CHL and Plain-Lip, within 2 h after their preparation. The liposomes were diluted with distilled water prior to analysis, and each measurement was carried out in triplicate. The polydispersity index (PI) was obtained by the software in-built in the instrument. For morphological characterization, TEM studies were carried out using phosphotungstic acid as negative stain (Philips, 400T, TEM, New Brunswick, Canada).

5.2.4.2 Percent entrapment efficiency (% EE) measurement:
Gel Exclusion Chromatography was employed to determine entrapment efficiency. The formulation was passed through Sephadex G-50 column to separate un-entrapped drug. The AmB loaded formulations were lysed with 0.1% v/v Triton X-100 and
AmB content was estimated by RP-HPLC method (van Etten, van den Heuvel-de Groot et al. 1993).

\[
\text{% Entrapment} = \frac{\text{Total amount of drug} - \text{Free drug}}{\text{Total amount of drug}} \times 100
\]

5.2.4.3 In vitro release study

The *in-vitro* release study was performed using dialysis tubing. For the release experiment, one ml of CHL and Plain-Lip formulations were pipetted out into a dialysis bag (molecular weight cut off 12,000Da; Sigma) at room temperature. The dialysis bags were kept in dissolution media (1% v/v Tween 80 in 500 ml PBS, pH 7.4). The temperature was maintained at 37°C and the shaking frequency at 150 rpm in dissolution apparatus. Samples (1 ml) were taken at 0, 15 and 30 min and 1, 2, 4, 6, 12 and 24 h and replaced with an equivalent volume of fresh dissolution media to maintain the sink condition. The concentrations of released AmB were determined by RP-HPLC (section 2.5.2) (van Etten, Otte-Lambillion et al. 1995).

5.2.4.4 Stability:

The stability of formulations in different storage conditions was evaluated. The samples were sealed in glass bottles and stored at 4°C or 25°C for a period of 30 days, and at various intervals aliquots of samples were withdrawn for particle size, zeta potential and % EE measurement.

5.2.5 In Vitro Studies

5.2.5.1 Nitrite analysis:

**Principle**

One means to investigate nitric oxide formation is to measure nitrite (NO$_2^-$), which is one of two primary, stable and nonvolatile breakdown products of NO. The griess reaction is one of sensitive technique to detect NO$_2$ in a variety of biological and experimental liquid matrices such as plasma, serum, urine and tissue culture medium. This assay relies on a diazotization reaction that was originally described by Griess in 1879. The Griess Reagent System is based on the chemical reaction shown in Figure 5.1, which uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions give colored azo compound.
Figure 5.1: Griess Reagent system for nitrite estimation

Procedure

J774A.1 macrophage cell lines (1.5x $10^6$ cells/well) were cultured for 3, 6 and 12 h with CHL and Plain-Lip liposomal formulations having concentration of AmB equivalent to 0.2µg/ml and the release of nitrite in culture extract was determined using the assay system previously described (Ding, Nathan et al. 1988; Tamis 2006). Briefly, 100 µl supernatant was removed from each well into an empty 96-well plate. After the addition of 100 µl Griess reagent (one part 0.1% naphthylethylenediamine dihydrochloride in distilled water plus one part 1% sulfanilamide in 5% concentrated H$_3$PO$_4$) to each well, absorbance at 540 nm was measured using a molecular device microplate reader. Nitrite concentration was calculated from a NaNO$_2$ standard curve. The levels of absorbance are indicative of NO production. The concentration of nitrite in the media related to the NO secreted by chitosan-activated macrophages was then determined. All experiments and determinations were done in triplicate.

5.2.5.2 TNF-α and IL-12 assays:

Macrophages J774A.1 (1.5x $10^6$ cells/well) in 0.2ml complete RPMI-1640 media were seeded in a 48 well plate. The cells were allowed to adhere for 24 h at 37°C, after which the medium was aspirated and replaced with 0.1 ml fetal bovine serum and phenol red free RPMI-1640 media 1 hr prior to treatment with CHL and Plain-Lip formulations. CHL and Plain-Lip formulations were diluted into complete RPMI-1640 medium and added to cells in 0.1 ml to achieve the desired final concentration. The cells were incubated with different
formulations (concentration of AmB equivalent to 0.2µg/ml) for 3 h, 6 h and 12 h separately. The 100 µl of cell supernatant was collected in well plate and kept at (-) 80°C until further assessment. The TNF-α and IL-12 in supernatants was determined by sandwich enzyme-linked immunosorbent assays (ELISAs) (R&D system, USA) according to standard procedures. Briefly, purified anti-cytokine antibodies of TNF-α and IL-12 were used as primary antibodies, biotinylated mouse anti-mouse cytokine antibodies clone, for TNF-α and IL-12 was used as secondary antibodies, and streptavidine-conjugated peroxidase as the developing reagent with 3,3,5,5-tetramethylbenzidine tablets (TMB) as substrate. All reagents used for ELISA were obtained from R&D System (San Diego, CA, USA). TNF-α and IL-12 concentration was determined by referring to standard curves constructed with fixed amounts of mouse recombinant TNF-α and IL-12. Optical densities (OD) were measured using an automated ELISA plate reader at 450 nm. Data from triplicate determinations were expressed as nanograms per ml (mean ± SD).

