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In vitro induction of extracellular neutrophil traps (NET) with Trichophyton rubrum

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Abstract

Tinea is a human dermatophytosis caused by *Trichophyton rubrum,* which infects keratinocytes, generates severe lesions in immunosuppressed individuals, and is a public Health problem. The immune system of mammals has kept his innate component based on the recognition of pathogen-associated molecular patterns through its receptor recognition patterns on the surface of immune cells, including macrophages and neutrophils, which besides its phagocytic function, are capable of destroying extracellular pathogen forming traps made of DNA, histones, and antimicrobial peptides (NET). The aim of this study was whether neutrophils isolated from peripheral blood of normal subjects by bringing them into contact with sonicated conidia and conidia of *T. rubrum,* form NET. by means of In vitro culture of *T. rubrum,* isolation and purification of conidia of *T. rubrum,* and obtaining a homogenate of *T. rubrum* conidia by sonication for the induction of Neutrophil extracellular traps (NET). The results shows that by putting in contact conidia and sonicated conidia of *T. rubrum* positively induced formation of NET. It is concluded that both conidia as the sonicated conidia of *T. rubrum* may have a protective function as a component of the innate immune response in individuals at risk of acquiring infection.

Innate Immune Response, Neutrophils Extracellular Traps (NET), *Trichophyton rubrum*

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1. Introduction

Humans have infectious diseases caused by fungal bacteria and pathogenic viruses that develop adaptation mechanisms to evade the Innate Immune Response (RII) and the Adaptive Immune Response (RIA) of the host, survive in it and generate these diseases that are becoming more frequent among the population, since they are resistant to treatment [2, 9, 22].

The most frequent fungal infections among the population are dermatophytosis or tinea infections caused by Trichophyton rubrum, among others. In Mexico this is a problem that occurs between 70 and 80% of all mycoses. Because it has a very high frequency as an infection in different parts of the body, it is believed that Trichophyton rubrum has developed survival modes to escape the host defense mechanisms, as a result the disease is expressed with bothersome and uncomfortable symptoms for the patient [4 , 24, 31].

1.1 Justification

The NETs are related to many infectious diseases, and possibly autoimmune as mentioned, and have shown affinity for pathogenic microorganisms destroying them. Therefore, this mechanism is essential for the innate immunity against T. rubrum, keeping the host free from Ringworm.

1.2 Problem

Trichophyton rubrum is a pathogen that mainly affects the keratinocytes, feeding on keratin, which is a necessary nutrient for it to develop, causing one of the most common fungal diseases in humans; ringworm and athlete's foot among others. This parasite shows to be rebellious to the treatments, since it has developed mechanisms of resistance and evasion [25, 19].

1.3 Hypotesis

The complete conidia and the sonicated extract of conidia of the fungus Trichophyton rubrum, induce the formation of extracellular networks of neutrophils of human peripheral blood in in vitro conditions.

1.4 Objectives

1.4.1 General objective

Experiment if the complete conidia and the sonicated extract of conidia of the fungus Trichophyton rubrum, are able to induce the formation of extracellular DNA networks of human peripheral blood neutrophils under in vitro conditions.

1.4.2 Specific objectives

- Isolation and Culture T. rubrum.
- Isolation of conidia from T. rubrum.
- Homogenate conidia of T. rubrum.
- Isolation and culture of polymorphonuclear neutrophils from human peripheral blood.
- Induction of extracellular neutrophil traps (NET).

2. Theoretical framework

Classically, physical barriers such as skin, mucous membranes, hair, body secretions, etc., constitute the first line of defense of the RII. Antimicrobial peptides, phagocytosis and the involvement of the complement system are important mechanisms of this response [30].

The RII is also largely integrated by the action of granulocytes and macrophages, its main function is to phagocytose fungi and destroy bacteria. They are very important cells for immunity [15, 32].

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Neutrophils are a type of granulocyte, whose function is to participate in the initiation of the inflammatory process and respond to the stimulation of fungi, bacteria, and other parasites to eliminate them, trying to make this mechanism effective [3, 23, 26, 27, 39, 41]. Recent research indicates that neutrophils under infection conditions can catapult extracellular networks or fibers at the site of an infection in response to pathogens, called traps.

