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Antidermatophytic activity of miconazole nanoformulation against *Trichophyton rubrum*Alka Gupta^{1*}, Hemanta Kumar Kar²¹University School of Medicine and Para Medical Health Sciences, Guru Gobind Singh Indraprastha University, Delhi, India²NDMC Medical College, Formerly at Post Graduate Institute of Medical Education and Research, Dr. Ram Manohar Lohia Hospital, Delhi, India

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ABSTRACT

Objective: To formulate and evaluate the antifungal activity of miconazole nitrate proniosomes against *Trichophyton rubrum*.**Methods:** Miconazole loaded proniosomal vesicles were prepared by coacervation phase separation method using surfactants, cholesterol and phosphatidylcholine as excipients. The *in vitro* antifungal activity of prepared vesicles was performed using agar disc diffusion technique.**Results:** The miconazole nitrate lipid vesicle preparation F5A and F5B showed good antifungal activity with high zones of inhibition *i.e.* (12.96 ± 0.63) mm and (14.06 ± 0.58) mm than nonvesicular plain drug gel *i.e.* (10.48 ± 0.79) mm after 3 days at 250 µg/mL concentration (For all comparisons $P < 0.05$ was considered as significant).**Conclusions:** Proniosomal preparation resulted in better permeation of the miconazole nitrate and there was a constant increase in zone of inhibition with increase in drug concentration. This study proves antifungal potential of novel proniosomal preparation of miconazole nitrate against *Trichophyton rubrum* in the treatment of tinea infections.

1. Introduction

Dermatophytes, being of ubiquitous occurrence in human habitat, are the most prevalent fungal pathogens in superficial fungal infections. Dermatophytosis or tinea is the superficial infection of keratinized skin, hair and nails caused by fungal genera *Trichophyton*, *Epidermophyton* and *Microsporum* and affecting global health at an alarming rate[1]. Dermatophyte infections are likely to infect every person at least once in their lifetime. The manifestation of tinea infection in an individual may be dependent upon the virulence of the dermatophyte, location of infection and the response of the host defense mechanisms[2]. Dermatophytes are causative agent of cutaneous mycoses which may be manifested as pruritus (itching), inflammation, glabrous skin, alopecia, hyperhidrosis (excess sweating) and damaged hair and nails. Transmission of tinea infection can occur by direct contact or from

exposure to desquamated cells. The considerably increasing trend seen since last decade in tinea infections is due to various factors such as population movements, use of immunosuppressive therapy for organ transplants, infection with HIV, lifestyle changes, hygiene levels and tropical climatic conditions[3]. Treatment of dermatophyte infections represents a significant cost burden to a nation as it can reach epidemic proportions if timely measures are not taken by health practitioners and physicians. It has been estimated that over US\$500 million per year is spent worldwide on drugs to address the problem of dermatophytoses[4]. Azoles have been recommended for superficial fungal infections and cutaneous diseases and have high affinity for keratin and thus lead to drug partitioning into stratum corneum. Miconazole nitrate is an azole class antifungal agent, lipophilic and characterized by relatively high molecular weight and melting point. It is a weak base with pKa 6.7, high log octanol/water partition coefficient and exhibits poor aqueous solubility (1.03 µg/mL). Miconazole is usually employed as 2% w/w in topical drug preparations for treatment of dermatophytosis, superficial mycoses, mixed infection and candida infections[5].

Trichophyton rubrum (*T. rubrum*) is the most frequently isolated anthropophile dermatophyte and accounts for 80%–90% of the

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dermatophytes adapted to infect keratinized tissues by virtue of its ability to utilize keratin as a nutrient source[6]. *T. rubrum* may cause clinical manifestations including onychomycosis, tinea corporis, tinea cruris, tinea manuum and tinea pedis, etc. that vary in degree and chronicity of inflammation. *T. rubrum* spreads through direct contact, survives on skin surface and releases mannans that suppress cell mediated immune reaction thus leading to persistent infection[7]. This pathogenic organism thrives in warm, moist conditions of tropic and subtropic regions of the world. As *T. rubrum* remains in the stratum corneum, it may evade immune surveillance, and complement and polymorphonuclear leukocytes that would attach the organism if it tried to invade into viable epidermis[8,9]. Studies suggest that the production of some endoproteases by *Trichophyton rubrum* break the bonds of the keratinized tissue, behave as antigens and induce various degrees of inflammation. As a result, tissue damage is a combination of the enzymatic action of the dermatophytes and the defense mechanisms activated during the inflammatory processes. Present work was undertaken to investigate effect of miconazole nitrate proniosomes against *T. rubrum* which leads to dermatological manifestations in pathogenic state.

