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ORIGINAL ARTICLE

Effect of fluconazole and terbinafine nanoparticles on the treatment of dermatophytosis induced by *Trichophyton mentagrophytes* in guinea pig

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ABSTRACT

Background and Objectives: Dermatophytosis induced by *Trichophyton mentagrophytes* is a major human and animal fungal contamination. Antifungals like terbinafine and fluconazole are widely used to treat dermatophytosis; nevertheless, the prevalence of drug resistance has increased. Hence, novel curative strategies are needed. In the present study, we compared the efficacies of conventional and nanoform of antifungals agents in guinea pig model of dermatophytosis.

Materials and Methods: Guinea pigs (n=36) were injected (the posterior dorsal portion) with *Trichophyton mentagrophytes* conidia. The guinea pigs were divided into 6 groups (positive control, negative control, fluconazole 0.5% treated group, nano-fluconazole treated group, terbinafine 1% treated group, and nano-terbinafine treated group), then were scored both clinically (redness and lesion intensity) and mycologically (microscopy and culture) until day 40 of inoculation. The treatment started 5 days after the inoculation and continued until day 40 of inoculation.

Results: Assessment of the mean score of clinical lesions in groups treated with nano-drug forms of fluconazole and terbinafine on the first day of treatment showed a score of 3 (significant redness with large scaling) and for the conventional form of terbinafine and fluconazole had a score of 4 (ulcer and scar). The decrease in lesion score in nano-drug treated groups was observed between days 15 and 20 and continued until day 40. On day 40, all groups had zero scores except the positive control group.

Conclusion: This study indicated that nano-drugs are more suitable for the treatment of dermatophytosis and could be considered as future alternatives for the treatment of dermatophytosis.

Keywords: *Trichophyton mentagrophytes*; Nano-drugs; Terbinafine; Fluconazole; Guinea pig

INTRODUCTION

Dermatophytosis is among major mycotic contaminations, affecting keratinized tissues like dermis, hair, and nails of farm animals, pets, wild animals, birds, and humans (1). The disease is one of the most prevalent zoonotic disease specially among farmers, livestock, domestic animals and pet owners (2). Zoophilic dermatophytes are classified into two genera, *Trichophyton* and *Microsporum*. Among them, *Microsporum canis* and *Trichophyton mentagrophytes* are most common isolated species from the clinical samples of domestic animals. Infections from dermatophytes may be transmitted from animal to human, animal to animal, and human to human (3) (4-6). Dermatophytes attack the stratum corneum or keratinized structures derived from the epidermis, which results in lesions on the dermatome or hair and nail (7). For all dermatophytes, the first stage of infection is colonization on the stratum corneum of the

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tissue. Since dermatophytes do not have the ability to invade living tissue, the only mechanism that can be attributed to the pathogenicity of these organisms is the synthesis and secretion of toxins, stimulants, or allergens (8, 9). These substances find their way from the living epidermis to the dermis, which has a vascular network and has the potential for response to the invasion of such toxins and allergens by an inflammatory reaction (9). It has been found that when a zoophilic organism infects human skin, severe inflammatory reactions occur (10). Treatment with antifungal agents, particularly fluconazole and terbinafine is considered as an effective treatment for dermatophytosis caused by *Trichophyton mentagrophytes* (11); however, there were some challenges including drug resistance and side effects. Thus, it is necessary to use newer therapeutic strategies (2). Nanoparticles are a new generation of antimicrobial agents being studied significantly in recent years (12). Nanoparticles can improve bioavailability, solubility, and permeability of drugs. Nano-liposomes has several benefits, such as enhanced penetration, selective delivery, prolonged release time, and high solidity of drugs (13). Besides, to assess the efficacy of medicines against dermatophytes, quantitative evaluation of dermatophyte growth is of crucial importance. Nearly all ingredients with *in vitro* antimicrobial properties do not affect the animal model, mainly due to their *in vivo* pharmacokinetics characteristics. Hence, assessing its therapeutic characteristics in animal models is of crucial importance for developing agents that are effective against fungal infections. Mammalian animals like the guinea pig are widely using to model skin fungal infections (14). In this study, the antifungal activities of nano-liposomal fluconazole and nano-liposomal terbinafine were compared to the conventional forms of fluconazole and terbinafine against dermatophytosis induced by *Trichophyton mentagrophytes* in the guinea pig model.

MATERIALS AND METHODS

Ethics approval. The study protocols and procedures had previously been confirmed by the Research Ethical Board of the Islamic Azad University, Science and Research Branch (code of ethics: 13982).