5.2.5.3 In vitro evaluation against intramacrophage amastigotes of *L. donovani* 

*In vitro* activity of different formulations (CHL, Blank CHL, Plain-Lip and Blank Plain-Lip) was determined against intramacrophage amastigotes in J774A.1 mouse macrophage cell line. Protocol for infecting J774A.1 using late log phase promastigotes has been already described in section “3.2.4.8” of this present thesis work. Briefly adherent mouse macrophage cell line J774A.1 was grown in 16-well chamber slides (Nunc, IL, USA) at 37°C with 5% CO₂. Late log phase promastigotes (10 parasites per macrophage) has been used for infecting macrophages. Infection was carried out with late log phase promastigotes [Expressing Green fluorescent protein (GFP)] at a multiplicity of infection of 10 parasites per macrophage. After 12 h, chamber slides were washed to remove non-phagocytosed promastigotes, re-supplemented with medium and kept with different concentration of AmB formulations for 3 h and macrophages were placed in medium for an additional 20 h. Post treatment slides were air-dried, fixed in methanol, stained with Giemsa and examined under oil immersion objective of the microscope. The infection level was
assessed by counting the percentage of infected macrophages and average number of amastigotes in each macrophage.

5.2.5.4 Macrophage uptake studies

Macrophage cell lines “J774A.1” was used for the present study using fluorescence activated cell sorter (FACS) instrument (BD Biosciences, FACS Aria, Germany). Aliquots (100 μL) containing J774A.1 cell lines at a density 1 × 10^5 cells/well were suspended in 24-well plates containing fresh RPMI-1640 medium supplemented with penicillin 10 U/mL, 10% fetal bovine serum, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 10 mM HEPES medium (Figueiro-Filho, El Beitune et al. 2008). After 24 h the culture medium was replaced with fresh culture medium containing CHL and Plain-Lip incorporating nile red (0.2% w/v). For separation of the internalized and surface bound liposomes, the cells were washed three times with acetate buffer (pH 4.0). The cell-associated fluorescence was measured by FACS at an excitation wavelength of 485 nm and an emission wavelength of 628 nm (Collazos 2008). The data has been represented as mean fluorescence obtained from a population of 10,000 cells.

5.2.5.5 Determination of haemolysis by chitosan decorated liposomes (CHL)

Haemolysis induced by CHL formulations was determined by above mentioned methods (section 3.2.4.7). Various chitosan coated liposomal formulations (0.2ml) containing equivalent concentration of AmB (50 μg AmB/ml) along with corresponding blank formulations having similar chitosan and lipid content were incubated with 1.0 ml of blood and processed as described above to determine percentage haemolysis by haemoglobin released into the supernatant.

5.2.6 In Vivo Studies

5.2.6.1 Antileishmanial activity testing

Laboratory bred male Syrian golden hamsters (Mesocricetus auratus, 45–50 g) from animal house facility of Central Drug Research Institute (CDRI) were used as the experimental host after approval from Institutional Animals Ethical Committee (IAEC) of CDRI. They were housed in plastic cages in climatically controlled rooms and fed with standard rodent food pellet (Lipton India Ltd, Bombay) and water ad libitum. The study was carried out with the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. The parasites were isolated from spleen of infected hamster and injected to naïve hamsters as described earlier (Gupta,
Dube et al. 2007). Infected hamsters having 37-40 amastigotes/100 macrophages nuclei were selected for the treatment with various formulations in the following manner (i) infected controls (no therapy was given), (ii) Plain-Lip, (iii) CHL, (iv) Blank Plain-Lip (without AmB), (v) Blank CHL (without AmB).

Infected hamsters were treated intracardially (i.c.) with 5 mg/kg of AmB in all formulations as single dose therapy (three in each group). After eight days post treatment splenic biopsies were performed and parasite burden was determined by counting number of amastigotes (Guru, Agrawal et al. 1989).

5.2.6.2 Estimation of expression of mRNA cytokines by real-time PCR in treated hamsters

To find the expression of mRNAs, real-time PCR was performed for various cytokines and inducible NO synthase (iNOS) in the splenocytes of hamsters on day 8 post treatment. Splenic tissues were taken from each of the three individual animals randomly chosen from different groups. Total RNA was isolated using TRI Reagent (Sigma-Aldrich). All RNA samples were DNase treated with TURBO DNA-free™ kit and quantified using Gene-quant (Bio-Rad). One microgram of total RNA was used for the synthesis of cDNA using a first-strand cDNA synthesis kit (Fermentas). For real-time PCR, primers were designed using Beacon Designer software (Bio-Rad) on the basis of cytokines and iNOS mRNA sequences (Melby, Chandrasekar et al. 2001). Real-time quantitative PCR was conducted as per the protocol described earlier. (Rama Iniguez, Dea-Ayuela et al. 2006) Briefly, it was carried out with 12.5 µl of SYBR green PCR master mix (Bio-Rad), 1 µg of cDNA, and primers at a final concentration of 300 nM in a final volume of 25 µl. PCR was conducted under the following conditions: initial denaturation at 95°C for 2 min followed by 40 cycles, each consisting of denaturation at 95°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s per cycle using the iQ5 multicolor real-time PCR system (Bio-Rad). cDNAs from normal hamsters were used as “comparator samples” for quantification of those corresponding to test samples. All quantifications were normalized to the housekeeping gene HGPRT. A no-template control c-DNA was included to eliminate contaminations or nonspecific reactions. Differences in gene expression were calculated by the comparative CT method (Rama Iniguez, Dea-Ayuela et al. 2006). This method compares test samples to a comparator sample and uses results obtained with a uniformly expressed control gene (HGPRT) to correct for differences in the amounts of RNA present in the two samples being compared to
generate a ΔCT value. Finally, results are expressed as the fold-changes compared to the control infected hamster.

