The susceptibility of trichophyton rubrum to photodynamic treatment

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Chapter VI

MORPHOLOGICAL CHANGES OF THE DERMATOPHYTE *TRICHOPHYTON RUBRUM* AFTER PHOTODYNAMIC TREATMENT: A SCANNING ELECTRON MICROSCOPIC STUDY

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Trichophyton rubrum microconidia, destroyed by photodynamic treatment with Sylsens B and red light

ABSTRACT

Treatment strategies for superficial mycosis caused by the dermatophyte *Trichophyton rubrum* comprise of topical or oral application of antifungal preparations. We have recently discovered a susceptibility of *T. rubrum* to PDT, with Sylsens B as a photosensitizer. The susceptibility depended on the fungal growth stage; the PDT efficacy was higher in microconidia when compared to mycelium.

The aim of this study was to investigate the morphological changes caused by a lethal PDT dose to *T. rubrum* grown on isolated human stratum corneum with the use of scanning electron microscopy. Corresponding dark treatment and a light treatment without photosensitizer were used as control. Sub-lethal PDT was also included. The morphologic changes were followed at various time points after the treatment of different fungal growth stages.

Normal fungal growth was characterized by a fibre-like appearance of the surface of the hyphae and microconidia with the exception of the hyphal tips in full mycelium and the microconidia shortly after attachment to the stratum corneum. Here, densely packed globular structures were observed. The light dose (108 J/cm^2) in the absence of Sylsens B or the application of the photosensitizer in the absence of light caused reversible fungal wall deformations and bulge formation. However, after a lethal PDT, a sequence of severe disruptions and deformations of both microconidia and mycelium was observed leading to extrusion of cell material and emptied fungal elements. In case of a non-lethal PDT, fungal re-growth started on the remnants of the treated mycelium.

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INTRODUCTION

Superficial dermatomycosis caused by the dermatophyte *Trichophyton rubrum* is one of the most common human skin infections worldwide (1-4). The treatment often comprises the use of an antifungal drug in either a topical or an oral application (5-7) or a combination of both (8) .The frequently used drugs can be divided into three different groups, the polyenes, the azols and the allylamines (9,10). Apart from these groups griseofulvin (10) and cyclopiroxolamine (11) are occasionally used. Furthermore, efforts have been made to use PDT for dermatophyte infections (12-14). We have recently demonstrated that *T. rubrum* is also susceptible to PDT with the synthetic porphyrin photosensitizer, Sylsens B, both *in vitro* (15) and *ex vivo* (16). PDT refers to the use of light-activated agents, called photosensitizers, in combination with light of an appropriate wavelength and molecular oxygen (17). This photochemical reaction results in the production of reactive oxygen species, namely singlet oxygen ($^1O_2$) and superoxide anion radial (O_2^-). As a consequence, the photodynamic effect can cause the injury of cells and different kind of pathogens if they are in close proximity to the photosensitizer (18), leading to an effective treatment for localised infections (19). It is generally agreed that $^1O_2$ is the key agent responsible for the cellular damage during PDT. However this reactive oxygen has a short life time and hence its diffusion distance is very small (< 200 nm) (20). A selective, tight binding of the photosensitizer to the target microorganism is thought to be necessary for PDT effectiveness (21-23). Several studies concerning the action mechanisms of antifungals have been published (10,24-27). Some of the morphological studies showed degenerative changes of fungal mycelium and spores (10,28,29). Some of these investigations also included *T. rubrum* (30-32). However, there have been few reports describing morphological changes caused by PDT to microbes in general (33,34) and to *T. rubrum*, in particular.

In the present work we used scanning electron microscopy (SEM) to examine the effect of lethal PDT on *T. rubrum* grown on human stratum corneum (SC). Sylsens B was used as a photosensitizer. Special attention was paid to the normal *T. rubrum* morphology under the selected conditions. Since a thorough binding of photosensitizer to the fungus is essential for successful PDT, we focused on the effects of morphological alterations of fungal wall on treatment effectiveness. We investigated not only the effect of the PDT but also, under similar conditions, the morphological changes caused by Sylsens B in the dark and the effect of the light dose alone on the fungal growth.

In addition, we visualized the growth of this dermatophyte after sub-lethal PDT. The application of the different treatments was performed on a novel *ex vivo* model developed by us (16). Since our previous studies revealed that the susceptibility of *T. rubrum* to PDT with Sylsens B depended on the fungal growth stage (23), it was decided to visualize the morphological changes at various growth stages and at different time points after the treatment. For the different treatments the selected fungal growth stages corresponded to 8, 48 and 72 hours after spore inoculation on isolated human SC present in the *ex vivo* model (16). Under the given conditions, the 8-hour growth stage was characterized by germinating microconidia and the absence of fungal hyphae. At 48 hours after spore inoculation, hyphae growth started, while the 72-hour growth stage was represented by full mycelium.

