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Short Communication

Topical nitric oxide releasing nanoparticles are effective in a murine model of dermal *Trichophyton rubrum* dermatophytosis

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Abstract

Systemic therapies are preferred for treating dermal dermatophytosis due to inadequate penetration of topical agents. However, systemic antifungals are associated with off-target effects and limited tissue penetration, and antimicrobial resistance is a growing concern. To address this, we investigated topical nitric oxide-releasing nanoparticles (NO-np), which have been used against superficial fungal infections and bacterial abscesses. In addition to enhanced penetration and permeation conferred by nanoparticles, nitric oxide, a broad-spectrum multi-mechanistic antimicrobial agent, offers decreased likelihood of resistance development. In the current study, NO-np inhibited *Trichophyton rubrum* *in vitro*, as well as in a murine model of dermal dermatophytosis. In mice, NO-np reduced fungal burden after three days, with complete clearance after seven. Furthermore, NO-np decreased tissue IL-2, 6, 10 and TNF α , indicating earlier attenuation of the host inflammatory response and decreased tissue morbidity. Thus, topical NO-np represent an attractive alternative to systemic therapy against dermal *T. rubrum* infection.

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Dermatophytoses afflict millions of people worldwide. Depending on infection depth and location, treatment failure may occur with both topical and systemic medications due to poor tissue penetration, side effects, lengthy treatment schedules, antifungal resistance, and failure to eliminate dormant spores.¹ Although cutaneous dermatophytoses are frequently limited to the epidermis, fungi may invade into the dermis and subcutaneous tissue,

especially when precipitated by trauma, immunosuppression, or use of topical corticosteroids. Deep cutaneous infection requires systemic therapy; however, conventional medications pose a range of side effects and undergo hepatic first-pass metabolism and extensive plasma protein binding, exemplifying inefficient drug delivery. Given these challenges, a topical antifungal capable of penetrating the stratum corneum and retaining potent activity in

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deeper skin layers while evading antimicrobial resistance mechanisms is highly desired.²

Nitric oxide (NO), a gaseous immunomodulator with broad-spectrum, multi-mechanistic antimicrobial activity, is an attractive agent to fill this need. Although NO is ubiquitous throughout the body, therapeutic delivery is challenging due to its short half-life and high reactivity.³ This can be overcome by a previously-characterized nanoparticle platform that generates and releases NO over time (NO-np).^{4–6} Nanoscaled particles (1–100 nm) offer enhanced cutaneous penetration and increased skin surface residence time (creating a depot effect), and their extremely large surface area increases their likelihood of interaction with fungal cells. When NO-np are exposed to moisture, NO is formed from encapsulated dinitrogen trioxide and released in a controlled, sustained manner, reaching a steady state between 184 and 196 ppb NO/mg within 40 min that lasts for several hours.^{5,7}

A previous study with fluorescently-labeled nanoparticles indicates that NO-np penetrate the epidermal barrier and localize within hair follicles, which may serve as a reservoir for NO-np and potentiate the sustained release benefit.⁸ Hair follicles are also the site of dermatophytosis in Majocchi's granuloma, a specific type of deep cutaneous dermatophytosis. Thus, NO-np may offer enhanced targeting of this infection.

In other prior investigations, NO-np have exhibited activity against yeast and filamentous forms of *Candida albicans*,⁹ as well as a variety of gram-positive and -negative bacteria,¹⁰ with limited toxicity in *in vitro* and *in vivo* evaluations.¹¹ Topical NO-np have also been used to treat murine dermal and intramuscular bacterial abscesses, deeper infections which would otherwise require systemic therapy. In abscess models, topical NO-np led to

decreased bacterial burden, as well as preservation of cutaneous and muscular architecture, respectively.^{12,13} Given their broad activity against fungal and bacterial species, as well as previous successes in treating deeper models of infection, the current study assessed topical NO-np against *Trichophyton rubrum*, both *in vitro* as well as in a murine model of dermal dermatophytosis.

Methods

NO-np were synthesized as previously described.⁶ Minimum inhibitory concentrations (MICs) were determined for two *T. rubrum* reference strains (ATCC MYA-4438 and ATCC 28189) and a clinical isolate using methods proposed by the Clinical and Laboratory Standards Institute (CLSI), modified by the use of a resazurin colorimetric assay.¹⁴ Minimum fungicidal concentrations (MFCs) were determined as previously described.¹⁵ Additionally, antimicrobial effects of the MIC of NO-np were visualized directly by transmission electron microscopy (TEM) following 96 h incubation with ATCC MYA-4438.