Extracellular neutrophils, by its acronym in English (NET), which are structures composed mainly of DNA, histones, elastase and antimicrobial peptides, which are present in the nucleus and granules of the cytoplasm of neutrophils [11, 12, 14, 34, 35, 37, 38, 43, 44, 47]. The DNA of the nucleus of the living cell is violently expelled to the outside of the membrane, and as a result the microorganisms are trapped in the network, eliminating them and initiating the process of healing and regeneration of the tissue, all at the cost of the death of the neutrophil. [5, 7, 8, 16, 17, 18, 20, 21, 33, 36, 42, 48].

There is still much to be deciphered regarding the role of NETs in the development or control of infectious diseases; however, the excessive formation of NET or its persistence would play a fundamental role in some infectious and autoimmune diseases. It is not clear if severe lung dysfunction or other diseases in conjunction with viral infections could be due to the formation of NETs as a consequence of the inflammatory response. Finally, it should be mentioned that the formation of NET is preferably induced by pathogenic microorganisms [28, 45, 46, 49].

3. Metodology

Isolation and Culture of T. rubrum.- Trichophyton rubrum was directly isolated from 1 patient diagnosed with dermatophytosis (at the Dermatological Institute of Jalisco, located at North Federalism Street # 3102, Atemajac del Valle, 45190 Guadalajara, Jalisco) according to the technique of Dr. Jorge Mayorga of the Mycology laboratory of the aforementioned institute. It was cultivated in glass tube with Agar Saboraud (J.T. Baker®) solid.

It was incubated at 37 ° C for 24 hrs (after this time it was left to incubate at room temperature between 7 and 15 days). The nichrome handle was introduced with a self-adhesive strip frame on the tube with Trichophyton rubrum, it was scraped and a part of the colonies was captured. The tape was placed in an object holder and two drops of the methylene blue dye (J.T. Baker®) were added, the object cover was placed and the identification of the microorganism was observed in the compound microscope.

The strain of T. rubrum was inoculated into the Petri dish containing the solidified Sabouraud Agar. Petri dishes were labeled with the name of the strain and incubated at 37 ° C for 24 hrs, the Petri dishes were left until their maximum growth at room temperature [19].

Conidial isolation of T. rubrum.- For the isolation of the conidia, 10mL of bidistilled water was used (obtained from the laboratory of Dr. Anne Santerre), sterilized by autoclave and added to a culture box with the microorganism in its maximum growth and scraped the agar surface with a spatula. The liquid was recovered and vortexed for 3 minutes (GENIES II®) three times to detach the conidia. The suspension was purified with sterile Wathman No.1 filters.

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The conidia were quantified with a Neubauer chamber (Loptik Labor®) obtaining 7X105 = 700,000 conidia of T. rubrum in 1 mL of water. The conidial solution was divided into two tubes of 15mL with a volume of 5mL per tube, one tube was cooled to -20 ° C and the second was placed in a sonicator (BRANSON®) to break the conidia [19].

Homogenate of conidia of T. rubrum.- Four sonication cycles of 30 minutes each were used. The solution was filtered through a sterile 0.22 μ membrane (Millipore®). The breaking of the conidia was confirmed by visualization in the microscope. The solution with the conidial homogenate was refrigerated at -20 ° C [19].

Isolation and culture of polymorphonuclear neutrophils from human peripheral blood. - 7mL (per donor) of human peripheral blood was obtained from 6 clinically healthy donors in heparin vacuator tubes (BD®), a tube of 15mL was prepared with 7mL of histopaque (Ficoll-SIGMA®) to perform the first density gradient. The 7mL of blood was slowly poured into the tube with the 7mL of ficoll, taking care not to break the gradient, centrifuged at 2500rpm for 20min, at room temperature.

The different phases were discarded: plasma, ficoll and mononuclear, the granulocyte phase was taken and placed in a new tube. 10mL of PBS pH 7.2 was added to the tube with the granulocytes and mixed by inversion, centrifuged at 1600rpm for 10min, (in a Jouan® centrifuge). At the end of the centrifugation, the supernatant was discarded and 4mL of PBS was added, the cells were resuspended. This solution was slowly passed to the tube containing 10mL of the percoll gradients *. Centrifuged at 2,500rpm for 20min, at room temperature. The polymorphonuclear ring was recovered and added to a new tube, 10mL of PBS was added and mixed by inversion, centrifuged at 1,600rpm for 10min, at room temperature.