Samples. Samples were taken from patients suspected of dermatophytosis, stored by the Mycology Laboratory affiliated to the Tehran University of Medical Sciences, Tehran (Iran) in January 2020. In addition, standard zoophilic strains of *Trichophyton mentagrophytes* ATCC 9533®TM were supplied by the Research and Production Company of ArkaTeb Roham (ATR).

Identification of isolates. All the *T. mentagrophytes* isolates were identified based on microscopic properties using KOH 10% and macroscopic properties after culturing on Sabouraud glucose agar (SDA) (Merck, Germany) in combination with chloramphenicol (50 mg/L) and cycloheximide (300 mg/L) (SCC). Then all the isolates were subjected to molecular identification. Briefly, genomic DNA of strains was isolated using the commercial kit Higher Purity™ fungi Genomic DNA Extraction (Canvax Biotech, Spain) according to the manufacturer's protocol. The quality and quantity of the extracted DNA were evaluated using spectrophotometry (Thermofisher, USA) and electrophoresis in 1% agarose gel. To distinguish *T. mentagrophytes* from other microorganisms, ITS-PCR was employed. The internal transcribed spacers (ITS) (ITS1 and ITS4) regions of ribosomal DNA (rDNA) by pan-fungal primers ITS 1 (5'- TCCGTAGGTGAACCTGCGG- 3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC - 3') were amplified in a thermocycler (Eppendorf, Germany). PCR products were sequenced (Bioneer, South, Korea), and results were checked in NCBI website using BLAST tool (15).

Animals. Thirty-six male guinea pigs, Pirbright strain, weighing 350-400 grams about 6 months old were used. they were randomly divided into 6 groups (positive control (n=6), negative control (n=6), fluconazole 0.5% treated group (n=6), nano-fluconazole treated group (n=6), terbinafine 1% treated group (n=6), and nano-terbinafine treated group (n=6)).

Preparation of inoculums. Clinical isolates of *Trichophyton mentagrophytes* were incubated at 25°C on Potato dextrose agar (PDA) (Merck, Germany) for 21 days. Then, all fungal cells were stored through floating samples in a solution composed of sterile water (12 mL), two drops of phosphate-buffered saline, containing 0.01% Tween 20 (Merck, Darmstadt, Germany), then a single-use sterile pipette was used for agitation. An 11-µm-pore-size filter (nylon net filters;

Millipore, Ireland) was applied to remove both macroconidia and large hyphal elements. The inoculum was made using a sterile saline solution with 95 % of transmission at 530 nm. Accordingly, the inoculum concentration ranging 1×10^6 CFU/mL was achieved (16).

Infection model. For cutaneous inoculation of dermatophytes, thirty-six non-predisposed guinea pigs were chosen and an area of 4 cm on their backs was shaved and few scratches (without causing a lesion) was done by a needle. Then out of five rings on each guinea pig (Portions of fungal suspension (10^6 conidia/mL)), four were inoculated. Using diameters of 4 cm and a Vaseline ring, all areas were marginalized. The samples were clinically assessed after 3, 7, 10, 14, and 17 days. It worth noting that all assessments were performed by a similar researcher. The clinical assessment comprised of a semi-quantitative score, in which the inoculated samples were assessed and compared with uninfected ring area taken from the back of the same animal; 0= no lesions, 1= hair loss only, 2= redness with scaling, 3= significant redness with large scaling, 4= ulcer and scar with signs of score 3, and 5= severe dermal lesions, redness, crust, ulcer and no hair growth (17).

Preparation of nano-liposomal fluconazole. In this part, we used Crude powder of FLC (Sigma- Aldrich, Steinheim, Germany), which was purchased from Sigma Company. Using the thin-film hydration method, FLC's liposomal formulation was prepared (18). In order to produce liposomes, 5.12 mg FLC, 5 mg cholesterol, and 50 mg lecithin were used. After dissolving these two substances, a narrow layer was prepared. To do this, an organic solvent chloroform-methanol mixture (1:1) comprised of FLC was used at a rate of 5.12 mg/mL. The eventual concentration of the drug was 5120 μg/mL (19).