MATERIALS AND METHODS

Materials

The fungus *T. rubrum* was purchased from the Centraalbureau voor Schimmelcultures (CBS, strain no: 304.60), Utrecht, The Netherlands. For the preparation of a microconidia suspension *T. rubrum* cultures were grown on Sabouraud Dextrose Agar (Sigma-Aldrich Chemie, Germany) at room temperature.

Sylsens B (mol wt: 769.16 g/mol) was synthesized by Buchem Holding BV (Lieren, The Netherlands; purity was more than 99 % as determined with NMR). Glutaraldehyde and OsO_4 were purchased from Electron Microscopy Sciences (Hatfield, Great-Britain), the cacodylate buffer from Sigma (Sigma-Aldrich Chemie, Germany), while all other chemicals were purchased from J.T.Baker (Deventer, The Netherlands). Stock solutions of 6 mM Sylsens B were prepared and stored at 4°C for no longer than one week.

Preparation microconidia suspension

The protocol for obtaining a suspension of microconidia produced by *T. rubrum* grown on Sabouraud Dextrose Agar was based on a method as described previously (16,35). The obtained microconidia suspensions (10– 40 x 10^6 colony forming units (cfu) /mL) were stored in liquid nitrogen for no longer than 6 months. Counting the number cfu on malt extract agar (MEA) dishes was used as a viability check.

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Preparation of the human SC

Abdominal or breast skin collected following cosmetic surgery was obtained from a local hospital. After removal of the fat tissue, the skin was cleaned with distilled water and dermatomed to a thickness of approximately 250 µm using a Padgett Electro Dermatome Model B (Kansas City, USA). The dermatomed skin was incubated at the dermal side with a 0.1 % trypsin solution in phosphate buffered saline of pH 7.4 (4°C) overnight. After 1 hour at 37°C, the SC was removed manually. The obtained SC was dried in the air for 24 hours and kept under nitrogen over silicagel for no longer than 3 months.

Light source

Illuminations were performed with a lamp from "MASSIVE" (no.74900/21), 1× max.500W-230 V-R7s, IP 44. To avoid heating of the samples during illumination, a 5 cm water filter was used to absorb infrared radiation. Light intensity was measured with an IL1400A photometer equipped with a SEL033/F/U detector (International Light, Newburyport, MA, USA). A red cut-off filter at 600 nm was used to obtain the red part of the spectrum of the light produced by the lamp. The light emitted by the lamp had a wavelength range of 580 – 870 nm. The irradiance at the sample of the infected human SC was 30 mW/cm^2.

The ex vivo model and photodynamic treatment

For the photodynamic, dark and light treatment, a *T. rubrum* microconidia suspension (45 to 450 cfu) was inoculated on human SC in the *ex-vivo* model as described before (16). Before the irradiation, the pieces of SC, with the microconidia inoculates were kept in an incubator at 28°C. The different treatments were performed in a water incubation medium of pH 5.2 supplemented with Sylsens B (with the exception of the light treatments). As described in the *ex-vivo* model, the membrane filter containing the SC and the treated fungus was transferred after every treatment to a malt extract agar (MEA) dish, and placed in an incubator at 28°C (16).

The fungus was subjected to the treatments at 8, 48 and 72 hours after spore inoculation. In case of PDT or dark treatment carried out 8 hours after spore inoculation, 20 µM Sylsens B was used. For PDT or dark controls performed at 48 and 72 hours after spore inoculation, 200 µM Sylsens B was used. Previous studies reported that PDT applied at 72 hours after spore inoculation with 200 µM Sylsens B resulted in 80 to 90 percent of the cases in a complete fungal inactivation. In the remaining cases we observed one

or two fungal colonies appearing at 5 days after the treatment (23). The described situation was defined as sub-lethal PDT. A light dose of 108 J/cm^2 was applied during all photodynamic and light treatments.

Scanning electron microscopy

In order to investigate structural alterations of *T. rubrum* following the different treatments, scanning electron microscopy was applied to visualise the (treated) fungus located on the sheets of human SC. Untreated *T. rubrum* inoculates on human SC, in different growth stages, were used as control. The samples were examined at 1, 20 and 40 hours after the treatment or at 8 days after spore inoculation. A JEOL JSM-6700F field emission SEM (Tokyo, Japan) was used at an acceleration voltage of 5 kV.
The SC sheets were fixed overnight at 4°C in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and post-fixed on ice during 1 hour in 1 % OsO_4 (in 0.1 M sodium cacodylate buffer of pH 7.2). Subsequently, the samples were rinsed (3 times) with PBS and dehydrated in increasing concentrations of ethanol (50-100%). The samples were than critical point dried with CO_2 using a Baltec CPD 030 (Balzers, Lichtenstein) and coated with gold-palladium using an Emitech K650X sputter coater (Ashford, England). Every experiment within a chosen growth stage and treatment was repeated at least 4 times (4 duplicates within every experiment) and representative images were selected (from approximately 150 to 200 for every condition and corresponding growth stage). Results were summarized and scored by three persons (one of them blinded).