The supernatant was removed and 6mL of RPMI medium (SIGMA®) was added, the cells were resuspended. The cells were quantified in a Neubauer chamber, concentration of 90μL of trypan blue (SIGMA®) with 10μL of the sample with the cells. [29, 1].

Formulation of the decreasing gradient of Percoll (SIGMA®) .- The solution for the percoll gradient was prepared: 36mL of percoll were mixed with 4mL of 10% PBS, to have a 100% solution. In a new 15mL tube, 1.5mL of Hank's solution was added with 8.5mL of percoll leaving an 85% solution, 2mL of Hank's solution was added with 8mL of percoll to another new tube leaving the 80% solution , in the next tube, 2.5mL of Hank's solution (SIGMA®) was placed with 7.5mL of percoll leaving the solution at 75%, in the following tube 3mL of Hank's solution was added with 7mL of percoll to remain a 70% solution, and 3.5mL of Hank's solution was added to the last tube with 6.5mL of percoll to be 65%. In a new tube of 15mL 2mL of 85% percoll was added, slowly to the same tube 2mL of 80% percoll, 2mL of 75% percoll, 2mL of 70% percoll and finally 2mL of 65% percoll were added. (the solutions were added as slowly to avoid breaking the gradient).

Induction of neutrophil extracellular traps (NET). - 6 covers were placed round objects 5 min in 70% ethanol, 5 min in 100% ethanol and 5 min in poly-L-lysine (SIGMA®) solution 1:10, subsequently They were placed on the 6-well plate (one covers per well).

It was washed with 1mL of PBS pH 7.2 at the periphery of the well to remove excess poly-L-lysine. 3X106 of neutrophils were added to each well, 200μL of autologous serum, two wells were left as negative control, no stimulus was received (C-), two were established as positive control (C +) with Forbol Miristato Acetate (for its acronym in English PMA SIGMA ®) at a concentration of 1μg / 1mL.

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And the last two wells were the experimental conditions and were stimulated with 70,000 complete conidia of the fungus T. rubrum in 100µL of bidistilled water each.

The plate was incubated for 3hrs at 37 ° C with 5% CO2 (in a Thermo® incubator) After the incubation time, each well was washed with 1mL of PBS to remove excess PMA and conidia. 500µL of 4% paraformaldehyde was added per well, incubated for 20min at room temperature. It was then washed with 1mL of PBS each well to remove the excess. 100µL of DAPI in concentration 1mg / 1mL was added to each well and incubated for 1h at room temperature. Was washed with 1mL of PBS each well to remove excess DAPI (SIGMA®), incubated for 24h at 4 ° C. The next day two drops of PBS pH 7.2 were placed in the holder and covers were mounted, samples were observed under the Karl Zeiss® fluorescence microscope) and the images were captured [10, 13].

4. Results

Isolation and Culture of T. rubrum.

As can be seen in Figure 1, it was possible to maintain the viability and maximum growth of the Trichophyton rubrum strain that was isolated directly from a patient.

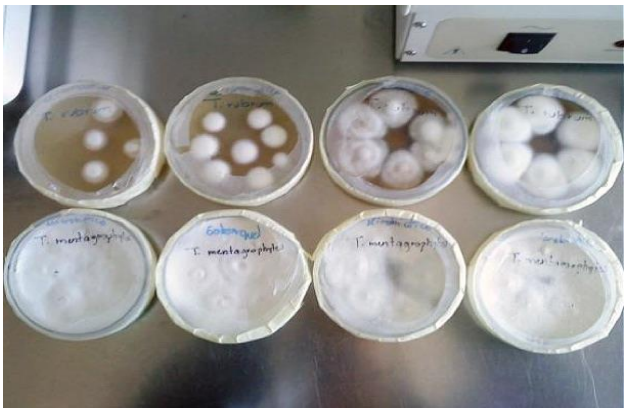

Figure 1 Maximum growth at room temperature of T. rubrum inside the Petri dish containing the solidified Sabouraud Agar. [19]

Induction of extracellular neutrophil traps (NET)

After isolating, culturing and stimulating the neutrophils of the seidonants with the different controls and experimental conditions, it was possible to observe, that the neutrophils of the first duplicate, of each of the 6 experiments (one from each donor), corresponding to the negative control (C-) remain intact when no stimulus is received, while the duplicates of each experiment with cells that were stimulated with PMA, corresponding to the positive control (C +), showed the DNA released and defragmented.

