Inhibitory effects of cold atmospheric plasma on the growth, ergosterol biosynthesis, and keratinase activity in *Trichophyton rubrum*

Atena Shapourzadeh [a, b], Neda Rahimi-Verki [b], Seyed-Mohammad Atyabi [c, *],
Masoomeh Shams-Ghahfarokhi [d], Zahra Jahanshiri [a], Shiva Irani [e],
Mehdi Razzaghi-Abyaneh [a, **]

[a] Department of Mycology, Pasteur Institute of Iran, Tehran, 13164, Iran
[b] Department of Biochemistry, Faculty of Basic Science, Islamic Azad University Damghan Branch, Damghan, Iran
[c] Department of Pilot Nanobiotechnology, Pasteur Institute of Iran, Tehran, 13164, Iran
[d] Department of Mycology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[e] Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran

ARTICLE INFO

ABSTRACT

Background: Dermatophytosis is the most important superficial fungal infection which affects nearly 20% of human population worldwide. Recurrence of disease and emerging resistance of *Trichophyton rubrum* to synthetic antifungals are the main problems in control of dermatophytosis. The purpose of this study was to evaluate the effect of cold atmospheric plasma (CAP) on *T. rubrum* growth, ergosterol biosynthesis and keratinase activity.

Methods: A CAP system, comprised of helium 98% − oxygen 2% (He/O_2), was used. *Trichophyton rubrum* conidia suspensions were treated with CAP in time periods of 90, 120, 150 and 180 s in 96-well microplates. Fungal growth was evaluated by counting the colony forming unit (CFU). Fungal dry weight, ergosterol biosynthesis and keratinase activity were evaluated in CAP-treated *T. rubrum* and untreated controls.

Results: *T. rubrum* growth was significantly inhibited by 62%–91%. CAP strongly suppressed fungal ergosterol biosynthesis by 27%–54%. The keratinase activity was increased by 7.30%–21.88% up to 120 s CAP exposure.

Conclusion: Our results demonstrated for the first time that CAP inhibits *T.* rubrum growth, suppresses ergosterol biosynthesis and increases moderately keratinase activity in a dose-dependent manner. Overall, CAP exposure could be a potentially useful method for treatment of clinical cases of human and animal dermatophytoses.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

Dermatophytes are a group of keratinophilic fungi which contain around 40 species classified into three main genera *Microsporum* (skin and hair), *Trichophyton* (skin, hair and nail) and *Epidermophyton* (skin and nail). Dermatophytosis is a superficial infection in keratinized skin tissue, in nails and hair which is prevalent worldwide. In the etiology of dermatophytosis, 5 major species involved are *Trichophyton rubrum*, *Trichophyton interdigitale*, *Trichophyton tonsurans*, *Microsporum canis*, and *Trichophyton violaceum* [1]. They have an arsenal of proteases aimed to digest the keratin network into absorbable oligopeptides or amino acids. Dermatophytes secrete multiple serine-subtilisins and metallo-endoproteases (fungalysins), formerly called *keratinases*, which are used for penetration into host cellular system [2–5]. The enzyme reduces disulfide bonds in keratins of skin, hair and nail. This reduction depends on a sulfite efflux pump, encoded by the *TruSsu*1 gene. Keratinase enzyme plays a major role in tinea diseases [6].

According to previous studies, the disease incidence is increasing every year and approximately 20%–25% of the world

population is infected by dermatophytes [7,8]. The prevalence of the disease in different parts of Iran is around 2.1%−74%. Although dermatophytes are not lethal diseases, they are the most widespread among human infections. Recently development of infectious fungi is on the increase because of various reasons, such as weakening of immunity factors, overdosing antibiotics, corticosteroids and etc. Consequently, it is essential to choose skillful treatments for the inflicted patients. The common treatments contain allylamine group drugs specially, terbinafine and various azoles such as itraconazole, fluconazole and voriconazole but these have some drawbacks such as long treatment time which may affect the liver, so it is necessary to periodically monitor liver function, drugs disability to vanish fungus entirely and other side effects for instance headache, nausea, dizziness and you name it. Moreover, it has been reported that some of the strains and dermatophyte species have developed resistance to drugs and hence, there is illness recurrence [9].

Todays, scientists try to find the best way to improve the quality of treatment. Recently, the cold plasma treatment has found many applications in biomedical fields.

The purpose of this research was to use a new method with high quality performance using cold atmospheric plasma (CAP) in order to treat different kinds of tinea diseases which are caused by *Trichophyton rubrum*. In the 1970s the first plasma was used in industrial applications.

Plasma is an ionized gas which is also made of photons, electrons, positive and negative ions, atoms, free radicals and excited or non-excited molecules that are referred to as the fourth state of matter (99% of the apparent universe is in the plasma state) [10−12].

Several groups worldwide are currently investigating the biomedical effects on cells and tissues induced by different CAP devices (plasma jets, torches and dielectric barrier discharges) including cell culture studies, animal models and clinical studies. Various investigations have shown the efficacy of CAPs against microorganisms including bacteria and fungi [13−21], biofilms [22−24] viruses [25], spores [26,27] and yeasts [28]. Nowadays usage of CAP in biomedical fields such as sterilization, cancer treatment [29], blood coagulation and tissue repair [30] are considerable [31−33].

In recent years there have been extensive investigations through high quality continuous operations and the benefits of atmospheric discharge, namely economical aspects.