RESULTS

The results of the PDT treated samples, dark and light controls, at different time points after spore inoculation, are presented and compared to normal fungal growth. The score results of the structural fungal wall alterations after the different treatment modalities are summarized in table 6.1.

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A

Treatment	*Minor wall Deformations*		*Bulge formation*		*Smooth wall appearance (loss of fibre-like Structure)*		*Extrusion of Cell material*		*Flattening*	
	I[2]	II[3]	I	II	I	II	I	II	I	II
PDT	100	100	45	23	0	0	8	73	16	93
Dark control	100	6	18	Sporadically	0	0	3	3	3	3
Light control	74	8	Sporadically	Sporadically	0	0	0	0	0	0
none	0	6	Sporadically	Sporadically	0	0	0	0	0	0

B

Treatment	*Minor wall Deformations*		*Bulge formation*		*Smooth wall appearance (loss of fibre-like Structure)*		*Extrusion of Cell material*		*Flattening*	
	I[2]	II[3]	I	II	I	II	I	II	I	II
PDT	100	100	30	28	45	88	4	65	13	87
Dark control	100	10	12	Sporadically	0	0	2	3	3	3
Light control	100	12	Sporadically	Sporadically	0	0	0	0	0	0
none	5	10	Sporadically	Sporadically	0	0	0	0	0	0

C

Treatment	*Minor wall Deformations*		*Bulge formation*		*Smooth wall appearance (loss of fibre-like Structure)*		*Extrusion of Cell material*		*Flattening*	
	I[2]	II[3]	I	II	I	II	I	II	I	II
PDT	100	100	30	21	43	86	5	57	10	95
Dark control	100	9	11	Sporadically	0	2	1	1	1	0
Light control	100	10	Sporadically	Sporadically	0	0	0	0	0	0
none	3	10	Sporadically	Sporadically	0	0	0	0	0	0

Table 6.1. Structural fungal wall alterations[1] observed as a result of PDT, light only treatment (108 J/cm^2) and dark treatment with the photosensitizer Sylsens B alone of *T. rubrum* applied at 8 (A), 48 (B) and 72 (C) hours after spore inoculation.

[1] The number of observed alterations is expressed as the percentage of the number of fungal elements scored in total. In total 60 to 80 fungal elements from different preparations were scored.

[2] Eight hours after treatment.

[3] Eight days after spore inoculation.

Eight hours after microconidia inoculation

SEM images of the *T. rubrum* growth stage corresponding to eight hours after spore inoculation are shown in figures 6.1 and 6.2. At this time point, the microconidia were attached to the SC and the germination process had started (Fig. 6.1). Application of PDT with 20 μM Sylsens B [known to cause complete fungal inactivation (23)] caused wall deformations and leakage of internal cell material shortly after the treatment (Fig. 6.2-A1 and 6.2-A2). Seven days later, only the remains of the successfully treated germlins could be observed (Fig. 6.2-A3). Control dark treatment with the same Sylsens B concentration resulted in minor wall deformations such as bulge formation (Fig. 6.2-B). Treatment with light alone (108 J/cm^2) at 8 hours after spore inoculation resulted in minor wall deformations, such as indented fungal wall (Fig. 6.2-C). Later fixation times showed fungal recovery in the case of control dark or light treatment. This indicates that the observed changes in the fungal wall appearance are reversible. Figure 6.3 provides a typical example of fungal growth at eight days after spore inoculation under *ex vivo* conditions in the absence of PDT. There is full and intense mycelium growth of *T. rubrum*. A similar growth was observed after a dark or light treatment only, indicating again that the morphological alterations due to dark or light treatment (observed shortly after the treatment) are not permanent, and normal fungal growth was observed at eight days after spore inoculation.

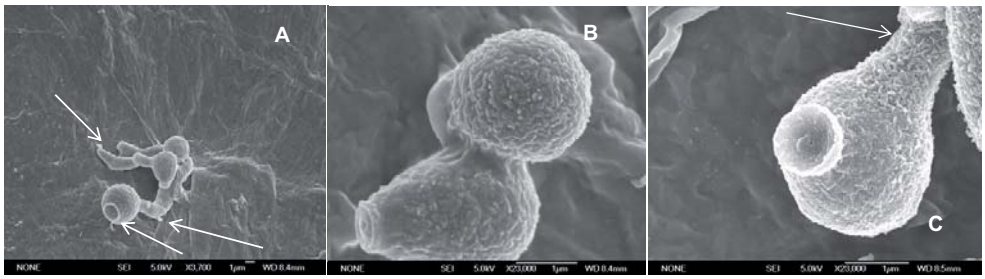

Figure 6.1. SEM images showing *T. rubrum* microconidia 8 hours after spore inoculation on human SC. In figure A a number of microconidia, just germinated, are depicted. Clearly shown are the adherences of the microconidium and germ tube to the SC can (white arrows). In figure B, the regular globular structure of the microconidia wall is noticed, while the wall structure in C (after microconidium germination) has a more fibre-like appearance. The white arrow points to the germ tube.

