

Effect of vacuum and thermal shock on laser treatment of *Trichophyton rubrum* (toenail fungus)

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ABSTRACT

The eradication of *Trichophyton rubrum* has been attempted via laser irradiation because it could result advantageous relative to current clinical therapies. Anticipating that the necessary thermal effects could unintentionally damage the underlying toe dermal layer, we have explored two auxiliary approaches: (a) laser irradiation under vacuum pressure, with and without water dousing and, (b) cooling followed by laser heating (thermal shock). The rationale is that at low pressures, the temperature necessary to achieve water evaporation/boiling is significantly reduced, thus requiring lower fluences. Similarly, a thermal shock induced by cooling followed by laser irradiation may require lower fluences to achieve fungus necrosis. For all experiments presented we use a Cooltouch, model CT3 plus, 1320 nm laser to irradiate fungi colonies. The *vacuum pressure* experiments exposed fungi colonies to a subatmospheric pressure of 84.7 kPa (25 inHg) with and without water dousing for 5 min, followed by irradiation with 4.0 J/cm² fluence and 40-90 J total energies. The *thermal shock* experiments consisted of three sections at 4.8 J/cm²: cooling the fungus to 0 °C at 0.39 °C/min and then irradiating to 45-60 °C; cooling to -20 °C at 1.075 °C/min and irradiating to 45 °C; and cooling to -20 °C at 21.5 °C/min and irradiating to 45 °C. Fungus growth rate over a 1-week period assessed the feasibility of these procedures. Results indicated both approaches hamper the growth rate of fungi colonies relative to untreated control samples, especially water dousing under *vacuum* conditions and slow cooling rate preceding irradiation for *thermal shock* effect.

1. INTRODUCTION

Onychomycosis is a fungal and most common infection of the nails¹⁻³. It is most commonly caused by dermatophytes, a common short hand label that encompasses three genera of fungi- Epidermophyton, Trichophyton, and Microsporum⁴. Each of these three genera is able to infect the skin, nail, and hair of humans and animals due to their need to obtain nutrients from keratinized materials^{1,2,4}. While there are other causes of onychomycosis, infection from dermatophytes is the most commonly seen and, of the three genera, *Trichophyton* is the most common by far, affecting an estimated 35 million people in the United States alone⁵ and 90% of fungal nail infections in the United State and Europe⁴.

The species most common to onychomycosis within the *Trichophyton* genera is *Trichophyton rubrum* (*T. rubrum*) and is observed to have a front that is cottony white or bright yellow and reverse that is red, dark red, or brown in color³. Not much is known about how *T. rubrum* propagates but it is suggested that it may have an imperfect sexual as well as asexual way to reproduce. It is known that *T. rubrum* does produce both conidium and arthrospores- asexually produced fungal spores that can withstand extreme heat, cold, and dryness and will germinate and grow when conditions are favorable. Because these spores can withstand extremely adverse environments, it makes destroying the fungus and permanently removing the infection very difficult⁶⁻⁹.

Current treatments of onychomycosis include antifungal medication, topical treatments such as nail paints and surgery, which requires the removal of the entire infected nail¹⁰. Of the current chemical treatments, oral antifungal treatments have shown the most success, with about 75% exhibiting a successful clinical outcome^{10,11}. However, there is also a 25-40% relapse rate^{2,4,10,12}. Even removing the entire nail combined with the use of antifungal treatments or medicine

does not guarantee the destruction of the fungus in its entirety^{2, 10, 13}. Obviously, there is a need for a safe, noninvasive, and efficient treatment for the permanent removal of onychomycosis from the patient.

Initial studies have been done by other groups suggesting the possible use of laser treatment for onychomycosis¹⁴⁻¹⁸. The purpose of this study is to further investigate the use of lasers in combination with vacuum pressures and thermal shock approaches with the overarching goal of improving the clinical outcome of onychomycosis treatments.