In the present study, we used cold atmospheric plasma jet treatment which had an electrical power supply with an operating frequency of 15 MHz, based on 98% helium gas mixed with 2% oxygen. The diameter of gas tube was 2 cm and the distance of electrodes was 4 cm. A transformer produced 10 kV (peak-to-peak) from the transistor. We studied the effect of CAP on growth, ergosterol biosynthesis and keratinase activity in *Trichophyton rubrum* as the most important etiologic agent of human dermatophytosis worldwide.

2. Materials and methods

2.1. Plasma device

The working mechanism involved mixes of two gases: helium and oxygen (volume ratio: 98% He and 2% O_2) and a power supply including electrodes which worked with a radio frequency of 15 MHz and 10 kV with 10 W output power using earth as an external electrode. The diameter of gas tube was 2 cm and the distance of electrodes was 4 cm. A transformer produced 10 kV (peak-to-peak) from the transistor. There was a manometer (a gauge) in order to check the gas output pressure. At atmospheric pressure, most plasmas are so hot (thousands of degrees centigrade) that they would immediately kill any living cells (healthy or unhealthy) they come into contact with. However, the CAP has developed techniques for producing low-temperature plasmas and has been used in this research. Gas flow and pressure are constant at 1 L/min and 1.5 bars, in the stated order. By utilizing the radio waves the electrical discharge began and produced various active chemicals which spread rapidly in its surrounding environment. Irradiation was carried out at a distance of 2 cm between the plasma jet nozzle and sample surface. The central electrodes accelerated the free electrons with the aid of radio frequency. These high-energy electrons under the non-elastic effect of charged particles stimulated the molecules, atoms, free radicals and paired electron-ions.

2.2. Fungal strain and culture conditions

Trichophyton rubrum PFCC 51431 (PTCC 5143; RI 613), isolated from human dermatophytosis, was prepared from Pathogenic Fungi Culture Collection of the Pasture Institute of Iran. The fungus was identified based on colony and microscopic morphology, urease test, hair perforation test, and ability to pigment production on corn meal agar (CMA) plus 2% dextrose [34]. To prepare and provide the testing stocks, inoculated cultures were grown for 2 weeks at 28 °C on Sabouraud dextrose agar (SDA) slants with chloramphenicol and cyclohexamide. Spore suspensions were prepared by gently scraping the culture surfaces using a sterile glass rod after adding adequate amounts of 0.1% aqueous solution of Tween 80. Afterward, the suspension cellular samples were prepared with density of 10^6 -10^8 cells/mL.

2.3. CAP treatment

For each independent experiment, suspended cellular samples were divided into 96-well plates and treated with cold atmospheric plasma jet for 0, 90, 120, 150 and 180 s.

2.4. Effect of CAP on T. rubrum growth

A prepared suspension of 100 μL was pipetted into selected wells of 96-well microtiter plates. Each well was treated with cold atmospheric plasma for 0, 90, 120, 150 and180 s. Next the samples were diluted by 5:95 μL into sterile water, and 5 μL of diluted suspension was cultured in SDA and incubated statically for 9 days at 28 °C until the colonies were formed.

2.5. Effect of CAP on T. rubrum weight

First, 100 μL of the fungal spores' suspension, at density of 2×10^6 cells/mL, was prepared and exposed to CAP for 0, 90, 120, 150, 180 s and inoculated in the Sabouraud maltose broth. It was finally incubated at 37 °C in 150 rpm for 3 days. Fungal mycelia were separated from the culture media by passing through Whattman filter No. 1. A known amount of thoroughly washed mycelia was placed on pre-weighed Petri plates and allowed to dry at 60 °C for 6 h to reach a constant weight [35].

2.6. Effect of CAP on T. rubrum ergosterol biosynthesis

Ergosterol content in the samples treated with cold atmospheric plasma and in control samples was measured. In these tests we used the suspension at density of 2×10^6 cells/mL. Then 100 μL of the *Trichophyton rubrum* suspension was added into the selected wells of 96-well microtiter plate and treated with CAP at different time intervals as follows: 0, 90, 120, 150 and 180 s. In the next step,

100 μL of untreated and treated suspensions were poured in 30 mL of Sabouraud dextrose broth. The cultures were incubated for 48−72 h at 30 °C. Then, each sample was washed 3 times and dried in oven for 1 h at 60 °C. The samples of equal weights were prepared after three milliliters of 25% alcoholic potassium hydroxide solution added into each and vortex mixed for 1 min and incubated in water bath of 85 °C for 1 h. Next, a mixture of 1 mL distilled water and 3 mL hexane was added into each sample and vortex mixed vigorously for 1 min to extract the sterols. The hexane layer was separated and stored at −20 °C for up to 24 h. Aliquots of the sterol extract were diluted by five-fold 100% ethanol and scanned between 200 and 300 nm with a Perkin Elmer UV/Vis spectrophotometer (EZ 301, USA). Ergosterol content was calculated as a percentage of the net weight of the mycelia mass by the following equations:

$$\text{Ergosterol (\%)} = [(A_{281.5}/290 \times F)/\text{sample weight}] - [(A_{230}/518 \times F)/\text{sample weight}]$$

Where, F is the dilution factor in ethanol and 290 and 518 are the E values [36].