2. Materials and methods

The drug miconazole nitrate was obtained as a gift sample from GlaxoSmithKline Pharma Ltd., Mumbai. Nonionic surfactants-Span 40, 60 (HPL chemicals), cholesterol (Loba Chemie) were used in the study. Phospholipon® 90 (phosphatidylcholine) was obtained as a kind gift from Lipoid GmbH, Ludwigshafen, Germany. All the chemicals and solvents used in the study were of analytical grade. Standard test strain of *T. rubrum* (MTCC 8477) was obtained from the Microbial Type Culture Collection Centre, Institute of Microbial Technology, CSIR, Chandigarh.

2.1. Preparation of vesicles

Miconazole proniosomal preparation was prepared by coacervation-phase separation technique using drug miconazole and excipients including surfactant, Phospholipon® 90 and cholesterol[10]. To prepare drug gel, a specific amount of carbopol 934 (1.5% w/w) was soaked in distilled water overnight. The required amount of drug (1%) was dissolved in this mixture with stirring at 500 r/min, by a magnetic stirrer for 1 h. Carbopol was then neutralized with 0.5% triethanolamine to obtain pH in range 5.5–6.0. Propyl paraben (0.05%, w/v) and methyl paraben (0.1%, w/v) were taken as a preservative in a beaker containing propylene glycol 10%. These were mixed properly by gentle stirring at 100 r/min and heating at 50 °C. This was then added slowly with stirring to prepared carbopol mixture to obtain a clear viscous gel[11,12].

The morphology and structure of niosomes formed after hydrating proniosome formulations were observed using a Tecnai G² Transmission Electron Microscope (Fei USA) with a 300 kV high tension and a field emission system. A combination of bright

field imaging at high resolution and diffraction modes were used to reveal the morphology and size of niosomes. Proniosome dispersions were suitably diluted with purified water. One drop of diluted sample was applied on a carbon-coated copper grid with 300 mesh size and left for one min to allow preparation to adhere to carbon substrate. The excess sample was removed using filter paper. After twice rinsing the grid with distilled water for 3–5 seconds, a drop of 2% aqueous solution of uranyl acetate was applied for 1 second, excess solution was removed and sample was air dried for 30 seconds before observation under the electron microscope[13].

2.2. In vitro antidermatophyte activity

Minimal inhibitory concentration (MIC) of the preparation was determined by Macro broth dilution method. Freeze dried and pure cultures obtained of *T. rubrum* were revived and maintained on Sabouraud glucose agar slant[14]. One loopful surface growth of test organism was scrapped off with sterile wire loop and inoculated in 50 mL Sabouraud glucose broth. Then it was incubated at (28 ± 2) °C for 48 h. Inoculum suspension that gave a concentration 1×10^5 CFU/mL was prepared for study. MIC of vesicle gel against *Trichophyton* was assessed by broth dilution method. Eight different concentrations of vesicle gel (100, 150, 250, 500, 750, 1000, 1250, 1500) in dimethyl sulfoxide (DMSO) in µg/mL were incorporated into Sabouraud broth in different test tubes. In each test tube, 1 mL of formulation of above mentioned concentration was added to 4.9 mL of broth and 100 µL of fungal culture. A +ve control tube containing the growth medium, fungal strain and an uninoculated tube of medium to serve as a negative growth control was set up. The mixtures were incubated at 25 °C for 48 h and analyzed for turbidity. The minimum concentration of gel preparation that inhibited the growth of the microorganism detected by lack of visual turbidity was determined as MIC[15].

Further, microbial growth inhibitory properties of test formulations were determined by disc diffusion method. Sterile Sabouraud dextrose agar medium was prepared using culture medium (dextrose 40 g, peptone 10 g, agar 2 g and distilled water 1 L) at temperature 25 °C and pH 5.5. The plates were inoculated with 100 µL standardized culture of test organism *T. rubrum* (aerobic, incubation time – 48 h) and exposed to air drying at room temperature under aseptic conditions for 15 min. The formulations (proniosomal preparation F5A and F5B; plain drug carbopol gel) were initially dissolved in DMSO and prepared at concentrations of 1000, 750, 500, 250 µg/mL. Then antifungal disks (6 mm in diameter) impregnated with 20 µL test formulation (prepared in DMSO) at concentrations mentioned were placed on surface of inoculated media plates. The agar plates were incubated at 25 °C for 3 days. Clear zones of inhibition on agar surface around the disc was measured in mm, including the diameter of the disc[16].