Preparing nano-liposomal terbinafine. The NanoZino Company prepared the liposomal formation of terbinafine (20). To do this, we used 1 mL Dimethyl Sulfoxide (DMSO) and 12.8 gram of terbinafine (Sigma, Germany). In the following a solvent of (6 mL) chloroform-methanol (1:1), and lecithin (Lipoid, Canada) and cholesterol (Sigma, Germany) were mixed. The evaporation was performed at 50°C. Sucrose contributed to hydration. A homogenizer set at 20,000 rpm (for 10 minutes) was used to conduct homogenization, while the temperature was set to be higher than liposomes phase transition (70°C). Afterward, we used an ice bath to perform Sonication of liposomal sample. In the following, the remaining ingredients were centrifuged at 4000×g for 4 minutes. Afterward, sterilization was performed for twice using a 0.22-micron filter needle.

Nanostructure analysis. Size, morphology, and zeta potential of nano-liposomal fluconazole and nano-liposomal terbinafine were evaluated (21). To calculate particle size, the produced liposomal nanoparticles were evaluated using photon correlation spectroscopy (PCS). To measure the particle in this device, the formulations that were diluted by distilled water were transferred to the instrument and measured. Zetasizer device was used to measure the Zeta potential, according to the laser light scattering. In addition, using the PDI (polydispersity index), the distribution of particle sizes was assessed. Scanning electron microscope (SEM) was applied to investigate nanoparticle formation. In the following, a small portion of the sample was put on a glass surface (1×1 cm), which was then incubated at 37°C until fully dried. Then, the particles were coated by gold; images were taken with 20000× and 40000× magnification (19).

Antifungals and antifungal susceptibility testing. According to the guideline published by the Clinical and Laboratory Standards Institute (CLSI) (M38-A2 manual) the *In-vitro* antifungal susceptibility was assessed (22). Average minimum inhibitory concentration (MIC) of fluconazole 0.5%, nano-fluconazole, terbinafine 1%, and nano-terbinafine was determined at concentrations of 1, 0.8, 0.6, and 0.5%, respectively (19).

Treatment of the inoculated guinea pigs. On the fifth day after inoculation, the first symptoms of the disease appeared and this day was considered as the first day of treatment. Fluconazole 0.5% nano-liposomal fluconazole, terbinafine 1%, and nano-liposomal terbinafine were applied to the lesions. According to previous researches, we started topical treatment every 12 hours on the 5th day (13). During the 40-day treatment, the nano-liposomal fluconazole and nano-liposomal terbinafine were sprayed by a sprinkler on and around the infected area. Following the same pattern, terbinafine% cream and fluconazole 0.5% cream were applied to the infected area. In positive and negative control groups, saline was used as the

placebo during the treatment period. Changes in lesion scaling, erythema, ulceration or alopecia were examined and recorded every 5 days.

Mycological examination. Mycological investigations were conducted on days 3, 7, 14, 17 and 40 after inoculation. We selected one of the four inoculation ring areas per animal for sampling. The entire area of each ring (diameter=4 cm) was scraped. A light (phase) microscope and 10% KOH were used for direct examination of samples. When hyphae, arthroconidia, and/or conidia were detected, microscopy was found as positive. In the following, all cultures were incubated for 4 weeks, while were assessing weekly. *T. mentagrophytes* was checked out as the true pathogen. It should be noted all animals except negative control group were mycological positive at 5^{th} day.

Statistical analysis. The analysis of variance (ANOVA) and Dunnett's test were applied for comparing the groups. Data were analyzed using SPSS version 21 (IBM SPSS Statistics for Windows, IBM Corp, Armonk, NY, USA). Statistical significance was defined as a P-value less than 5%.

RESULTS

PCR assay. Using a homology of 99% and Blast 2 Sequences Tool, the sequence of DNA of ribosomal ITS region of the fungus was *Trichophyton mentagrophytes* (data not shown).

Scanning electron microscopy. To examine the construction of nano-liposomal fluconazole and nano-liposomal terbinafine, scanning electron microscopy was applied.

After magnifying images of SEM (both higher and lower), it was found that the structures of nano-liposomal fluconazole and nano-liposomal terbinafine were spherical and the particles sizes were 88.9 ± 12.14 and 360.2 ± 0.293 nm, respectively (Fig. 1).

Zeta potential. According to the findings, for fluconazole nano-liposomal, zeta potential was 20.12 mV. Also, for terbinafine nano-liposomal formation, zeta potential was 37.6 mV (Fig. 2).