2.7. Effect of CAP on keratinase activity in T. rubrum

2.7.1. Screening of keratinolytic activity on agar plates

The isolates were screened for keratinase production based on the method of Wawrzkiewicz et al. [37]. Chicken feathers were used as a source of keratin. The keratin, soluble in dimethyl sulfoxide (DMSO), was precipitated with acetone. The precipitate, which was the only source of carbon and nitrogen, was added to the sterile agar medium at a final concentration of 0.06%. This medium consisted of Bacto agar (15 g), $MgSO_4.7H_2O$ (0.5 g), KH_2PO_4 (0.1 g), $FeSO_4.7H_2O$ (0.01 g), $ZnSO_4.7H_2O$ (0.005 g), NaH_2PO_4 (3.86 g), Na_2HPO_4 (3.97 g), cycloheximide (0.5 g) and chloramphenicol (0.05 g) in one liter of distilled water. The agar plates were inoculated with 20 μL of fungal suspensions of 5×10^5 cells/mL (prepared by gently rubbing of slants in presence of 0.01% Tween 80). Keratinolytic activity of the isolates was detected as a clear zone around the colony after incubation at 25 °C for 6 days. The diameter of the clear zone was measured to quantify the enzyme activity.

2.7.2. Submerged cultivation

A mineral liquid medium, containing all the ingredients of solid medium except agar, was transferred into 250 mL flasks at 50 mL aliquots and sterilized at 121 °C for 15 min. At the start, 100 μL of *T. rubrum* suspension at density of 1×10^6 cells/mL was added into the selected wells of 96-well microtiter plate and treated with CAP in 0, 90, 120, 150 and 180 s. The flasks were inoculated with each sample (treated and untreated) and were incubated at 28 °C for 14 days.

2.7.3. Keratinase activity assay

Keratinolytic activity of culture filtrates was measured spectrophotometrically according to the method of Takiuchi et al. [38], with some modifications using keratin powder instead of chicken feathers as a keratin source. A 2.0 mL culture filtrate that had been treated with cold atmospheric plasma in 0, 90, 120, 150 and 180 s, keratin powder (20 mg) and 3.0 mL phosphate buffer (28 mM, pH 7.8) was incubated in a shaker water bath of 150 rpm at 37 °C for 1 h. After the addition of 10% trichloroacetic acid (TCA) and centrifugation at 10,000 g for 15 min, the optical absorption of the supernatant was measured at 280 nm wavelengths using a double-beam UV/Vis 1601 Shimadzu spectrophotometer against the blank in the absence of TCA. The increase of 0.1 unit absorption was equal to one unit of enzyme activity as calculated by the following formula:

$$\text{Total activity (U/mL)} = \Delta \text{ OD}/0.1$$

Protein content was measured following the Bradford method. First, the Bradford profile was obtained by the absorption of various concentrations of bovine serum albumin and its corresponding curve was plotted through Excel program. Next, the absorption of each plasma-treated sample was recorded and its protein concentration (mg/mL) was calculated. In the following step, the enzyme specific activity was obtained through Bradford equation.

$$\text{Specific activity (U/mg)} = \text{Total activity (U/mL)/Protein content (mg/mL)}$$

2.8. Statistical analysis

In order to compare the analytical tests between CAP's different time flows, a one-way ANOVA statistical test was performed, based on triplicates which had been tested on each sample, there was statistically significant difference between various times of plasma irradiation ($P < 0.05$).

3. Results

3.1. Effect of CAP on growth and weight of T. rubrum

After incubation for 9 days the number of colonies was counted. The results (Table 1) indicated that, in comparison to untreated sample, by increases in cold atmospheric plasma jet flow time the number of colonies dropped substantially. There were by average 16.11 colonies in untreated samples. After the treatment with cold atmospheric plasma for 90 s, this counting was reduced to an average of 6.11and the number of colonies decreased to 2.44 in 120 s. The difference was that at 150 s the number of colonies had unexpectedly increased to 3.88, though the reduction in the number of colonies, compared to untreated sample, had been continued. Finally, the number of colonies reached 1.44 during 180 s irradiation. According to Fig. 1A, depicted from the results of triplicate tests, significant differences were observed between the treated and untreated samples ($P < 0.05$). At the CAP exposure over the time 180 s (210 s), fungal growth was completely inhibited.

Also, we have observed the results of the samples' dry weights which have been obtained as in similar colony counting test. The average dry weight was 96.55 mg in untreated samples. By increases in CAP flow time, dry weights of the samples were decreased to an average of 83.55 mg and 52.88 mg in 90 s and 120 s. Like the colonial test a slight increase of 62.55 mg was obtained for samples treated for 150 s. Nevertheless the results have been lower than the control samples. Eventually, the lowest dry weight was 42.11 mg in the CAP flow of 180 s samples (Table 1). There have been significant differences between the dry weights of control and each of the treated samples ($P < 0.05$) (Fig. 1B). Finally our results showed that CAP significantly inhibited the growth of *T. rubrum* by 62%−91% and dry weights by 13%−57% at the established times.

3.2. Effect of CAP on ergosterol biosynthesis

As shown in Table 1 the percentage of ergosterol was 33.44 in the control sample. After plasma treatments during 90 s and 120 s, the amounts of ergosterol were 24.44% and 18.44%, respectively. At 150 s treatment time there was an increase of 19.77% observed although it was a drop compared to untreated sample. Fig. 2 shows that the ergosterol content and inhibition percentage level in

Table 1
The effect of cold atmospheric plasma on the growth and virulence factors of *Trichophyton rubrum*.

