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RESEARCH ARTICLE

Transungual delivery of terbinafine by iontophoresis in onychomycotic nails

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Abstract

Trans-nail permeability is limited due to the innate nature of the nail plate and the recent investigations indicated the potential of iontophoresis in enhancing the transungual drug delivery in normal nails. However, the onychomycotic nails differ from the normal nails with respect to the anatomical and biological features. The current study investigated the effect of iontophoresis (0.5 mA/cm^2 for 1 h) on the transungual delivery of terbinafine in onychomycotic finger and toe nails. The presence of fungi in the onychomycotic nails was diagnosed by potassium hydroxide (KOH) microscopy. Passive and iontophoretic delivery of terbinafine across the infected nail was studied in Franz diffusion cell. Further, the release profile of terbinafine from the drug-loaded nails was investigated by agar diffusion method. KOH microscopy confirmed the presence of fungi in all the nails used. The amount of drug permeated across the nail plate was enhanced significantly during iontophoresis over passive delivery, that is, by 21-fold in case of finger and 37-fold in case of toe nails. Further, the total drug load in the onychomycotic nail was enhanced by ~12-fold (in both finger and toe nails) due to iontophoresis. Release of terbinafine from the iontophoresis-loaded nails into agar plates exhibited two phases, a rapid phase followed by a steady release, which extended >2 months. This study concluded that the drug delivery in onychomycotic nails did not differ significantly when compared with normal nails, although the extent of drug permeation and drug load differs between finger and toe nails.

Keywords: Drug release, iontophoresis, microscopy, onychomycotic nails, terbinafine

Introduction

Onychomycosis accounts for one-third of all nail infection in the nail plate and nail bed, which affects over 13% of the world population[1]. This fungal infection in the nails is primarily (~90%) caused by the *Trichophyton rubrum* and *Trichophyton mentagrophytes*, anthropophilic dermatophytic pathogens, and occasionally by non-dermatophytic molds and yeasts[2]. Among the nails, toe nails are more prone to onychomycosis infection when compared with finger nails (4:1) and required prolonged treatment[3,4]. Based on the route of fungal invasion in the nails, onychomycosis is classified into four categories namely distal subungual onychomycosis, white superficial onychomycosis, proximal subungual onychomycosis, and total dystrophic onychomycosis[1]. The predisposing factors for this disorder are the presence of tinea pedis, cancer, immunodeficiency, psoriasis, diabetes, and age[5]. Onychomycosis become severe and leads to thickness of nail, onycholysis, disturbs the physical mobility and activity due to pain, in addition leads to secondary bacterial infection, if untreated[6]. Reports also indicated that this disorder generally leads to patient–social isolation and negatively impacts the quality of life[7].

Existing treatment modalities in onychomycosis and recalcitrant dermatophytosis are oral and topical therapies, where the former is considered to be more effective when compared with the topical therapy[8,9]. However, the clinical and mycological cure rate with newer oral antifungal agents was not optimum (<70%) and the failure and recurrence rate was high[3,10,11]. On the other hand, efficiency of the topical therapy is much low due to the innate low permeability of nails[12]. As both the therapies have limitations, the successful treatment in onychomycosis remains elusive. However, topical therapy

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has an edge over the conventional oral treatment with respect to higher patient compliance, better proximity to the infected site, and minimal systemic side effects[13]. Recently, we demonstrated the utility of iontophoresis in the rapid delivery of a potent antifungal agent, terbinafine, into and through the normal human cadaver nail plate[14,15].