Clinical evaluation of treatment efficacy of nano-liposomal fluconazole and nano-liposomal terbinafine in the guinea pig model. Five days after the infection, symptoms appeared and treatment began and continued until day 40. Assessment of the mean score of clinical lesions in groups treated with nano-drug forms of fluconazole and terbinafine on the first day of treatment showed a score of 3 (significant redness with large scaling) and for the conventional form of terbinafine and fluconazole a score of 4 was recorded (ulcer and scar with what was observes in grade 3). The decrease in lesion score in nano-drug treated groups occurred between days 10 and 15. The downward trend continued until day 40 and on day 40, all groups were scored zero except the positive control group (Figs. 3-5). Concerning the mean score of clinical lesions, we found a statistically significant difference among the groups treated with conventional forms of the drugs (fluconazole 5% and terbinafine 1%) and the negative control group ($p < 0.05$) in all days. Also, we found a significant difference among those treated with nano-liposomal form of terbinafine and fluconazole, and the positive control group from day 5 until the end of treatment ($p < 0.05$). The mean score of clinical lesions in the two nano-fluconazole and nano-terbinafine treated groups significantly decreased between days 10 and 15 compared to the two groups of guinea pigs treated with terbinafine 1% and fluconazole 0.5% ($p<0.05\%$). There were no statistically significant increases or decreases in the mean score of clinical lesions in the two groups that were treated with the conventional form of the drug between days 10 and 15 (Figs. 3-5).

Mycological evaluation of treatment efficacy of nano-liposomal fluconazole, and nano-liposomal terbinafine in the guinea pig model. Fungal cultures on days 30, 37, and 42 indicated the effect of fluconazole, terbinafine, nano-liposomal fluconazole, and nano-liposomal terbinafine on different guinea pig groups (positive control, negative control, fluconazole 0.5% treated group, nano-fluconazole treated group, terbinafine 1% treated group, and nano-terbinafine treated group). Treatment was discontinued when at least 2 consecutive cultures had negative results in all animals in each group (apart from those in the negative control group) (Table 1).

DISCUSSION

Dermatophytosis is a major skin disorder, that re-

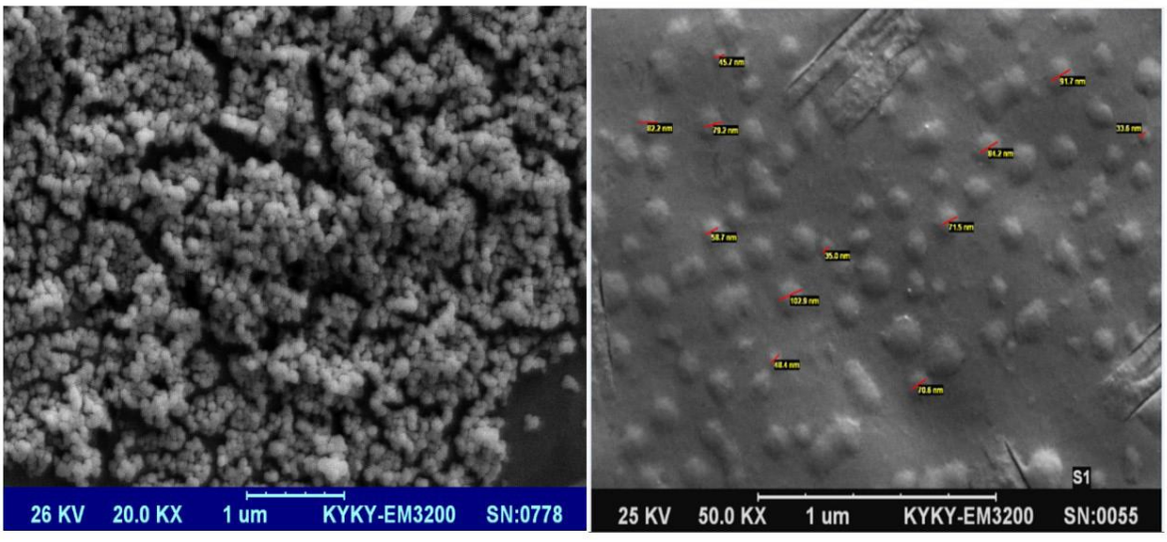


Fig. 1. Scanning electron microscopy of nano-liposomal fluconazole (Left) and nano-liposomal terbinafine (Right)