5.3 STATISTICAL ANALYSIS

All results are given as means ± SD (n = 3). Differences between formulations were compared using one-way analysis of variance (ANOVA) followed by the Turkey–Kramer multiple comparison test, using Graph pad Instat software (Graph Pad Software Inc. CA, USA). P < 0.05 denotes significance in all cases.

5.4 RESULTS

5.4.1 Formulation development and characterization

5.4.1.1 Preparation and characterization:

Novel modified ethanol injection method was found to be excellent for preparation of amphotericin B liposomes. This is the simplest method in comparison to other reported methods i.e. freeze thaw method, hand shaking method, emulsification and ether injection methods. The amount of solvents i.e. DMA and ethanol used in the present study is in accepted limits for intravenous human use. Size of Plain-Lip and CHL is 104.3± 4.6 and 132.6 ± 3.2 nm respectively with very low polydispersity index. Plain-Lip shows zeta potential of (−) 27.2 ± 2.4 mV while CHL shows zeta potential value and (+) 38.3 ± 3.1 mV (Table 5.1).

5.4.1.2 Coating efficiency of chitosan

Chitosan with 0.5% concentration was found to be optimum as size of the liposomes was in the range of 132.6±3.2 nm with narrow size distribution. At higher conc. of chitosan (1%) the particle size was increased significantly and aggregation was also observed due to higher viscosity and therefore this formulation was discarded.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>CH Liposome</th>
<th>Zeta binding</th>
<th>CH binding over Binding</th>
</tr>
</thead>
</table>

Table 5.1: Physicochemical properties of CHL with different chitosan concentrations, each value represents the mean±S.D. (n = 3).
<table>
<thead>
<tr>
<th>conc. (%w/v)</th>
<th>size ± S.D. (nm)</th>
<th>potential ± S.D. (mV)</th>
<th>liposomes (mg/mg of lipid)</th>
<th>efficiency ± S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>104.3± 4.6</td>
<td>−27.2± 2.4</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.25</td>
<td>118.6± 4.1</td>
<td>+31.2 ± 2.9</td>
<td>2.06/40</td>
<td>82.5±7.4</td>
</tr>
<tr>
<td>0.5</td>
<td>132.6 ± 3.2</td>
<td>+38.3 ± 3.1</td>
<td>3.91/40</td>
<td>78.2±7.4</td>
</tr>
<tr>
<td>1.0</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

CH, chitosan; % Binding efficiency= (quantity of chitosan coated over liposomes/total quantity of chitosan used for coating)*100

5.4.1.3 Surface morphology & %EE:

Particles are spherical in appearance. Separate layers are visible in surface decorated liposomes which proved that polymer is uniformly coated on liposomes (Figure 5.2). Both CHL and Plain-Lip showed more than 95±2.3% entrapment efficiency (Table 5.2).

![Figure 5.2](image.png)

**Figure 5.2:** Visualization of (A) Plain-Lip and (B) CHL liposomes by Transmission Electron Microscopy (TEM). Outer dark layer covering on CHL liposome clearly shows coating of chitosan. Scale bar = 200nm. Magnification, ×15,000

5.4.1.4 In vitro release study:

The cumulative percent release of AmB from liposomal formulations over varying time intervals in dissolution media was examined. AmB concentration in the dialysis cassettes was assayed at periodic intervals using a RP-HPLC method. The release percentage of AmB from Plain-Lip and CHL was 38.2±2.7 and 26.5±1.9% respectively at 6h, while it was 64.1±4.2 and 47.6±3.4% respectively after 24h (Figure 5.3). The release rate of CHL was significantly lower (P<0.05) than that of Plain-Lip at all time points.
Figure 5.3: Release profiles of AmB lipid preparations in 1% v/v Tween 80 in PBS, pH 7.4 at 37°C. (mean±SD) (n = 3). *Depicts comparison of AmB release from CHL formulations vs Plain-Lip. P<0.05.

5.4.1.5 Stability of formulations:
Stability of CHL and Plain-Lip was tested for 30 days at 4 and 25°C months (Table 5.2). Particle size and zeta potential did not change significantly after 30 days for both the formulations. However, in case of Plain-Lip the %EE was decreased significantly to 88.3 ± 1.7 from 96.9 ± 2.1% after 30 days at 25°C while % EE remain unchanged in case of CHL.

Table 5.2: Storage stability of formulations (CHL and Plain-Lip) for 30 days at different storage conditions. Each value represents the mean±S.D. (n = 3).