The nail anatomy of onychomycotic nails differs from the normal nails. Onychomycosis causes the nail plate to become thick, deform, hyperkeratize, crack, discolor, and undergo onycholysis[16]. Due to the difference in anatomical and biological features of the onychomycotic nails, the transungual drug delivery during iontophoresis is likely to alter. The study results using onychomycotic nail is believed to give better insight into the drug delivery *in vivo*, when compared with the normal nails. The objective of the current investigation was to assess the *in vitro* transungual iontophoretic terbinafine delivery in onychomycotic nails. Different from our earlier investigations, here we used nail specimen of both finger and toe nails diagnosed for onychomycosis (tested by KOH chlorazol black E microscopy method). A gel-based formulation, which was evaluated in our earlier study, was used to evaluate the effectiveness of the iontophoretic technique in the delivery of terbinafine in the diseased nail plates. This study is believed to give better insight into the drug delivery *in vivo*, when compared with the normal nails. To our knowledge, this is the first comprehensive report using onychomycotic nails as model for *in vitro* evaluation of transungual iontophoretic drug delivery of terbinafine.

Experimental

Materials

The composition (w/w %) of the TH formulation (pH 3.2) used in the current study comprised terbinafine HCl (4%) [MW = 327.90 Da, log octanol/water partition coefficient = 3.3, aqueous solubility = 1 mg/mL], 95% ethanol (21%), polysorbate 80 (5%), glycerin (40%), hydroxyethyl cellulose (0.3%), benzoic acid (0.2%), butylated hydroxyl toluene (0.01%), and disodium EDTA (0.01%). The composition in the gel formulation is suitable for iontophoretic delivery and optimized for pH, solubility, stability, and penetration enhancement from various surfactants, alcohols, antioxidants, and permeation enhancers. The gel formulation was prepared by weighing the required quantities of EDTA, benzoic acid, and butylated hydroxytoluene (BHT) and dissolved in water by constant stirring and heating (~50°C). Tween 80 and PEG were added with continuous mixing until a clear solution was obtained. The solution was then cooled to room temperature and added ethanol and terbinafine with continuous stirring. Hydroxyethyl cellulose dispersion was prepared in glycerin and added to the terbinafine solution and stirred for 12 h. The pH of the gel was adjusted to 3.5. Human cadaver onychomycotic/finger/toe nails, both male and female, aged between 49 and 86 years, with a thickness range of 0.4–0.7 mm were procured from Science Care (Phoenix, AZ) and were stored at 4°C until used. All other chemicals and reagents used were of analytical grade. All solutions were prepared in deionized water.

Analytical method

The amount of terbinafine in the samples was quantified by high-performance liquid chromatography (HPLC) system (Waters, 1525) with an autosampler (Waters, 717 plus) consisting of a Phenomenex C18 (2) 100 R analytical column (4.6 × 150 mm, Luna, 5.0 µm) and a variable wavelength dual λ absorbance detector (Waters, 2487). The mobile phase consisted of aqueous solution (0.096 M triethyl amine, 0.183 M orthophosphoric acid) and acetonitrile (60:40) adjusted to pH 2 with orthophosphoric acid. Elution was performed isocratically at 32°C at a flow rate of 1.0 mL/min. The injection volume was 20 µL and the column effluent was monitored at 224 nm. The method was validated by determination of linearity, precision, and accuracy. The range for the calibration curve was 2–1000 ng/mL ($R^2 = 0.99$). The coefficient of variation and the accuracy ranged 1.03–6.08% and –0.54–6.96, respectively.

Potassium hydroxide microscopy

Specimens were collected from the ventral surface of the each nail and placed on glass plate. Few drops of potassium hydroxide (KOH) solution (25% KOH, 5% glycerin, and chlorazol black) were added, heated at 50°C for 1 h, and examined under microscope (20× magnification—Zeiss MI, Thornwood, NY).