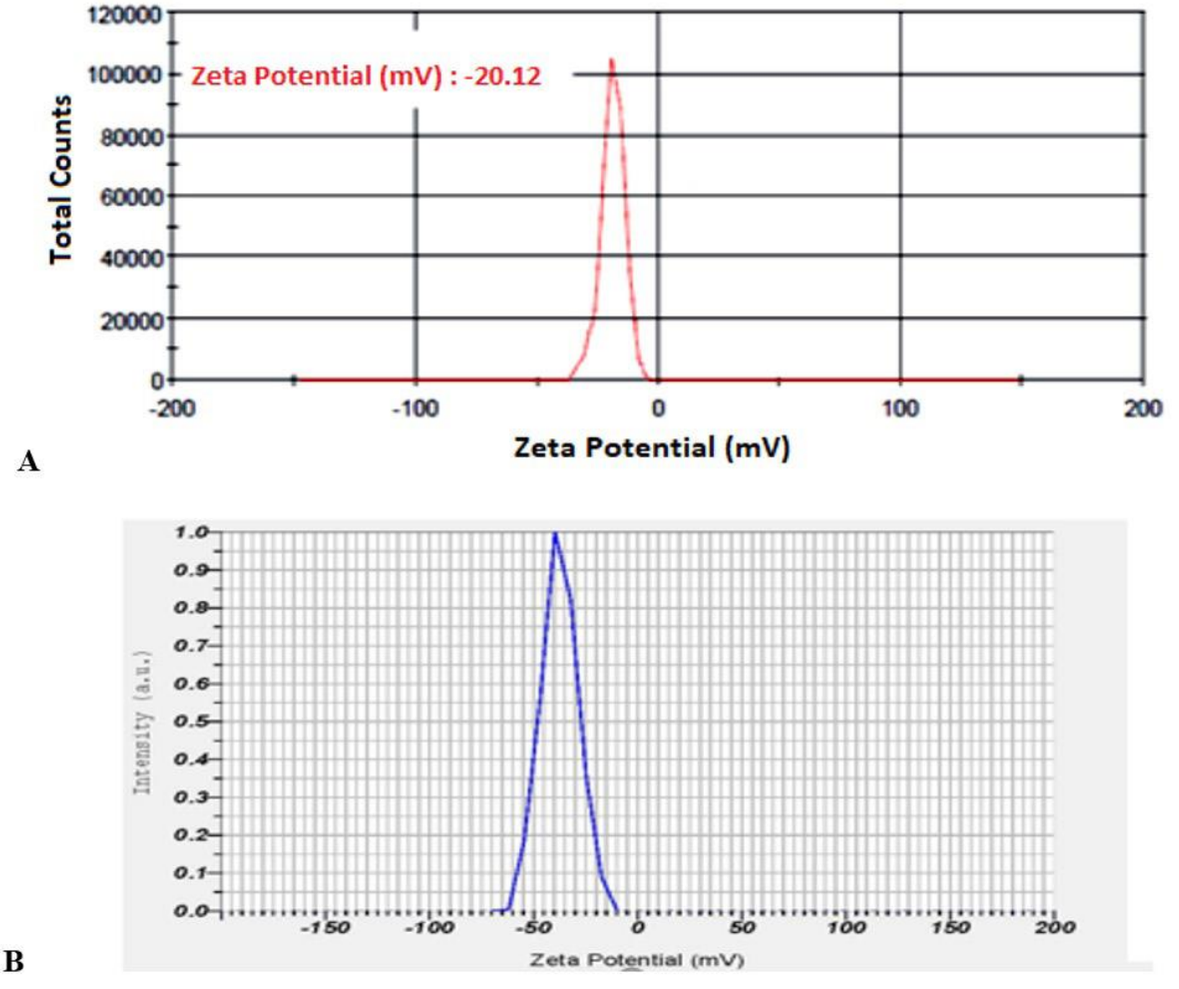


Fig. 2. The Zeta potential of nano-liposomal fluconazole (A) and nano-liposomal terbinafine (B)

