

The effect of K101 Nail Solution on *Trichophyton rubrum* and *Candida albicans* growth and ultrastructure

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Summary

K101 Nail Solution (trademarks Emtrix[®], Nalox[™], Naloc[™]) is a combination of propylene glycol, urea and lactic acid in a topical formulation for the treatment of nails affected by onychomycosis. The aim of this study was to investigate the Minimal Cidal Concentration (MCC) of K101 Nail Solution against *Trichophyton rubrum* and *Candida albicans* as well as the effect of K101 Nail Solution on the micromorphology of these fungi. The MCC of K101 Nail Solution against *T. rubrum* and *C. albicans* was 1.0% after 60-min exposure time. A MCC of 1.0% for K101 Nail Solution means that K101 Nail Solution diluted with e.g. water to 1.0% will totally kill the fungi tested. In the scanning electron microscope *C. albicans* cells, treated with 1.0% K101 Nail Solution, showed a shrunken surface. *T. rubrum* cells were severely damaged shown as collapse and degradation of the cells. In the transmission electron microscope most *C. albicans* cells, treated with 1.0% K101 Nail Solution exhibited destroyed organelles and many necrotic cells were found. The cell wall was clearly degraded and the contact between the cell wall and the inner membrane was punctured. In *T. rubrum* most cells were necrotic. Some cells were clearly collapsed and the content in the cytoplasm was degraded shown as small membrane vesicles and many big vacuoles. The cell wall was clearly degraded and the membrane was punctured. In conclusion, this *in vitro* study documents the efficacy of K101 Nail Solution against *T. rubrum* and *C. albicans*.

Key words: K101 Nail Solution, *Candida albicans*, *Trichophyton rubrum*, Minimal Cidal Concentration, scanning electron microscopy, transmission electron microscopy.

Introduction

Onychomycosis is a fungal infection that affects the nails of the hand and foot. Infection rates in Western adult populations range from 2 to 14%, although onychomycosis may affect up to 10% of people over

70 years of age.¹ Prevalence of onychomycosis is also higher in the immuno-compromised and patients with diseases that affect the peripheral circulation, such as diabetes mellitus.^{2,3} Onychomycosis is often associated with pain and discomfort coupled with a significant negative impact on emotional health and social image.^{4,5} The dominating fungi in onychomycosis of the toenails are dermatophytes and *Candida* spp. are the dominating fungi in onychomycosis of the fingernails. Onychomycosis can be treated pharmacologically with both systemic and topical agents.⁶ Systemic antifungal drugs such as terbinafine and itraconazole are effective treatments; although their use must be balanced against the risk of side effects that include

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gastrointestinal disorders, skin rashes and headache.⁷ There is a definite but very low rate of drug toxicity and serious side effects occur in less than 1% of patients.⁸ Topical agents are usually formulated as lacquers that adhere to the nail plate and include antifungal drugs such as amorolfine, tioconazole and ciclopirox.⁷ Topical application allows targeted delivery to infected areas, minimising the risk of secondary effects related to systemic exposure. Topical treatment should only be used when there is no matrix involvement. However, this is not always the case and this could be one explanation for the poor result reported in some studies.⁷

K101 Nail Solution (available under trademarks such as Emtrix[®], Nalox[™], Naloc[™]) is a combination of propylene glycol, urea and lactic acid in a topical formulation for the treatment of nails affected by onychomycosis. The concept of using propylene glycol solutions of urea and lactic acid to treat onychomycosis has been confirmed in a placebo-controlled study that documented the efficacy and tolerability of K101 Nail Solution vs. placebo in 493 patients with onychomycosis. A greater number of patients who received K101 Nail Solution experienced mycological cure after 26 weeks of treatment (27% vs. 10%).⁹ Also, almost half the patients who received K101 Nail Solution considered that their condition had shown at least some improvement from week 2, and approximately 7% from week 8 of treatment onwards. This study was performed in order to study the mycological efficacy of K101 Nail Solution compared to placebo for a treatment period of 24 weeks. However, longer treatment studies are planned.

In another study 7 patients with onychomycosis were treated with K101 Nail Solution for 8 weeks.¹⁰ The aim of this study was to follow the effect of K101 Nail Solution on nail thickness, discoloration, brittleness and softening of the nails in the short term. Already after 2 weeks 77% of the patients reported at least some improvement increasing to 92% after 8 weeks.