Permeation studies

Nails were cleaned and adherent tissues were removed with a pair of scissors and a scalpel followed by a water rinse. Each of the nail plates were soaked in 0.9% w/v saline for 1 h immediately prior to use and mounted on a nail adapter (PermeGear, Bethlehem, PA). The whole assembly was sandwiched between the two chambers of a Franz diffusion cell with an active diffusion area of 0.2 cm^2 (Logan Instruments Ltd., Somerset, NJ). Ag/AgCl wire electrodes (0.5 mm diameter; Alfa Aesar, Wardhill, MA) were fixed at a distance of 2 mm from the nail surface in donor and receiver chambers. A custom-made power supply was used for the application of a constant DC current. The anode was connected to the donor and the cathode to the receiver chamber and iontophoresis was carried out for a period of 1 h at a current density of 0.5 mA/cm^2. The gel formulation (500 µL) was placed in the donor chamber. The receptor compartment, which had a capacity of 5 mL and was filled with acidified water (adjusted to pH 3 using 0.01 N HCl), provides sink conditions due to increased drug solubility and contributes chloride ions to help maintain current flow. The receiver compartment was stirred at 600 rpm with a 3-mm magnetic stir bar at room temperature. Similarly, passive permeation experiments were run in parallel with iontophoresis at 0 mA/cm^2 current density for 1 h, which served as control for iontophoresis experiments (1 h). After application of current or

passive delivery, the receptor compartment was analyzed for terbinafine concentration and the cumulative amount of terbinafine permeated into the receiver chamber normalized to the surface area exposed to the drug was expressed as μg/cm^2.

Drug load

After the *in vitro* diffusion studies, the nail plate that was directly in contact with the formulation was easily discriminated and marked for active diffusion area (0.2 cm^2) using permanent marker and metric punch, before the washing protocol was followed. The nail plate was washed with water and alcohol five times each using a standardized protocol to avoid the washout of drug loaded into the nail while removing surface drug. In brief, washing was carried out by holding the nail with forceps and shaking twice by placing in 2 mL of water (pH 3). Such five washings were performed in fresh 2 mL of pH 3 water each time. The nail surface was cleaned using a cotton swab soaked in 95% ethanol and rinsed with 1 mL ethanol (95%)—this procedure was repeated five times. The nail surface was wiped with Kim wipe®. Further, to prevent deviation in the border between the active diffusion and the peripheral region of the nail plate, the active diffusion area and peripheral region were precisely separated using a metric punch with diameter of 2 mm. The active diffusion area of the nail plate was weighed (~5–8 mg and 7–12 mg in the case of finger and toe nails, respectively) and dissolved in 1 M sodium hydroxide (1.5 mL) by constant overnight stirring. Extraction of drug was carried out by a slight modification of the method described by Dykes et al.[17] In brief, after dissolving the nails in the vials, 200 μL of 5 M hydrochloric acid was added to neutralize the mixture. Terbinafine was extracted by adding hexane (3 mL) to the vial and shaking manually for 30 min. The mixtures were transferred into centrifuge tubes and centrifuged at 4000 rpm for 10 min. The hexane layer was collected, 1 mL of 0.5 M sulfuric acid–isopropyl alcohol mixture (85:15) was added, and the mixture was shaken vigorously for 30 min. The lower acidic aqueous layer, which holds the majority of terbinafine, was collected separately and the amount of drug in the nail was determined. This extraction procedure was validated by spiking different drug concentrations (2–20 μg/mL) into sodium hydroxide solution in which the nail was previously dissolved. The recovery was found to be 86 ± 5%.

The amount of terbinafine, which had diffused into the peripheral region (4–5 mm surrounding the active diffusion area; nail not directly in contact with the donor chamber), was determined by dissecting the peripheral nail area, which was then washed, dried, and weighed (~40–75 mg and 85–105 mg in the case of finger and toe nails, respectively). The amount of drug loaded into the nail was determined as described before.

Drug release studies

Release of terbinfaine from drug-loaded finger and toe nails was carried out by agar diffusion method after loading the drug into the nails by iontophoresis (0.5 mA/cm^2) or passive processes for 1 h. Initially the drug-loaded nail plates (~3 × 3 mm) were placed, such that the dorsal side was facing up, on a potato dextrose agar (PDA) plate and a standard 4-day protocol was followed at 30°C incubation. At the end of every 4 days, the nail samples were transferred to new PDA plates and this procedure was followed up until the release reached plateau. The amount of terbinafine released into the agar was determined by extracting the drug from the agar plate. After the study period, the agar was transferred into a centrifuge tube, added 10 mL of 0.1 M HCl, and kept overnight at room temperature in dark. Samples were vortexed (4 min), centrifuged (4000 rpm for 10 min), and the supernatant was analyzed by HPLC.

