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Biological evaluation of pyrazinamide liposomes for treatment of *Mycobacterium tuberculosis*

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Abstract

Pyrazinamide liposomes were prepared employing the phospholipid molar ratios; dipalmitoyl phosphatidyl choline (7):cholesterol (2) neutral and dipalmitoyl phosphatidyl choline (7):cholesterol (2):dicetyl phosphate (1) negatively charged. Swelling at 52 °C led to higher trapping efficiencies. An optimum sterilizing dose of 25 kGy was exhibited by gamma (γ)-irradiation. Neutral pyrazinamide liposomes (7:2), swollen for 24 h, were employed in biological evaluation for treatment of mice infected by *Mycobacterium tuberculosis*. Liposomal pyrazinamide could effect highly significant reduction in bacterial counts (colony forming units/g lung), 10, 20 and 30 days after the last treatment dose. Histopathological examination of mice lungs showed highest severity of infection in drug-free liposomes (control) group > pyrazinamide liposomes > free pyrazinamide 6 days/week. The results indicate high therapeutic efficacy of pyrazinamide liposomes, injected twice weekly, in treatment of *M. tuberculosis* in mice.
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1. Introduction

Mycobacterium tuberculosis infects approximately one-third of the world's population and causes about 3 million deaths each year (Raviglione et al., 1995; Raviglione, 2003; Aziz and Wright, 2005). Multidrug resistant strains are on the rise. The slow growth of *M. tuberculosis* is responsible for the chronic nature of infection and necessitates long-term drug treatment (Kaufmann, 2001). The intracellular mycobacteria pathogens can survive within macrophage phagosomes, thereby evading host defence strategies (Sturgill-Koszycki et al., 1994; Mellman et al., 1985; Ferrari et al., 1999).

Pyrazinamide (PZA) is an important component of the current 6 month short-course TB chemotherapy. This therapy which consists of isoniazid, rifampin, PZA and ethambutol is also called for directly observed treatment, short-course (DOTS), is recommended by the World Health Organization (WHO) for treatment of every TB patient (WHO Report, 1995). PZA plays a unique role in shortening the therapy from a period of 9 to 12 months down to 6 months, because PZA kills a population of semidormant tubercle bacilli residing in an acidic environment (occuring during active inflammation) which are not killed by other TB drugs (Mitchison, 1985; Heifets and Lindholm-Levy, 1992). Acidic pH enhances the intracellular accumulation of pyrazinoic acid (poa), the active derivative of PZA after conversion of PZA by pyrazinamidase. Strains of *M. tuberculosis* that are resistant to PZA are often defective in pyrazinamidase activity (Konno et al., 1967; Mc Clatchy et al., 1981; Trivedi and Desai, 1987). *M. tuberculosis* pyrazinamidase gene mutation is a major mechanism of PZA resistance in *M. tuberculosis* (Scorpio et al., 1997).

A primary goal in modern drug therapy is site-specific drug delivery or drug targeting (Kreuter, 1991). Colloidal drug carriers, as liposomes, are easily taken up by phagocytic cells of the reticuloendothelial system (RES) and find access to the lysosomes. Therefore, such carriers may be very useful antibiotics delivery systems for the treatment of infections of these cells. In the lungs, liposome-loaded monocytes subsequently migrate to the alveoli to become alveolar macrophages (Kreuter, 1991). Liposome encapsulation has

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been shown to improve the therapeutic efficacy of pharmaceutical drugs (Sharma and Sharma, 1997; Pinto-Alphandary et al., 2000; Tomii, 2002; Allen and Martin, 2004; Ramsay et al., 2005). Justo and Moraes (2003) incorporated isoniazid, pyrazinamide, rifampicin, ethionamide and streptomycin in extruded distearoylphosphatidyl choline/cholesterol liposomes designed for administration through inhalation. Promising results were reported by Kurunov et al. (1995) concerning the efficacy of liposomal formulations containing antibiotics when administered through inhalation against tuberculosis in BALB/c mice.

In this work we enhanced the targeting properties and monitored the intracellular degradation of PZA liposomes, by manipulation of the surface properties of the carriers as well as their wall or matrix composition.