2. MATERIALS AND METHODS

An isolate of *T. rubrum* was obtained from American Type Culture Collection (Manassas, VA) and was cultivated on potato dextrose agar. Four-millimeter biopsy punch samples of the primary colonies were then transplanted to new plates containing pure potato dextrose agar as medium, four colonies per plate, and immediately subjected to the treatments described below. Figure 1 shows a typical arrangement of the 4 fungi colonies on a Petri dish. One colony of each plate was used for one of 3 types of controls: (1) completely untreated, (2) exposed to 84.7 kPa (25 in Hg) vacuum pressure, non-irradiated, (3) cooled to approximately 0°C, non-irradiated. All control samples were left to grow inside an incubator at 30°C temperature with no O₂ or CO₂ control. Treated samples were also introduced into the incubator after the procedures and allowed to grow under the same conditions.

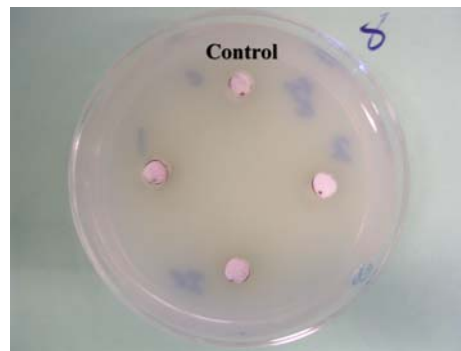


Figure 1. Example of plate set up with new samples of fungus. The number 8 refers to the experiment number for categorization purposes only.

The *vacuum* and *thermal shock* procedures were as follows:

- (a) *vacuum-treated* samples (Figure 2) were divided into two subsets. Those denoted “V”, were dry samples placed under - 84.7 kPa (25 inHg) pressure for approximately 5 min and subsequently exposed to laser irradiation, which was provided by a CoolTouch Q-switched, Nd:YAG laser, 1320nm, 6-350µs pulses forming a 50 ms pulse envelope at 20 Hz, and 4 mm beam diameter, using a fluence of 4.0 J/cm² per pulse and an exposure time of 2-20 seconds. Those denoted “VW” followed the same procedure as “V” except that they were first heavily doused in water before being exposed to vacuum pressure and laser irradiation.
- (b) *thermal shocked* samples were placed on top of 2 Alpha Heatsinks (Figure 3) and allowed to cool down following three different protocols and each protocol was irradiated with the same laser as noted in *vacuum-treated*:
 - (b.1) *Cooling Control*: A subset of samples was surrounded in ice until samples were approximately 0°C at a rate of < 0.39 °C/min. *Proof of cooling concept*: Another subset of these samples were cooled in the same way and then irradiated as described above for approximately 7-15 seconds, until the samples reached 45-60 °C.
 - (b.2) *Slow Cooling*: A subset of samples was cooled down at a rate of 1.075 °C/min until they reached a minimum temperature of -20 °C. Then they were introduced into the incubator and allowed to rewarm to 30 °C. *Slow Cooling, 1320*: Another subset of these samples was cooled in the same way and then irradiated for approximately 2-4 seconds until a maximum temperature of 45 °C was reached.
 - (b.3) *Quick Cooling*: cooling at a rate of 21.5 °C/min until they reached a minimum temperature of -20 °C. Then they were allowed to rewarm to 30 °C in the incubator. *Quick Cooling, 1320*: Another subset of these samples

was cooled in the same way and then irradiated for approximately 3-6 seconds until a maximum temperature of 45 °C was reached.

Standardize photographs were taken with Nikon CoolPix 3100 digital camera from 8 cm above the surface of the sample, 24 hours after the experiment and up to 7 subsequent days thereafter. Assessment of colony growth was made by converting standardized digital images into bitmap format, counting the amount of pixels per colony and converting this count to an average surface area in mm² using Microsoft Paint Program (Microsoft, Seattle, WA).

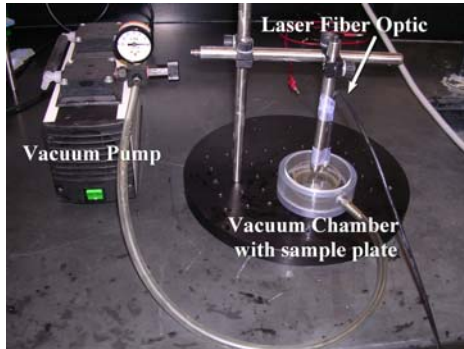


Figure 2. Set up for vacuum procedure.