Data analysis

Delivery enhancement of TH was calculated as the ratio of the iontophoresis value to the corresponding passive value. Statistical analysis was carried out by Kruskal–Wallis test or Mann–Whitney *U* test (Statistica software). *P*-value < 0.05 was considered statistically significant. The data points provided in the graphs are an average of three trials. The error bars represent the standard deviation.

Results and discussion

Numerous methods have been described to diagnose the nail infection, onychomycosis. These include direct microscopy techniques with KOH, KOH with dimethyl sulfoxide, or KOH with chlorazol black E and fungal culture on dermatophyte test medium or mycobiotic and inhibitory mold agar. However, KOH with chlorazol black E was reported to have higher sensitivity (94%) and

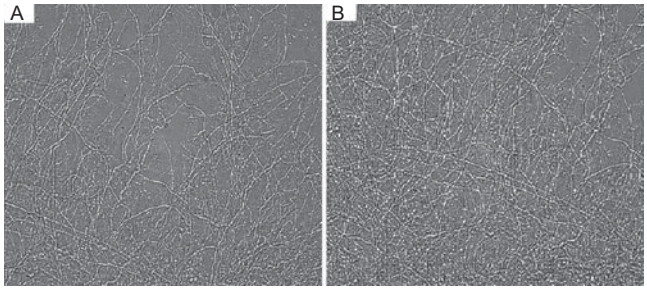

Figure 1. Light microscopy pictures of onychomycotic finger (A) and toe (B) nail specimens by KOH microscopy counterstained using chlorazol black E showing hyphae.

is cost-effective[3]. Hence, in the current study, presence of fungus in all the onychomycotic nails was tested by KOH chlorazol black E microscopy method. The KOH specimens were counterstained using chlorazol black E, which is chitin-specific, stains hyphae blue–black in color for easy visualization[1]. Figure 1 shows the KOH microscopy pictures of the onychomycotic nails showing the presence of hyphae, a long, branching filamentous cell of a fungus.

It is well-known that the effective therapy in topical nail delivery depends on the ability of antimycotic agents to permeate into the deeper stratums of the infected nail and to accomplish its activity. Unfortunately, most of the existing antifungal molecules possess the inherent property to permeate across the keratinized nail plate. Iontophoretic permeation studies using TH was carried out across the onychomycotic nails at a constant current (0.5 mA/cm^2) for a period of 1 h. The current density of 0.5 mA/cm^2 was chosen as it is reported to be well-tolerated *in vivo* during transdermal drug delivery applications[18]. Figure 2 represents the amount of terbinafine permeated through the onychomycotic finger and toe nails by passive process and iontophoresis. The enhancement in drug permeation in finger and toe nails by iontophoresis was 21- ($P = 0.001$) and 37-fold ($P = 0.003$) higher than the passive process, respectively. The extent of permeation across the finger nails was higher than across the toe nail in both passive and iontophoretic delivery, which is likely due to the thicker and denser nature of the toe nails.

