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Effects of Environmental Factors and Selected Antifungal Agents on Arthroconidia Production in Common Species of *Trichophyton* Genus and *Epidermophyton floccosum*

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Abstract: The aim of this study was to examine the environmental factors and selected antifungal which affect arthroconidia production in *T. verrucosum*, *T. violaceum*, *Epidermophyton floccosum*, *T. rubrum* and *T. mentagrophytes*. Arthroconidia production was studied on different media and under conditions of varying temperature, pH and CO₂ concentrations. The effect of the addition of antifungal to media was also examined. Arthroconidia formation was occurred optimally on sabouraud dextrose agar under CO₂ 10% tension at 37°C at pH 7.5 and was maximal at 10 days. At 42°C and in media plus NaCl 3% or more than it, no growth occurred. The rate of arthroconidia production was different in these species. Griseofulvin, clotrimazole and betamethasone induced the formation of arthroconidia and itraconazole and particularly terbinafin decrease the rate of arthroconidia production. This result emphasis to effective role of environmental factors and some of antifungals on arthroconidia formation. Also, this result may be clinically significant, if sufficiently fungicidal concentrations are not attained in some areas of the lesion, the antifungal themselves may enhance the conversion of hyphae to arthroconidia.

Key words: Dermatophytes, arthroconidia, environmental factors, antifungals

INTRODUCTION

Dermatophytosis is a mycotic infection that affects the superficial keratinize (skin, hair and nail) of human and other animals (Evans, 1989) and studies estimate that between 10 and 15% of population world wide is affected (Matsumoto, 1996). In the alternative mode, they produce spores by the division of unspecialized vegetative hyphae into cylindrical or spherical cells that termed arthroconidia. The characteristic macro and micro conidia produced by dermatophytes in cultures and those living as saprobes are not produced in any dermatophytes lesions observed to date (Rashid, 2001). It seems that they have an important role in pathogenesis of disease (Rippon, 1988). Arthroconidia formation, characteristic of dermatophyte infection of skin, hair or nails (Miyazi and Fujiwara, 1971). One of the most important concerns of patients treatment from dermatophyte infections is the persistence of fomite, in their surrounding area of their life that can be a cause of reinfection (Gupta *et al.*, 2001). It is generally suspected that arthroconidia may be the dormant form responsible of establishing that chronic recurrent form the infection. Arthroconidia are seen within nail-bed infections, nail plate infection and in infections of scalp hair and skin (Rashid, 2001). In spite of rapid improvement in treatment

during the past decade, many aspects of dermatophyte infections remain poorly understood (Evans, 1999). There is difference between susceptibility of arthroconidia and micro conidia and it can be one of the causes of therapeutic failure and various level of resistance to the antibiotics. Coelho *et al.* (2008) showed that arthroconidia were more resistance to fluconazole, griseofulvin and itraconazole than micro conidia. Success rate of treatment is between 80-85% in dermatophytosis, especially in onychomycosis. Possible reasons for the 20% failure rate include the development of antifungal resistance, poor pharmacokinetics due to the characteristics of the nail or skin and presence of resistant structures such as subungual dermatophytomas (Evans and Roberts, 1989). Arthroconidia are frequently found in active ringworm lesion. They have long been thought to be the sole means of reproduction in parasitic stage in hair and their presence in pathological materials in considered diagnostic (Ajello, 1974). Growth in the stratum corneum of skin, nails and hairs occurs in the form of hyphae, which may than form arthroconidia (Miyazi *et al.*, 1971). Invasion was typically at different sites from that of micro conidia and arthroconidia attachment. Duek *et al.* (2004) showed that fungal elements penetrating occur in between the layers of stratum corneum and separating them. There is little information in the literature about

arthroconidia formation in dermatophyte. They are resistant to adverse environmental conditions (Hashimoto and Blumenthal, 1978). Arthroconidia production in *Trichophyton mentagrophytes* has been observed *in vitro* by number of authors (Bibel *et al.*, 1977; Emyamitoff and Hashimoto, 1979; Weigl and Hejtemanek, 1979; Wright *et al.*, 1984) and in *T. rubrum* (Miyazi and Fujiwara, 1971; Yazdanparast and Barton, 2006). Peano *et al.* (2008) reported that *Trichophyton verrucosum* in the microscopic pattern showed a reduced sporulation. They reported that any micro conidia did not observe. Gupta *et al.* (2003) showed predominance of arthroconidia in *T. raubitschekii* cultures isolated from clinical materials. There is not enough information about arthroconidia production in *T. verrucosum*, *T. violaceum* and *Epidermophyton floccosum*. Here, we describe a detailed examination of the environmental factors and selected antifungal which affect arthroconidiation *in vitro* in *T. verrucosum*, *T. violaceum*, *Epidermophyton floccosum*, *T. rubrum* and *T. mentagrophytes*.