<table>
<thead>
<tr>
<th>Temp.</th>
<th>Formulation</th>
<th>Vesicle size (nm±S.D.)</th>
<th>Zeta potential (mV±S.D.)</th>
<th>%EE±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial 15 days 30 days</td>
<td>Initial 15 days 30 days</td>
<td>Initial 15 days 30 days</td>
</tr>
<tr>
<td>25°C</td>
<td>Plain-Lip</td>
<td>104.3±4.6 105.8±5.9 141.3±2.8</td>
<td>−27.2±2.4 −27.8±1.3 −29.2±0.5</td>
<td>96.9±2.1 95.2±3.6 88.3±1.7</td>
</tr>
<tr>
<td></td>
<td>CHL</td>
<td>132.6±3.2 135.6±2.1 139.8±5.3</td>
<td>38.3±3.1 37.4±1.4 38.2±0.2</td>
<td>96.6±2.3 95.4±1.4 96.9±2.2</td>
</tr>
<tr>
<td>4°C</td>
<td>Plain-Lip</td>
<td>104.3±4.6 107.7±1.5 112.8±3.7</td>
<td>−27.2±1.4 −28.9±0.9 −27.6±0.7</td>
<td>95.9±1.1 97.6±3.2 94.0±1.5</td>
</tr>
<tr>
<td></td>
<td>CHL</td>
<td>132.6±3.2 137.4±1.2 140.7±4.0</td>
<td>38.3±3.1 38.1±1.2 38.9±0.8</td>
<td>97.6±1.3 97.8±2.6 97.5±3.2</td>
</tr>
</tbody>
</table>

CHL=Chitosan coated liposomes, Plain-Lip=Plain undecorated liposomes

5.4.2 In vitro studies

5.4.2.1 NO generation in infected macrophages:
It has been observed that CHL induces the production of NO. With CHL the level of NO was found to be 16.7±1.1, 18.9±1.3 and 23.4±1.9 µM while in case of Plain-Lip formulation it was 7.1±0.6, 8.5±0.7, 9.9±1.0 µM at 3, 6 and 12 hours respectively (Figure 5.4).

**Figure 5.4:** NO production by phosphate buffer saline (PBS), uncoated liposomes (Plain-Lip) and chitosan coated liposomes (CHL) at 3, 6 and 12 hours (mean±SD) (n = 3). *Depicts comparison of NO production of CHL formulations with Plain-Lip and PBS. P<0.05.

### 5.4.2.2 TNF-α and IL-12 generation in infected macrophages:

When the macrophages were activated with CHL the level of TNF-α was found to be 873.2±29.1, 1023.4±32.7 and 1289.6±52.7 pg/ml after 3, 6 and 12 hours respectively while with Plain-Lip the level was 45.3±2.4, 78.7±3.7 and 104.5±4.9 pg/ml (Figure 5.5). Similarly, CHL activated macrophages released 11.2±0.5, 13.7±0.7 and 15.5±1.1 pg/ml of IL-12 at 3, 6 and 12 hours respectively while 1.2±0.08, 2.2±0.12 and 3.1±0.16 pg/ml of IL-12 was released with Plain-Lip (Figure 5.6). Concentrations of both IL-12 and TNF-α with CHL are significantly higher (P<0.05) from respective concentration released with Plain-Lip after 3, 6 and 12 hours.
Figure 5.5: TNF-α production by phosphate buffer saline (PBS), uncoated liposomes (Plain-Lip) and chitosan coated liposomes (CHL) at 3, 6 and 12 h (mean±SD) (n = 3). *Depicts comparison of TNF-α production of CHL formulations with Plain-Lip and PBS. P<0.05.

Figure 5.6: IL-12 production by phosphate buffer saline (PBS), uncoated liposomes (Plain-Lip) and chitosan coated liposomes (CHL) at 3, 6 and 12 h (mean±SD) (n = 3). *Depicts comparison of IL-12 production of CHL formulations with Plain-Lip and PBS. P<0.05.
5.4.2.3 % Parasite inhibition:
For % parasite inhibition CHL was compared with Plain-Lip, blank CHL and blank Plain-Lip at various concentrations of AmB (0.03, 0.08, 0.13 and 0.2 µg/ml) loaded formulations. As shown in Figure 5.7 the % parasite inhibition in case of CHL formulation was more than 2.5 and 1.5 times as compared to Plain-Lip at 0.03 and 0.08 µg/ml of AmB. However, at higher concentration of AmB (0.2µg/ml) the % parasite inhibition was found to be 95.1±2.3 and 82.7±3.1% for CHL and Plain-Lip respectively. The CHL formulation was found to be more effective at lower concentration of AmB as compared to Plain-Lip formulation. The percent parasite inhibition was also observed with blank CHL formulation and it was almost two fold higher than Plain-Lip formulation (Figure 5.7).

![Figure 5.7: In vitro activity against intracellular amastigotes in macrophages (J774A.1). Different formulations containing 0.03, 0.08, 0.13 and 0.2 µg/ml of AmB (Blank formulations having the same composition) i.e. CHL, Plain-Lip, blank CHL and blank Plain-Lip were added to infected macrophages cell culture inside the chamber slide wells in triplicate. The number of amastigotes in 100 treated and untreated macrophages was counted after incubation with drug formulations and percent parasite inhibition in treated wells was calculated in comparison to the parasite burden of untreated macrophages (mean±SD) (n = 3). *Depicts comparison of % parasite inhibition of CHL formulations with Plain-Lip, blank CHL and blank Plain-Lip at all concentrations. P<0.05.](image-url)
5.4.2.4 **In-vitro uptake studies:**

As observed in the parasite inhibition studies, CHL have more pronounced effect on intramacrophagic amastigotes than Plain-Lip formulations. This indicates active uptake of CHL in comparison to Plain-Lip. In order to confirm specificity of these particles towards macrophages, an *in-vitro* uptake study was carried out using nile red labeled CHL and Plain-Lip formulations. As shown in Figure 5.8, cellular uptake was higher in chitosan decorated liposomes (CHL) in comparison to Plain-Lip. From these data it seems that enhanced cellular uptake depends upon the outer coating of chitosan polymer on macrophages. Uptake of the CHL was more than two fold than Plain-Lip in macrophages. These observations agree with the enhanced efficacy of CHL against intramacrophagic amastigotes, providing evidence of cationic polymer mediated enhanced macrophage cell uptake of liposomes. These results also indicated that chitosan might affect the function of macrophages for the production of various mediators.