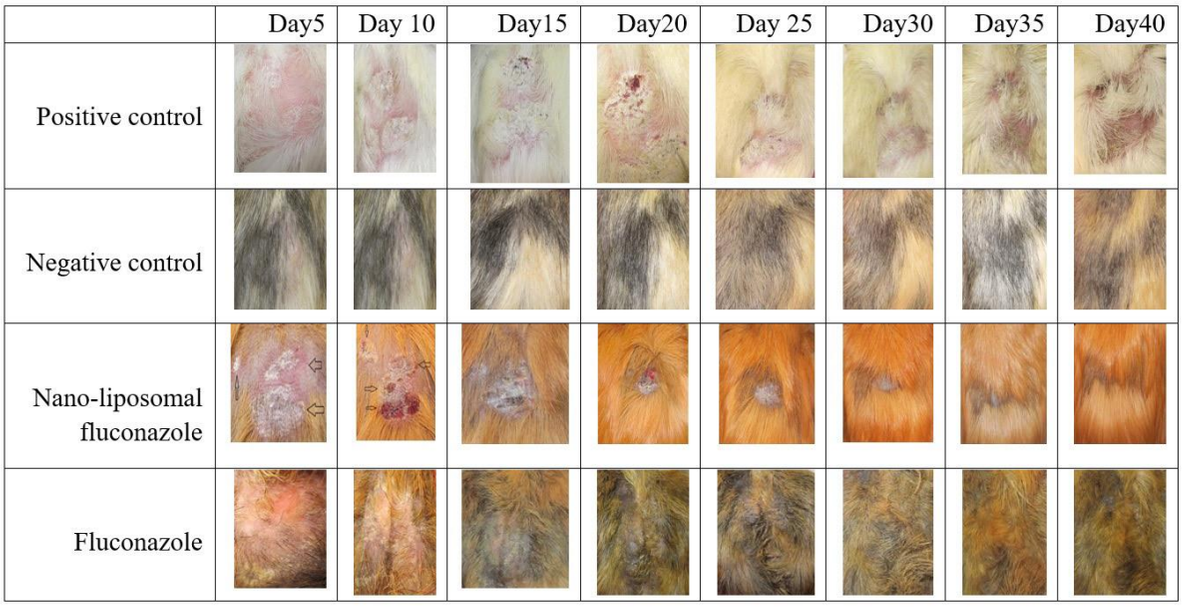


Fig. 3. Comparison of the recovery process between fluconazole 0.5% treated group and nano-fluconazole treated group

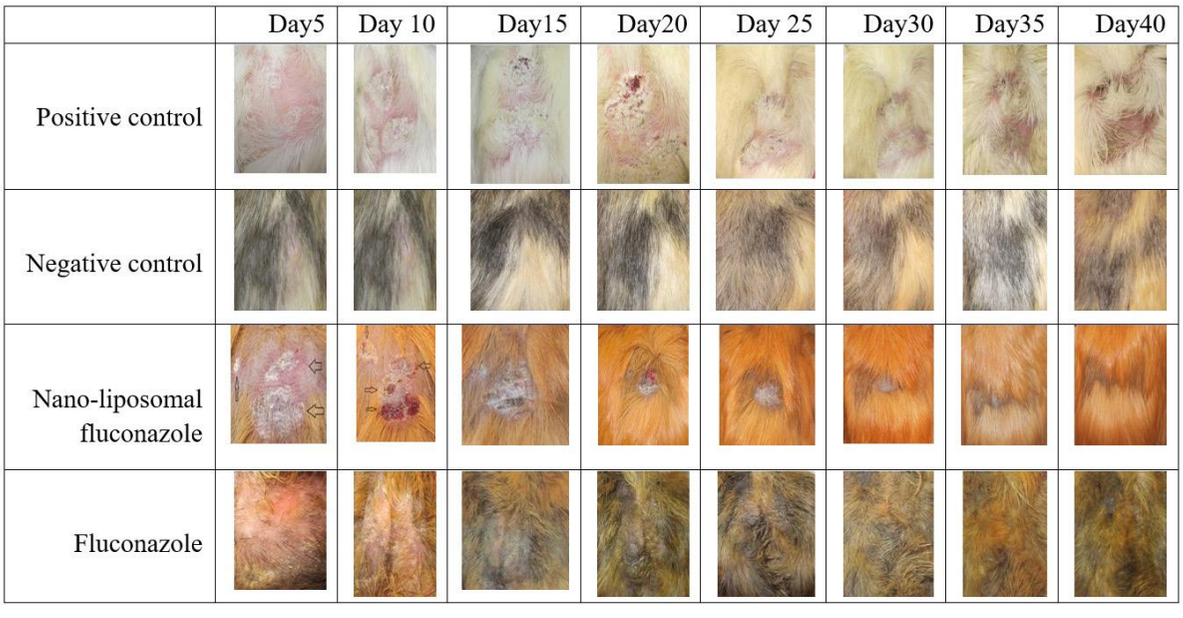
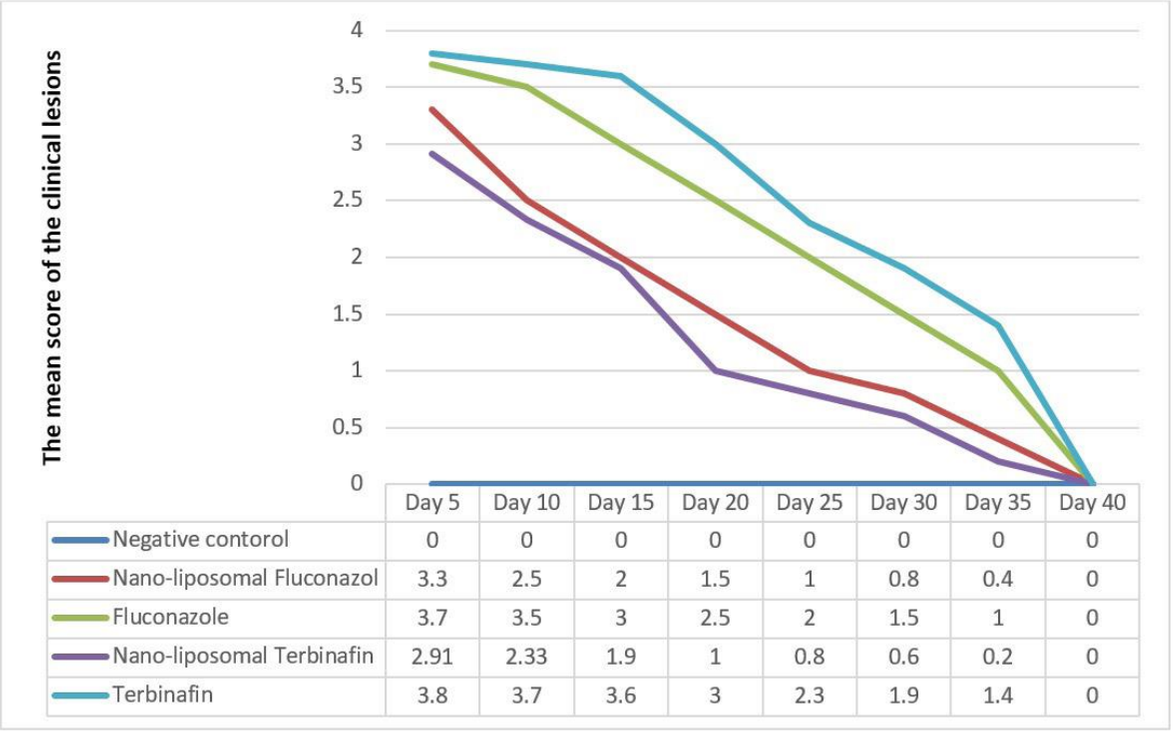


