Prophylactic Role of Liposomized Chloroquine Against Murine Cryptococcosis Less Susceptible to Fluconazole

Masood A. Khan,[1,2,3] Rukhsana Jabeen,[1] and Owais Mohammad[1]

Received June 27, 2004; accepted August 26, 2004

Purpose. The prophylactic role of liposomized chloroquine (lip-CQ) has been assessed against less susceptible *Cryptococcus neoformans* infection in murine model.

Methods. In the current study, we investigated the antifungal activity of lip-CQ against *C. neoformans* in macrophages cell line (J 774) and murine model. Mice were pretreated with free as well as liposomized formulations of CQ at various doses. The anticryptococcal activity of fluconazole was compared in mice with or without CQ pretreatment. The efficacy of CQ prophylaxis was assessed by survival as well as colony forming units (cfu) in brain and lungs of treated mice.

Results. Fluconazole alone was not found significantly effective against *C. neoformans* in both *in vitro* and *in vivo* studies. However, the antifungal activity of fluconazole increases in chloroquine-pretreated mice. Lip-CQ was found to be more effective in comparison to the same dose of free chloroquine in reducing fungal burden from macrophages *in vitro* and lungs and brain of *C. neoformans* infected mice.

Conclusions. The enhanced prophylactic activity of lip-CQ seems due to rapid uptake of drug-containing liposomes by macrophages. The liposome-mediated accumulation of CQ in macrophages makes the environment unfavorable (alkaline) for the intracellular multiplication of *C. neoformans*. Moreover, the increased incidence of multi-drug resistance and diversity of pathogenic microorganisms inhibited or killed by CQ makes it the drug of choice for prophylactic therapy.

KEY WORDS: chloroquine; cryptococcosis; fluconazole; liposomes.

INTRODUCTION

Cryptococcus neoformans is one of the major causes of morbidity and mortality in persons with impaired cell mediated immunity, especially those with AIDS or undergoing chemotherapy for organ transplantation and neoplastic diseases (1,2). The key point behind the success of *C. neoformans* as a parasite in macrophages is its ability to survive within the acidic environment of phagolysosomes (3).

The treatment of fungal diseases such as cryptococcosis is based on the use of polyene and azole groups of antifungal chemotherapeutic agents. Drugs from polyene class of antifungal agents, especially amphotericin B, have long been considered the most effective of the systematically administered antifungal agents. Unfortunately, infusion related toxicities and the frequent association of renal dysfunction with the use of Amp B have limited its utility for longer duration (4). The azole antifungal agents (e.g., fluconazole and itraconazole), because of their relative safety and ease of delivery, have subsequently become a critical component of the antifungal armamentarium. Resistance in pathogenic fungi against the less toxic azoles has been reported with rapid rate in recent years (5). To cope with current rate of antifungal resistance, it becomes mandatory to search for safe and broad spectrum therapeutic agents for prophylaxis and treatment of fungal infections.

Various pathogens adopt different mechanisms to avoid the low pH of phagolysosomes; for example, *Mycobacterium tuberculosis* and *Mycobacterium avium* modulate the internal environment of phagosomes by selective blocking of vacuolar proton-ATPase (6). *Toxoplasma gondii* and *Legionella pneumophila* avoid acidification by inhibiting the fusion of residing vesicles with lysosomes (7,8).

Chloroquine (CQ) has widely been available drug for prophylaxis and therapy of malaria (9). The lipophilic nature of CQ makes it easy to diffuse freely into membrane in the unprotonated form, but on reaching into intracellular acidic environment becomes protonated and thus raises the intravacuolar pH (9). It inhibits the proliferation of *L. pneumophilia, H. capsulatum* and *F. tularensis* by limiting availability of crucial nutrients required for the growth of these microbes (10–12). It has been demonstrated that CQ inhibits the growth of *C. neoformans* in macrophages by a mechanism not dependent on iron deprivation but by alkalinizing the pH of mononuclear phagocytes (13).