Dermal dermatophytosis was simulated with ATCC MYA-4438 on dorsal surfaces of BALB/c mice using a previously described model.¹⁶ Seven days post-infection, mice began daily topical treatment with 100 μ L of either saline, coconut oil, 10 mg/mL empty nanoparticles, 10 mg/mL NO-np, or 1% terbinafine. Nanoparticles and terbinafine were delivered in coconut oil. Following three and seven days of treatment, homogenized tissue samples were filtered with a 40 μ m cell strainer and plated on potato dextrose agar. Colony forming units (CFUs) were quantified after 72 h. Following three days of treatment, cytokine analysis was performed on supernatants

	C-np		NO-np		TRB		FLZ		
	MIC (mg mL)	MFC (mg mL)	MIC (mg mL)	MFC (mg mL)	MIC (mg L)	MFC (mg L)	MIC (mg L)	MFC (mg L)	
Reference strain 1 ATCC MYA-4438	>20	>20	10	>20	0.03	-	4	-	A
Reference strain 2 ATCC 28189	>20	>20	10	>20	0.06	-	64	-	
Clinical Strain	>20	>20	5	>20	0.25	-	32	-	

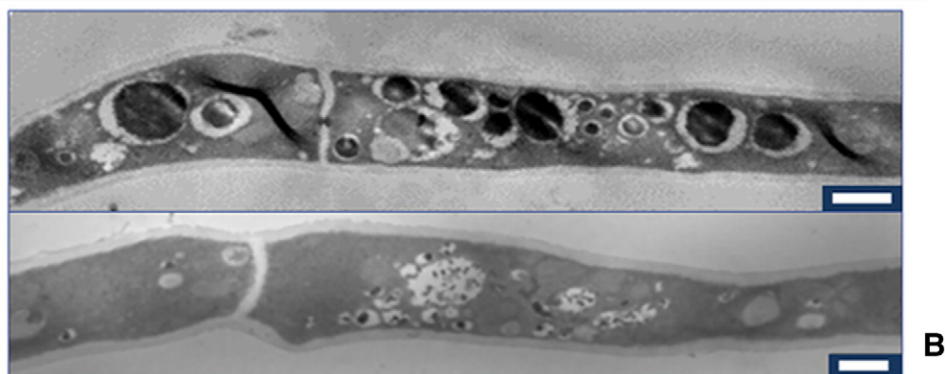


Figure 1. NO-np inhibit *Trichophyton rubrum* *in vitro*. (A) Minimum inhibitory and fungicidal concentrations (MICs and MFCs) of NO-np against *T. rubrum* reference strains (ATCC MYA-4438; ATCC 28189) and a clinical strain performed in triplicate. C-np denotes control (empty) nanoparticles. Quality control tests were performed with terbinafine (TRB) and fluconazole (FLZ), as proposed by the Clinical and Laboratory Standards Institute.¹² (B) TEM of untreated *T. rubrum* reference strain (upper panel) versus the same strain with 10 mg/mL NO-np (lower panel) shows accumulation of material suggestive of intracellular vesicles and black granules within the vacuoles at the MIC of NO-np. Scale bars = 1 μ m.

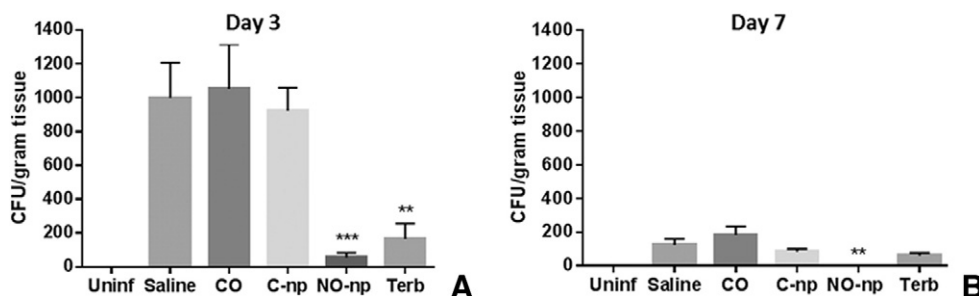


Figure 2. NO-np decrease fungal burden in a murine model of dermal dermatophytosis. Quantification of colony forming units (CFUs) from mouse tissue following 3 (A) and 7 (B) days of treatment. Groups include uninfected mice (Uninf), saline, coconut oil (CO), control (empty) nanoparticles (C-np), NO-np and terbinafine 1% (Terb) treated mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $N = 14$. Error bars denote SEM.

from homogenized tissue using a BD Cytometric Bead Array Mouse Cytokine Kit and BD LSR II flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed with FlowJo software (FlowJo, Ashland, OR). All procedures for animal experimentation were approved by the Institutional Animal Care and Use Committee at Albert Einstein College of Medicine (protocol #20150403), and international standards were followed to ensure humane treatment of animals.

Results

The MICs of NO-np for the *T. rubrum* reference strains and clinical isolate were 10 and 5 mg/mL, respectively. MFC results demonstrated fungistatic behavior of NO-np, and empty nanoparticles did not demonstrate antifungal activity at concentrations up to 20 mg/mL (Figure 1, A). On TEM, accumulation of material suggestive of intracellular vesicles and black granules within

vacuoles was observed in the fungal cells following incubation with the MIC of NO-np (Figure 1, B).