**Figure 5.8:** Flow cytometry diagram of macrophage J774A.1 cells exposed to Nile red-labelled liposomes. X-axis represents fluorescence inside the cells. A: Blank, B: Uncoated (Plain-Lip) and C represents chitosan decorated liposomes (CHL).
5.4.2.5 *Determination of haemolysis by polymer decorated liposomes (CHL)*

The degree of haemolysis with CHL and Plain-Lip formulations is 6.8 ± 1.3 and 5.8 ± 1.2%, respectively suggesting that formulations have no deleterious effects on erythrocytes (Figure 5.9).

![Graph showing % haemolysis for different formulations](image)

**Figure 5.9:** Determination of haemolysis of CHL and Plain-Lip formulations. n=3

5.4.3 *In Vivo Studies*

5.4.3.1 *In vivo antileishmanial activity testing:*

Splenic biopsies showed 89.2±3.1% parasite inhibition with CHL, in comparison to 71.3±4.6% with Plain-Lip as represented in Figure 5.10. Similarly blank CHL showed 25.9±1.9% parasite inhibition in comparison to 12.7±2.3% with blank Plain-Lip indicating immuno-stimulatory effect of chitosan for fighting against leishmaniasis. Giemsa stained splenic smears of Leishmania donovani infected hamsters from control and treated groups are shown in Figure 5.11.
Figure 5.10: Antileishmanial activity of AmB formulations against established infection of Leishmania donovani in hamsters. Drug formulations and formulations without drug were injected intracardially into each hamster on day 31st post infection. Parasite burden was estimated by splenic biopsy on day 8 post treatment and percent parasite inhibition was calculated in comparison to the parasite burden of untreated animals (mean ± SD) (n = 3). *P<0.05 (*Depicts comparison of CHL vs. Plain-Lip and Blank CHL vs. Blank Plain-Lip).
Figure 5.11: Photomicrographs showing Giemsa stained splenic smears of Leishmania donovani infected hamsters from control and treated groups (X 100). [a] Blank Plain-lip treated group, [A] Plain-Lip treated group, [b] Blank CHL treated group, [B] CHL treated group.

5.4.3.2 Estimation of expression of mRNA cytokines by real-time PCR:
A] The expression of iNOS was significantly higher in CHL and blank CHL treated, compared to infected hamsters (P<0.01). There was no significant difference (P>0.05) in relative fold expression level of iNOS between Plain-Lip treated and infected hamsters group (Figure 5.12).
B] The relative expression level of IFN-γ was significantly higher in CHL and blank CHL treated, compared to infected hamsters (P<0.01). There was no significant difference (P>0.05) in relative fold expression level of iNOS between Plain-Lip treated and infected hamsters group (Figure 5.13).
Figure 5.12: Splenic iNOS mRNA expression profile analysis of normal, infected, and formulation treated hamsters after 8 day post treatment by quantitative real-time RT-PCR. (mean ± SD) (n = 3). P<0.01. **Depicts comparison between infected vs CHL and between infected vs blank CHL.

Figure 5.13: Splenic IFN-γ mRNA expression profile analysis of normal, infected, and formulation treated hamsters after 8 day post treatment by quantitative real-time RT-PCR. P<0.01. **Depicts comparison between infected vs CHL and between infected vs Blank CHL.
C] The relative expression level of TNF-α was significantly higher in CHL and blank CHL treated, compared to infected hamsters (P<0.01). Plain-Lip treated group showed significantly lower (P<0.05) relative expression level of TNF-α compared to infected hamsters (Figure 5.14).

![Figure 5.14: Splenic TNF-α mRNA expression profile analysis of normal, infected, and formulation treated hamsters after 8 day post treatment by quantitative real-time RT-PCR. (*, p<0.05; **, p < 0.01). **Depicts comparison between infected vs CHL and between infected vs Blank CHL. *Depicts comparison between infected vs Plain-Lip relative fold expression level.](image)

D] The relative expression level of IL-12 was significantly higher in CHL and blank CHL treated, compared to infected hamsters (P<0.01). Plain-Lip treated group showed significantly higher (P<0.05) relative expression level of TNF-α compared to infected hamsters (Figure 5.15).
**Figure 5.15:** Splenic IL-12 mRNA expression profile analysis of normal, infected, and formulation treated hamsters after 8 day post treatment by quantitative real-time RT-PCR. (*, p<0.05; **, p < 0.01). **Depicts comparison between infected vs CHL and between infected vs Blank CHL. *Depicts comparison between infected vs Plain-Lip relative fold expression level.