Liposomes have been proved to be very useful in treatment of macrophage-based intracellular infections (14). Previously, we have shown that antigens entrapped in liposomes are avidly taken up by macrophages (14). Keeping in view this property of liposomes, we used them for the targeting of CQ to macrophages, which may prove more effective in prophylaxis and therapy of *C. neoformans* infection. They not only reduce the toxicity of free drug but also capable of targeting the substantial part of drug to macrophages. The current study clearly shows that prophylactic use of liposomized chloroquine (lip-CQ) is more effective than that of free chloroquine alone or in mice injected with fluconazole for treatment of *C. neoformans* infection.

MATERIALS AND METHODS

All the reagents used in the study were of the highest purity available. Cholesterol was bought from Centron Research Laboratory (Bombay, India) and used after crystallization with methanol. Fluconazole (Flu) was procured from Roerig-Pfizer (New York, NY, USA). Chloroquine, morpholinepropanesulphonic acid (MOPS) and RPMI 1640 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Egg phosphatidylcholine (egg PC) was isolated and purified according to the published procedure (15).

Liposomes

Chloroquine-containing liposomes were prepared from egg PC (49 μmols) and cholesterol (Chol; 21 μmols) as described earlier (16). Briefly, all the ingredients including chloroquine (drug:lipid 1:20) were dissolved in a round-bottomed

[1] Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India.
[2] Department of Immunology and Medical Microbiology, IUPUI, Indianpolis, Indiana, USA.
[3] To whom correspondence should be addressed. (e-mail: alammasood1@rediffmail.com)

flask in a minimum volume of chloroform/methanol (1:1, v/v). The solvents were carefully evaporated under reduced pressure to form thin lipid film on the wall of the flask. The final traces of the solvents were removed by subjecting the flask to vacuum for overnight at 4°C. Subsequently, the dried lipid film (consisting of egg PC/Chol, chloroquine) was hydrated by vortexing with 2.0 ml of 150 mM sterile saline. The suspension of lipid-drug formulation was sonicated (1 h, 4°C) in a bath type sonicator under N_2 atmosphere. The sonicated preparation was centrifuged at 10,000 × *g* for 30 min at 4°C to remove traces of undispersed lipid. The upper 2/3 volume was taken out and dialyzed against normal saline for 24 h at 4°C in the dark. Free drug was separated from liposomized drug by passing the preparation through Sephadex G-50 column. The liposomized formulation of chloroquine was used in the study.

Estimation of Liposome Intercalated Chloroquine

The intercalation efficiency of chloroquine in the liposomes was estimated spectrophotometrically. A standard curve of the chloroquine was plotted at 342 nm as described earlier (16). The amount of drug associated with the liposome was determined by dissolving the formulation in 1% triton X-100 and determining the absorbance at 342 nm against a corresponding amount of lipid in final solution of 1% triton as a blank. The amount of chloroquine entrapped in liposomes was calculated using the standard curve of the drug. The amount of CQ associated with liposomes was found to be 135 ± 10 μg of CQ/ μmol of lipid P.

Animals

Female Swiss mice weighing 20 ± 4 g were used in the study. The animals were given a standard pellet diet (Hindustan Lever Ltd.) and water *ad libitum*. Mice were checked daily for their mortality and moribundity. The techniques used for bleeding, injection as well as sacrifice of animals were approved by the animal ethics committee [Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA], Govt. of India).

Test Strain

The strain of *C. neoformans* (JMCR 102) was obtained from leukemia patient of Jawaharlal Nehru Medical College (JNMC), Aligarh Muslim University, Aligarh. SD Agar/broth was used for growing patient isolates of *C. neoformans*. The identity of clinical isolate of *C. neoformans* was confirmed in the mycology section of the Department of Microbiology, JNMC, Aligarh, India.

Macrophages Cell Lines

The murine macrophage cell line J 774 was maintained in minimal essential medium as described earlier (17). J 774 macrophages were mechanically collected with a cell lifter (Costar Italia, Milan, Italy).