MATERIALS AND METHODS

A total of 5 species of dermatophytes (including *T. verrucosum*, *T. violaceum*, *Epidermophyton floccosum*, *T. rubrum* and *T. mentagrophytes*) isolates from the skin and nail of 50 patients have referred to Razi Hospital in Tehran in 2006-2007 were used. Cultures were maintained at 27°C by monthly subculture on Sabouraud Dextrose Agar (SDA) and distilled water. Slideculture was used for microscopic examination of dermatophyte.

For arthroconidia production a standard pH of 5.6 was used for SDA; other pH were obtained by the addition of Sodium Hydroxide or Hydro Chloride acid (HCL). Other media such as Brain Heart Infusion (BHI), *Trichophyton* No.1 (Difco) and SDA plus 1, 3, 5 and 10% NaCl were also used. For investigating the effect of antifungal on arthroconidia formation, we added itraconazole, terbinafine, griseofulvin, clotrimazole and betamethasone 17-valerate to SDA. All of the antifungal were dissolved in DMSO and DMSO at the same concentration was used as a control. Cultures were incubated for up 10 days in different conditions such as various temperatures, CO₂ concentrations and under aerobic condition. The culture was examined by slideculture technique with or without staining with lactophenol cotton blue. For arthroconidia purification species were selected based on arthroconidia production and were grown on SDA for 10 days until a good yield

of arthroconidia was available. Plates were harvested at 2 weeks and the resulting growth removed by gently scraping the surface of the agar with a glass rod with PBS. The cells removed, were resuspended in PBS, washed and gently agitated. The suspension was allowed to settle for 30 min, the supernatant decanted and filtered through a 12 µm pore size nucleopore filter (Millipore, membrane filter). The concentration of units (single arthroconidia, plus small arthroconidia chains) was standardized at 1×10⁷ unit mL⁻¹, using a heamocytometer. The number of arthroconidia was determined after examining four randomly selected fields a view under 40×magnifications. Arthroconidia were defined as any hyphea compartment <4 µm long or any spherical cell representing a disarticulated arthroconidium that was not obviously an aleuroconidium (microconidium).

RESULT

Arthroconidia formation occurred in 5 species used in this study, when cultures incubated under 10% CO₂ concentration on SDA media at 37°C after 10 days (Fig. 1). Rate of arthroconidia production was different in species. These conditions were used as standard in the comparison of the effect of different factors on arthroconidia production. Under aerobic condition, the frequency of arthroconidia production was zero. Optimal CO₂ concentration was found 10% (Fig. 2) and for time

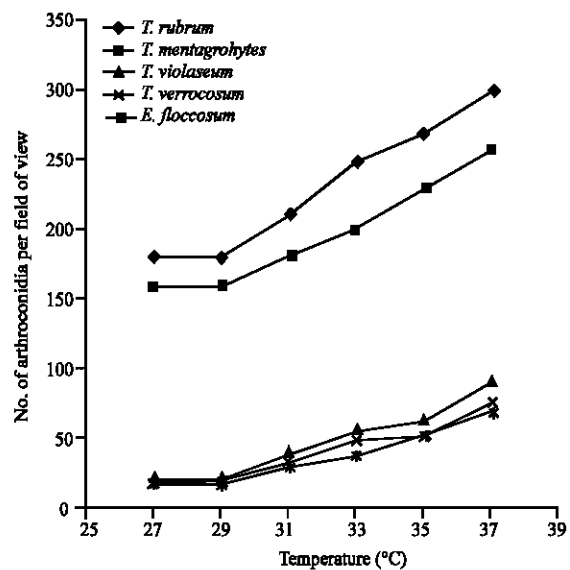


Fig. 1: Effect of temperature on arthroconidia formation after 10 days on SDA at 37°C. Means and 95% confidence limits are shown (n = 3)