### 5.5 DISCUSSION:

Ethanol injection method has been suitably modified for development of AmB liposomes which is reproducible with minimum variations. Narrow size distribution, spherical morphology and more than 95% entrapment efficiency has been achieved with minimum variations. During liposomal formulation, EPG was added as a negatively charged lipid which provided the binding force to the positively charged chitosan. Liposomes were surface modified with chitosan under stirring. The non-coated liposome (Plain-Lip) was negatively charged, and after surface modification with chitosan the zeta potential was changed from \((-\) 27.2 ± 2.4 to \((+) 38.3 ± 3.1 \text{ mV}\), as a result of mutual interaction between chitosan and negatively charged liposome surface. AmB loaded Plain-Lip formulation was coated with different conc. of chitosan, and its effects on liposome physicochemical properties were evaluated. Surface modification of liposomes was carried out with different concentration of chitosan (i.e. 0.25%, 0.5% and 1%) to achieve uniform coating without aggregation. Chitosan with 0.5% concentration was found to be optimum as size of the vesicles was in the range of 132.6±3.2 nm with narrow size distribution (Table 5.1). The slight
increase in vesicle size might be due increase in the wall thickness due to polymer coating. But further aggregation was hindered by higher surface zeta potential and steric turbulence due to chitosan chain. At higher conc. of chitosan (1%) the vesicle size was increased significantly and aggregation was also observed due to higher viscosity and therefore this formulation was discarded.

There was no significant change in %EE after coating liposomes with chitosan (Table 5.2). This ensures that integrity of drug loaded liposomes is not affected by chitosan coating and loaded drug is well retained inside the vesicles. The in-vitro release study suggests that AmB release profile was prominently prolonged by the liposome encapsulation, and further by chitosan coating. Very low burst release justifies the complete entrapment of drug into liposomes. In case of liposomes, the rate of drug release is mainly affected by membrane permeability which is influenced by the fluidity of lipid bilayer (Gupta, Dube et al. 2007). At the experimental conditions (37°C), the lipid bilayer is in liquid crystalline phase which permits the drug permeation. In case of CHL, the rate of drug permeation was reduced by the presence of chitosan coating. This can be justified as chitosan is strongly adhered and adjacently linked to liposome surface which constructs a uniform shell, which probably restricts the bilayer fluidity. Consequently, the membrane was consolidated and the permeability was decreased.

The physicochemical stability of CHL with different coating levels of chitosan has been shown in Table 5.2. At 25°C, CHL displayed superior stabilities as compared to Plain-Lip. At 25°C the EE of Plain-Lip was reduced to 88.3 ± 1.7% and vesicle size increased significantly after 30 days. This can be attributed to hydrolysis and oxidation of the lipids at room temperature which consequently induce decomposition and clustering of the liposome vesicles. This factor would have led drug leakage and slight changes in zeta potential. However, in case of CHL, there was no significant change (P>0.05) in vesicle size and % EE as compared to Plain-Lip. This indicates that lipid decomposition has been reduced due to jacketing by chitosan layer over liposomes. As chitosan has stability in water, it can execute as protective covering over liposomes. (Li, Zhuang et al. 2009). On the other hand, at 4°C, both coated and non-coated liposomes demonstrated significantly improved stability profile. This suggests that the decomposition of liposome was inhibited at low temperature. The CHL formulation was found to be more stable than Plain-Lip in terms of vesicle size, indicating stabilizing effect of chitosan coating layer.
Combination of antimicrobial chemotherapy along with immuno-stimulation could lead to paradigm shift in approach to treat leishmania due to synergistic effect, however, limited literature is available till now. Macrophages exhibit either direct cytotoxicity against parasite or show indirect cytotoxicity by secreting cytokines that causes activation of natural killer (NK) cells, cytotoxic lymphocyte (CTL) and dendritic cell (DC) to start the anti-leishmanial immune response (Figure 5.16). Macrophages has been reported to constitute both host and the effector cells against Leishmania infections (Rittig and Bogdan 2000). Irrespective of host T-cell responsiveness, the in-vivo efficacy of antileishmanial drugs can be ameliorated by concurrently triggering pro-inflammatory Th1 immune responses with the secretion of cytokines such as NO, TNF-α, IL-12 and IFN-γ (Murray 2001; Masihi 2003; Murray, Brooks et al. 2003). NO produced by activated macrophages has been shown to control antimicrobial and antitumor activities (de Lima, de Sa Lima et al. 2006) and its significance in leishmanicidal activity has been established by increase in parasite load upon retarding its release with NO antagonists (Evans, Thai et al. 1993). It has been reported that Leishmania mitochondrial respiration is repressed by NO production in macrophages and this could be an important feature to control Leishmania disease (Lemesre, Sereno et al. 1997). Combining drugs with immuno-stimulation (immune-chemotherapy) can be beneficial for development of efficacious formulation against leishmaniasis (Buates and Matlashewski 1999; Arevalo, Ward et al. 2001).
Various researchers have reported macrophage activation potential of low molecular weight chitosan which triggers release of various pro-inflammatory cytokines as NO, TNF-α, IL-12 and IFN-γ (Feng, Zhao et al. 2004; Yu, Zhao et al. 2004; Wu and Tsai 2007). Previously immunological effect of chitin was evaluated by Shibata et al (1997) who demonstrated activation of lung macrophages to release various cytokines as IL-12, TNF-α and IFN-γ (Shibata, Metzger et al. 1997). It was proved that cytokine release was mediated through mannose-receptor-dependent phagocytic process (Feng, Zhao et al. 2004). Lee (2009) further demonstrated that chitin stimulates macrophages by interacting with different cell surface receptors such as macrophage mannose receptor, toll-like receptor-2 (TLR-2), C-type lectin receptor Dectin-1, and leukotriene 134 receptor (BLT1) (Lee 2009). Based on above investigations, we have developed an idea of combining AmB loaded liposomes (rather than plain AmB) along with agent having potential of immuno-stimulation like CHL. As AmB liposomal therapy has been very successful due to its preferential
uptake by macrophages in liver and spleen, its efficacy can further be augmented by modifying its surface with a polymer having immuno-stimulatory potential like chitosan. Although AmB lipidic formulations are very successful in delivering higher amount of drug with minimum side effects by preferential uptake in RES systems but they also reduce the ability of the free drug to activate macrophages (Larabi, Legrand et al. 2001). Based on successful immuno-chemotherapy for leishmaniasis and macrophagic activation potential of chitosan, AmB liposomal formulation coated with chitosan has been evaluated for synergistic activity against leishmaniasis.