Nail plates generally retain antifungal agents during long-term systemic delivery and eventually release the drug from reservoir into the nail bed. The maintenance of effective drug levels in the nail apparatus is crucial in determining the efficacy of the treatment. Therefore, drug loaded into the nail plate holds a lot of clinical significance. The actual amount of drug loaded (μg) into the diffusion area of the nail plates during iontophoresis and passive processes is summarized in Table 1. Figure 3 represents the amount of drug loaded (μg/mg; normalized to nail weight) into the onychomycotic finger (10.20 ± 3.74 μg) and toe nails (6.53 ± 1.50 μg) during iontophoretic delivery, which was found to be significantly higher (~12-fold) than their respective passive process (0.83 ± 0.10 μg and 0.57 ± 0.30 μg in finger and toe nails, respectively). These data signify that the amount of drug loaded into the finger nails and toe nails differs (in both passive and iontophoretic processes). Further, the amount of drug loaded due to iontophoresis (0.5 mA/ cm^2 for 1 h) in finger and toe nails is three and two orders of magnitude, respectively, higher than the amount in nail by oral therapy (0.5 μg/g) after daily administration of 250 mg for 4 weeks[19]. On the other hand, a moderate amount of drug was loaded in the passive experiments as well (0.83 ± 0.10 μg and 0.57 ± 0.30 μg in finger and toe nails, respectively). However, the total drug load in the nail plate during passive process does not necessarily correlate with antifungal efficacy as it has been reported by several research groups that the higher amount of drug in passive process is due to the retention of drug in the upper dorsal surface[15,20]. Nevertheless, iontophoresis was demonstrated (both qualitative and quantitative) to drive significant drug into the deeper nails, which resulted in uniform drug distribution in all the layers of the nail[14]. It is most likely that the iontophoresis leads to significant distribution of drug in all the layers of onychomycotic nails as well.

Generally the whole nail apparatus, including the nail that is not exposed due to overlapping by the nail fold, will be affected in nail diseases. The drug loaded in the active diffusion area represents the area of nail that is in direct contact with the formulation. *In vivo*, this would be the exposed part of the nail available for application of drug formulation. Therefore, the drug loaded in the active diffusion area is required to diffuse laterally into the area that is unexposed to formulation; this in turn would play a crucial role in the success of

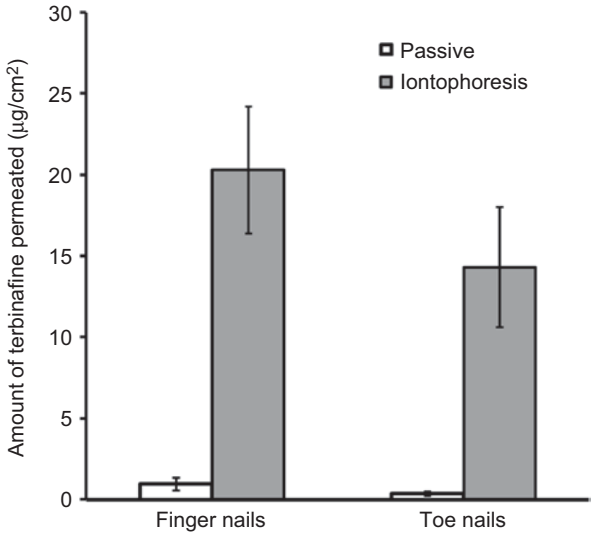

Figure 2. Comparison of terbinafine permeation across the onychomycotic finger and toe nails with iontophoresis and passive studies after 1 h. Data expressed as means ± SD ($n = 3$).

Table 1. Amount of terbinafine loaded (μg) into the nail regions of onychomycotic finger and toe nails with iontophoresis (0.5 mA/cm^2) and passive processes after 1 h.

	Amount of drug in nail (μg)			
	Finger nail regions		Toe nail regions	
Mode of drug loading	Active diffusion	Peripheral region	Active diffusion	Peripheral region
Passive	0.827 ± 0.095	0.130 ± 0.047	0.573 ± 0.299	0.099 ± 0.039
Iontophoresis	10.204 ± 3.744	1.18 ± 0.328	6.526 ± 1.496	0.898 ± 0.214

Data expressed as means ± SD ($n = 3$).