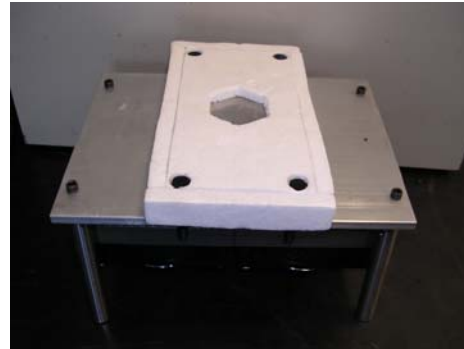


Figure 3. Set up of Alpha heat sinks with cooling plate surrounded by Styrofoam on top.

3. RESULTS

Figure 4 shows preliminary results of the average growth rate and standard deviation of all control samples (those not irradiated). As seen, the growth rate of the vacuum and cooling controls was slower than that of the untreated control samples. However, the trend of the vacuum and cooling controls towards the last days of this study seem to suggest that all control samples could have reached the same average size given long enough periods of time.

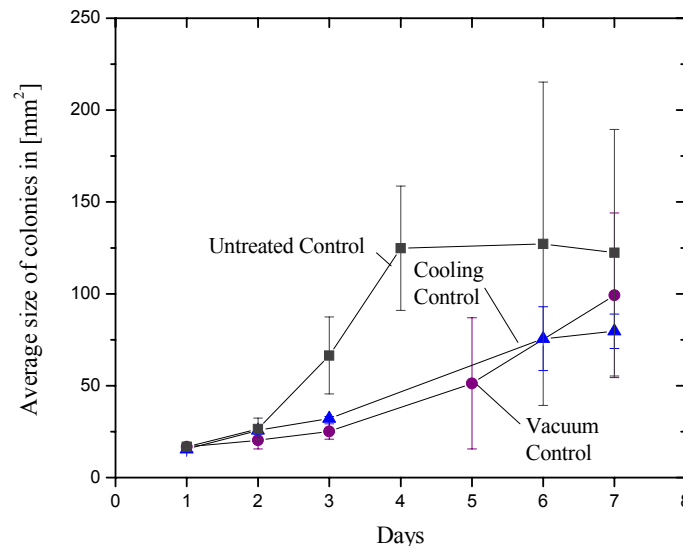


Figure 4. Comparison of average size of control samples in mm². Squares: untreated control samples; circles: vacuum control samples; triangles: cooling control samples.

3.1 Vacuum

Figure 5 shows the main results for the tests involving vacuum pressure. The curve labeled V corresponds to the samples placed under vacuum, irradiated with 40-90J, and left to grow. The curve labeled VW corresponds to those that were first doused in water, placed in vacuum, and then irradiated with 40-90J.

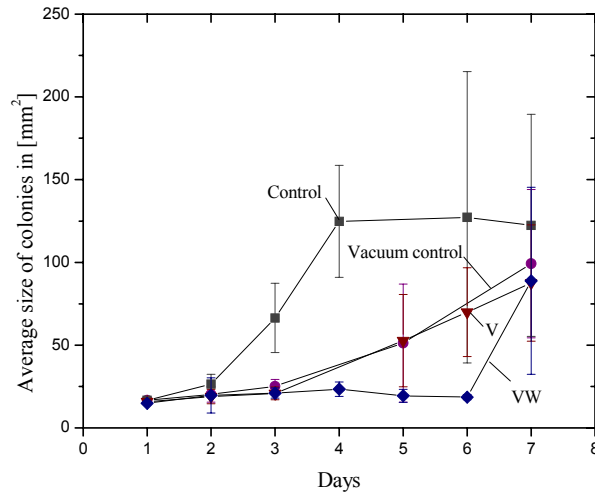


Figure 5. Comparison of average size of colonies in mm² of control (squares), vacuum control (circles), vacuum procedure without water dousing (V, upside down triangle) vacuum procedure after water dousing (VW, diamond).

As seen, while vacuum alone seems to hamper the colony growth rate relative to untreated controls, there was no significant difference between the vacuum control and V samples. Thus, irradiation alone does not appear to change the size of the colonies or growth rate once placed in vacuum. However, when the samples were doused with water and irradiated (VW) the growth rate was significantly suppressed for up until day 6, but for many of these samples, significant growth was observed by day 7, regardless of the energy input.