3. Results

Miconazole was encapsulated in proniosomal preparation

using nonionic surfactants – Span 40, Span 60, and cholesterol and phosphatidyl choline. The morphology was studied using transmission electron microscopy which was observed as spherical vesicles with smooth surface. The vesicles were discrete and separate with no aggregation (Figure 1).

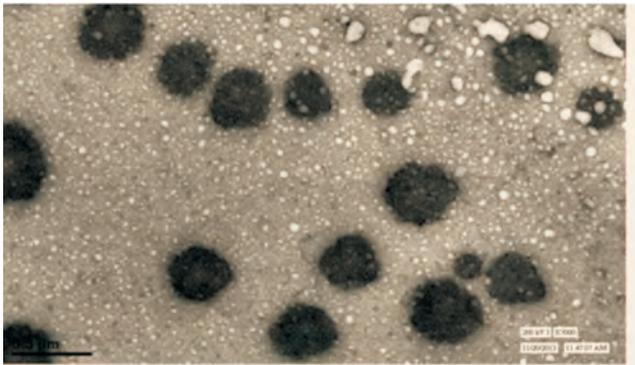


Figure 1. TEM photomicrograph of proniosomal preparation showing spherical nano vesicles

Macro broth dilution test showed that the minimum concentration which inhibited the growth of test organism was 250 $\mu\text{g/mL}$. The study was performed using four different concentrations of miconazole (0.25, 0.50, 0.75 and 1.00 mg/mL) in proniosomal formulations. The concentration higher than 1000 $\mu\text{g/mL}$ led to overlapping of zone of inhibitions. The microbiological tests revealed that during the study period of 3 days, the formulations F5A, F5B showed gradual increase in zone of inhibition with no overlapping at 250, 500 and 750 $\mu\text{g/mL}$ due to the controlled release of medicament. In F5A, it is observed that at 1000 $\mu\text{g/mL}$, there was an initial burst release of drug leading to overlapping of inhibition zone with 750 $\mu\text{g/mL}$ concentration due to free drug which could not be entrapped in vesicles. However, in F5B, at 1000 $\mu\text{g/mL}$, drug was better entrapped in vesicles due to surfactant and cholesterol and leading to controlled inhibition. There was an increase in zone of inhibition with increased concentration of miconazole in all tested formulations (Table 1).

The drug release was also found to be more potent in vesicular preparation than drug carbopol gel *i.e.* non-vesicular form of topical preparation at each concentration. The maximum zone of inhibition was observed as constant in plain drug carbopol gel in 24 h itself. The antifungal activity of vesicle gel prepared showed

sustained release of medicament by regular intervals when compared to plain carbopol gel (Figure 2). However, a significantly higher antifungal activity was observed with F5A, F5B (in vesicular form) in comparison to plain drug carbopol gel (in non-vesicular form) at concentration of 250, 500, 750 and 1000 $\mu\text{g/mL}$.

Table 1

Zone of inhibition against *T. rubrum* MTCC 8477 (mm).

No.	Concentration ($\mu\text{g/mL}$)	Formulation F5A	Formulation F5B	Plain Drug Carbopol gel
1	1000	28.16 \pm 1.16	22.36 \pm 1.16	18.17 \pm 1.36
2	750	20.35 \pm 0.59	22.16 \pm 0.47	18.14 \pm 0.28
3	500	16.91 \pm 0.89	18.01 \pm 0.82	13.57 \pm 0.52
4	250	12.96 \pm 0.63	14.06 \pm 0.58	10.48 \pm 0.79

F5A = Span 40: Cholesterol: Phospholipon 90 (6:1:3), miconazole nitrate 1%; F5B = Span 60: Cholesterol: Phospholipon 90 (6:1:3), miconazole nitrate 1%. Values are expressed as mean \pm SD, $n=3$.