It can be seen that the cell loses all its structural properties, releasing their genetic and cytoplasmic material to the extracellular space in the form of a network (NET). The following duplicates correspond to the stimulation of complete conidia (3 first donors) and finally with conidial homogenate (donors 4, 5 and 6), the induction and release of the genetic material was also observed, with the formation of NETs. (See Figures 2-7).

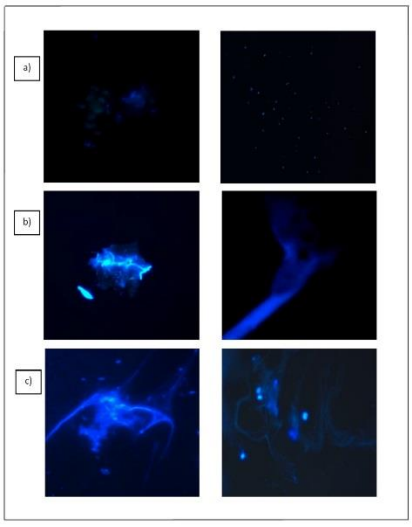

Figure 2. Induction of NET in vitro with conidia of Trichophyton rubrum, images obtained by fluorescence microscopy (40X) (a). Neutrophils without stimulus (C-). (b) Neutrophils stimulated with PMA (C +). (c) Neutrophils stimulated with complete conidia of Trichophyton rubrum.

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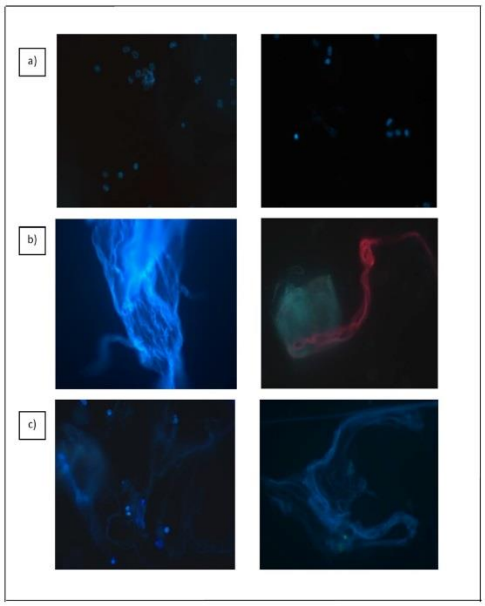

Figure 3 Induction of NET in vitro with conidia of Trichophyton rubrum, images obtained by fluorescence microscopy (40X) (a). Neutrophils without stimulus (C-). (b) Neutrophils stimulated with PMA (C +). (c) Neutrophils stimulated with complete conidia of Trichophyton rubrum

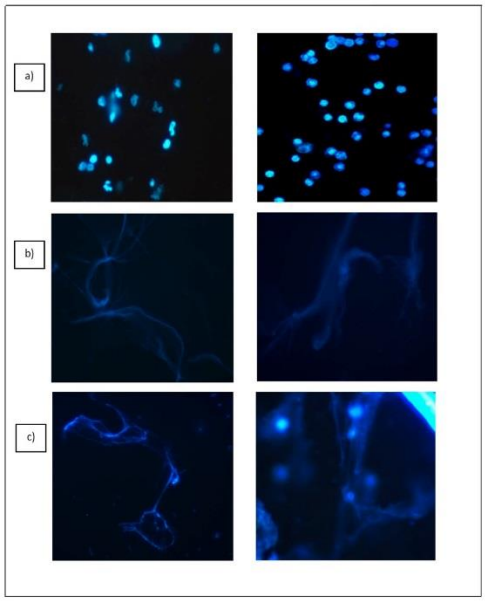

Figure 4 Induction of NET in vitro with conidia of Trichophyton rubrum, images obtained by fluorescence microscopy (40X) (a). Neutrophils without stimulus (C-). (b) Neutrophils stimulated with PMA (C +). (c) Neutrophils stimulated with complete conidia of Trichophyton rubrum