The aim of this study was to investigate the Minimal Cidal Concentration (MCC) of K101 Nail Solution against *T. rubrum* and *C. albicans* as well as the effect of K101 Nail Solution on the micromorphology of these fungi.

Materials and methods

Test for MCC

This study was performed in the Mycology laboratory at the Department of Clinical Microbiology, Karolinska

University Hospital, Stockholm, Sweden. *T. rubrum* ATCC 22402 and *C. albicans* ATCC 90028 were used. They were maintained on Sabouraud's glucose agar at 30 and 37 °C respectively.

Test substance

K101 Nail Solution was obtained from Moberg Pharma, Bromma, Sweden.

Experimental procedure

K101 Nail Solution was instilled in glass tubes, either in the form of the original solution (100%) or mixed with distilled water to obtain a desired concentration of 10, 20 and 10% of K101 Nail Solution.

Trichophyton rubrum was harvested after 1 week of growth. The mycelium was dispersed in PBS (phosphate-buffered saline, pH 7.2) and crushed in a sterile mortar. The suspension was then filtered through filter gauze and adjusted to a transmittance of 6% at a wavelength of 330 nm (=conidia/hypha count by haemocytometer $\times 10^6$ per ml). From this suspension 100 μ l was added to the glass tubes with the various concentrations of K101 Nail Solution and mixed for 30 s. The final volume in the tube was one ml.

Candida albicans was harvested after 2 days of growth. *C. albicans* was then suspended in a glass tube with PBS, vortexed and diluted to obtain samples with a concentration of 10^6 cells ml⁻¹ (transmittance 76% at 330 nm). From this suspension 100 μ l was added to the glass tubes with the various concentrations of K101 Nail Solution and mixed for 30 s. The final volume in the tube was one ml.

K101 Nail Solution, in the various concentrations, was allowed to react with the *T. rubrum* and *C. albicans* cell suspensions for 60, 240 min and 24 h. After exposure time 20 μ l aliquots of the mixture (fungi in K101 Nail Solution) were plated onto Sabouraud's glucose agar in duplicate and the plates were incubated at 30°C for *T. rubrum* and 37°C for *C. albicans*. Plates with *T. rubrum* were read after 2, 6 and 14 days of incubation. Plates with *C. albicans* were read after 1 and 2 days of incubation.

Electron microscopy (EM)

Samples for EM were initially prepared at the Mycology Laboratory, Department of Dermatology, Sahlgrenska University Hospital, Gothenburg, Sweden and then sent to the Department of Laboratory Medicine, Karolinska Institute, Stockholm, Sweden for EM. *T. rubrum* from Culture Collection University of Gothenburg (CCUG) no. 36833 and *C. albicans* CCUG no. 32723

were used. They were maintained on Sabouraud's glucose agar at 32°C.

Experimental procedure

K101 Nail Solution was instilled in glass tubes, either in the form of the original solution (100%) or mixed with distilled water to obtain a desired concentration of 20% K101 Nail Solution.

In order to have enough material for EM the concentration of fungal cells/spores/hyphae had to be increased.

Trichophyton rubrum was harvested after 1 week of growth. The mycelium was dispersed in PBS (phosphate-buffered saline, pH 7.2) and crushed in a sterile mortar. The suspension was then filtered through filter gauze and adjusted to an optical density of 4.0 or 1×10^8 cells hyphae⁻¹ ml⁻¹. From this suspension 1 ml was added to the glass tubes containing 1 ml of the various concentrations of K101 Nail Solution and mixed for 30 s. The final volume in the tubes was 2 ml and the final concentration of K101 Nail Solution was 10% or 20%.

Candida albicans was harvested after 2 days of growth. *C. albicans* was then suspended in a glass tube with PBS, vortexed and diluted to obtain samples with a concentration of 10^8 cells ml⁻¹. From this suspension 1 ml was added to the glass tubes containing 1 ml of the various concentrations of K101 Nail Solution and mixed for 30 s. The final volume in the tubes was 2 ml and the final concentration of K101 Nail Solution was 10% or 20%. The above procedures were performed in duplicate, one sample for EM and one for culture.