Treatment times(s)	Colony number (CFU) (Mean ± StDev)	Fungal dry weight (mg) (Mean ± StDev)	Ergosterol (Mean ± StDev) Content (%)	Inhibition (%)	Keratinase activity (Mean ± StDev) Total (U/mL)	Specific (U/mg)
0	16.11 ± 1.36	96.55 ± 2.55	33.44 ± 4.69	0.00	8.90 ± 0.01	25.54 ± 0.58
90	6.11 ± 0.60*	83.55 ± 2.78*	24.44 ± 1.13*	26.92	8.92 ± 0.01	25.64 ± 0.31
120	2.44 ± 0.72*	52.88 ± 1.96*	18.44 ± 0.72*	44.86	9.55 ± 0.01*	31.13 ± 0.47*
150	3.88 ± 0.60*	62.55 ± 2.40*	19.77 ± 0.66*	40.88	9.52 ± 0.01*	30.61 ± 0.12*
180	1.44 ± 0.88*	42.11 ± 1.76*	15.55 ± 0.88*	53.50	9.48 ± 0.01*	30.20 ± 0.13*

* Statistically significant difference between plasma-treated samples with non-treated control at the 0.05 level.
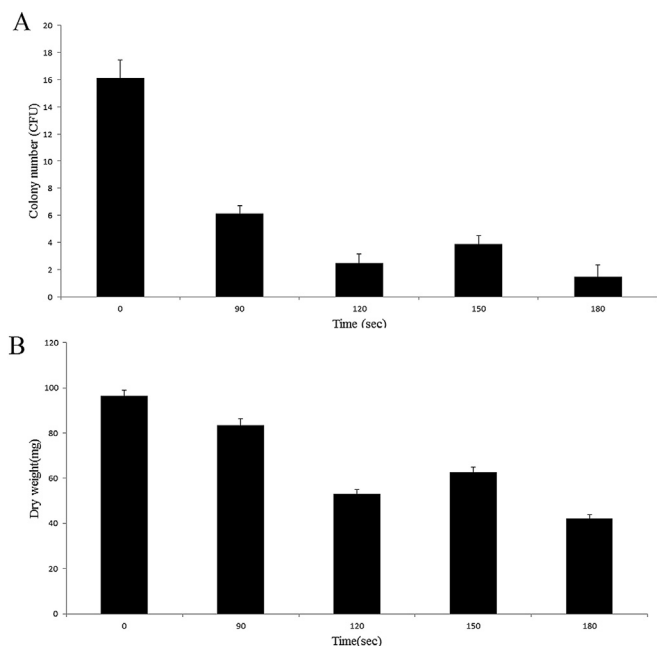

Fig. 1. Comparison of *Trichophyton rubrum* colony numbers after exposure to cold atmospheric plasma for 0, 90, 120, 150 and180 s then cultured in SDA and incubated statically for 9 days at 28 °C. Colony numbers (CFU) significant differences were observed between the treated and untreated samples (P < 0.05) (Fig. 1A). Samples(100 μL) of the *T. rubrum* spores' suspension exposed to CAP for 0, 90, 120, 150, 180 s and inoculated in the Sabouraud maltose broth then incubated at 37 °C in 150 rpm for 3 days. Fungal mycelia were dried at 60 °C for 6 h to reach a constant weight. Dry weights significant differences were observed between the treated and untreated samples (P < 0.05) (Fig. 1B).

T. rubrum were significantly reduced by CAP treatment. CAP strongly prevented the fungus from ergosterol biosynthesis by 27%–54%, though this response was increased at 150 s, which was significantly different from the control and all other treatment durations (P < 0.05) (Fig. 2).

3.3. Effect of CAP on keratinase activity

3.3.1. Agar plate screening

Screening of *T. rubrum* isolates for keratinase production on solid mineral medium showed that the examined isolates were able to produce extracellular keratinase. Keratinolytic activity was assessed based on the observation of a clear zone around the fungal colony of the plate (Fig. 3).

3.3.2. Optimization of keratinolytic activity in submerged cultures

The best production of extracellular keratinase was studied in mineral liquid medium. The maximum keratinolytic activity was
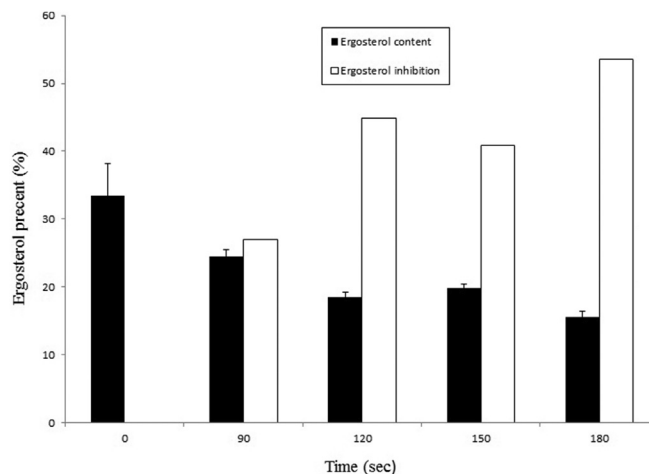