The basis of our study lies in macrophage activation potential by low molecular weight chitosan polymers. We have compared macrophage activation potential of uncoated liposomes (Plain-Lip) and chitosan coated liposomes (CHL) on J774A.1 macrophage cell lines at various time intervals i.e., 3, 6 and 12 hours. Plain-Lip releases 7.1±0.6, 8.5±0.7, 9.9±1.0 µM of NO while CHL releases significantly higher amount (P<0.05), i.e., 16.7±1.1, 18.9±1.3 and 23.4±1.9 µM of NO respectively at 3, 6 and 12 hours after macrophage activation. Chitosan, on interaction with immune cells lead to generation of various cytokines, and thus shows anti-tumor, anti fungal and anti-leishmanial activity. Recent studies have shown that NO plays a key role in eliminating parasites from the body (Liew and O'Donnell 1993). It is interesting to note that biomaterial induced inflammation similar to pathogens activate immune system. In any case, it is encouraging to observe that the CHL formulation were apparently have stimulatory effect and causes substantial activation of macrophage to handle *leishmania* parasite. The nitric oxide production was found to be higher in CHL treated group as compared to Plain-Lip.

Augmented TNF-α production is also a indicative of macrophage activation and essentially involved in NO mediated killing of *Leishmania* parasites and various other pathogens (Roach, Kiderlen et al. 1991). Therefore we have considered to evaluate the effect of CHL on macrophages for activation and release of TNF-α and IL-12 cytokines compared to Plain-Lip. Release of both the cytokines (TNF-α and IL-12) was significantly higher (P < 0.05) with CHL activated macrophages compared to Plain-Lip. Thus CHL role as macrophage activator and subsequent release of cytokines which mediates killing of leishmania parasites encourages us to assume this as potential delivery systems for the conditions of leishmaniasis.

To evaluate the effect of chitosan coating over liposomes, comparative uptake studies on macrophage cell lines were performed using nile red as fluorescent moiety.
Figure 5.8 shows comparative uptake of CHL and Plain-Lip formulations by flow cytometry. This figure indicates almost two fold uptake of CHL in cell lines compared to Plain-Lip. This finding suggests that positively charged CHL formulation undergo electrostatic interactions with negatively charged cell membrane. Chitosan has been known to interact with cell membranes through nonspecific attractive electrostatic forces (Schipper, Olsson et al. 1997; Huang, Ma et al. 2002; Yang, Shim et al. 2009). Similarly, Tahara (2009) has shown better uptake of chitosan surface-modified nanospheres compared to unmodified one (Tahara, Sakai et al. 2009). They have observed that cellular uptake of chitosan modified nanospheres display saturable pattern similar to that of unmodified nanospheres. Therefore, it can reasonably be assumed that chitosan modified nanospheres are also transported by the adsorptive endocytic pathway. There are several endocytic pathways i.e clathrin, caveolae, macropinosomes or other clathrin-independent pathways that regulate cellular trafficking and helps in transporting molecular cargo (Panyam, Zhou et al. 2002; Panyam, Sahoo et al. 2003; Panyam and Labhasetwar 2004).

Chitosan is suitable as a material for surface modification of negatively charged liposomes for intracellular targeting because it increased the interaction between the cell membrane and liposomes without showing cytotoxicity. Thus, CHL has been found to have potential to achieve higher cellular uptake resulting from their strong interaction with cells without any cytotoxicity. These results indicated that chitosan might affect the function of macrophages for the production of various mediators. Since 0.5% w/w chitosan concentration for liposome coating was the appropriate concentration that affected immune system efficiently with no effect on survival rate as viability of infected macrophages was more than (90%) under experimental conditions. In addition, the augmented phagocytosis reaffirmed that CHL could activate the function of macrophages without affecting the viability of cells. It can therefore be implied that the CHL would have first been internalized into macrophage via non-specific endocytotic pathways, followed by AmB release inside the cytoplasm, inhibiting the growth of the cells. Thus, the targeting nature of the chitosan polymer may lead to higher selectivity to intramacrophagic parasite and enhance their cellular uptake, with a consequent decrease in systemic toxicity.