During the irradiation of many of the VW samples, it was observed that steam was formed and bubbles would form inside the medium and remained trapped for the duration of the experiment. The higher the energy input, the more steam and bubbles were produced. Also, a third of the Petri dishes used were affected in an unforeseen way. The consistency of the entire medium changed becoming thicker and grainy as shown in Figure 6. This new medium greatly inhibited fungus growth.

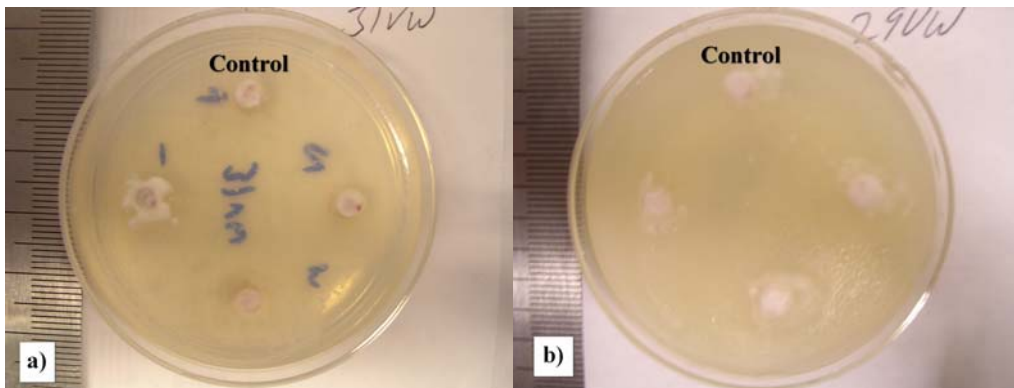


Figure 6. a) Example of normal medium 3 days after VW treatment. b) Example of medium that was adversely affected by the VW treatment shown in day 3 of post treatment.

When the Petri dishes that showed the thicker and grainy medium were removed from the analysis, the VW curve ended up matching the curves of the vacuum control and V samples, as seen in Figure 7.

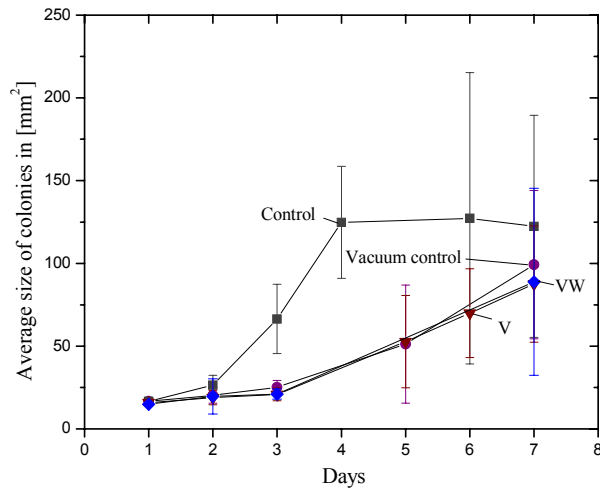


Figure 7. Comparison of average size of colonies in mm^2 of control (circles), vacuum control (squares), vacuum procedure without water dousing (V), and vacuum procedure with water dousing (VW), after the samples where the VW procedure adversely affected the medium were removed.

3.2 Thermal shock

Figure 8 shows the results of the proof of cooling concept experiments. As seen, this aggressive protocol was effective in hampering the growth of the colonies for all 7 days. Only a few samples showed a small amount of growth over the week. It is unclear, however, if the effect on the growth rate is due to the temperature gradient, minimum and/or maximum temperature reached, or the total amount of energy administered by the laser. Clearly, this procedure would be unsuitable for clinical use due to the extreme temperatures involved.

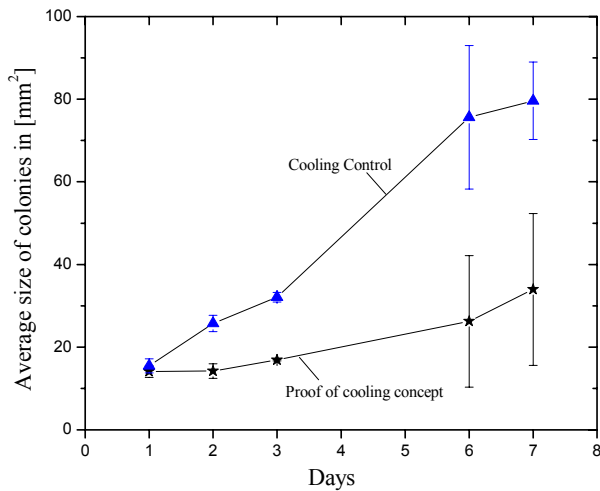


Figure 8. Comparison of average size in mm^2 of cooling control samples (triangles) and proof of concept cooling samples (stars) which were subjected to >100 J and the temperature $45\text{-}60^\circ\text{C}$.