Forty-eight hours after microconidia inoculation

The figures 6.4 and 6.5 cover the results obtained at this fungal growth stage. At 48 hours after spore inoculation, hyphae proliferation was in full development. In Fig.

6.4 we focused the attention on the appearance of the outer wall of the fungal hypae in this proliferation stage. The structure of the hyphal wall resembled that of a fibre mesh, resulting in a rough appearance. One and twenty hours after the lethal PDT, bulge formation and a ruptured wall were observed (Fig. 6.5-A1 and A2) as well as small smooth appearing hyphal wall areas (inlay in Fig. 6.5-A2). Here, the loss of the wall fibres had started. At a later time point (forty hours after the treatment) and in extreme cases the fibre-rich structure disappeared, rendering a complete smooth appearance of the outer wall (Fig. 6.5-A3). Eventually, after the successful PDT (8 days after the original spore inoculation), flattened and dented hyphae were observed (Fig. 6.5-$A4_a$ and 6.5-$A4_b$).

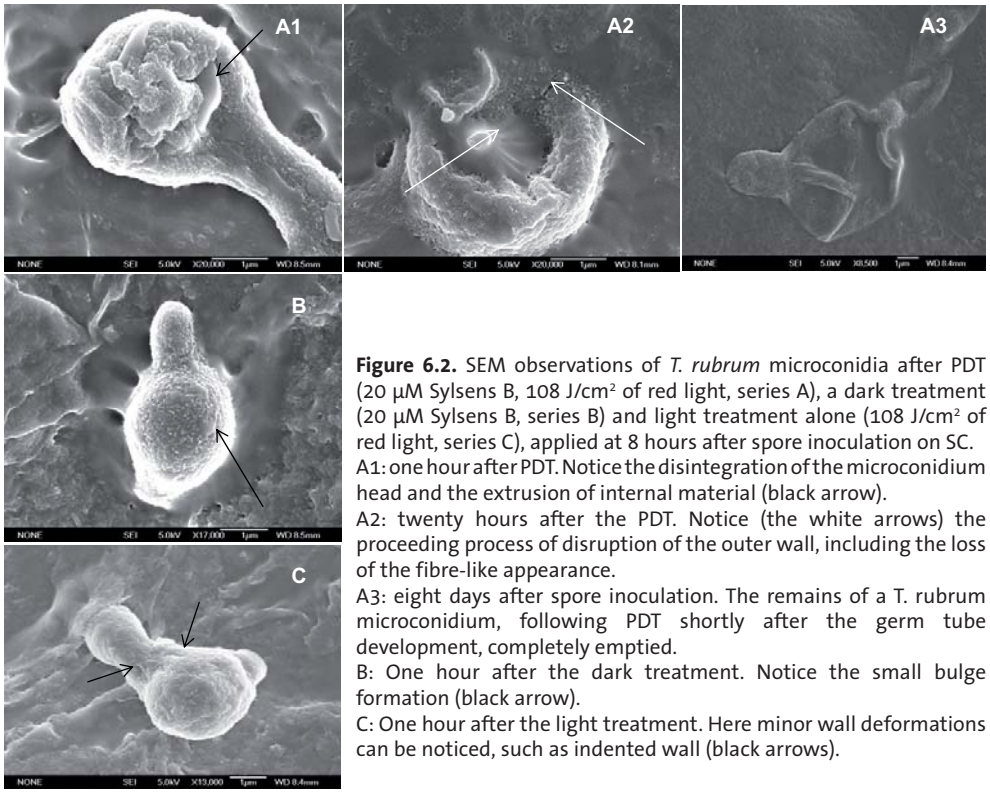

Figure 6.2. SEM observations of *T. rubrum* microconidia after PDT (20 µM Sylsens B, 108 J/cm² of red light, series A), a dark treatment (20 µM Sylsens B, series B) and light treatment alone (108 J/cm² of red light, series C), applied at 8 hours after spore inoculation on SC.
A1: one hour after PDT. Notice the disintegration of the microconidium head and the extrusion of internal material (black arrow).
A2: twenty hours after the PDT. Notice (the white arrows) the proceeding process of disruption of the outer wall, including the loss of the fibre-like appearance.
A3: eight days after spore inoculation. The remains of a T. rubrum microconidium, following PDT shortly after the germ tube development, completely emptied.
B: One hour after the dark treatment. Notice the small bulge formation (black arrow).
C: One hour after the light treatment. Here minor wall deformations can be noticed, such as indented wall (black arrows).

Occasionally, we observed a severely damaged fungus after treatment with 200 µM Sylsens B in the dark (Fig. 6.5-B). However, in most cases, dark treatment resulted in similar (but less severe) deformations, while *T. rubrum* treated with 108 J/cm² of red light alone had an almost normal appearance (Fig. 6.5-C).