Fig. 4. Comparison of the recovery process between terbinafine 1% treated group, and nano-terbinafine treated group

sult from infection of the skin by fungal organisms that usually remain localized to the superficial layers of human and animal hair, skin, or nails (23). It can be transmitted through direct contact with contaminated material (e.g., skin and hair of either infected humans or animals). Topical therapies reduce infectiousness and contagiousness, as well as zoonotic risk of dermatophytosis through disinfecting the hair coat and minimizing contaminating the environment and are considered the basis for the treatment of this disease. Among topical antifungal agents, fluconazole and terbinafine are known as effective treatments for dermatophytosis (24). However, there are challenges such as drug resistance and side effects. Thus, it seems necessary to find newer and more effective antifungal agents to address these problems. For example, alternative approaches including applying commercial drugs like nano-scaled particles as a novel drug delivery system may be considered as new approaches to treat this contagious disease. A



	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30	Day 35	Day 40
Negative contorol	0	0	0	0	0	0	0	0
Nano-liposomal Fluconazol	3.3	2.5	2	1.5	1	0.8	0.4	0
Fluconazole	3.7	3.5	3	2.5	2	1.5	1	0
Nano-liposomal Terbinafin	2.91	2.33	1.9	1	0.8	0.6	0.2	0
Terbinafin	3.8	3.7	3.6	3	2.3	1.9	1.4	0

Fig. 5. The mean score of the clinical lesions in different groups of guinea pigs (positive control, negative control, fluconazole 0.5% treated group, nano-fluconazole treated group, terbinafine 1% treated group, and nano-terbinafine treated group) 5 days after infection until day 40

Table 1. Mycological examinations in different groups of guinea pigs (positive control, negative control, fluconazole 0.5% treated group, nano-fluconazole treated group, terbinafine 1% treated group, and nano-terbinafine treated group) on days 30, 37, and 42 after infection