Antifungal Susceptibility Testing

The minimum inhibitory concentration (MIC) of fluconazole (Roerig-Pfizer) was determined by broth macrodilution method according to the guidelines of National Committee for Clinical Laboratory Standards (NCCLS) document M-27 A (18). Stock solution of fluconazole was prepared in water at 10 times the highest concentration tested. Stock solutions were diluted with RPMI 1640 medium (Sigma Chemical Co.) supplemented with L-glutamine, without bicarbonate, buffered to a pH 7.4 with 0.165 M MOPS (morpholinepropanesulfonic acid; Sigma). The final concentration ranges were from 0.125 to 128 μg/ml for fluconazole. Antifungal susceptibility testing was performed in 96-well round-bottom microtitration plates. Yeast inocula were prepared in sterile water and were diluted in RPMI 1640 medium to give a final inoculum concentration of approximately 5×10^2 to 2.5×10^3 cells/ml. The plates were incubated at 37°C, and were read after 72 h. MIC was defined as the lowest concentration at which there was 80% inhibition of growth of *C. neoformans* compared with that in a drug free control. The MIC of fluconazole for *C. neoformans* (JMCR 102) was found to be 16 μg/ml.

Preparation of *C. neoformans* Cells for Infection

Stock culture of an encapsulated strain of *C. neoformans* was maintained on Sabouraud Dextrose Agar. Yeast cells were harvested from agar plates into YPD (1% yeast extract, 2% peptone, 5% dextrose) medium at 37°C for 36 h. The cells were washed with normal saline in low speed centrifugation (2000 rpm) and diluted to the appropriate concentration in saline prior to be used in both *in vitro* as well as *in vivo* studies.

Antifungal Activity of Macrophages in Presence of Chloroquine

Antifungal activity of macrophages in presence of chloroquine was determined as described earlier (13). J774 macrophages were seeded in triplicate in 96-well Costar plates with 2×10^5 cells/well in complete medium supplemented with 5% human serum and incubated at 37°C in 5% CO_2 for 24 h. The macrophages were then treated with free chloroquine and liposomized-CQ at indicated concentrations for 1 h. *C. neoformans* (1×10^5 cells/well) was added to the wells containing macrophages and drug. After two hours of incubation nonphagocytized yeast cells were washed out and fluconazole (8 μg/ml or 0.5 MIC) was added to the wells. After 24 h and 48 h of incubation, macrophages were lysed with 0.1% Tween-20 and phagocytized yeasts were recovered and centrifuged. The number of colony-forming units (cfu) of *C. neoformans* was determined by dilution and spread plates on Sabouraud dextrose agar after incubation at 37°C for 18 h in triplicates.

Treatment with Fluconazole

Each animal was infected with 7×10^5 viable cells of *C. neoformans* in 0.2 ml of saline through intravenous route. Various doses of fluconazole (5, 10, 20, and 50 mg/kg of body weight) were used to treat *C. neoformans* infection in mice. The drug was administered on days 1, 3, and 5 after infection via intravenous route. The mice (n = 10 in each group) were divided into following groups:

Saline
Flu (5 mg/kg)
Flu (10 mg/kg)
Flu (20 mg/kg)
Flu (50 mg/kg)

Prophylactic Effect of Chloroquine Against *C. neoformans* Infection in Mice

The prophylactic role of chloroquine was investigated against systemic infection of *C. neoformans* in mice. The animals were treated with free as well as lip-CQ at various doses (5, 10, 15, and 20 mg/kg) for 3 consecutive days before infection through intravenous route. Each animal was infected with 1×10^5 cells of *C. neoformans*. Mice (n = 10 in each group) were divided into following groups:

Saline
Free CQ (5 mg/kg)
Free-CQ (10 mg/kg)
Free-CQ (15 mg/kg)
Free-CQ (20 mg/kg)
Lip-CQ (5 mg/kg)
Lip-CQ (10 mg/kg)
Lip-CQ (15 mg/kg)
Lip-CQ (20 mg/kg)

Dose dependent efficacy of CQ-pretreatment in various groups was assessed by culturing tissue homogenates of brain and lungs of *C. neoformans* infected mice for fungal burden.

Efficacy of Fluconazole Against *C. neoformans* Infection in CQ-Pretreated Mice

For prophylactic study, the animals were pretreated with lip-CQ (10 mg/kg) for 3 consecutive days by intravenous route. After CQ pretreatment mice were challenged with *C. neoformans* infection (7×10^5 cells/mouse). The treatment with fluconazole was started after 24 h of *C. neoformans* infection on days 1, 3, and 5. The animals were divided into following groups and each group contained 10 mice.