Percent parasite inhibition with CHL was determined on intramacrophagic amastigotes of leishmania and compared with Plain-Lip to check if released cytokines would have synergistic effect with AmB. In intramacrophagic amastigotes, % parasite
inhibition with CHL was 2.5 and 1.5 times higher as compared to Plain-Lip at 0.03 and 0.08 µg/ml concentrations, respectively. It was really surprising to observe that immuno-modulatory effect of chitosan synergizes with AmB and increases parasite inhibition significantly (almost two fold) at similar concentration compared to plain liposomes. Blank formulations were also tested for activity against intramacrophagic amastigotes to check effect of formulation ingredients i.e. DMPC, Chol, EPG and chitosan. Blank CHL showed significant higher inhibition (P<0.05) compared to blank Plain-Lip which justifies the improved efficacy imparted by chitosan against intramacrophagic amastigotes. A difference in antileishmanial activity of the CHL with uncoated formulation is attributable to enhanced uptake, interaction with host cells rather than intracellular distribution and most importantly immuno-modulatory activity. Chemotherapeutic activity of AmB synergizes with immuno-modulatory activity of chitosan jacketed over liposomes. Generation of nitric oxide, TNF-α and IL-12 have antileishmanial activity which synergizes with AMB loaded CHL formulation in killing intramacrophagic amastigotes.

Murray et al (2003) has carried out study which they have combined exogenous IL-12 and agonist anti-CD40 monoclonal antibody (MAb) to induce IFN-γ and maintain IL-12 with AmB against visceral leishmaniasis. They have shown 7.5 fold efficacy with immuno-interventions compared with AmB alone (three injections of 5 mg/kg; total dose, 15 mg/kg) (Murray, Brooks et al. 2003). It has been proved by these investigations that AmB can be used in low dose with immune-interventions. In parallel to this, we have carried out a study in our lab to test if AmB liposomes coated with immune-modulatory polymer (i.e. chitosan) have better efficacy than uncoated liposomes (Plain-Lip). Recently, single dose AmB liposome was tested for treatment against visceral leishmaniasis at the dose of 10 mg/kg.(Sundar, Chakravarty et al. 2010) In contrast to this, we have used single dose therapy at half of the AmB dose in liposomes (5 mg/kg) coated with chitosan (CHL) and compared with unmodified AmB liposomes. Parasite inhibition in spleen was reduced to 89.2±3.1% with CHL formulation while it was only 71.3±4.6% with Plain-Lip formulation. To test, if significantly enhanced efficacy of AmB against visceral leishmaniasis in-vivo is coupled with immuno-stimulation with CHL formulation, cytokine profile (TNF-α, IL-12 and IFN-γ) along with iNOS level was determined by real time-PCR and compared with unmodified one. Significantly higher level (P<0.001) of TNF-α, IL-12, IFN-γ has been observed in hamsters treated with CHL and Blank CHL
formulations compared with infected untreated hamsters which clearly indicates immuno-stimulatory potential of chitosan. While insignificant levels of TNF-α and IFN-γ (P>0.05) with Blank Plain-Lip and Plain-Lip compared to untreated infected hamsters, further supports the immune-modulatory activity of CHL formulations. It has been shown that IFN-γ along with other cytokines was the mediator of protective immunity induced against leishmaniasis (Liew and O'Donnell 1993; Reiner and Locksley 1995; Liew, Wei et al. 1997; Alexander, Satoskar et al. 1999). IFN-γ has been reported to play a vital role in antileishmanial defense which is released by leishmania-infected, activated macrophages (Muller, Freudenberg et al. 1997). The synergistic effect of IFN-γ and TNF-α on the production of NO and anti-microbial activity has been reported (Green, Crawford et al. 1990; Langermans, Van der Hulst et al. 1992). However, higher levels of IL-12 and iNOS (P<0.05) was observed with Plain-Lip compared to untreated infected animals but the overall parasite inhibition was much lower as compared to CHL. These interesting investigations showed that complete eradication of parasite would be possible at much lower dose of AmB (5-7 mg/kg) when loaded in CHL formulations compared to Plain-Lip (10mg/kg of AmB).

Such synergistic approach tested with lower dose of AmB as single dose therapy can be beneficial in decreasing AmB’s toxicity and could have shorter treatment duration without the need of hospitalization and above all it is cost effective which is a major concern for large sum of populations as most of the patients are residing in developing or poor countries. Further to this investigation, it may require cautious interpretation since it may not necessarily extrapolate the treatment of human visceral leishmaniasis with polymer decorated liposomes. There are, nevertheless, interesting pointers to the promising role of this delivery system in our results and its application is in offing.

**Conclusion:**

Impetus to this data it has been revealed that improved chemotherapy can be achieved by integrating AmB delivery in novel structured formulations along with intervention with immune system for the conditions of leishmaniasis. The conventional therapy has limited scope for patients with visceral leishmaniasis because parasites induce immuno-suppression. The immunological effects of chitosan coated on liposomes have stimulated renewed interest in the development of not only the AMB products, but also in other drug delivery products. Having thorough understanding of immune-pharmacology of chitosan decorated liposomes, new
formulations may have high tolerability, improved anti-leishmanial efficacy and reduced toxicity and better ability to circumvent drug resistance mechanisms.