The results of the three cooling protocols (proof of concept, slow and quick) with and without irradiation, along with the untreated and cooling controls are shown in Figures 9 and 10.

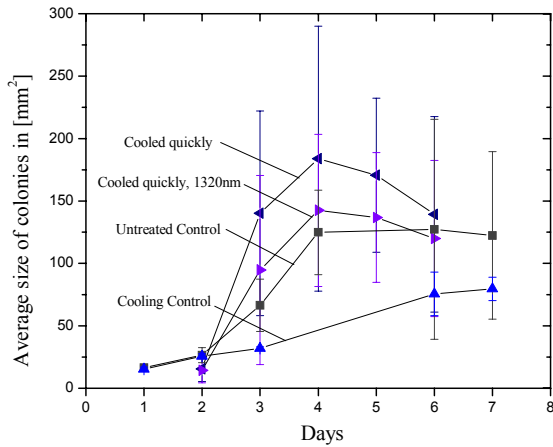


Figure 9. Comparison of average size in mm² of different samples: untreated control (squares), cooling control (triangles), and quick cooled with and without irradiation (left and right facing triangles respectively).

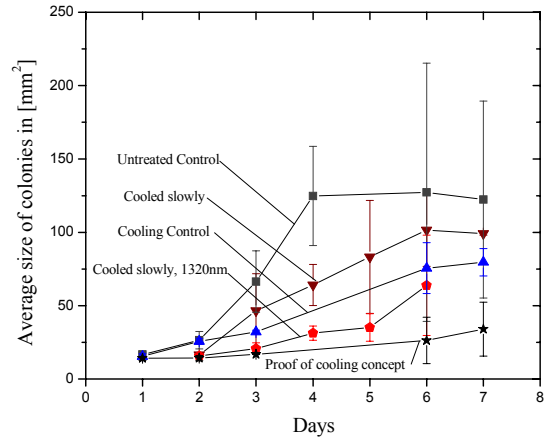


Figure 10. Comparison of average size in mm² of different samples: untreated control (squares), cooling control (triangles), proof of cooling concept (stars), and slow cooled with and without irradiation (hexagon and upside down triangle respectively).

Several observations can be made based on these experiments:

- (1) Relative to the untreated controls, the slow cooling procedure both with and without irradiation demonstrated a slower growth rate and smaller average size.
- (2) Both the irradiated and non-irradiated samples that were quick cooled demonstrated at least the same if not higher growth rates than the untreated control.
- (3) Laser irradiated samples show a reduced growth rate relative to their non-irradiated counterparts.
- (4) Relative to the cooling control, the slow cooled samples that were not irradiated had larger than average colony sizes. On the other hand, the slow cooled and irradiated samples had smaller colony sizes. All three showed similar growth rates.
- (5) The results that showed the slowest growth rate were the *proof of cooling concept*. The second slowest were the *cooled slowly*, irradiated samples.

4. DISCUSSION

4.1 Vacuum

The objective of using vacuum pressure in conjunction with laser irradiation was, amongst other purposes, to take advantage of the reduced boiling temperature of water and either make more efficient use of the heat imparted via the laser or reduce the fluence required for fungus necrosis. The standard dry samples were very dry and while not overly desiccated, they did not have much excess water to alter with the vacuum pressures. Dehydrated conidia, one of the main types of spores that *T. rubrum* uses to infect and reproduce, can resist up to 124 °C for up to 3 minutes while still remaining largely viable⁸. Thus, making the vacuum system no more effective than simply irradiating the samples while under standard temperature and pressure initial conditions, as shown in Fig 5. Water dousing appeared to have an important effect at first, but its effectiveness seem to be correlated with inexplicable changes we observed in the media (Fig. 6) which, once removed, appeared to have no effect (Fig. 7). One of the factors that needs to be highlighted is that vacuum alone appeared to hamper the growth rate of the colonies with little to no effect from irradiation when the samples with deformed medium were removed (Fig. 7) which leads us to believe that humidity may be a very important factor. In 1976, Schmit proposed that *conidia* viability was affected by humidity. Storing *conidia* at 100% humidity killed the samples after only 9 days at 22 °C⁸. Other works indicated that *Trichophyton mentagrophytes*, the other main