Effective and targeted treatment of the latent TB infection, using liposome-encapsulated PZA, is imperative to reduce the risk of dissemination of TB in the population.

2. Materials and methods

2.1. Chemicals and drugs

PZA (El-Nasr Chem. Co., Egypt), dipalmitoylphosphatidylcholine (DPPC), synthetic approximately 99%, cholesterol (Chol.), min. 99% and dicetylphosphate (DCP) (Sigma Chem. Co., St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Biological materials

Male albino mice, *Mus musculus*, strain CD_1, were supplied by the animal house of the Biological Supply Centre, Theodore Bilharz Research Institute (TBRI), Egypt, originally obtained from Charles River Laboratory, USA. *M. tuberculosis* bacteria, strain $H_{37}Rv$, were supplied by VSVRI, Egypt. Biological indicator for sterilization, *Bacillus pumilus* E_{601}, was supplied by the Control Department of the Statens Serum Institute, Copenhagen, Denmark, in the form of test-pieces which are dried droplets of the spores of *B. pumilus* suspended in horse serum broth and enveloped in polyethylene bags.

2.3. Preparation of pyrazinamide liposomes

PZA liposomes of the molar ratio 7 (DPPC):2 (Chol.) neutral and 7 (DPPC):2 (Chol.):1 (DCP) negatively charged, were prepared by the Vortex Dispersion Method (Szoka and Papahadjopoulos, 1981). Free PZA was then separated from entrapped PZA by cooling centrifugation at 20,000 × *g*. Free PZA was then estimated by spectrophotometric measurement at 268.4 nm (British Pharmacopoea, 1998). Entrapped PZA was determined by difference from the amount added at the start of the experiment. Some PZA liposomes underwent swelling by incubation for 6 and 24 h at 52 °C, the transition temperature of the main phospholipid (DPPC). Thereafter, PZA liposomes were centrifuged then assayed for entrapped drug.

2.4. Sterilization of pyrazinamide liposomes

PZA liposomes were prepared under hygienic conditions, by washing all instruments with ethanol 99.9% prior to use. After centrifugation, liposomal pellets were stored in glass vials, then firmly sealed by rubber and metal closures. These were sterilized by gamma (γ)-irradiation. Two γ-irradiation doses were attempted, viz.; 15 and 25 kGy. Eight PZA liposomal vials were exposed to each irradiation dose, while eight vials were left unirradiated as control. The irradiation facility used was Cobalt-60 Gamma Chamber 4000-A, BHABHA Atomic Research Centre, India. The biological indicator used was *B. pumilus* E_{601}, in the form of test-pieces.

In the sterility testing, the medium employed was sterile Brewer's thioglycollate medium. One-half of the irradiated PZA liposomes were employed in the sterility testing. These as well as the biological indicator and *Escherichia coli* and *Clostridium sporogenes*, aerobic and anaerobic bacteria, used as positive control, each was inoculated on sterile thioglycollate slants. Negative control was made of uninoculated thioglycollate slants. They all were incubated at 32 °C for 14 days and examined regularly to monitor any growth.

The other half of irradiated PZA liposomes was utilized in carrying out the stability testing of the irradiated product. This was achieved by measuring drug retention in the liposomal vesicles, after the irradiation process. An aliquot sample was taken from each vial after mixing, acetate-buffered saline added, then separation and washing carried out. PZA content was assayed by spectrophotometric measurement at 268.4 nm (British Pharmacopoea, 1998).

2.5. Biological evaluation of pyrazinamide liposomes

The biological evaluation of the chemotherapeutic efficacy of PZA liposomes compared to free PZA in treating *M. tuberculosis* infected mice was accomplished by: (a) bacterial counts CFU/g lung of infected and treated mice and (b) histopathological examination of selected mice lungs.

The PZA liposomal formulation used in this study was 24 h swollen 7:2 neutral PZA liposomes for showing the highest trapping efficiency. Drug-free liposomes of the same molar ratio were used as control.

Experimental infection took place by using a suspension of *M. tuberculosis*, strain $H_{37}Rv$, maintained on Lowenstein–Jensen medium, in sterile saline. Mice were infected by injecting 0.1 ml of mycobacterial suspension intravenously. 0.1 ml of this suspension is equivalent to a load of about 10^6 bacilli.