Fig. 2. Comparison between the ergosterol percent content and inhibition percent in *Trichophyton rubrum* when suspensions were CAP-treated during times of 90, 120, 150 and 180 s. It was significantly different from the control and all other treatment durations (P < 0.05) (■ ergosterol content; □ ergosterol inhibition).
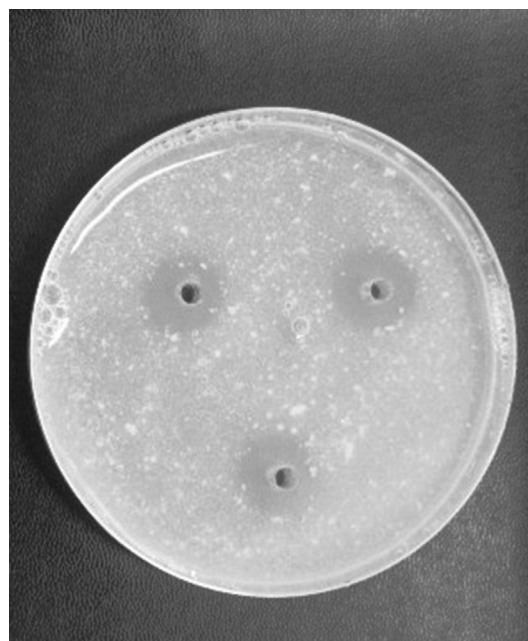

Fig. 3. Keratinase production of *T. rubrum* PFCC 51431 on solid mineral medium evidenced by clear zones around the fungal colonies.

obtained for 14 days cultures maintained at 28 °C. The total (U/mL) and specific activity (U/mg) of keratinase in treated and control samples were evaluated. Total and specific activities of keratinase in

untreated sample were an average of 8.90 U/mL and 24.54 U/mg. After the treatment with CAP for 90 s and 120 s the keratinase total activities were higher, reaching 8.92 U/mL and 9.55 U/mg and that of specific activity of keratinase acquired 25.64 U/mg and 31.13 U/mg. Nevertheless, by cold atmospheric plasma flow, at longer durations of 150 s and 180 s, the keratinase total activities began to fall by 9.52 U/mL and 9.48 U/mg at specific activities of 30.61 U/mg and 30.20 U/mg. The total and specific activities of keratinase enzyme were increased meaningfully by additional time of 120 s under plasma exposure by 7.30% and 21.88% (Table 1). However, there was statistically significant difference between different plasma flow durations ($P < 0.05$) (Fig. 4).

4. Discussion

In this experiment the growth inhibition, ergosterol content and keratinase enzyme activity (total and specific evaluation methods) in *Trichophyton rubrum* were studied by cold atmospheric plasma (CAP) device. Since it was considered very important that dermatophytes, triggered by fungus, especially *Trichophyton rubrum*, as one of the main causes of relatively high worldwide occurrence of superficial infections in human and animals would require a reliable method for their treatments. Recently, scientists have studied plasma antifungal properties extensively. Therefore, a series of attempts have been made on the development and performance of various atmospheric pressure plasma techniques, such as plasma jets, dielectric barrier discharges, and plasma needles to combat fungi causing diseases. In 2013, Heinlin et al. (2013) used SMD plasma containing ambient air without any active flow on single colony of *Trichophyton rubrum* and *Microsporum canis*, in one application and daily treatment with single CAP for 5, 8 and 10 min showing less fungal growth inhibition as compared to a daily CAP treatment only [19]. In another study, Daeschlein et al. (2011) showed that with atmospheric pressure plasma jet (APPJ), using argon gas flowing on colonies of *Trichophyton rubrum*, *T. interdigitale*, and *M. canis* and the yeast *Candida albicans*, in vitro could kill more than 90% of the microorganisms during 30 s [39]. Less than two years ago, Ali et al. (2014) using FE-DBD plasma, with argon as a working gas, investigated its antifungal activity on *Trichophyton mentagrophytes* and *T. rubrum* in saline and in an infected skin model which showed that a significant inhibition occurred by both strains in conidia and hyphal growth, in the infected skin

mimicking model. They also produced a time-dependent correlation between the intracellular reactive species (ROS) and the fungal cell viabilities after plasma treatment [40]. Specially, many research reports have been published on plasma effect on Candida species. Sun et al. (2011) used cold plasma microjet that contained helium and oxygen 2% on *Candida albicans*, *Candida krusei*, and *Candida glabrata* with fluconazole resistance in air and in water. Their result showed that 90% inactivation was achieved in 10 min in air and 1 min in water [16]. In 2013, Keidar et al. used cold plasma, working with helium gas, in cancer therapy. They found that the cold plasma application selectively destroyed cancer cells in-vitro without damaging the normal cells and reduced tumor size in-vivo considerably [33]. "Plasma functions selectively", implied that it was able to select between healthy and unhealthy cells in our body; a characteristics which can help treatments of many diseases.

In the present study, we have examined a number of factors such as growth, weight changes, ergosterol content and keratinase activity enzyme in *Trichophyton rubrum* by cold atmospheric plasma containing helium (98%) and oxygen (2%) working gases. In most published articles there is reference made to the attractive properties of plasma and its effects on fungi, though the biochemical system and its performance is still in question and hence we tried to examine the biochemical effects of plasma on fungi.

Moreover in this project, plasma treatment has been performed on fungal suspensions instead of direct fungal colonization, to understand the growth and the enzyme activity and ergosterol biosynthesis more clearly. The data show that, CAP could affect suspensions and inhibit the growth of *Trichophyton rubrum* on SDA which has been almost stopped by 180 s.

Also, the results of dry weight tests, for indication of CAP treated non-growing spores, were detected by minimum weight in 180 s samples. In the following tests, ergosterol biosynthesis experiment demonstrated that ergosterol percentages were decreased by higher flow of cold atmospheric plasma. Although as it was stated and concluded earlier that at 150 s there were higher average values of all the factors observed relative to 90 s and 120 s treatments, but it was still lower than the control samples. It should be noted that occurrence could be related to the specific behavior of cold atmospheric plasma on fungus. In 2015 Rahimi-verki et al. studied the effects of CAP on *Candida albicans*. They reported that within 120 s the decreasing trend in number of fungal colonies has displayed an ascending and descending currents during irradiation of plasma in every 30 s, whereas in the current finding this variation has been observed in 150 s treatments. Since, plasma is the mixture of ions, radicals, atoms and molecules it can play critical role in microbial inactivation. In our cold atmospheric plasma, the He (98%) and electronegative O_2 (2%) working gases easily produced reactive oxygen species (ROS) and hydroxyl radical (•OH).