K101 Nail Solution, in the various concentrations, was allowed to react with the *T. rubrum* and *C. albicans* suspensions for 60 min. When K101 Nail Solution had reacted with *T. rubrum* and *C. albicans* 20 µl of the mixture was transferred to Sabouraud's glucose agar and incubated at 32 °C. Plates with *T. rubrum* were read after 2, 6 and 14 days of incubation. Plates with *C. albicans* were read after 1 and 2 days of incubation.

Transmission electron microscopy (TEM)

The 2 ml were centrifuged at 2900 g for 5 min. The pellet was added to 1 ml mixture of 3% glutaraldehyde and 1% paraformaldehyde in 0.1 mol l⁻¹ sodium cacodylate buffer and 0.1 mol l⁻¹ sucrose, pH 7.4 and stored in a refrigerator. The suspensions were centrifuged to a pellet and rinsed in 0.1 mol l⁻¹ phosphate buffer (0.1 mol l⁻¹ PB) pH 7.4 followed by postfixation

in 2% osmium tetroxide in 0.1 mol l⁻¹ PB at +4°C for 2 h, dehydrated in ethanol followed by acetone and embedded in L -112 (Ladd, Burlington, VT, USA). Ultrathin sections (approximately 40-50 nm) were cut by a Leica EM UC 6 (Leica, Wien, Austria). Sections were contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 12 Spirit Bio TWIN TEM (FEI Company, Eindhoven, The Netherlands) at 100 kV. Digital images were taken by using a Veleta camera (Olympus Soft Imaging Solutions, GmbH, Münster, Germany).

Scanning electron microscopy (SEM)

Specimens were fixed as described above. The specimens were briefly rinsed in distilled water and placed in 70% ethanol for 10 min, 90% ethanol for 10 min, absolute ethanol for 10 min at room temperature and in pure acetone for 10 min and then dried using a critical point dryer (Balzer, CPD 010, Lichtenstein) using carbon dioxide. After drying, specimens were mounted on an aluminium stub and coated with Carbon (Bal-Tec MED 010, Lichtenstein). The specimens were analysed in an Ultra field emission scanning electron microscope (Zeiss, Oberkochen, Germany) at 3 kV.

Control suspensions with only *T. rubrum* and *C. albicans* in PBS were included for both growth control and for electron microscopy.

Results

Nail Solution was effective against both *T. rubrum* and *C. albicans*. However, it was more effective against *T. rubrum* with a MCC of 1.0% after 60 min and 20% after 240 min of incubation. The MCC of K101 Nail Solution against *C. albicans* was 1.0% after 60 min. A MCC of 1.0% for K101 Nail Solution means that K101 Nail Solution diluted with e.g. water to 1.0% will totally kill the fungi tested.

When we performed the MCC test with 10^8 cells conidia⁻¹ hyphae⁻¹ the results were

Table 2 The Minimal Cidal Concentration of K101 Nail Solution against *Candida albicans* and *Trichophyton rubrum* with an inoculum of 10^8 cells conidia⁻¹ hyphae⁻¹ per ml.

Fungus	K101 Nail Solution	60 min (CFU dish ⁻¹)
<i>Candida albicans</i> CCUG 32723	50%	2×10^3
	20%	$>10^6$
	Control	$>10^6$
<i>Trichophyton rubrum</i> CCUG 36833	50%	0
	20%	$>10^6$
	Control	$>10^6$

different with a much higher MCC (Table 2). However, this concentration is far too high to be used in a correct MCC setting. We performed it as a growth and activity control for the EM studies.

Scanning electron microscopy

Both untreated *C. albicans* (Fig. 1a and Fig. 1b) and *T. rubrum* (Fig. 2a and Fig. 2b) controls showed a normal morphology. In *C. albicans*, treated with 1.0% K101 Nail Solution, the cells showed a shrunken surface (Fig. 1c and Fig. 1d). The cells in *T. rubrum*, treated with 1.0% K101 Nail Solution, were severely damaged shown as collapse and degradation of the cells (Fig. 2c and Fig. 2d).