topical therapy of nail diseases. In the present permeation experiments, the amount of drug loaded into the surrounding of the active diffusion nail area (~4–5 mm) was also assessed. The amount of drug reaching the peripheral nail area depends on the concentration gradient between the active diffusion area and peripheral nail area. Figure 4 compares the amount of terbinafine loaded (μg/mg; normalized to nail weight) in the peripheral region of the nail during iontophoresis and passive processes. It is apparent from the figure that the peripheral drug load was higher during iontophoresis (~9-fold) when compared with their respective passive process. However, it is evident from Table 1 that

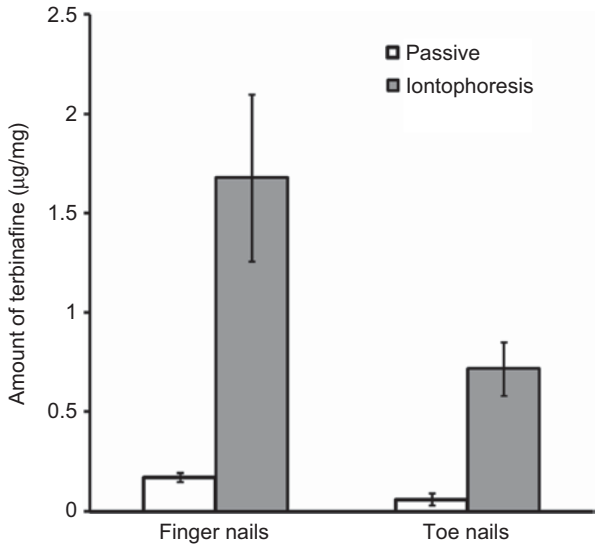

Figure 3. The amount of TH loaded (μg/mg) in the diffusion area of onychomycotic finger and toe nails with iontophoresis and passive studies after 1 h. The diffusion area was 0.2 cm^2. Iontophoresis was carried out by applying different current density of 0.5 mA/cm^2. Data expressed as means ± SD (n = 3).

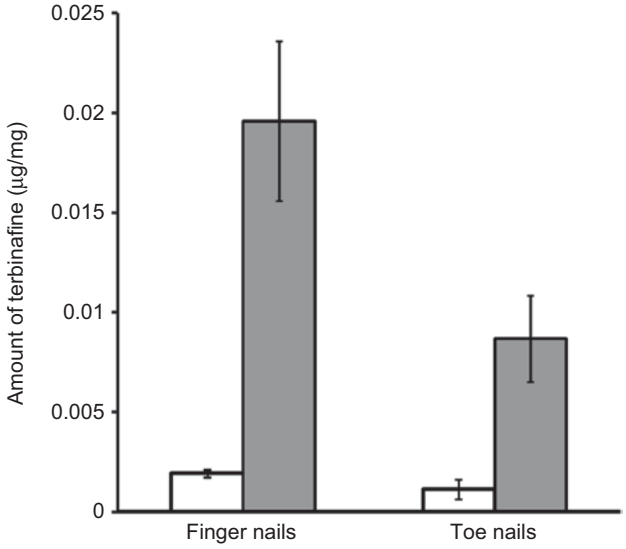

Figure 4. The amount of TH loaded (μg/mg) in the peripheral area of onychomycotic finger and toe nails with iontophoresis and passive studies after 1 h. The peripheral area was 4–5 mm surrounding the active diffusion area. Iontophoresis was carried out by applying different current density of 0.5 mA/cm^2. Data expressed as means ± SD (n = 3).