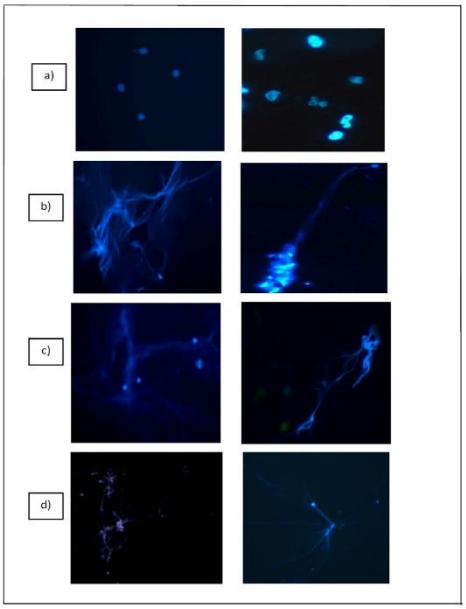

Figure 5 In vitro induction of NET with sonic extract of conidia of T. rubrum: images obtained by fluorescence microscopy (40X). (to). Neutrophils without stimulus (C-). (b) Neutrophils stimulated with PMA (C +). (c). Neutrophils stimulated with complete conidia of Trichophyton rubrum. (d) .Neutrophils stimulated with sonic extract of conidia

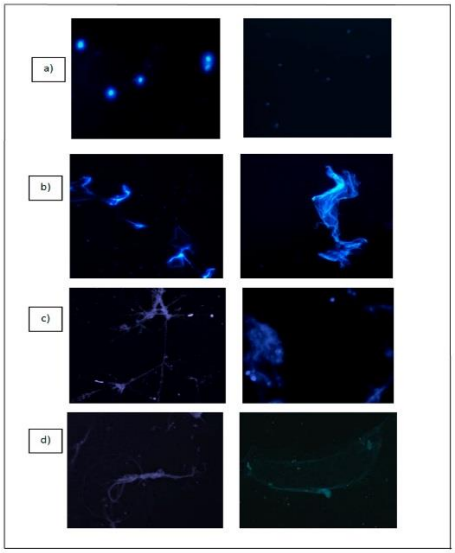

Figura 6 Inducción in vitro de NET con extracto sonicado de conidias de *T. rubrum:* imágenes obtenidas por microscopía de fluorescencia (40X). (a). Neutrófilos sin estímulo (C-) . (b). Neutrófilos estimulados con PMA (C+). (c). Neutrófilos estimulados con conidias completas de *Trichophyton rubrum.* (d).Neutrófilos estimulados con extracto sonicado de conidias.

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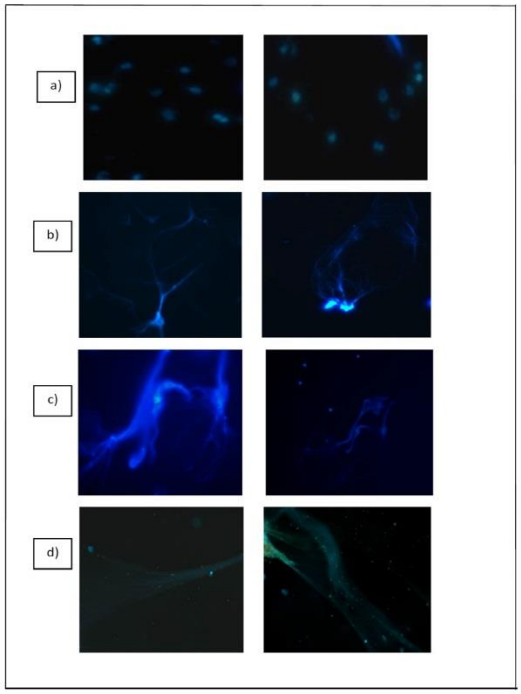

Figura 7 Inducción in vitro de NET con extracto sonicado de conidias de *T. rubrum:* imágenes obtenidas por microscopía de fluorescencia (40X). (a). Neutrófilos sin estímulo (C-) . (b). Neutrófilos estimulados con PMA (C+). (c). Neutrófilos estimulados con conidias completas de Trichophyton rubrum. (d).Neutrófilos estimulados con extracto sonicado de conidias.