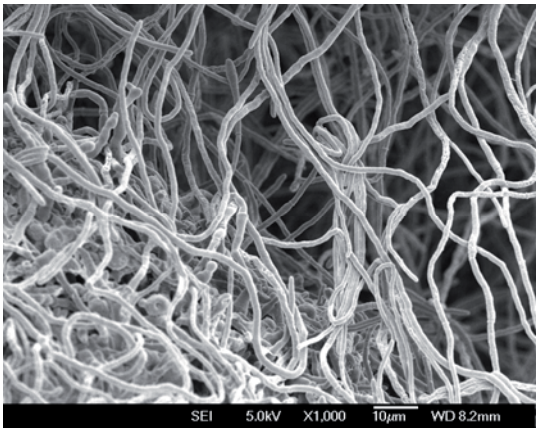

Figure 6.3. A SEM visualization of *T. rubrum* growing on human SC under normal *ex vivo* model conditions, eight days after spore inoculation.

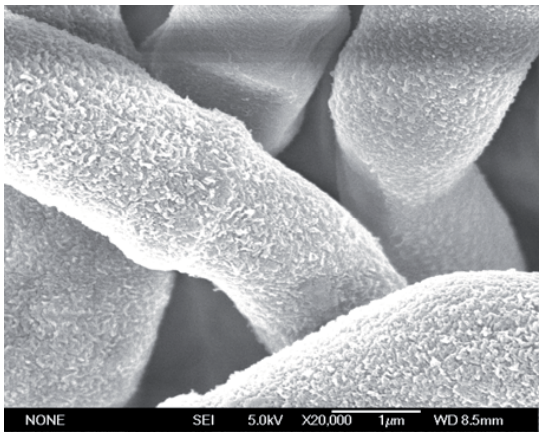

Figure 6.4. A SEM image showing the normal appearance of *T. rubrum* hyphae, displaying a fibre mesh, at 48 hours after inoculation of a microconidia suspension on human SC. Notice the rough surface of the fungal hyphae.

Seventy-two hours after microconidia inoculation

The growth of *T. rubrum* on SC in the *ex vivo* model at 72 hours after spore inoculation can be seen clearly in Fig. 6.6. In 6.6-A the full mycelium is clearly shown. Sporulation of the dermatophyte had occurred under the given *ex vivo* conditions, within three days (see Fig. 6.6-B and 6.6-C). Of particular interest is the difference in fungal wall structure in the middle of hyphae compared to the hyphal tips (Fig. 6.6-D and 6.6-E).

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The tips were more densely packed with material of globular appearance, a difference that could not be observed in the other growth stages. The morphology of the hyphal wall in the branching areas is shown in Fig. 6.6-F. Close inspection of the branching point showed a densely packed, globular structure.