Transmission electron microscopy

Untreated control cells of *C. albicans* examined in TEM (Fig. 3a, 3c and 3e) displayed a normal morphology with intact cell walls and cytoplasm (Fig. 3a). The cytoplasm contained few but visible organelles

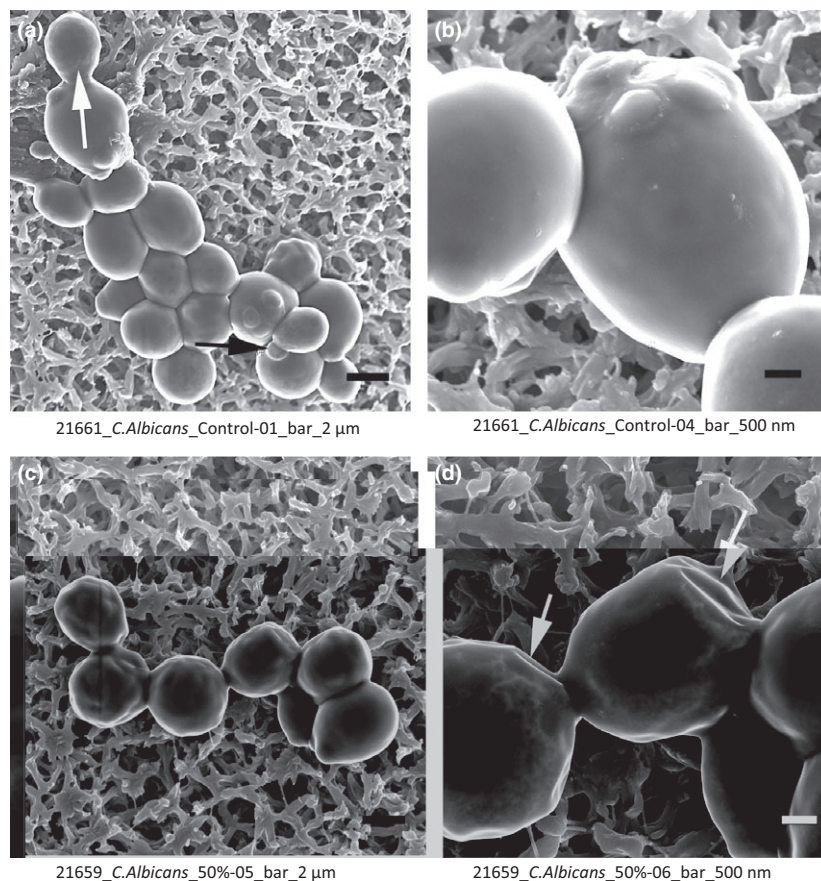


Figure 1 Scanning electron microscope (SEM) of *Candida albicans*. Untreated control images showing a cluster of well-preserved cells, some at dividing state (arrows) (a). Higher magnification of untreated cells showed a smooth and even surface of the cells (b). Cells treated with 1.0% K101 Nail Solution (c). The shapes in cells treated with 1.0% K101 Nail Solution: the cells are changed and the surfaces are shrunken (arrows) (d). Bars = 2 μm (a, c) and 500 nm (b, d).