Discussion

The nature of DNA as a polynucleotide that contains deoxyribose and complementary nitrogenous bases linked by phosphodiester bonds and hydrogen bonds within its structure, locate it as a hierarchical molecule, which contains the genetic information of all biological organisms. In this work, it was demonstrated that not only is it a molecule that stores and protects information, but it is also a utilitarian molecule since it participates in the protection of the organism against infectious agents, and can be released into the extracellular space of neutrophils and mast cells, in a of network in conjunction with antimicrobial accessory molecules [11, 50, 51].

Therefore the knowledge of this function, has demonstrated this novel role of DNA in granulocytes, and opens the possibility of new hypotheses for the realization of research on this mechanism different from apoptosis, called "netosis", developed evolutionarily as protection against infection, in different biological models of invertebrates and vertebrates as an adaptation mechanism.

Previous results of our work group revealed that human keratinocytes in vitro, respond to the antigenic stimulus of conidia and the homogenate of the fungus T. rubrum, proliferating and differentiating in response to this stimulus, also by means of TLR-2 receptors, TLR-4 and TLR-6 induce the production of antimicrobial peptides such as beta-defensin 2 and interleukins 1β and 8 [19]. So it shows local immunity.

Here it is shown that the neutrophils of human peripheral blood also respond to the stimulus of the complete conidia of T. rubrum, forming the DNA networks, but importantly, in this work it is demonstrated that the sonicate-homogenate of these conidia also induces the NET. Other works report that NET traps and eliminates bacteria. Using Staphylococcus aureus, it is reported that it reacts with neutrophils and these suffer netosis, subsequently releasing the NETs [17].

It has also been shown that DNA is the main component of the NET since in an experiment with Streptococcus pyogenes and DNAse treatments it is not possible to trap the bacteria, and therefore eliminate them, because the networks are disintegrated by the DNAse [12, 13]. The foregoing indicates the importance of this mechanism. Furthermore, interestingly, in other experiments with Staphylococcus aureus, the results showed that pathogens are trapped, but do not die by NETs, because they promote the premature death of the immune cell, as a mechanism of evasion [40].

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It is demonstrated that Streptococcus pneumoniae escapes NETs because they produce DNADs and allow the diffusion of pneumococci from the upper airways of the lungs and from the lungs into the bloodstream during pneumonia [6]. A similar study was conducted with Mycobacterium tuberculosis, and the result showed that mycobacteria stimulate the neutrophils that release the NETs but the networks fail to destroy the bacteria with their accessory molecules [36]. So this important mechanism can be evaded.

Several experiments have been carried out to directly or indirectly induce the NETs with different microorganisms, but it has never been done with the conidia or with any structure of T. rubrum, nor with the sonic extract of conidia, so this would be the main contribution of this work. It is likely that when the conidia are destroyed by sonication, they release the molecular patterns of the fungus, and in this way the ability of the fungus to be able to parasitize is lost.

That complete conidia is needed among other conditions in order to perpetuate its species. The above, together, allows us to hypothesize that the sonicate-homogenate of conidia can have an effect similar to a vaccine, since when homogenized by sonication, the conidia breaks and does not have the danger of parasitizing, but it does have the advantage of start an immune response, what which can be applied prophylactically in the case of the prevention of infections in the skin and other sites or of infectious diseases involving neutrophils.

5. Conclusions

The conidia and their sonicate-homogenate of the pathogenic microorganism Trichophyton rubrum were placed in contact with neutrophils isolated from human peripheral blood.

The above markedly induced in vitro the neutrophils of human peripheral blood to the production of NET. It is important to conclude that the homogenate of T rubrum conidia that does not cause infection could be an immunizing and therefore protective prophylactic agent in healthy subjects exposed to the causative agent of tinea Trichophyton rubrum.

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