0.25 ml of PZA liposomal suspension, pH 5.4, equivalent to one treatment dose (25 mg/kg) (Anitha et al., 1994) was injected subcutaneously (s.c.)/20 g mouse. Free PZA solution was prepared in acetate-buffered saline, pH 5.4. Each single treatment dose/20 g mouse (25 mg/kg), was equivalent to 0.1 ml. Drug-free liposomal suspension was used as the control group.

Two weeks after infection, mice were randomly divided into five groups. The first group, 10 mice, was s.c. injected with PZA liposomes (therapeutic dose, 25 mg/kg (Anitha et al., 1994)), twice weekly. A total of seven treatment doses were given. The

second group, 10 mice, received free PZA s.c. (25 mg/kg), twice weekly, in a total of 7 treatment doses. The third group, 10 mice, was given free PZA (25 mg/kg), 6 days/week, in a total of 18 s.c. treatment doses. The fourth group, 6 mice (control 1), was given drug-free liposomes, twice weekly, in a total of 7 s.c. doses. The fifth group, 5 mice (control 2), was given acetate-buffered saline, 6 days/week, in a total of 18 s.c. doses.

2.5.1. Bacterial counts

Three different dates: 10, 20 and 30 days after the last treatment dose, were considered for carrying out bacterial count in mice lungs. At each of these times, two to three mice were chosen randomly from each group, and sacrificed. The animals were dissected and the lungs of each group, in a pooled design, were separated, weighed and kept in tightly closed containers at 4 °C. The lungs were processed, in a pooled design (Agarwal et al., 1994), by homogenization by a method modified from that applied by Marks (1972). The lung homogenates were diluted with sterile saline and centrifuged at 2000 rpm for 20 min. The supernatant of the lung homogenates was cultured on a duplicate set of sterile Lowenstein–Jensen slants. The slants were incubated at 37 °C for 8 weeks and examined weekly for visible growth. The number of live bacilli was counted as number of colony forming units (CFU)/g lung, 8 weeks after culture.

2.5.2. Histopathological study

Histopathological examination of mice lungs was performed considering three mice groups: those receiving PZA liposomes twice weekly, those receiving free PZA 6 days weekly and the control group receiving acetate-buffered saline. Examination was conducted on the mice sacrificed 30 days after the last treatment dose. The organs were preserved in 10% formaline/normal saline solution. The organs were fixed with paraffin, stained with H.E. stain and examined for the severity of lesions. Histopathological commentary was reported blind to the regime the animals received.

3. Results

3.1. Preparation and swelling of pyrazinamide liposomes

Table 1 illustrates the results of PZA entrapment in liposomes as well as the effect of swelling in promoting the percentage drug encapsulation in liposomes.

Table 1
Effect of swelling time on liposomal pyrazinamide entrapment

Molar ratio	Surface charge	Swelling time (h)	Entrapment (%)[a] (mean ± S.D.)
7:2	Neutral	0	8.833 ± 2.4
		6	10.752 ± 2.2
		24	11.190 ± 0.7
7:2:1	Negative	0	7.883 ± 1.9
		24	9.625 ± 2.3

[a] Results are the mean of 13–19 batches.

3.2. Sterilization of pyrazinamide liposomes

This study aimed at investigating the optimum sterility conditions required to provide a sterile and stable parenteral PZA liposomal formulation which could be safely injected. The study was conducted utilizing 7:2 neutral and 7:2:1 negatively charged PZA liposomes, in duplicate, for each sterilizing dose of γ-irradiation.

Table 2 demonstrates the results of sterility testing of irradiated PZA liposomes. The data presented show no growth in the negative control group. Growth is observed in the two positive control groups. The tubes inoculated with the biological indicator show detected growth at the radiation doses 0 and 15 kGy while no growth appears at 25 kGy; the dose known to stop the viability of this microorganism. For PZA liposomes, no growth is observed at 25 kGy.

Stability testing for the irradiated PZA liposomes, was carried out 2 months post-irradiation. The results are presented in Table 3. It is clear that there is no significant difference in PZA retention in liposomal vesicles at the two radiation doses selected.