CFU quantification revealed decreased fungal burden in NO-np treated mice compared to saline controls following 3 and 7 days of treatment (Figure 2, A and B). After 7 days, no fungal growth was observed only in the NO-np group. Significant decreases in IL-2, 6, 10 and TNF α (Figure 3–D) were detected in NO-np treated mice, whereas terbinafine led to decreased IL-6 only.

Discussion

Our results present topical NO-np as a potential treatment for dermal *T. rubrum* infection. NO exerts direct fungistatic activity *via* DNA damage, lipid peroxidation, and enzyme inactivation,³ as well as indirect activity *via* upregulation of macrophage phagocytic activity.¹⁷ Of note, NO-np exhibit trans-nitrosylation

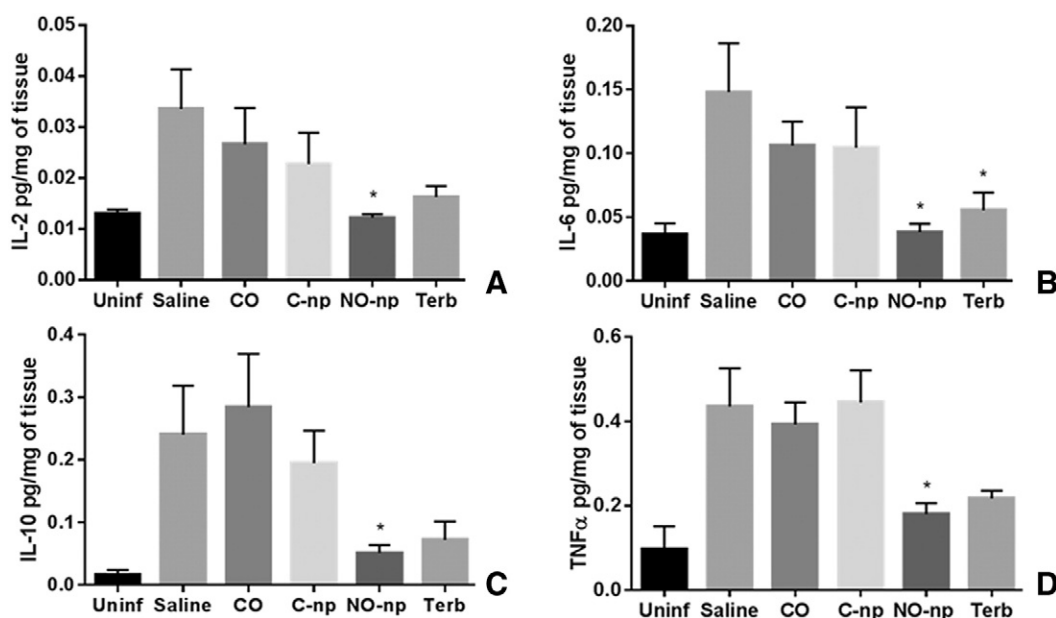


Figure 3. NO-np attenuates the host inflammatory response in a murine model of dermal dermatophytosis. IL-2 (A), 6 (B), 10 (C) and TNF α (D) as measured by enzyme linked immunosorbent assay following 3 days of treatment. Groups include uninfected mice (Uninf), saline, coconut oil (CO), control (empty) nanoparticles (C-np), NO-np and terbinafine 1% (Terb) treated mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $N = 7$. Error bars denote SEM.

activity in addition to NO release, which likely explains the significant effects visualized on TEM.¹⁸

In addition to fungal death, NO-np were associated with decreased IL-2, 6, 10 and TNF α , consistent with a prior study in which exogenous NO downregulated TNF α and IL-2 from murine macrophages and Th1 cells, respectively, without affecting Th2 cytokines.¹⁹ As NO is an immunomodulator, significant cytokine attenuation in the NO-np group compared to terbinafine is not surprising.^{17,20,21} Although antifungal activity associated with TNF α downregulation may seem counterintuitive, another study showed that while *T. rubrum* infection upregulates TNF α and IL-10 from murine macrophages, autocrine activity was not observed in this setting.²² Rather, the authors postulated, the induction of TNF α may play immunoinflammatory roles favoring fungal growth, thereby enhancing *T. rubrum* virulence.^{22,23}

While cytokines' roles in *T. rubrum* infection and NO's complex, often paradoxical immunomodulatory functions continue to be investigated, our study demonstrates 95% *T. rubrum* clearance by day 3 of NO-np treatment coupled with significant cytokine downregulation, indicating an overall earlier attenuation of the host inflammatory response not observed with terbinafine. Given the enhanced inflammatory milieu and fungal clearance, NO-np may reduce local tissue morbidity associated with dermal dermatophytosis.

The effectiveness of topical NO-np is important given that systemic therapy is currently the only option against dermal dermatophytosis. Future studies should aim to translate these findings to human disease, as patients could benefit from shorter treatment courses, lack of systemic side effects and decreased risk of antimicrobial resistance. Furthermore, additional animal models may be pursued to determine NO-np utility in other models of dermatophytosis, such as tinea unguinum or tinea capitis, where inadequate penetration of conventional agents poses similar barriers to topical therapy.

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