dermatophytes related to onychomycosis, has a very narrow humidity range of 95-98% and that different levels of humidity are better or worse for different stages of *T. mentagrophytes*—high humidity is necessary for arthrospore formation but reduced humidity necessary for maturation¹⁹. While *T. mentagrophytes* and *T. rubrum* are not the same, they are similar enough to warrant further studies investigating the effect of humidity and spore creation and growth^{6, 19, 20}.

In relation to our experiments, humidity comes into question when the effects of the VW technique are studied. The energy imparted to the sample during V and VW techniques was the same as the initial experiments of simple irradiation of a dry or wet sample with no other environmental factors (data not shown). Even though the energy was the same, the vacuum pressure reduces the boiling temperature of the water and thus it was reached sooner. This boiling, while enclosed in the small vacuum chamber, produced steam that would raise the relative humidity of the environment. Samples that received more energy also created more steam which may have further inhibited the growth rate of the samples. It is also possible that the humidity reached fits within the narrow band necessary for the efficient production of arthrospores while also destroying the main section of the fungus, thus minimizing the thermal effect that the laser irradiation would have on the growth rate. The current experiments cannot differentiate the effect of energy, overall temperature, or humidity from the results of the growth rate or colony size, so this should be investigated further.

As discussed above, there were many effects to the medium that could also change the outcome of the results. The water when placed on the sample did not only soak up into the fungus, but it also surrounded the fungus even going so far as to filter through the medium to get underneath the sample or in the crevices at the edge of the Petri dish. While irradiating the sample, the area directly around the sample would also become heated, as well as the water trapped within or around the medium. This caused the medium to change. Sometimes small bubbles would form in the medium that could not dissipate. For one third of the cases the medium was irrevocably changed for unknown reasons which greatly inhibited fungus growth as seen by the change between Figures 6a and 6b.

4.2 Thermal shock

T. rubrum is incredibly resistant to many extreme environments including heat, cold, and dryness. Dormant conidia and arthrospores, which are considered the main way that *T. rubrum* spreads and stays alive, have been known to survive at 4 °C for at least 3 years, with no morphological changes or mutations²¹. They can also withstand -70 °C for up to 6 months with no significant morphological changes^{22, 23}. *T. rubrum* has also been known to be extremely resistant to heat. Mature conidia can withstand 55 °C for 10 minutes with no loss in viability and more than 90% of dehydrated conidia can resist up to 124 °C for as much as 3 minutes⁸. Dropping the initial cooling temperature to -20 °C and then raising it quickly to 45 °C is well within the range that many of the conidia can withstand, however, the quick cooling and heating rates may expose the fungus to extreme conditions that it may not be able to withstand. Further studies are required to address this issue.

The proof of cooling concept worked well because the samples were brought above 55 °C in a small amount of time and the sample was not completely desiccated due to the medium that it was growing on, making it more susceptible to the heating process. But it also explains why even those samples were not completely destroyed. All it takes is one viable conidia spore to create a whole new colony and the current procedure that is bounded by clinical pain boundaries is not enough to kill the entire sample. The samples that were more significantly affected out of the clinical temperature samples were the ones that were cooled slowly and then immediately irradiated to 45 °C. The growth rate was about the same as the cooling control but the sample sizes were smaller overall for the first half of the week. Later, growth rate sped up and growth continued as normal. This may be an indicator that more of the sample was in a dormant stage due to the lower initial temperatures but that it was able to sufficiently recover and continue its growth. Multiple treatments following the same procedure or the introduction of topical or oral antifungal medications after initial thermal shock may continue to hamper and possibly eliminate the fungal growth.