Groups	Days after infection		
	day 30	day 37	day 42
Positive control	6/6 (100%)	5/6 (83.3%)	5/6 (83.3%)
Negative control	0/6 (0%)	0/6 (0%)	0/6 (0%)
Fluconazole	2/6 (33.3%)	1/6 (16.6%)	0/6 (0%)
Terbinafine	2/6 (33.3%)	1/6 (16.6%)	0/6 (0%)
Nano-liposomal fluconazole	1/6 (16.6%)	0/6 (0%)	0/6 (0%)
Nano-liposomal terbinafine	1/6 (16.6%)	0/6 (0%)	0/6 (0%)

variety of chemical and physical technologies have been developed to improve drug delivery through the skin. In order to treat cutaneous fungal contaminations, the drug must penetrate into the deep areas of the skin (25). Small size of the lipid nanoparticles improves the presence of nanoparticles in direct contact with stratum corneum and ensures the entry of encapsulated drugs into the skin (26, 27). Compounds such as surfactants in nanoparticle formulation have been known to optimize the absorption of substances through the skin. That is why in the structure of most locally available nanoparticles (such as lipid nanoparticles and polymer nanoparticles), surfactants are used. Until now, no study has investigated the impact of terbinafine and fluconazole nanoparticles on the treatment of dermatophytosis in animal models. In the present study, the liposomal formulation of fluconazole and terbinafine were developed and demonstrated more effective antifungal properties on dermatophytosis induced by *Trichophyton mentagrophytes* in guinea pigs compared with the conventional forms of these drugs. In consistent with

our results, Bafrui et al. (2019) assessed the antifungal sensitivity of nano-fluconazole in comparison to fluconazole on 47 zoophilic dermatophyte samples and reported that the minimum inhibitory concentration (MIC) value for fluconazole was higher than nano-fluconazole; hence, in lower concentration of the drug, nano-fluconazole was associated with decreased growth of dermatophytes, more effective than fluconazole (28). Similarly, Motedayen et al (2018) evaluated the antifungal activity of terbinafine and terbinafine nano-drug against clinical isolates of dermatophytes and demonstrated that the MIC values of nano-liposomal terbinafine against *T. rubrum* and *M. canis* were 0.0156 to 0.25 µ/mL and 0.0078 to 0.125 µ/mL, respectively. In addition, MIC values of terbinafine against *T. rubrum* and *M. canis* strains were 0.0625 to 1 µ/mL and 0.0313 to 0.5 µ/mL, respectively (29). That study showed that the nanoparticles of terbinafine had a greater antifungal activity against the *T. rubrum* and *M. canis* isolates in comparison with free terbinafine, which is in line with our findings.

To the best knowledge of the authors, the current research is the first study to apply nano-liposomal terbinafine successfully to dermatophytes. In the present study, all *Trichophyton mentagrophytes* isolates were identified using sequencing the rDNA and ITS regions. Exact identification of species is essential for epidemiological studies and treatment. Our results showed that particle size and zeta potential for nano-liposomal fluconazole was 88.9 ± 12.14 nm and 20.12 mV, respectively. Also, particle size and zeta potential for nano-liposomal terbinafine was 360.2 ± 0.293 nm and 37.6 mV, respectively. Particle size and distribution width were considered the crucial quality-related variables which affect other macroscopic properties of the nano-particle. The enhanced frequency of particles bigger than 1 µm can show their physical instability (30). Zeta potential is a key factor in determining the steadiness of the colloidal system and is the best indicator for determining the surface electric status of dispersions. In this study, a particle size<1 µm for both nano-liposomal fluconazole and nano-liposomal terbinafine, and the appropriate zeta potential (20.12 mV for nano-liposomal fluconazole, and 37.6 mV for nano-liposomal terbinafine) indicated and confirmed the stability of these formulated nano-drugs. The mean score of the clinical lesions in two nano-fluconazole and nano-terbinafine treated groups significantly decreased between days 10 and 15 compared to the two groups of guinea pigs treated with terbinafine 1% and fluconazole 0.5%. There were no statistically significant increases or decreases in the mean score of the clinical lesions in the two groups that were treated with conventional form of the drug between days 10 and 15. This suggests that the speed of recovery in two nano-fluconazole and nano-terbinafine treated groups is faster than that of the groups treated with the conventional drugs.

CONCLUSION

The primary significance of this study is that nano-liposomal fluconazole and nano-liposomal terbinafine could speed the recovery process of dermatophytosis better than the conventional forms of these drugs. To the best of our knowledge, the current research is the first study that examined the effect of terbinafine and fluconazole nanoparticles on the treatment of dermatophytosis in animal models.

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