Based on our investigation, it may be concluded that there is a correlation between ergosterol and plasma treatment in the *Trichophyton rubrum*, because the fungal plasma membrane has a three-phase composition, consisting of a double layer of phospholipids, large amounts of proteins and sterols. Therefore, the membrane can reduce the amount of ergosterol by cold plasma by direct effect on the wall and the plasma membrane of the fungus.

The ionization of plasma generates ions, free radicals and hydroxyl which lead to disruption of membrane redox system and potassium ion leaving through the cell wall; followed by cells lysis and cell death. Finally the growth rate, weight and amount of ergosterol are low in all samples [41].

In 2011, Peres et al. described the increasing of ergosterol content by exposed fungi to growth inhibitory activity of antifungal azoles. Azoles target the ergosterol biosynthetic pathway by inhibition of a key enzyme, the lanosterol 14α-demethylase, encoded as
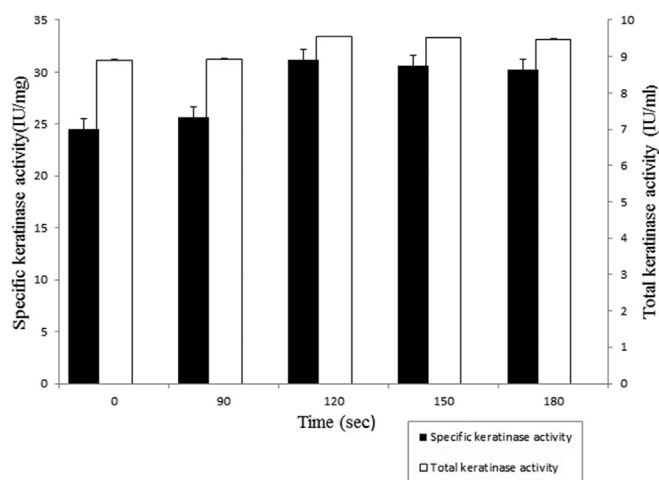

Fig. 4. Comparison of extracellular keratinase activity in *Trichophyton rubrum* PFCC 51431 cultured at 28 °C for 14 days in control group (time 0) and those exposed to CAP for 90, 120, 150 and 180 s (■Specific keratinase activity; □ total keratinase activity).

ERG11 gene. It seems that exposed fungi try to increase ergosterol content with overexpression of the major genes of ergosterol biosynthesis pathway like as *ERG11* as a compensatory mechanism which eventually results in the inducing resistance to the azoles [41].

In the present study, CAP has inhibition role in 90 and 120 s, while increasing the CAP flow time to 120 s the fungi resistance increased. As a result fungi produced and replaced ergosterol however not reaching the zero control time. The fungi resistance failed at beyond 120s and died. Similarly, it was observed in growth and weight results. It is very important that fungi resistance to cold atmospheric plasma is less than its resistance toward drug.

In the last part of our investigation, we studied the effect of plasma on keratinase enzyme activities which have reduced di-sulfide bridges inside the compact keratin network that constituted keratinized tissues. It has been recently suggested that in *T. rubrum* this reduction depends on a sulfite efflux pump, codified by the *TruSsu*1 gene. However, these enzymes could not act before. The sulfite secretion by this transporter allowed the cleavage of the cystine present in keratin [6].

Martinez − Rossi in 2008 showed that *T. rubrum* could resist terbinafin through greater expression of efflux pumps such as ATP binding cassette transporters [42]. In *T. rubrum*, two ATP binding cassette transporters were identified showing importance in enzyme secretion and probably in the pathogenicity of this dermatophyte [6].

The present results showed that keratinolytic activity (total and specific) in all samples increased meaningfully. *T.rubrum* resisted to flowing CAP and tried to survive. Therefore, the fungus tried to use last resort and began to release keratinase enzyme. For instance through over expression of efflux pumps such as ATP binding cassette transporters or other agents that needed to secrete en-zymes the keratinase activity was increased.

Ahmad Khan et al. (2014) investigated the effects of *Carum copticum* and *Thymus vulgaris* oils on the growth of *Trichophyton rubrum* and *Aspergillus* spp. However, these oils appeared to be less effective in reducing the keratinase activity but it could be inhibited on virulence in both fungi [42]. Hence in our studies, it was obvious that potency of CAP inhibits *T. rubrum* although keratinase enzyme increased and fungi resistance occurred in 150 s.

Our experiment has shown that our CAP has antifungal speci-ficity and is able to inhibit the amount of growth in ergosterol. Today, the use of laser is on the rise despite its complications, such as inflammation and burning of diseased cells and healthy cells around the infection; while the cold atmospheric plasma research so far has shown no side effects and it can cure the infected cells. With the development of plasma science and further research in the treatment of patients with cutaneous fungal agent this alter-native method is very suitable for a variety of medical complications.