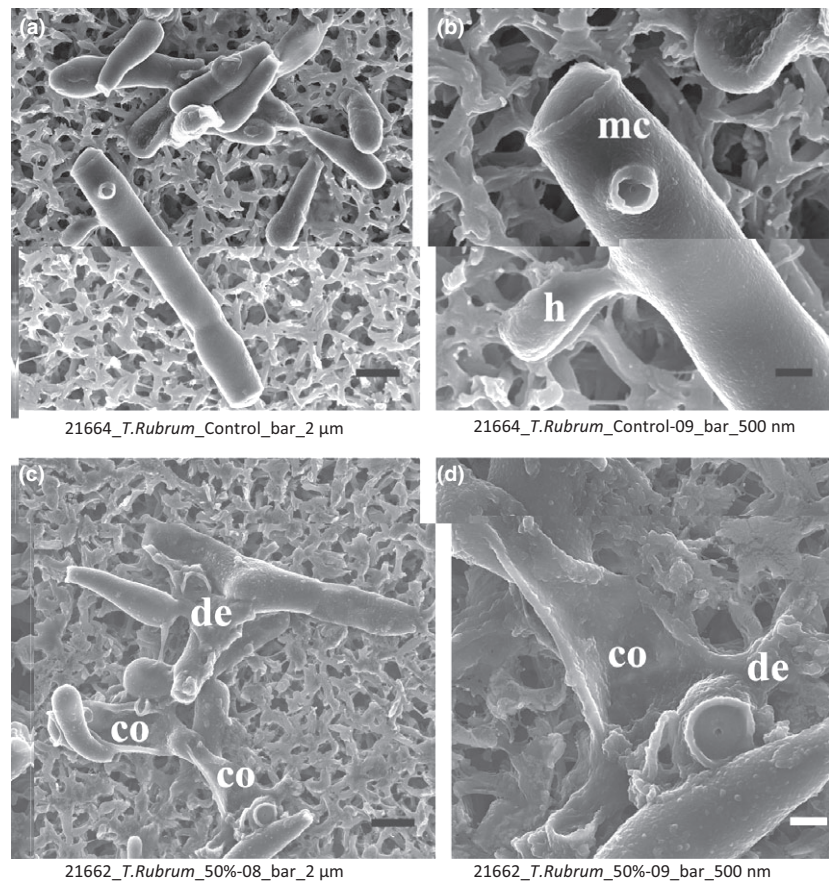


Figure 2 Scanning electron microscope (SEM) of *Trichophyton rubrum*. Untreated control image shows well-preserved cells (a) some with hyphae (h) and microconidia (mc) (b). Cells treated with 50% K101 Nail Solution demonstrate both collapsed (co) and degraded (de) cells (c, d). Bars = 2 μm (a, c) and 500 nm (b, d).

including mitochondria (Fig. 3c). The cell wall and the inner plasma membrane was intact (Fig. 3e). In *C. albicans*, treated with 50% K101 Nail Solution, the changes were prominent. In most cells the cytoplasm was degraded and the organelles were destroyed and many necrotic cells were found (Fig. 3b). The cell wall was clearly degraded and the contact between the cell wall and the inner membrane was punctured (Fig. 3) and remnants of membranes were found as vesicles in the cytoplasm (Fig. 3f).

Untreated control cells of *T. rubrum* (Fig. 4a, 4c and 4e) displayed a normal morphology with intact cell walls and cytoplasm (Fig. 4a and 4c). The cytoplasm contained few but visible organelles including mitochondria (Fig. 4e). The cell wall and the plasma membrane was intact (Fig. 4e). In *T. rubrum* treated with 50% K101 Nail Solution, the morphology was very prominent. Most cells were necrotic, shown as membrane debris (Fig. 4b). Some cells were clearly collapsed and the content in the cytoplasm was degraded shown as small membrane vesicles and many big vacuoles (Fig. 4d). The cell wall was clearly degraded and the membrane was punctured (Fig. 4d). Only

remnants of membranes were found as vesicles in the cytoplasm (Fig. 4f).

Discussion

The most important fungus in toenail onychomycosis is *T. rubrum* and in fingernail onychomycosis *C. albicans*. This study clearly documented the efficacy of K101 Nail Solution against these fungi *in vitro* with very good MCC values. Other studies have shown the clinical efficacy of K101 Nail Solution in the treatment of nails affected by onychomycosis.^{9,10}

The mechanism of action for diols (glycols) against microorganisms is primarily an effect on the cell wall and cell membrane. The permeability is thought to be increased and liquid can pass freely over the cell barrier giving rise to osmotic changes and collapses. TEM and SEM studies were performed in this study in order to document this process.

All untreated controls examined both in SEM and TEM showed a normal ultrastructure including cell division and organelles in the cytoplasm, intact cell walls and plasma membranes. In *T. rubrum* a distinct