Saline
Empty-lip
Lip-CQ (+) Flu (–)
Lip-CQ (+) Flu (5 mg/kg)
Lip-CQ (+) Flu (10 mg/kg)
Lip-CQ (+) Flu (20 mg/kg)
Lip-CQ (+) Flu (50 mg/kg)
Lip-CQ (–) Flu (50 mg/kg)

Assessment of Anticryptococcal Activity

The role of chloroquine alone or with fluconazole in protection against *C. neoformans* infection was assessed by survival data and fungal burden in the brain and lungs of mice. The animals were observed till day 30 postinfection. For cfu determination, three mice from each group were sacrificed and their lungs and brains were analyzed as described earlier (19). Briefly, weighed portions of the given organ were homogenized in 5 ml of sterile normal saline, and an aliquot of the suspension was plated on SD agar plates containing chloramphenicol and gentamicin after appropriate dilution. The plates were incubated for 48–72 h at 37°C. The numbers of fungal colonies (cfu) were counted and the fungal load in various organs was calculated by multiplying with the dilution factor.

Statistics

Statistical significance (p value) of survival data was ascertained by performing Mann Whitney-Wilcoxon test and fungal burden (CFU) in organs was analyzed by paired *t* test.

RESULTS

Liposomized-Chloroquine Shows Increased Antifungal Activity Against *C. neoformans* in Murine Macrophages

Cryptococcus neoformans multiplies inside macrophages, as evidenced by increase in cfu after 48 h of culture (Table I). Treatment of J 774 cells with chloroquine (10 μM) induced a significant inhibition of *C. neoformans* growth as compared to the macrophages without drug treatment. Table I shows that macrophages treated with lip-CQ show remarkable reduction in *C. neoformans* proliferation as compared to macrophages treated with free CQ or without any drug treatment.

Fluconazole Shows Reduced Efficacy Against *C. neoformans* Infection in Mice

Various doses of fluconazole (5, 10, 20, and 50 mg/kg) were evaluated to treat *C. neoformans* infection in murine model. The animals treated with higher dose of fluconazole (50 mg/kg) were showing some response but it was not satisfactory both in terms of survival as well as fungal burden. Mice treated with a dose of 50 mg/kg and 20 mg/kg on days 1, 3, and 5 postinfection show only ~30% and ~10% survival till day 30 of observation. While mice from all other groups died

Table I. Effects of Liposomized-Chloroquine on the Intracellular Growth of *C. neoformans* in J 774 Macrophages

Treatment	cfu (10^2) ± SD on various time intervals		
	2 h	24 h	48 h
Baseline	115.4 ± 12		
Medium		154.8 ± 24	386 ± 50
Free chloroquine (10 μM)		78.2 ± 15.8	90.6 ± 12.4
Lip-chloroquine (10 μM)		36.2 ± 6.0	48 ± 8.0
Fluconazole (8 μg/ml)		96.8 ± 12.4	152 ± 28
Fluconazole + free CQ		32.4 ± 9.6	54 ± 12
Fluconazole + lip-CQ		16 ± 4.4	11.4 ± 3.6

Macrophages (J 774) were pretreated with free chloroquine as well as lip-CQ (10 μM) for one hour at 37°C. *C. neoformans* cells were phagocytized by macrophages at 1:2 ratios in complete MEM for 2 h. Cell monolayers were washed to remove nonphagocytized yeast cells followed by incubation with fluconazole. The number of cfu recovered from the lysis of J 774 cells (without drug treatment) after 2 h of phagocytosis was considered the initial inoculum (baseline). At the end of 24 and 48 h of incubation, cells were lysed and intracellular yeasts were plated on Saboraud Dextrose Agar plates for 48 h at 37°C. The experiments were repeated three times.
Groups: Medium vs. free chloroquine (24 h) ($p = 0.024$); medium vs. lip-chloroquine (24 h) ($p = 0.010$); free chloroquine vs. lip-chloroquine (24 h) ($p = 0.014$); fluconazole vs. free CQ + fluconazole ($p = 0.0021$); fluconazole vs. lip-CQ + fluconazole ($p = 0.0003$); free CQ + fluconazole vs. lip-CQ + fluconazole ($p = 0.0213$).

before day 30 after infection (Fig. 1). Results show that treatment with fluconazole (50 mg/kg) was superior to that control and lower doses of fluconazole ($p < 0.05$).