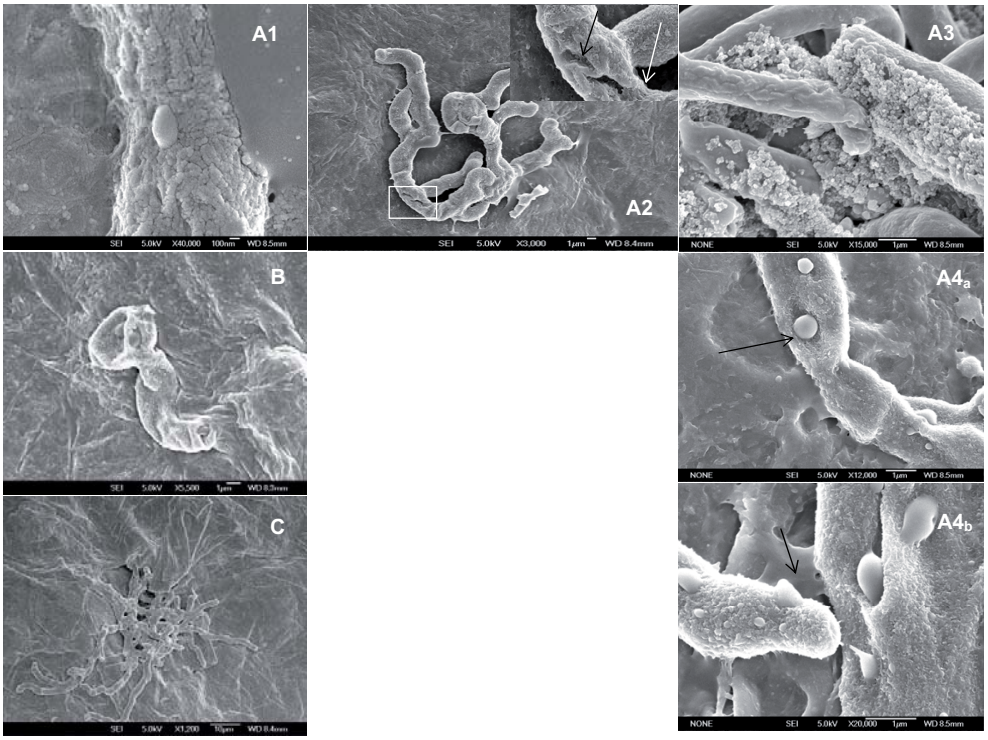

Figure 6.5. SEM images of *T. rubrum* after lethal PDT (200 µM Sylsens B, 108 J/cm² of red light, series A), dark treatment (200 µM Sylsens B, series B) and light treatment alone (108 J/cm² of red light, series C), applied at 48 hours after spore inoculation on SC.
A1: a hyphae close-up, one hour after the PDT. Notice the bulge formation in the middle.
A2: twenty hours after PDT. As can be seen in the inlay (magnification: 20.000 X), a complete rupture of the hyphae wall occurred (black arrow). Notice the disappearance of the rough fibre-rich structure rendering a smooth appearing wall surface (white arrow in the inset).
A3: forty hours after the PDT. We observed a complete loss of the wall fibre-mesh material rendering smooth, naked, hyphae elements.
A4: eight days after spore inoculation. At this time point after PDT the fungal hyphae show a flattened appearance and areas of bulge formation (black arrow in $A4_a$) and leakage (black arrow in $A4_b$) are observed.
B: twenty hours after the dark treatment, showing a severely damaged (wall flattening, loss of structure and bulge formation) fungal element.
C: one hour after the light treatment.

The application of a lethal PDT dose at 72 hours after spore inoculation resulted not only in fibreless fungal hyphae (one hour after the treatment, Fig. 6.7-A1$_a$ and 6.7-A1$_b$), flattened and emptied mycelium structures (Fig. 6.7-A2) twenty hours after the treatment, but also in other time related morphological changes. Worth mentioning is the abundant bulge formation, observed 8 days after the original spore inoculation (5 days after PDT), on the already smooth appearing hyphae (Fig. 6.7-A3). As a result of the PDT, the hyphae were deprived of their fibre-like, grainy wall material giving them a smooth appearance.

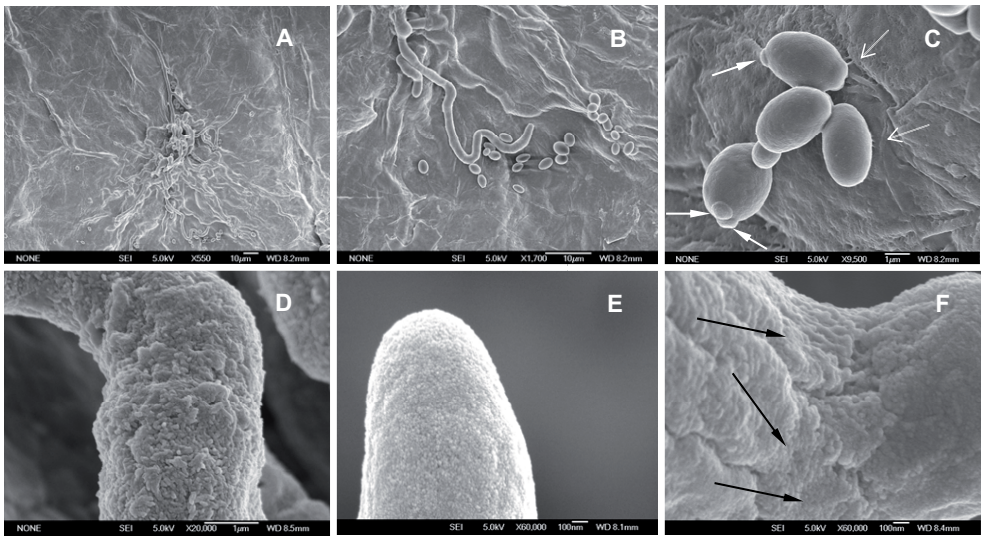

Figure 6.6. Different SEM images all showing *T. rubrum* growing on human SC 72 hours after spore inoculation. In A full mycelium growth can be seen. Notice in B, and in close-up in C, the formation of new microconidia within three days after spore inoculation. In C the closed white arrows point to the places of germ tube formation and the open arrows point to the tender points of attachment of the microconidia to the SC. In figure D a close-up is shown of the wall surface in the middle of a fungal hyphae and in E of a growing tip (notice the closely packed globular appearance here). In F a close up was taken of new hyphal branching. The arrows indicate areas displaying a more globular structure.

The dark treatment (Fig. 6.7-B) caused some wall deformations, while for the light treatment hardly any changes could be observed (Fig. 6.7-C). From both images in Fig. 6.8, it is clear that in the case of an ineffective PDT, fungal re-growth started on the remains of the fungal mycelium. As can be observed during a close inspection, re-growth appeared to start not only on the growing tips but also in other areas of the "old" fungal hyphae.