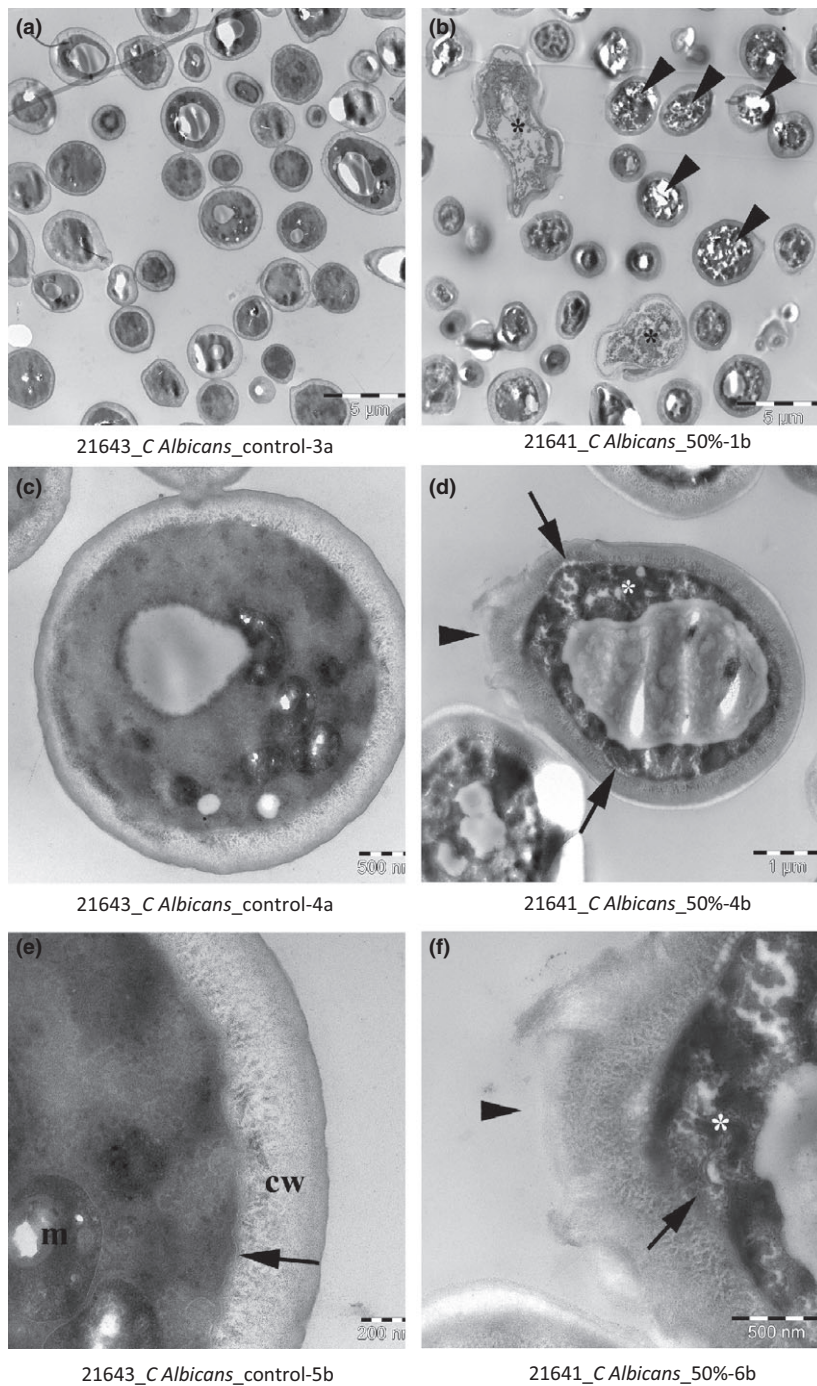


Figure 3 Transmission electron microscope (TEM) images of untreated control from *Candida albicans* (a, c, e) displayed a normal morphology with intact cell walls and cytoplasm (a). The cytoplasm contained few but visible organelles including mitochondria (m) (c). The cell wall (cw) and the inner plasma membrane was intact (arrow) (e). In *C. albicans*, treated with 50% K101 Nail Solution (b, d, f), the changes were prominent. In most cells the cytoplasm were disrupted and the organelles were destroyed (arrowheads) and many necrotic cells (*) were found (b). The cell wall was clearly degraded (arrowhead) and the contact between the cell wall and the inner membrane was disrupted (arrows) (d) and only remnants of membranes were found as vesicles in the cytoplasm (*) (f).

‘outer’ double membrane was observed (Fig. 4c). This ‘outer’ membrane has been described by Mares *et al.* [11] supporting our observation. The cell wall in *C. albicans* is seen as a multi-layered structure with a varying ultrastructure morphology depending of age and culture media but in our observation lacking the ‘outer’ membrane as seen in *T.rubrum*.¹² However, an

electron-dense small outer layer is clearly seen in the *C. albicans* also described by Bizerra *et al.* [13].