3.3. Biological evaluation of pyrazinamide liposomes

3.3.1. Bacterial counts of lung homogenates

Bacterial counts in homogenized lungs of mice sacrificed 10 days after the last treatment dose, calculated as log CFU/g lung, decline in the following order: the control group receiving acetate-buffered saline shows the highest count > the drug-free liposomes group > free PZA, twice weekly, group > the group receiving liposomal PZA > group receiving free PZA 6 days/week (Fig. 1). The statistical analysis done by Student's *t*-test indicates a very highly significant ($P < 0.005$) bacterial count reduction conferred by both PZA liposomes in 7 doses

Table 2
Results of sterility testing of pyrazinamide liposomes

Radiation dose (kGy)	Pyrazinamide liposomes on thioglycolate		Biological indicator *Bacillus pumilus E601* on thioglycolate	Control tests		
	7:2 (neutral)	7:2:1 (negative)		Positive control (*E. coli*)	Positive control (*C. sporogenes*)	Negative control
0	+	+	+			
15	+	+	+	+	+	−
25	−	−	−			

(+), growth; (−), no growth; *E. coli*, *Escherechia coli*, aerobic bacteria; *C. sporogenes*, *Clostridium sporogenes*, anaerobic bacteria.

Table 3
Results of stability testing of irradiated pyrazinamide liposomes

Radiation dose (kGy)	Percentage pyrazinamide retained (mean ± S.D.)			
	Molar ratio			
	7:2 (neutral)	*P* value (<)[a]	7:2:1 (negative)	*P* value (<)[a]
0	97.804 ± 0.2	–	97.185 ± 0.8	–
15	97.179 ± 0.5	NS	96.032 ± 0.2	NS
25	97.432 ± 0.4	NS	97.969 ± 0.2	NS

[a] Statistics were performed using unpaired Student's *t*-test.

(mean log CFU/g lung = 7.411 ± 0.1) and free PZA in 18 doses (7.045 ± 0.1) compared to untreated control (9.291 ± 0.1). On comparing the effects conferred by PZA liposomes with free PZA 6 days/week as well as free PZA twice weekly, non-significant difference ($P < 0.2$) is observed in both comparisons.

Bacterial counts 20 days after the last treatment dose reveal the highest value displayed by the group receiving acetate-buffered saline. The same sequence is observed for the groups as in the 10 days experiment. The statistical significance of the data of the groups receiving liposomal PZA twice weekly and those receiving free PZA 6 days/week, shows $P < 0.005$ for each of the two groups compared with the control group (mean log CFU/g lung = 8.053 ± 0.1, 7.664 ± 0.2 and 9.869 ± 0.1, respectively). Non-significant difference ($P < 0.02$) is observed between liposomal PZA and free PZA 6 days/week, while a significant difference in bacterial count ($P < 0.05$) is obtained when comparing liposomal PZA to free PZA twice weekly (8.053 ± 0.1 and 9.037 ± 0.2, respectively) (Fig. 1).

The data of 30 days bacterial counts, presented in Fig. 1 are consistent with those of 10 and 20 days, regarding comparison of treatment groups with the control. Non-significant difference ($P < 0.02$) is obtained on comparing liposomal PZA twice weekly with free PZA 6 days/week (mean log CFU/g lung = 8.705 ± 0.1 and 8.465 ± 0.1, respectively). Significant difference ($P < 0.05$) is observed between liposomal PZA and free PZA twice weekly (8.705 ± 0.1 and 9.179 ± 0.1, respectively). It is noteworthy that the group receiving free liposomes exhibits a significant change ($P < 0.05$) from the control group after 10 and 20 days while after 30 days non-significant change from the control group is observed.