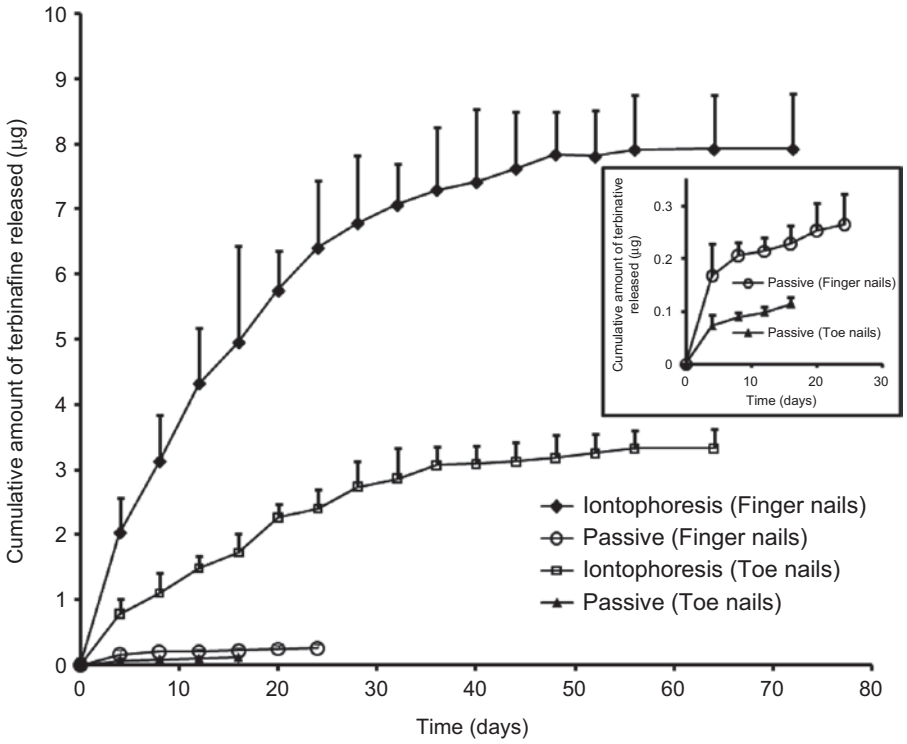

Figure 5. Amount of terbinafine released into the agar from loaded onychomycotic finger and toe nails. The drug loading was carried out by iontophoresis (0.5 mA/cm^2 for 1 h) and passive delivery for 1 h, respectively. The insert figure has been provided for better clarity of drug release profile from nails loaded by passive delivery. Data expressed as means ± SD (n = 3).

the amount of drug diffused into peripheral area is ~10 times lesser than the amount of drug diffused into their respective active diffusion areas (0.2 cm^2).

The drug-load data (in nail plate) obtained in the current study indicates that a higher amount of terbinafine was loaded into the nail during the iontophoresis process as compared with passive delivery. Hence, to assess whether the terbinafine loaded in the onychomycotic nail would eventually be discharged over a period of time, release studies were carried out by agar diffusion method. Figure 5 represents the release profile of terbinafine from drug loaded by various treatments. It is obvious from the figure that iontophoretically loaded nails exhibited a two-phase release, consisting of an initial rapid release (up to 16 days) phase, which was followed by a slower release phase, up to 72 and 64 days in finger and toe nails, respectively (Figure 5). The higher release in the initial stage may be due to the free drug in the nail, whereas the slow release phase may be due to the diffusion of drug molecules that were entrapped in the nail or bound to keratin. Further, higher amount of drug (~90% of the total released) was released within 32 days, in both finger and toe nails. The percentage of drug released in finger and toe nails were 65.94 ± 2.87% and 45.69 ± 2.87%, respectively. However, the passive loaded nails released much less drug in a single slow phase, and the amount released from the total drug loaded were 28.03 ± 10.20% (24 days) and 13 ± 2.69% (16 days) in finger and toe nails, respectively. This study concluded that the drug loaded in the nail due to iontophoresis could preserve drug levels in the nail apparatus over long time, which in turn is likely to extend the duration of activity of drug. Terbinafine permeation across and drug load into the onychomycotic finger nails by passive and iontophoretic delivery when compared with our earlier normal nail studies under the similar experimental condition indicated no significant difference[14]. Hence, the current study concluded that the drug permeation, drug load, and release of antifungal agents did not vary significantly between the onychomycosis and normal nails. Further, the utility of iontophoresis in enhancing the transungual delivery of antifungal agents is substantiated.

Declaration of interest

The authors report no declarations of interest.

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