Treatment with 50% K101 Nail Solution clearly influenced the preservation of the cells. The most prominent changes were observed in *T. rubrum*. Here, a collapsed surface and degraded content of cells were observed. Evaluation by TEM showed numerous

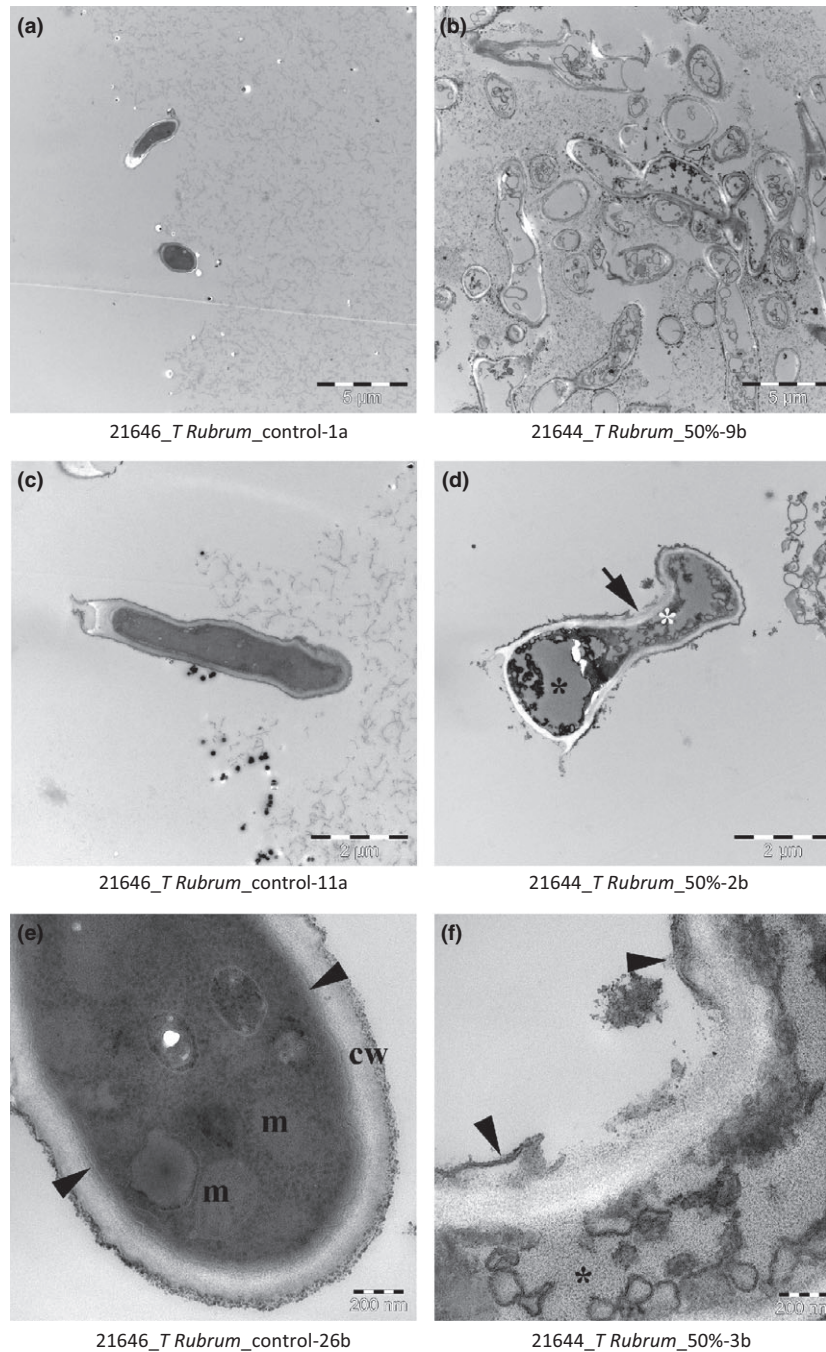


Figure 4 Transmission electron microscope (TEM) images of untreated controls from *Trichophyton rubrum* (a, c, e) displayed a normal morphology with intact cell walls and cytoplasm (a, c). The cytoplasm contained few but visible organelles including mitochondria (m) and the cell wall (cw) and the outer (arrow) and inner plasma membrane (arrowheads) was intact (e). In *T. rubrum* treated with 50% K101 Nail Solution (b, d, f), the morphology was very prominent. Most cells were necrotic, shown as membrane debris (b). Some cells were clearly collapsed and the content in the cytoplasm was degraded shown as small membrane vesicles and many big vacuoles (*) (d). The cell wall was clearly degraded and the membrane was punctured (arrow) (d). Higher magnification showing punctured membrane (arrowheads) and only remnants of membranes were found as vesicles (*) in the cytoplasm (f).