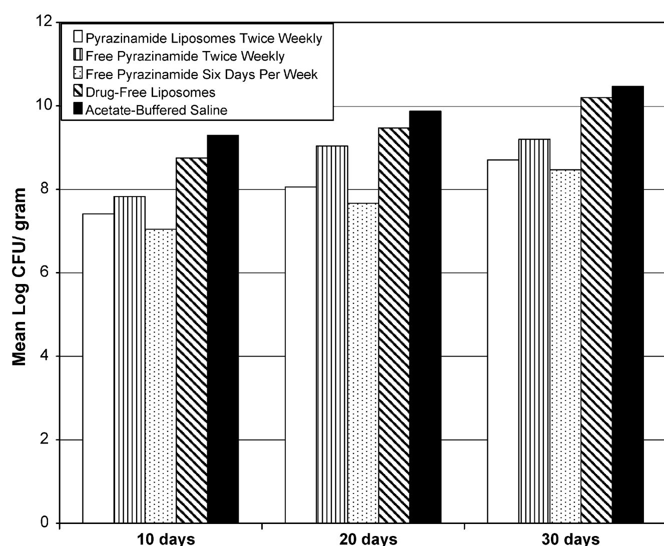

Fig. 1. Bacterial counts of mice lung homogenates.

3.3.2. Histopathological study

Mice lungs treated with PZA liposomes are qualitatively described. They show focal areas of granulomatous reactions (++), consisting of epitheloid cells, lymphocytes, macrophages and plasma cells, around the bronchioles, bronchi, pulmonary vasculature and within the lung alveoli (Fig. 2a).

The lungs of mice receiving free PZA 6 days/week, show focal areas of granulomatous reactions (+) surrounded with numerous large foamy macrophages, while most of the lung tissue is clear (Fig. 2b).

The lungs receiving drug-free liposomes (control) show the severest reaction with marked granulomatous lesions (+++), consisting of plasma cells, epitheloid cells and lymphocytes distributed interstitially and around the blood vessels, associated with foamy macrophages (Fig. 2c). These results agree with those of bacterial counts.

4. Discussion

4.1. Preparation and swelling of pyrazinamide liposomes

Preparation of liposomes by the Vortex Dispersion Method (Szoka and Papahadjopoulos, 1981), leads to incorporation of the drug in both the lipophilic anf hydrophilic phases of liposomes (El-Ridy, 1988) and also results in pronounced enhancement of drug entrapment in the liposomal vesicles (El-Ridy, 1988; Ghorab, 1991; Mostafa, 1995). The Vortex Dispersion Method produces relatively large multilamellar vesicles (MLV). These are a prerequisite for long-lasting effect, as they provide protection of the drug from the hydrodynamic stress and lipoprotein binding effect encountered in the circulation (Juliano, 1981; El-Ridy et al., 1997).

The swelling process was conducted in agreement with the respective conditions reported previously. Alving et al. (1993) stated that the amount of swelling solution is dictated by the amount of liposomal phospholipid, which is always 10 mM with respect to the swelling solution. The swelling procedure led to higher trapping efficiency (Table 1). This could be explained