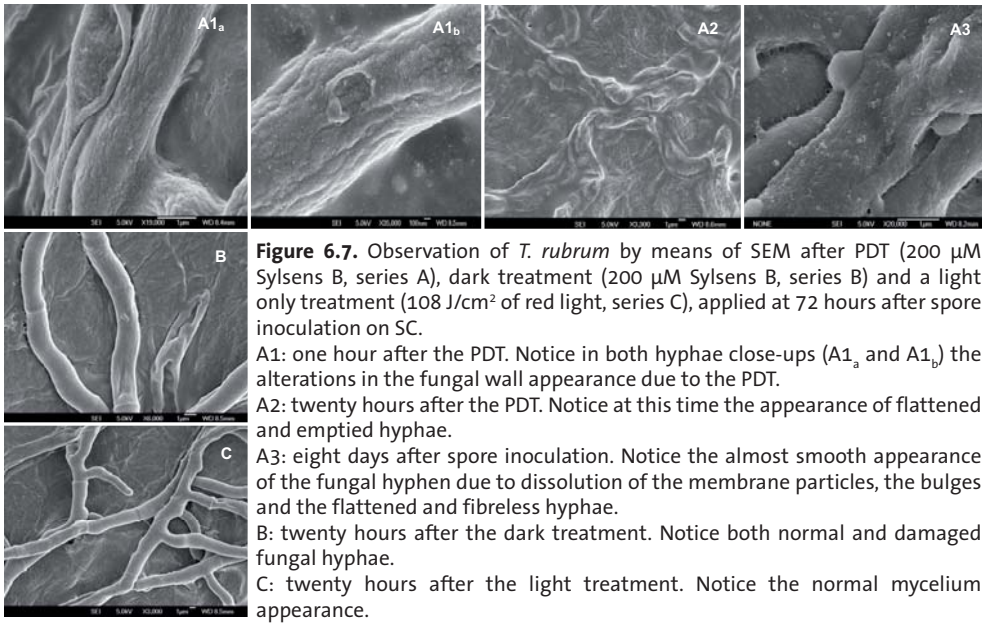

Figure 6.7. Observation of *T. rubrum* by means of SEM after PDT (200 µM Sylsens B, series A), dark treatment (200 µM Sylsens B, series B) and a light only treatment (108 J/cm² of red light, series C), applied at 72 hours after spore inoculation on SC.
A1: one hour after the PDT. Notice in both hyphae close-ups ($A1_a$ and $A1_b$) the alterations in the fungal wall appearance due to the PDT.
A2: twenty hours after the PDT. Notice at this time the appearance of flattened and emptied hyphae.
A3: eight days after spore inoculation. Notice the almost smooth appearance of the fungal hyphen due to dissolution of the membrane particles, the bulges and the flattened and fibreless hyphae.
B: twenty hours after the dark treatment. Notice both normal and damaged fungal hyphae.
C: twenty hours after the light treatment. Notice the normal mycelium appearance.

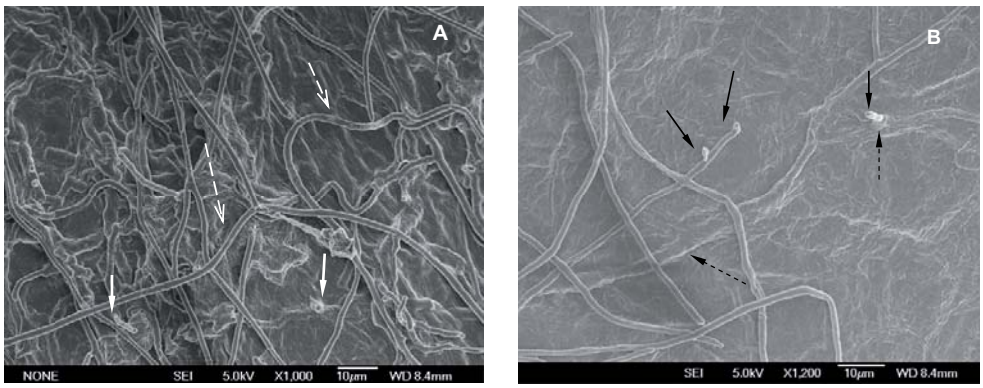

Figure 6.8. Visualisation of *T. rubrum* after a PDT with 200 µM Sylsens B displaying an incomplete fungal inactivation. Notice in A (white closed arrows) the points were fungal re-growth starts on certain parts of the treated fungus, visible here at the background. In front of this image normally growing fungal hyphae can be detected as well (white dashed arrows). In B the black dashed arrows point to a wrinkled, dented mycelium part, inactivated by PDT, while the closed black arrows point to the places where fungal re-growth started.

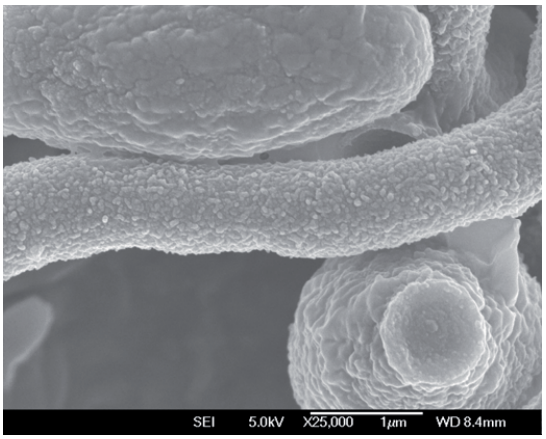

Figure 6.9. A SEM observation of *T. rubrum* fungal hyphae between two microconidia that are not yet germinated.