5. Conclusion

Taken together, our results showed that CAP inhibits the growth of *Trichophyton rubrum* and suppresses the ergosterol biosynthesis while increases moderately keratinase activity probably as a compensatory mechanism by the fungus for CAP-mediated fungal growth inhibition. Thus, it can be considered as a potentially useful device to treat clinical cases of superficial dermatophytosis, espe-cially those not-responding to known synthetic antifungal drugs.

References

[1] I. Weitzman, R.C. Summerbell, The dermatophytes, Clin. Microbiol. Rev. 8 (1995) 240−259.
[2] S. Vermout, Pathogenesis of dermatophytosis, Mycopathologia 166 (2008) 267−275.
[3] R. Tainwala, Y.K. Sharma, Pathogenesis of dermathophytose, Indian J. Der-matol 56 (2011) 259−261.
[4] M.V. Dahl, Dermatophytosis and the immune response, J. Am. Acad. Dermatol 31 (1994) 34−41.
[5] A.J. Zapata Garrido, A.C. Romo, F.B. Padilla, Terbinafine hepatotoxicity A case report and review of literature, Ann. Hepatol. 2 (2003) 47−51.
[6] P. Vandeputte, S. Ferrari, A.T. Coste, Antifungal resistance and new strategies to control fungal infections, ijmicro 1 (2011) 2012.
[7] K.J. Kwon-Chung, J.E. Bennett, Medical Mycology, Lea & Febiger Press, 1992, pp. 105−161.
[8] D.H. Howard, Fungi Pathogenic for Humans and Animals. Part B: Pathogenicity and Detection, Marcel Dekker Inc, 1983, pp. 267−271.
[9] N. Amirrajab, M. Shams-Ghahfarokhi, A. Ghajari, M. Razzaghi-Abyaneh, *In vitro* antifungal activities of griseofulvin and terbinafine against common dermatophytes, Hakim 9 (2006) 28−33.
[10] S. Arndt, P. Unger, E. Wacker, T. Shimizu, J. Heinlin, Y. Li, H.M. Thomas, G.E. Morfill, J.L. Zimmermann, A. Bosserhoff, S. Karrer, Cold Atmospheric Plasma (CAP) changes gene expression of key molecules of the wound healing machinery and improves wound healing in vitro and in vivo, PloS One 8 (2013) e79325.
[11] R. Hippler, H. Kersten, M. Schmidt, K.H. Schoenbach, Low Temperature Plasmas: Fundamentals, Technologies and Techniques, Wiley VCH, Berlin, Germany, Weinheim, 2008.
[12] R. d'Agostino, P. Favia, Y. Kawai, H. Ikegami, N. Sato, F. Arefi-Khonsari, Advanced Plasma Technology, Wiley-VCH Verlag GmbH & Co. KGaA, Wein-heim, 2008. ISBN: 978-3-527-40591-6.
[13] Y.F. Hong, J.G. Kang, H.Y. Lee, H.S. Uhm, E. Moon, Y.H. Park, Sterilization effect of atmospheric plasma on *Escherichia coli* and *Bacillus subtilis* endospores, Appl. Microbiol. 48 (2009) 33−37.
[14] A. Ali, Y.J. Hong, J. Park, S.H. Lee, E.H. Choi, G.C. Kwon, B.J. Park, A novel approach to inactivate the clinical isolates of *Trichophyton mentagrophytes* and *Trichophyton rubrum* by using non-thermal plasma, J. Microb. Biochem. Technol. 6 (2014) 314−319.
[15] N. Rahimi-verki, A. Shapoorzadeh, M. Razzaghi-Abyaneh, S.-M. Atiaby, Cold atmospheric plasma inhibits the growth of *Candida albicans* by affecting ergosterol biosynthesis and suppresses the fungal virulence factors *in vitro*, Photodiagnosis Photodyn. Ther. (2016), http://dx.doi.org/10.1016/j.pdpdt.2015.12.007.
[16] P. Sun, Y. Sun, H. Wu, W. Zhu, J.L. Lopez, Atmospheric pressure cold plasma as an antifungal therapy, J. Appl. Phys. 98 (2011) 021501.
[17] N. Bai, P. Sun, H. Zhou, H. Wu, F. Liu, W. Zhu, J.L. Lopez, J. Zhang, J. Fang, Inactivation of *Staphylococcus aureus* in water by a cold ,He/O2 atmospheric pressure plasma microjet, Plas Proc. Polym. 8 (2011) 424−431.
[18] T. Shimizu, B. Steffes, R. Pompl, F. Jamitzky, W. Bunk, K. Ramrath, M. Georgi, W. Stolz, H.U. Schmidt, T. Urayama, S. Fujii, G.E. Morfill, Characterization of microwave plasma torch for decontamination, Plas Proc. Polym. 5 (2008) 577−582.
[19] J. Heinlin, T. Maisch, J.L. Zimmermann, T. Shimizu, T. Holzmann, M. Simon, J. Heider, M. Landthaler, G. Morfill, S. Karrer, Contact-free inactivation of *Trichophyton rubrum* and *Microsporum canis* by cold atmospheric plasma treatment, Future Microbiol. 8 (2013) 1097−1106.
[20] M. Selcuk, L. Oksuz, P. Basaran, Decontamination of grains and legumes infected with *Aspergillus* spp and *Penicillum* spp by cold plasma treatment, Biores Technol. 99 (2008) 5104−5109.
[21] Z. Xiong, X.P. Lu, A. Feng, Y. Pan, K. Ostrikov, Highly effective fungal inacti-vation in He+O2 atmospheric-pressure nonequilibrium plasmas, Phys. Plasm. 17 (2010) 123502−123506.
[22] X. Pei, X. Lu, J. Liu, D. Liu, Y. Yang, K. Ostrikov, P.K. Chu, Y. Pan, Inactivation of a 25.5μm *Enterococcus faecalis* biofilm by a room-temperature, battery-oper-ated, handheld air plasma jet, J. Phys. D. Appl. Phys. 45 (2012) 165205−165210.
[23] G. Brelles-Mariño, Challenges in Biofilm Inactivation: the use of cold plasma as a new approach, J. Bioproces Biotech. 2 (2012) 4.
[24] T. Maisch, T. Shimizu, G. Isbary, J. Heinlin, S. Karrer, T.G. Klämpfl, Y.F. Li, G. Morfill, J.L. Zimmermann, Contact-free inactivation of *Candida albicans* biofilms by cold atmospheric air plasma, Appl. Environ. Microbiol. 78 (2012) 4242−4247.
[25] J.L. Zimmermann, K. Dumler, T. Shimizu, G.E. Morfill, A. Wolf, V. Boxhammer, J. Schlegel, B. Gansbacher, M. Anton, Effects of cold atmospheric plasmas on Adenoviruses in Solution, J. Phys. D. Appl. Phys. 44 (2011) 505201.
[26] S. Moreau, M. Moisan, J. Barbeau, J. Pelletier, A. Ricard, Using the flowing afterglow of a plasma to inactivate *Bacillus subtilis* spores: influence of the operating conditions, J. Appl. Phys. 88 (2000) 1166−1174.
[27] M.K. Singh, et al., Inactivation factors of spore-forming bacteria using low-