4. Discussion

With the advent of novel biocompatible and biodegradable materials like phospholipids, surfactants and advanced drug delivery technologies *i.e.* proniosomes, solid lipid nanoparticles, ethosomes, and nanoemulsions, the possibility to improve the efficacy and safety of the topical products has increased manifold. Vesicular systems are considered very promising in topical drug delivery as they exhibit flexibility in their structural characterization and are patient compliant due to better drug retention in stratum corneum[17]. Present study was aimed at preparation of vesicle based formulation of miconazole nitrate using coacervation technique as per available literature reports[18].

In vitro antifungal susceptibility tests are used to determine clinical outcome based upon an optimization of antifungal therapy. Multicenter studies to develop a standardized antifungal susceptibility assay were initiated by the Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards, NCCLS) in 1983. Micro broth dilution tests M38 A, M44 A2 and diffusion tests are methods used for antifungal susceptibility testing. However, NCCLS methods may not be the most practical procedures for use in the routine clinical laboratory for dermatophytes[19]. The disc diffusion method is a practical, reliable and reproducible agar-based *in vitro*

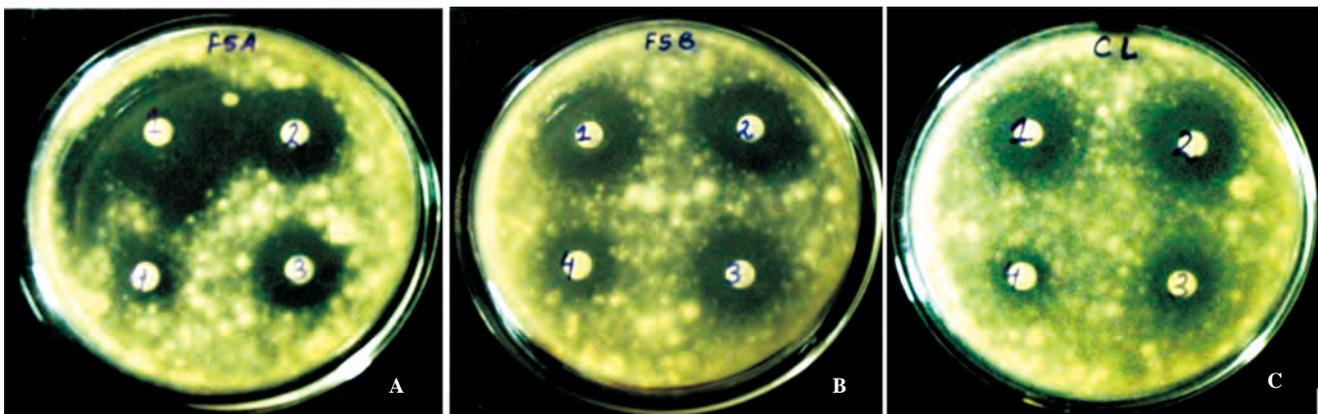


Figure 2. Antifungal susceptibility testing of formulation against *T. rubrum*. A: Formulation F5A; B: Formulation F5B; C: Plain drug carbopol gel.

assay method which enables the determination of the activity of various antifungal drugs against dermatophytes[20]. In this method, diffusion of drug from disc through a solidified agar layer in a Petri dish occurs to an extent such that growth of added micro-organism is prevented entirely in a zone around the disc containing antimicrobial drug. This method of fungal susceptibility assessment yields data consistent with results obtained from dilution method and is also recommended for routine use[21,22]. The lowest concentration at which the isolate is completely inhibited (as evidenced by the absence of visible fungal growth) is recorded as the MIC. The drug microbiological inhibition data obtained for test formulations F5A, F5B was subjected to statistical Mann–Whitney U test for pairwise comparison with control preparation (plain drug carbopol gel). For all comparisons, $P < 0.05$ was considered as significant. The data were reported as mean \pm SD ($n=3$).

Miconazole nitrate was successfully entrapped within the lipid bilayers of the proniosomal vesicles which are capable of releasing drug through the skin due to permeation enhancing effect of non-ionic surfactants. From the findings of antimycology research study, it is indicated that developed preparation has significant potential for topical delivery of miconazole nitrate against rapidly evolving dermatophyte *T. rubrum* and could contribute to drug delivery in terms of better efficacy and sustained release in clinical medicine.

Conflict of interest statement

We declare that we have no conflict of interest.

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