The efficacy of fluconazole was also evaluated on its effect on the establishment of the infection by culturing tissue homogenates of lungs and brain. cfu data shows that fluconazole in high dose (50 mg/kg) was effective in reducing the fungal load in comparison to other lower doses (5, 10, and 20 mg/kg). However, fluconazole even at higher dose (50 mg/kg) was not completely eliminating the infection from the tissue of infected animals (Fig. 2).

Liposomal Chloroquine Shows Prophylactic Effect Against Murine Cryptococcosis

Both free as well as lip-CQ was intravenously administered to mice at various doses (5–20 mg/kg) for analyzing its prophylactic role against *C. neoformans* infection in mice. Among the various doses, lip-CQ at the dose of 10 or 20 mg/kg was more effective in controlling the severity of *C. neoformans* infection in mice and was also suitable for *in vivo* study in terms of both efficacy as well as toxicity. Lower dose of lip-CQ (5 mg/kg) did not show very significant outcome of prophylaxis. Pretreatment with lip-CQ at the dose of 10 or 20 mg/kg showed remarkable reduction in fungal load of brain and lungs homogenates of pretreated animals. Free-CQ at the dose of 10 or 20 mg/kg also showed some prophylactic effect but it was inferior to that of lip-CQ at equal dose (Fig. 3).

Antifungal Activity of Fluconazole Increases in Lip-CQ Pretreated Mice

The prophylactic effect of lip-CQ was assessed in mice infected with *C. neoformans* and consequently treated with various doses of fluconazole (5, 10, 20, and 50 mg/kg). The efficacy of fluconazole remarkably increases in CQ-pretreated mice in comparison to mice without CQ treatment.

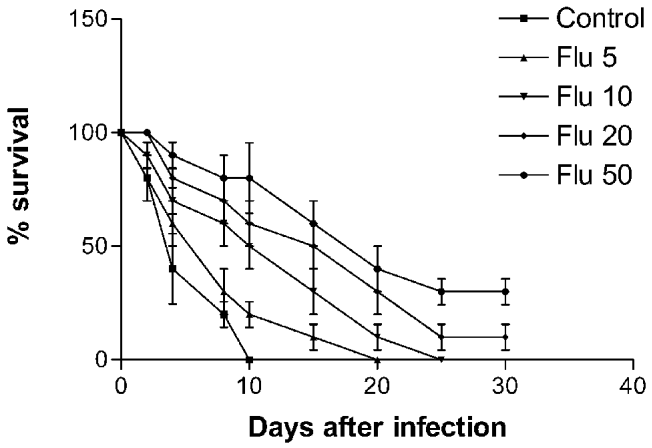

Fig. 1. Effect of fluconazole on survival of mice infected with less susceptible strain of *C. neoformans*. Swiss mice (no. of animals in each group = 10) were challenged with less susceptible strain of *C. neoformans* (7×10^5 spores/animal by i.v. route) as described in "Materials and Methods." Mice were treated with different doses of fluconazole (5, 10, 20, 50 mg/kg) (i.v.) on days 1, 3, and 5 postinfection. Survival was monitored over 30 days. The groups are (■) saline, (▲) fluconozole (5 mg/kg), (▼) fluconazole (10 mg/kg), (♦) fluconazole (20 mg/kg), (●) fluconazole (50mg/kg). The data is mean of three independent experiments ± SD.