Table 1: Effect of different media on arthroconidia production

Species of fungi	BHI	SDA	SDA +1% Nacl	Trichophyton agar No.1
<i>T. rubrum</i>	90.0±16.5	287.3±16.7	189.0±13.5	38.7±4.6
<i>T. mentagrophytes</i>	84.0±20.0	258.0±61	176.0±9.70	29.4±4.2
<i>T. violaceum</i>	38.2±12.6	88.0±2.3	60.2±7.10	11.2±3.5
<i>T. verrocosum</i>	33.9±9.8	73.9±8.3	55.4±11.0	8.3±1.4
<i>E. floccosum</i>	27.4±5.3	65.0±7.0/0	46.9±8.30	12.0±4.1

The mean and 95% confidence limits are shown (n = 3)

Table 2: No. of arthroconidia per field of view in SDA plus antifungals

Antifungal agent	Concentration (µg L ⁻¹)	Mean No. of arthroconidia per field of view				
		<i>T. rubrum</i>	<i>T. Mentagerophyte</i>	<i>T. violaseum</i>	<i>T. verrocosm</i>	<i>E. floccosum</i>
Griseofulvin	0.25	358±13.6	334±12.1	77±9.9	94±7.2	70±4.5
	0.50	299±17.8	287±14	68±7.0	86±3.4	69±7.6
Clotrimazole	0.10	375±8.2	299±10.1	81±3.2	89±6.5	76±9.4
	0.50	319±9.4	303±9.8	71±5.0	94±2.4	71±5.4
Itraconazole	0.25	199±14.0	166±7.6	44±6.2	64±7.1	58±6.5
	0.50	175±12.3	157±11	40±5.1	59±3.2	50±3.3
Terbinafine	0.001	123±7.4	118±8.5	33±1.2	44±2.3	38±4.4
	0.05	83±2.1	80±14	28±0.4	39±3.1	29±2.0
Betamethasone	100	350±3.4	310±5.2	84±6.1	96±4.0	74±3.0
DMSO (10%)	NA	279±4.9	280±6.1	71±1.3	85±4.0	60±6.1
Sabouraud broth control	NA	295±9.8	287±2.3	74±2.4	86±7.1	62±1.6

NA: Not applicable, The mean and 95% confidence limits are shown (n = 3)

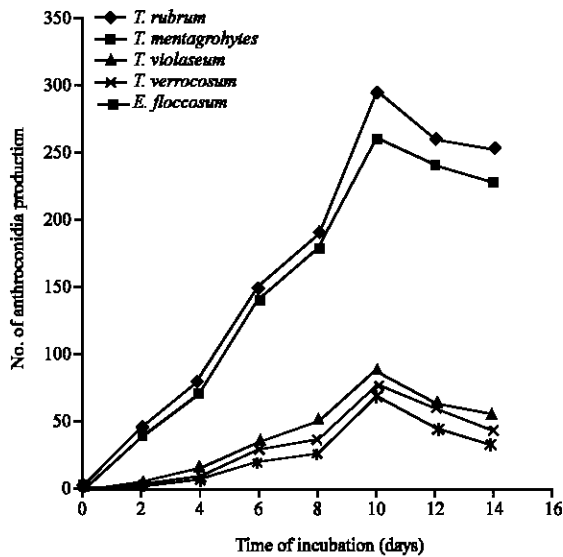


Fig. 2: Effect of time of incubation on arthroconidia formation after 10 days on SDA at 37°C. Means and 95% confidence limits are shown (n = 3)

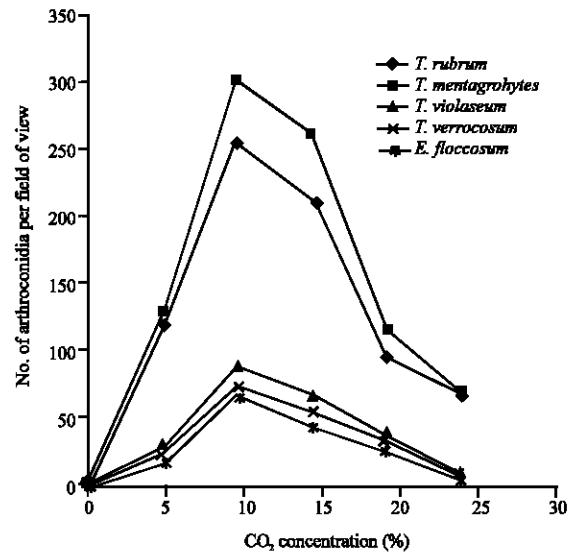


Fig. 3: Effect of CO₂ concentration on arthroconidia formation after 10 days on SDA at 37°C. Means and 95% confidence limits are shown (n = 3)