pressure microwave plasmas in an N_2 and O_2 gas mixture, New J. Phys. 11 (2009) 115027.

[28] Y. Song, D. Liu, L. Ji, W. Wang, P. Zhao, C. Quan, J. Niu, X. Zhang, The inactivation of resistant *Candida albicans* in a sealed package by cold atmospheric pressure plasmas, Plasma process. Polym. 9 (2012) 17—21.

[29] Irani S, Shahmirani Z, Atyabi S M, Mirpoor S. Induction of growth arrest in colorectal cancer cells by cold plasma and gold nanoparticles. Arch. Med. Sci. doi: 10.5114/aoms.2015.48221.

[30] Z. Shahmirani, S. Irani, S.M. Atyabi, S. Mirpour, S. Shadpour, M. Ghorannevis, M. Daliri Joupari, Effect of cold atmospheric pressure plasma and gold nanoparticles on cell viability, Ann. Res. Rev. Biol. 4 (2014) 3108—3118.

[31] G. Fridman, M. Peddinghaus, H. Ayan, A. Fridman, M. Balasubramanian, A. Gutsol, A. Brooks, Blood coagulation and living tissue sterilization by floating-electrode dielectric barrier discharge in air, Plasma Chem. Plasma Proc. 26 (2006) 425—442.

[32] G. Isbary, J. Köritzer, A. Mitra, Y.-F. Li, T. Shimizu, J. Schroeder, J. Schlegel, G.E. Morfill, W. Stolz, J.L. Zimmermann, Ex vivo human skin experiments for the evaluation of safety of new cold atmospheric plasma devices, Clin. Plasma Med. 1 (2013) 36—44.

[33] M. Keidar, Volotskova O. ShashurinA, M. Ann Stepp, P. Srinivasan, et al., Cold atmospheric plasma in cancer therapy, Phys. Plasmas 20 (2013) 057101.

[34] L. Ajello, A taxonomic review of the dermatophytes and related species, Sabouraudia 6 (1968) 147—159.

[35] I. Rasooli, M. Razzaghi Abyaneh, Inhibitory effects of Thyme oils on growth and aflatoxin production by *Aspergillus parasiticus*, Food control. 15 (2004) 479—483.

[36] B.A. Arthington-Skaggs, H. Jradi, T. Desai, Morrison CJ.Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of *Candida albicans*, J. Clin. Microbiol. 37 (1999) 3332—3337.

[37] K. Wawrzkiewicz, T. Wolski, J. Lobarzewski, Screening the keratinolytic activity of dermatophytes in vitro, Mycopathologia 114 (1991) 1—8.

[38] I. Takiuchi, D. Higuchi, Y. Sei, M. Koga, Isolation of an extracellular proteinase (keratinase) from *Microsporum canis*, Sabouraudia 20 (1982) 281—288.

[39] G. Daeschlein, S. Scholz, T. Woedtke, M. Niggemeier, E. Kindel, R. Brandenburg, K.-D. Weltmann, M. Jünger, In vitro killing of clinical fungal strains by low-temperature atmospheric-pressure plasma jet, IEEE Trans. Plasma Sci. 39 (2011) 815—821.

[40] A. Ali, Y. June Hong, J. Park, S. Lee, E. Choi, G. Chung Kwon, B. Park, A novel approach to inactivate the clinical isolates of *Trichophyton mentagrophytes* and *Trichophyton rubrum* by using non-thermal plasma, Microb. Biochem. Technol. 6 (2014) 314—319.

[41] N.T. Peres, F.C. Maranhão, A. Rossi, N.M. Martinez-Rossi, Dermatophytes: host-pathogen interaction and antifungal resistance, An Bras Dermatol 85 (5) (2010) 657—667.

[42] M.S. Khan, I. Ahmad, S.S. Cameotra, *Carum copticum* and *Thymus vulgaris* oils inhibit virulence in *Trichophyton rubrum* and *Aspergillus* spp, BJM 45 (2) (2014) 523—531.