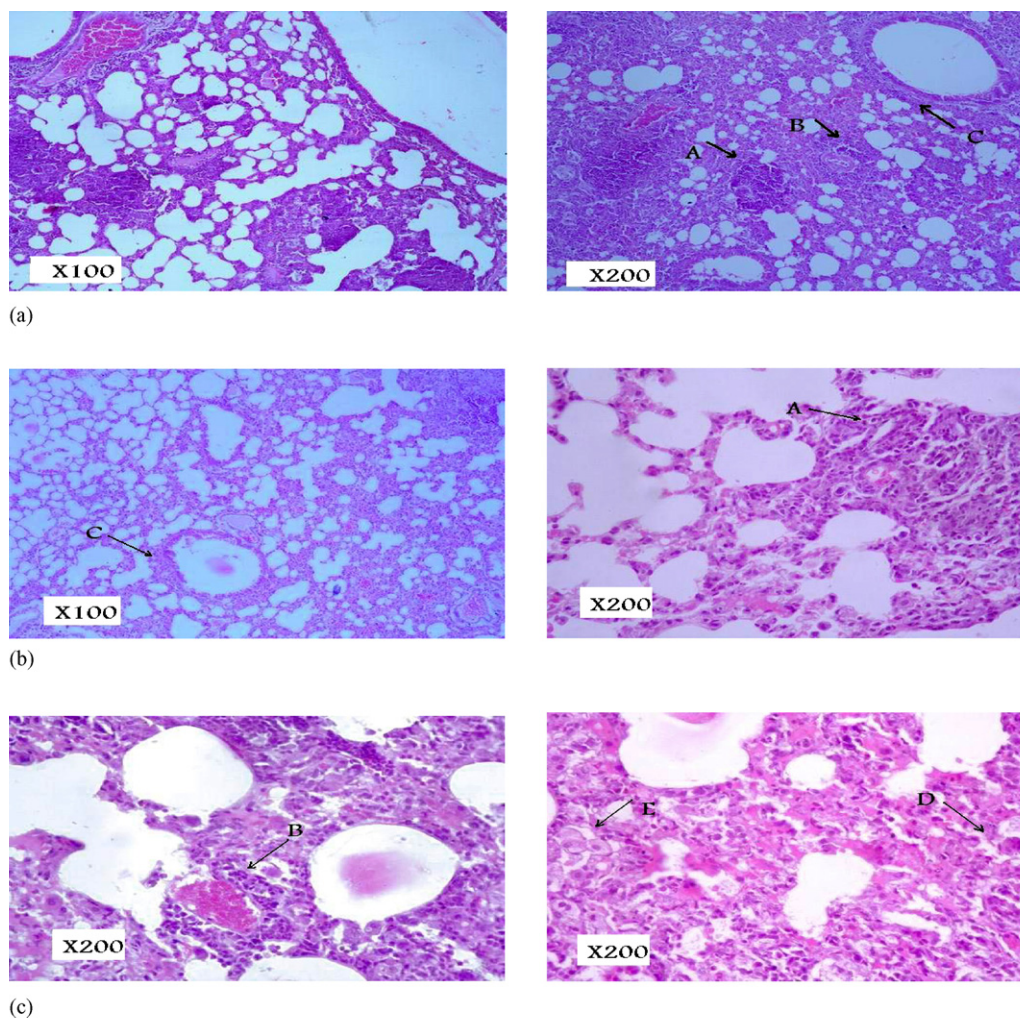

Fig. 2. Histopathological examination of lung tissue in mice. (a) Pyrazinamide liposomes group, twice weekly. Granulomatous reaction distributed interstitially (Arrow A), around pulmonary vusculature (Arrow B) and around bronchus (Arrow C). (b) Free pyrazinamide group, 6 days/week. Few grauloma interstitially (Arrow A) and around brochus (Arrow C). (c) Drug-free liposomes, twice weekly (control). Distributed granulomatous reaction around lung vusculature (Arrow B), few giant cells (Arrow D) and numerous large foamy macrophages (Arrow E).

by the fact that at the transition temperature of the main phospholipid (DPPC), 52 °C, the liposomes are in fluid state. In addition, the solution surrounding the liposomes contains a relatively higher concentration of the drug. Accordingly, feasible diffusion of the drug will occur into the interior of the liposomal compartments providing greater drug entrapment.

4.2. Sterilization of pyrazinamide liposomes

The negative control showed no growth denoting sterility of the culture medium. The positive control showed bacterial growth signifying suitability of the medium for growth of aerobic and anaerobic bacteria. Sterility of PZA liposomes was achieved at 25 kGy (Table 2). It has been reported sterility of liposomes will be achieved if a low degree of contamination, during preparation, is combined with an effective sterilization step after the preparation procedure (Van Winden et al., 1998). In addition, the statistically non-significant change in drug retention in the irradiated and non-irradiated PZA liposomes (Table 3), indicates that sterilization by γ-irradiation is safe and does not induce any adverse effect on the integrity of the liposomal vesicles. Kapanin et al. (1988) reported that the most effective way to protect liposome dispersions from irradiation damage, is freezing. This method is called cryoradiation. With cryoradiation, the capacity to inactivate viruses or microorganisms upon exposure to a dose of 25 kGy is maintained. By freezing, the damaging effect by the indirect irradiation action is suppressed. The damage by the indirect action is not caused by direct 'hits' on the bilayer structure by photons, but it is caused indirectly, by reactive agents outside the bilayer that are generated by γ-irradiation (Zuidam et al., 1995). Accordingly, 25 kGy is the optimum sterilizing dose for the frozen product yielding sterile and stable PZA liposomes, safe for parenteral administration.