course of arthroconidia production was 10 days (Fig. 3). No growth was seen above 3% NaCl at 42°C. Various levels of arthroconidia production were seen with other media (Table 1). On SDA and in the presence of 10% CO₂ at 37°C was the optimal condition of arthroconidia production for most species. Optimal pH was found to be 7.5 (Fig. 4). The rate of arthroconidia production increased

when we used griseofulvin (0.5 µg mL⁻¹) and clotrimazole (0.1 µg mL⁻¹) in addition to SDA under 10% CO₂ concentration at 37°C. Itraconazole and particularly terbinafine suppressed arthroconidia production at the same condition. The steroid betamethasone at 1mg mL⁻¹ caused stimulation of arthroconidia formation (Table 2).

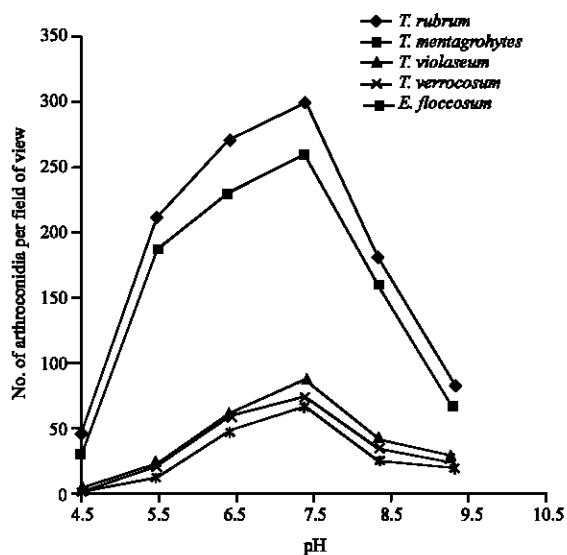


Fig. 4: Effect of pH on arthroconidia Formation after 10 days on SDA at 37°C. Means and 95% confidence limits are shown (n = 3)

DISCUSSION

Over the past few decades rate of incidence of infections by dermatophytes has increased considerably and such infections are often recalcitrant to therapy (Gupta, 2000). Text books in medical mycology usually describe the spores found in ringworm lesions of the skin or infected hairs as arthroconidia (Rippon, 1988; Emmons *et al.*, 1977) that they are considered to be the primary cause of skin and nail infection in humans and animals (Gupta *et al.*, 2003). The infection caused by these parasitic spores is the important public health problem as yet unresolved (Fernandez-Torres *et al.*, 2003). A variety of conditions were found which either supported or stimulated arthroconidia formation in these species, including temperature, CO₂ tension and composition of complex media. Temperature of incubation was found to markedly affect arthroconidia formation in SDA medium. Optimal temperature was seen at 37°C in all species. Some investigators have reported that favored temperature for arthroconidiation of *T. mentagrophytes* on SDA is between 32-39°C and no or less arthroconidia formation were seen at 30°C. Similar to our result, arthroconidia production on sabouraud dextrose broth in *T. rubrum* has been reported by Miyazi and Nishimura (1971) and Yazdanparast and Barton (2006) and in *T. mentagrophytes* by Emyanitoff and Hashimoto (1979). Arthroconidia production in the most of species seen at 37°C, not 25°C. This is similar to the result that Miyazi and Nishimura have reported about arthroconidia formation in *T. rubrum*.

Mares (1999) showed that the treatment with 1-amino-6-methyl-4-phenylpyrazolo (3, 4-d) - 1, 2 and 3-triazole suppressed the various forms of saprophytic conidia, induced the formation of chlamydo spores and accelerated the formation of arthroconidia in *T. rubrum*. For the optimal arthroconidia production, the temperature should be 37°C which is higher than those recorded for the body surface, typically 33°C±1°C and near the upper limit for growth for these species, especially in *T. rubrum*. However, the temperature is higher than the intermediate and ventral layers of nails and skin which typically contain arthroconidia, so that the temperature for growth and arthroconidia formation in dermatophyte especially in *T. rubrum* in onychomycosis maybe due to its pathogenic adaptation *in vivo*.