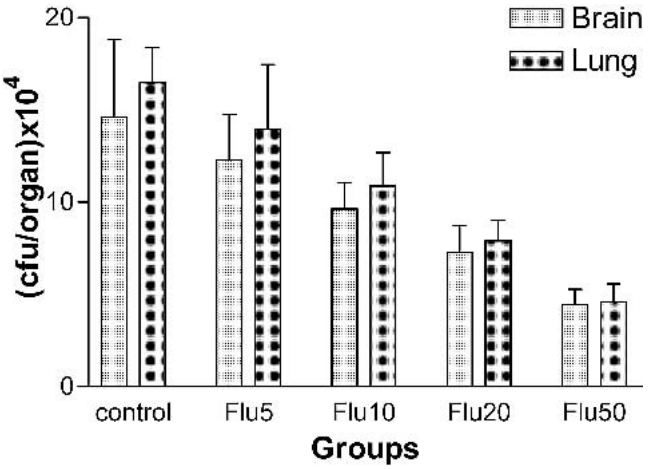

Fig. 2. Dose-dependent effect of fluconazole on fungal burden in brain and lungs of *C. neoformans* infected mice. Mice infected with *C. neoformans* (7×10^5 spores/animal) were treated with various doses of fluconazole (5, 10, 20, and 50 mg/kg) for 3 consecutive days. On day 4 postinfection, three mice from each group were sacrificed and their brains and lungs were taken out aseptically and processed as described in "Materials and Methods." The fungal load among various groups was compared by paired *t* test: saline vs. fluconazole (5 mg/kg) ($p = 0.440$), saline vs fluconazole (50 mg/kg) ($p = 0.014$), fluconazole 5 vs. fluconazole 50 ($p = 0.0057$), fluconazole 10 vs. fluconazole 50 ($p = 0.0049$), fluconazole 20 vs. fluconazole 50 ($p = 0.036$).

Lip-CQ pretreated mice followed by fluconazole treatment (50 mg/kg) show increased survival (~70%) followed by those treated with fluconazole at the dose of 20 mg/kg (~40%). Fluconazole at lower doses (5 or 10 mg/kg) also shows enhanced anticryptococcal activity in CQ-pretreated mice but it is not very significant (Fig. 4). Fluconazole treatment at the dose of 50 mg/kg in lip-CQ pretreated mice shows superior efficacy to all other treatment combinations ($p < 0.05$).

CFU Burden in Lungs and Brain

The prophylactic role of lip-CQ in the elimination of *C. neoformans* infection from tissues was assessed by quantifi-

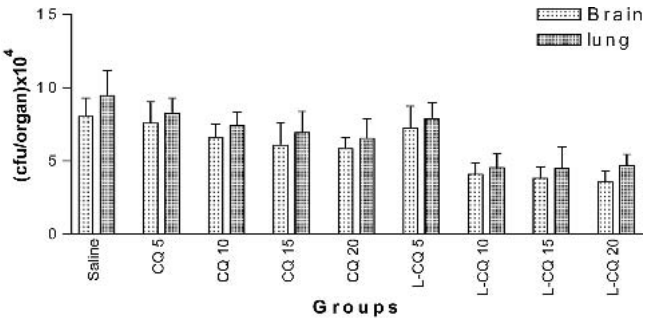

Fig. 3. Prophylactic effect of liposomized-CQ on establishment of *C. neoformans* infection in mice. The groups of mice were treated with various doses of free and liposomized-CQ for 3 consecutive days via intravenous route. The animals were infected with *C. neoformans* (1×10^5 cells/mouse) intravenously. On day 2 after *C. neoformans* infection, three mice from each group were sacrificed and their brains and lungs were taken out and processed for cfu determination as described in "Materials and Methods." Saline vs. free-CQ (10 mg/kg) ($p = 0.192$), saline vs. lip-CQ (10 mg/kg) ($p = 0.0190$), free-CQ vs. lip-CQ (10 mg/kg) ($p = 0.0207$), free-CQ vs. lip-CQ (20 mg/kg) ($p = 0.0246$).