DISCUSSION

Dermatophytes exhibit various morphologies depending on their growth stage and environmental conditions (36). According to Vasquez et al., the surface of micro- and macroconidia of the genus *Trichophyton*, when cultivated under *in vitro* conditions, appears to be smooth. Bibel et al. described the ruffled surface of *Trichophyton mentagrophytes* when cultivated *in vitro* on Sabouraud dextrose agar (37), while Osumi described a granular surface cell wall on *T. rubrum* (38). In our studies, the surface of both the germinated microconidia and the hyphae had a rough, granular and fiber-mesh like appearance. Shortly after the microconidia adherence to the SC (but before germ tube development) the spore wall appeared to be more globular with a lack of fiber-like material and coated with a clear membrane (see Fig. 6.9 to illustrate this difference). This different structure could be necessary to enable attachment to the SC (39). The fungal wall structure on hyphal tips also differed from the rest of the hyphae i.e. a densely packed globular structure. We noticed this typical structure of the tips only at 72 hours after spore inoculation. This might indicate an increased metabolic activity at the tip of the hyphae.

In this study, we showed that morphological damages occurred not only after PDT but also after treatment with Sylsens B in the dark and to lesser degree after light treatment without the photosensitizer. The similarities for the different growth stages are discussed below.

In general, in all the different fungal growth stages, the observed damage due to the different treatments occurred shortly after the treatment. This is, in the case of PDT, to be expected because of the short lifetime of the reactive $^1O_2$. Although similar damage was observed after all the different treatments, only after PDT was the extent and severity such that it resulted in fungal death. The main consequences of an effective PDT were indented and deformed fungal elements, bulge formation, rupture of the fungal hyphae and, eventually, the development of areas displaying leakage of internal cell material. This sequence of events must have been caused at first by both the binding of the cationic porphyrin Sylsens B in the dark (during the incubation) to the fungal wall, followed by the production of $^1O_2$ after light application. Indeed, from the results of the dark treatments a minor bulge and leakage formation was observed, giving rise to some decrease in wall stability. Binding of Sylsens B in the dark to the fungal wall could decrease the binding possibilities for other cationic molecules involved in wall stability and also transport across the cell. This could also explain the growth delay after dark treatment with Sylsens B and the dark toxicity sporadically observed in earlier studies when Sylsens B was used at 200 μM concentration (16). Treatment with 108 J/cm^2 of red light alone (i.e. no photosensitizer) however, did not cause a growth delay in our previous experiments (16). This indicates that the observed injury induced by the red light had only a minor influence on fungal life.

As regards the bulge formation on the fungal hyphae after a dark treatment (and occasionally after a light treatment), it must be mentioned that similar structure alterations we also noticed (sporadically) on untreated fungal hyphae growing on human SC in the *ex vivo* model.

We also looked more closely at the effect on fungal structure of only partly effective PDT (Fig 6.8). The SEM study confirmed our previous observation, that the unsuccessful PDT (80 to 90 % fungal kill) caused an arrest of the fungal growth (23). However, the fungal body was able to recover from certain surviving regions of the hyphae. As illustrated in Fig. 6.8 re-growth was observed on hyphal tips and in other hyphal areas. These observations can be of practical importance to PDT of *T. rubrum* applied at the time of full mycelium growth. The metabolically active fragments of hyphae are probably more resistant to PDT, either because of their better anti-oxidative defense due to the altered binding capacity for Sylsens B, or because of an increased keratinase enzyme production during this growth stage. Recently, we have shown that the inhibition of keratinase activity increased PDT efficacy when applied on *T. rubrum* mycelium at 72 hours after spore inoculation (23). In the present study, we also demonstrate a

difference in wall structure of the hyphal tips in full mycelium at 72 hours after spore inoculation when compared to the rest of the fungal hyphae and the tips during earlier stages. A consequence of these structural differences may be a decreased binding capacity of Sylsens B to these hyphal tips. This may result in lower local PDT effect and, consequently, the observed fungal re-growth. A similarly low binding capacity for Sylsens B might be present in the hyphae branching areas. Our SEM study indicated a difference in wall structure in branching areas (more globular and dense) compared to other hyphae regions. In other types of fungi, such differences were also observed; these morphologic differences were comparable with those found for the hyphal tips (40,41). These observations in fungal wall structure differences may be of importance for the treatment strategy. We hypothesize that better fungicidal effect could be achieved by application of a second treatment within 24 to 48 hours, a time interval in which hyphal tip morphology could not yet change into more resistant globular structures. However, further study is warranted to confirm this hypothesis.
Finally, it must be mentioned that although the photosensitizer concentrations used were known (from previous work) to be lethal and this was checked again in the present study at 8 days after spore inoculation, the present research has no correlation to clinical data.

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