The effects that were seen may also be due to the damage to the medium more than the sample itself. Freezing the Petri dish had the possibility of shrinking the entire plate of medium due to its high water content thereby inherently changing the fungi's ability to grow. For this experiment the samples that were dropped to only 0 °C were frozen as one dish while the samples that were reduced to -20 °C were frozen individually. Therefore, the shrinkage was only a possibility for the 0 °C experiments and not for the revised experiments thus mitigating the medium problem.

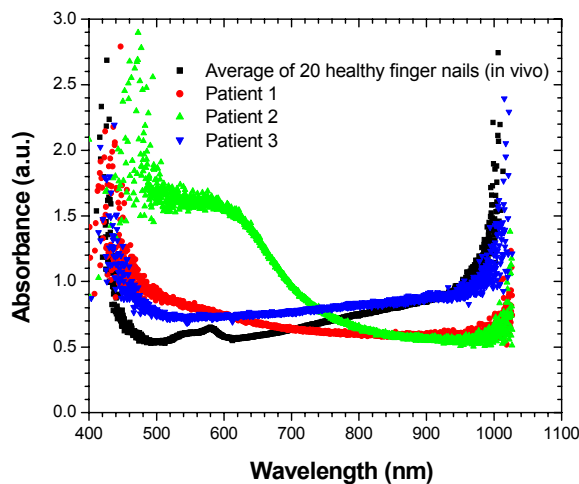
5. CONCLUSIONS

Our results indicate that the *vacuum* approach hampers the growth rate of fungi colonies relative to untreated control samples, especially the combination of water dousing prior to laser irradiation under *vacuum* conditions. Thermal shock can also reduce the growth rate of fungi colonies when slow cooling is applied followed by rapid laser irradiation, while quick cooling preceding laser irradiation shows little effect.

Exposing fungi to vacuum alone appears to deter the fungus growth rate, even without laser irradiation. However, when fungus water dousing precedes laser irradiation, the growth rate is hampered even more. Overall, the vacuum samples showed some promise as they inhibited the growth of the samples but the results were not consistent and it is not entirely clear as to whether it is the fungus or the medium which is greater affected. Further studies must be done to distinguish the effects of humidity, as well as the effect of both thermal shock and vacuum combined and in multiple applications.

Most of the cooling results showed minimally effective at inhibiting the growth rate of *T. rubrum*. The best results so far are contained within the proof of cooling concept samples but it was unclear whether or not it was the temperature gradient, maximum temperature, or amount of energy that had the dominant effect. The cooling experiments that dropped the initial cooling temperature to $-20\text{ }^{\circ}\text{C}$ recreated the same temperature gradient as the *proof of cooling concept* samples but the results were universally worse producing a faster growth rate and larger sample size. This ruled out the temperature gradient, thus leaving the maximum temperature or the total amount of energy as the only feasible parameters to explain the difference. While the quick cooling samples at $-20\text{ }^{\circ}\text{C}$ produced unfavorable results, the results of the *slow cooled, 1320* samples were promising. They reduced the growth rate and colony size beyond that of the *cooling control* while still staying within suitable temperature ranges for clinical use. The combination of thermal shock with vacuum or topical chemicals to improve upon the current results should be investigated in the future.

Since laser heating is still the underlying procedure, we have initiated studies aimed at characterizing optically, both healthy and diseased human nails. Figure 11a shows the absorbance spectra of the average of 20 healthy finger nails and three diseased human toe nails, the apparent absorbance was obtained from reflectance measurements as the negative of the logarithm base 10 of the reflectance. These optical measurements could be used to determine which wavelength would be better absorbed by the diseased nail and use that wavelength to increase the nails temperature thus affecting more of the fungus overall and having a better chance to affect the fungus trapped on the edges of the nail or inside the nail itself. The difference in the absorbance spectrum between diseased nails could relate to different subtypes of onychomycosis or different stages of the disease, a nail in an advanced stage of onychomycosis is thicker and more opaque than a nail in an earlier stage of the disease, this thickness and opaqueness can be seen as an increase in absorbance in the visible portion of the spectrum as can be seen in the absorbance spectrum of Patient 2 in Figure 11a.



a



b



c

Figure 11. (a) Average absorbance of 20 healthy in-vivo finger nails (squares) and the diseased ex-vivo toe nails, corresponding to subfigures 11a (circles), 11b (triangles up), and 11c (triangles down).

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