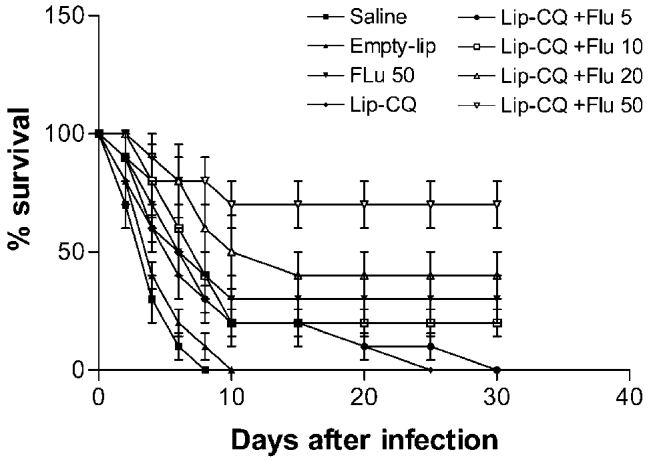

Fig. 4. Prophylactic use of lip-CQ increases the efficacy of fluconazole in mice infected with less susceptible strain of *C. neoformans*. Mice were pretreated with liposomized-CQ intravenously (10 mg/kg) before infecting them with *C. neoformans* (7×10^5 spores/animal). After 24 h of infection, the animals were treated with doses of fluconazole (5, 10, 20, 50 mg/kg) on days 1, 3 and 5. Survival was monitored over 30 days after infection. The groups are (■) saline, (▲) empty-lip, (♦) lip-CQ (+) flu (–), (●) lip-CQ (+) flu 5, (□) lip-CQ (+) flu 10, (△) lip-CQ (+) flu 20, (∇) lip-CQ (+) flu 50, (▼) lip-CQ (–) flu 50. The data is mean of three independent experiments ± SD.

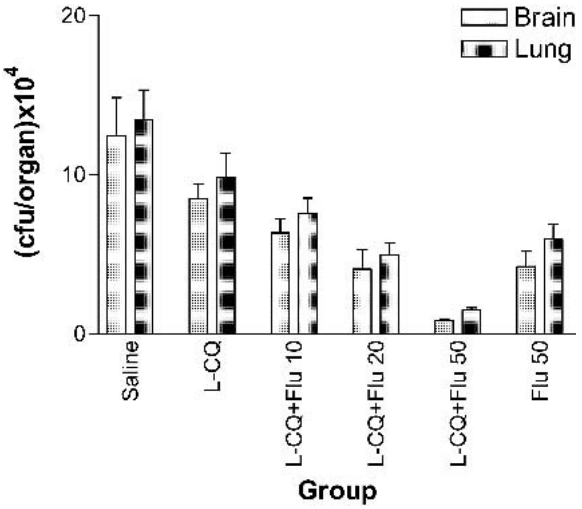

Fig. 5. Prophylactic role of lip-CQ in combination with fluconazole treatment on the establishment of *C. neoformans* infection in mice. Mice were pretreated with lip-CQ (10 mg/kg) for 3 consecutive days via i.v. route. Each animal of all groups was infected with *C. neoformans* (7×10^5 cells) through lateral tail vein. After 24 h of infection, the animals were treated with various doses of fluconazole (5, 10, 20, and 50 mg/kg) intravenously. On day 4 postinfection, three animals from each group were sacrificed for cryptococcal load (cfu) in brain and lung homogenates. Saline vs. lip-CQ (+) flu (–) ($p = 0.058$), saline vs. flu (50 mg/kg) ($p = 0.0053$), lip-CQ (+) flu 10 vs. lip-CQ (+) flu 50 ($p = 0.0001$), lip-CQ (+) flu 20 vs. lip-CQ (+) flu 50 ($p = 0.0099$), lip-CQ (–) flu 50 vs. lip-CQ (+) flu 50 ($p = 0.0039$).

cation of fungal burden in vital organs (i.e., lungs and brain). Fluconazole shows dose dependent response in CQ-pretreated mice also. Remarkable reduction in fungal load was observed in the organs of lip-CQ pretreated animals followed by fluconazole (50 mg/kg) treatment in comparison to those treated with lower doses of fluconazole (Fig. 5).

DISCUSSION

In recent years, the incidence and prevalence of opportunistic fungal infections have increased dramatically as a result of alterations in immune status associated with the AIDS epidemic and the extensive use of novel immunosuppressive agents for cancer chemotherapy and organ transplantation (1).