necrotic cells indicated by remnants of plasma membranes with a collapsed appearance. The cytoplasm contained big vacuoles and membrane vesicles. No organelles were visible. The cell wall was severely degraded and part of the plasma membrane was clearly punctured. These changes are in line with other studies showing similar changes. López-Villegas *et al.* [14] studied the ultrastructural changes by

Saponin SC-2 on *T. rubrum* showing main alterations on the cytoplasmic membrane including the cell wall and degradation of organelles and cellular death. A study on *T. rubrum* after treatment with 4-amino-3-methyl-1-phenylpyrazolo-(3,4-c) isothiazole, G8, showed autolytic phenomena and acting by breaking down the endomembrane system including the 'outer' membrane.¹¹ The effect of TDT067, a novel carrier-based

dosage form of terbinafine, on *T. rubrum* showed extensive ultrastructural changes such as cell collapsing, cell membrane deterioration and disruption of cellular materials and accumulation of lipid droplets. The later finding, accumulation of lipid droplets was not observed in this study. Use of conventional terbinafine resulted in similar morphological changes, however, not as severe compared with TDT067.

The changes observed in *T. rubrum* in this study were also seen in *C. albicans*, however, not to the same extent. The changes in *C. albicans* were similar; the cell wall showed degradation and the plasma membrane was partly punctured and the cytoplasm contained numerous small, membrane bound vesicles indicating autolysis. Treatment with Caspofungin (CAS) on four different *Candida* isolates showed alteration only in the cell wall but no major changes in the cytoplasm supporting the present cell wall changes.¹³

Our hypothesis is that K101 Nail Solution disturbs the cell wall integrity, in both *C. albicans* and *T. rubrum*, by degrading the contents in the cell wall as a first target and this is followed by degrading the inner plasma membrane. At this stage, the environment in the cytoplasm is dramatically changed since water can now freely pass the membrane thereby changing the osmotic environment in the cytoplasm giving rise to autolysis.

Pharmacologically-active antifungal substances used in medicinal products typically interact with cell metabolism by inhibiting the synthesis of specific enzymes (azoles, allylamines, amorolfine), bind to certain molecules in the cell membrane (polyenes) or components of the cell wall (e.g. caspofungin).¹⁶ Acquired resistance to these antifungal agents has been described and is an increasing problem. As for antibacterial resistance, the mechanism of resistance can very commonly be ascribed to the specific mode of action. Antimicrobial resistance against chlorhexidine has also been found¹⁷ and recent data strongly suggests a molecular basis for its antimicrobial action.¹⁸

In contrast, the observed structural changes following treatment with K101 Nail Solution implies an unspecific mode of action. Antimicrobial resistance has never been described with propylene glycol or K101 Nail Solution. This is probably because the mechanism of action of glycols and K101 Nail Solution is less specific: with physical degradation of the cell wall and cell membrane, and finally cytoplasmic organelles.

K101 Nail Solution is a mixture of propylene glycol, lactic acid and urea. The pH is approximately 4 which may contribute to its efficacy, compared with other topical agents.⁹ It is well known that a low pH in skin and nails is important for the antimicrobial protection

against both pathogenic fungi and bacteria.¹⁹ The 'acid mantle' of the skin will protect against infections from pathogenic microorganisms. Although a low pH may not inhibit growth, it can change the pathogenicity of the microorganisms. This has been shown for *C. albicans* where a low pH will not inhibit growth but will reduce the change from the blastospore form to the mycelium form.²⁰ This will reduce the risk for *C. albicans* infections. For *T. rubrum* the effect of pH is even more complex.²¹ Initially *T. rubrum* will respond to the environmental pH by de-repressing an offensive number of genes encoding for various proteins and enzymes that have optimum activity at acidic pH values. The hydrolysis of skin proteins will lead to a secretion of ammonia shifting the extracellular pH towards alkaline. This has also been shown *in vitro*.²² A topical formulation that will diminish this shift from acidity to alkaline will therefore reduce the risk for *T. rubrum* infections. Even if the growth of *T. rubrum* is not influenced by an acidic pH the production of arthroconidia is dependent on pH.²³ The highest production was seen at pH 7, and an 8% reduction was seen at pH 4.

In conclusion, this *in vitro* study documents the efficacy of K101 Nail Solution against *T. rubrum* and *C. albicans*.

Conflict of interest

This study was made possible through a grant from Moberg Pharma AB. Kjell Rensfeldt is an employee at Moberg Pharma AB.

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