4.3. Biological evaluation of pyrazinamide liposomes

The results of bacterial counts encountered (Fig. 1), signify very highly significant reduction in bacterial load in mice lungs of the group that received PZA liposomes (25 mg/kg),

twice weekly, in seven doses. The same is observed for the group receiving free PZA (25 mg/kg) 6 days/week, in a total of 18 doses, compared to the untreated control receiving acetate-buffered saline. The statistical analysis showed no significant difference between the effects of the two groups: PZA liposomes and free PZA 6 days/week. These results are observed at the three stated time intervals: 10, 20 and 30 days after the last treatment dose.

Application of liposomes to achieve site-specific drug delivery in infected tissues may be of great value. Antibiotic treatment failure may be related to the intracellular location of the microorganism (Bakker-Woudenberg and Van Etten, 1998). Another cause of unsuccessful antibiotic treatment is the failure of the host defence system to provide adequate support for antibiotic therapy (Bakker-Woudenberg and Lokerse, 1991). The application of liposomes can achieve high intracellular cocentrations of antibiotic in the infected cells. Liposomes also act as immune adjuvants (Allison and Gregoriadis, 1974). They can stimulate the immune response of the body independent of the carrier function (Daeman et al., 1998). In addition, liposomes as carriers of antibiotics can provide site-avoidance drug delivery, thus reducing toxic side-effects and effecting efficient clinical efficacy (Bakker-Woudenberg and Van Etten, 1998). Reduced toxicity allows administration of a relatively high antibiotic dose, thereby creating therapeutic advantages. Avid internalization of liposomes by microphages allowing high drug concentrations at the site of infection might also be beneficial in overcoming drug resistance (Emmen and Storm, 1987). Liposomes were tested in various in vivo models of intracellular bacterial infections, as reviewed by Bakker-Woudenberg and Lokerse (1991). The results showed that, compared to the free agents, liposome-entrapped agents were superior in reducing the number of intracellularly located bacteria. Pandey et al. (2004) reported the superior pharmacokinetic and chemotherapeutic efficacy of lung specific stealth liosomes, encapsulating rifampicin and isoniazid in guinea pigs, compared to free drugs. The study indicated that 7 i.v. doses of liposomal antitubercular drugs were likely to be more and/or equally acceptable than 46 conventional oral or i.v. doses. Pandey et al. (2005) could incorporate antitubercular drugs (rifampicin, isoniazid and pyrazinamide) in solid lipid nanoparticles SLNs. The chemotherapeutic potential of antitubercular drugs loaded in SLNs was found superior to free orally administered drugs in *M. tuberculosis* H37 Rv infected mice. Forty-six conventional oral doses of antitubercular drugs could be reduced to five doses of SLNs loaded with antitubercular drugs (Pandey et al., 2005). Liu et al. (1991) synthesized pyrazinamide derivatives, one of which was *N*-palmitoyl pyrazinamide. This was incorporated in liposomes consisting of soy phosphatidyl choline and dipalmitoyl phosphatidyl glycerol (7:3, molar ratio). In vitro, *Mycobacterium avium*-intracellulare MAI was susceptible to the formulation at concentrations of 12.5 and 25 μg/ml although MAI was not susceptible to the parent drug. Several studies support the fact that reducing the dosing frequency, through liposomal drug encapsulation, is more and/or equally acceptable by patients than conventional daily doses (Pandey et al., 2004). Therefore, the liposomal drug carrier system offers promising potential as an alternative chemotherapy for treatment of tuberculosis, by improving patient compliance and lowering cost, dosage and toxic effects of therapy with higher safety (Labana et al., 2002).

The blind histopathological examination (Fig. 2) of mice lungs receiving treatment and control medications are consistent with the results of bacterial counts.

Based on the results obtained, statistical analysis conducted and literature reviewed, it is possible to conclude that liposomal PZA in two-fifths the dose of daily free PZA, is superior over free PZA in management of TB infection in experimntal animals. This would pave the way for future studies on formulating PZA in liposomal or other suitable delivery systems to obtain effective targeting of the drug to subcellular organelles where the mycobacterial bacilli reside in infected macrophages, viz.; phagosomes, with anticipated eradication of the disease.

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