The present strain of *C. neoformans* was not responding well to fluconazole alone both *in vitro* and *in vivo* studies. Fluconazole was found to be ineffective in lower doses (5 and 10 mg/kg) and moderately effective in higher doses (20 and 50 mg/kg) against *C. neoformans* infection in murine model. There was ~30% and ~10% survival rates in mice treated with fluconazole at the doses of 50 and 20 mg/kg, respectively, whereas the animals treated with lower doses (5 and 10 mg/kg) did not survive till day 30 of observation. This is in agreement of our *in vitro* results of MIC of fluconazole (16 μg/ml), which clearly shows the less susceptibility of *C. neoformans* to fluconazole.

Chloroquine has previously been shown to exert *in vitro* and *in vivo* inhibitory effects on growth of *H. capsulatum* (11) and *C. neoformans* (13). In *H. capsulatum*, CQ impedes the pH-dependent acquisition of iron either from the transferrin-transferrin receptor complex in the endosome or from the ferritin in the lysosomes (11). On the other hand, the inhibition of *C. neoformans* growth by CQ is independent of iron acquisition but is related to other phenomenon of the pH increase in the subcellular acidic compartments (13). In the current study, we demonstrate the prophylactic role of lip-CQ against *C. neoformans* both *in vitro* as well as *in vivo*. Liposomized-CQ was found to impart more protection at lower doses as compared to the previous report of free chloroquine in higher doses (20). This is supported by the fact that lip-CQ was taken up by macrophages more efficiently, which results in accumulation of substantial amount of CQ in macrophages in comparison to free formulation of CQ. It has been shown that *C. neoformans* shows increased proliferation at pH ~5.0 (20). Liposome-mediated delivery of CQ increases the pH of acidic compartment of macrophages that creates long-lasting hostile environment for growth of *C. neoformans*. Thus, macrophages respond with enhanced antifungal activity upon treatment with same dose of CQ in liposomized form. The liposome-mediated CQ targeting to macrophages also reduces the chances of drug induced toxic manifestations to other cells even in higher concentration.

The increased therapeutic potential of fluconazole in mice pretreated with lip-CQ can be attributed to the CQ-induced unfavorable physiologic conditions for the proliferation of *C. neoformans* inside macrophages. Chloroquine alkalizes the acidic constitution of phagosomes, which results into inhibition of various biochemical reactions crucial for the survival of intracellular pathogens. Macrophages preferably engulf the liposomes and thus most of the drug associated with liposomes accumulates into macrophages. Thus, it can be speculated that liposome mediated increased level of CQ inside macrophages may directly affect the growth of *C. neoformans*. It is further supported by the fact that CQ in higher concentrations directly kills *C. neoformans* (21). Furthermore, the mobilization of iron from transferrin and ferritin,

two major sources of iron in mononuclear phagocytes, is dependent on acidic environment. Iron dissociates from transferrin at pH <6.0 and becomes available to the pathogen (11). Iron availability from ferritin occurs after proteolysis in lysosomes at lower pH. Chloroquine inhibits both these processes by creating the alkaline medium inside macrophages and thus restricts the supply of crucial nutrients to the microbes. This ultimately results into poor growth of *C. neoformans* at higher pH.

CQ-pretreatment of mice prior to *C. neoformans* infection increases the efficacy of fluconazole in treatment of murine cryptococosis not responding to fluconazole in CQ untreated mice. Again, it can be ascribed to CQ mediated alkaline environment, which does not allow the pathogen to proliferate within the cells. At this stage, the administration of fluconazole proves to be more effective in controlling the weakly proliferating fungal cells and eliminates *C. neoformans* infection, which was not responding to fluconazole in absence of CQ treatment.

Opportunistic fungi and their products have been shown to stimulate HIV replication in latently infected macrophages and lymphocytes (22). *C. neoformans* exacerbates the AIDS by activating HIV replication via secretion of proinflammatory cytokines such as tumor necrosis factor (TNF)-α (20). CQ has been found to inhibit the secretion of proinflammatory cytokines such as TNF-α (23). Besides its direct and indirect anticryptococcal effect, CQ treatment might have extra advantage in blocking secondary infection–induced HIV replication. Thus, strategies for prevention and treatment of fungal infections could result in improved outcome by decreasing morbidity from both the fungal as well as HIV infection.

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