An increased CO₂ tension, 10% being optimal, was shown to be essential for *in vitro* arthroconidia formation in these species. Arthroconidia production in *T. mentagrophytes in vitro* was also induced by Bible *et al.* (1997) by cultivation in an atmosphere with higher CO₂ tension at 31°C. Whether the stimulation of arthroconidiation is due to the presence of CO₂ itself or reduced oxygen tension has been the subject of some controversy (Berrera, 1986). Yazdanparast and Barton (2006) showed that arthroconidia production occurred in *T. rubrum* when 10% of the incubation atmosphere was replaced by CO₂ on SDA media at 37°C after 10 days. Emyanitoff and Hashimoto (1979) have shown that exogenous CO₂ is not absolute requirement and that replacement of air by either N₂ or CO₂ stimulates arthroconidiation in *T. mentagrophytes* to the same extent.

However, Wright *et al.* (1984) showed that arthroconidia of *T. mentagrophytes* were obtained in the presence of increased CO₂ tension but not increased N₂ tension. Helium and methan like CO₂ tend to inhibit growth of *T. mentagrophytes*; they do not induce arthroconidiation (King *et al.*, 1976). CO₂ diffuses physiologically from normal skin (Frame *et al.*, 1972). At the skin surface CO₂ concentration under on occlusion bandage reaches values of 5-8% (King *et al.*, 1978). Such concentrations are also found when the skin is damaged by infection or trauma (Malten and Theiele, 1973).

So, arise of CO₂ tension can stimulated the transformation of dermatophyte hyphea in to arthroconidia chains (Allen and King, 1978). However, Gupta *et al.* (2003) have shown that *T. raubitschekii* can produce arthroconidia chain under normal aerobic conditions without stimulation by 10% CO₂. This study showed that some arthroconidia formation occurred at levels as low as 5% CO₂.

Arthroconidia formation in Sabouraud Dextrose Broth (SDB) in *T. rubrum* has been also reported by Miyazi and

Nishimura (1971) and in *T. mentagrophytes* by Emyniatoff and Hashimoto (1979). In this study, SDA was found to be optimal for the maximal formation of arthroconidia in these species arthroconidia was also produced on SPA with 1% nail by these species at 10% CO₂ but to a lesser extent. Yazdanparast and Barton (2006) have found that SDA was the optimal medium for arthroconidiation in most strains of *T. rubrum*.

A great number of arthroconidia productions were seen on BHI at 37°C than other media and that presence of 0.85% nail. (Miyazi and Nishimura, 1971). It is possible that the pH of the local environment within the skin or nail is an important factor in arthroconidia formation for example in *T. rubrum*. Meevootism and Niederpruem (1979) reported that dermatophyte posses a high keratinase activity which has an alkaline pH optimum. The most appropriate pH for arthroconidia production have been reported 7.5 (Yazdanparast and Barton, 2006).

The optimal pH of the medium for arthroconidia formation in *T. rubrum* was found to be 7.4 by Miyazi *et al.* (1971). Although, generally, slightly acidic conditions favored arthroconidiation, the optimal pH for arthroconidia formation was found to be 7.5 low levels of several antifungal agents at sub-inhibitory concentration stimulated arthroconidia formation in these species. In present study, similar effects were seen in *T. mentagrophytes* where griseofulvin (0.5 µm mL⁻¹), clotrimazole (0.1 µm mL⁻¹) and particularly amphotericin B (5 µm mL⁻¹) stimulated a low level of arthroconidiation. This is very similar pattern to that which has been shown here in these species. Interestingly, in present study itraconazole and particularly terbinafine suppressed arthroconidia production and that two antifungals also have significantly increased rate of cure and shorter treatment times for dermatophytosis (Roberts, 1999).

Dormant arthroconidia of *T. mentagrophytes* are resistant to common antifungal such as clotrimazole, miconazole, nistatin and griseofulvin. These result maybe clinically significant, if sufficiently fungicidal concentrations are not attained in some areas of lesion, the antifungal may stimulated transformation of hyphae to arthroconidia. Steroids may influence either directly or indirectly the development of dermatophyte infection. An early report showed an inhibition of the growth of *T. rubrum* by different steroids (Chattaway *et al.*, 1959). The steroid betamethasone at 1 mg mL⁻¹ caused stimulation of arthroconidia formation. This phenomenon may explain how many cases of treatment failures and relapses, which occur, with all currently available therapeutic agents. The thick walls of arthroconidia may resist antifungal treatment, only to germinate on infection following the cessation of treatment.

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