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STUDY OF STRATUM CORNEUM FREE AMINO ACIDS IN PATIENTS WITH DERMATOPHYTOSIS AND NORMAL SUBJECTS

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ABSTRACT

Dermatophytes are a group of fungi that cause infections in keratinized human and animal tissues. Physical and chemical agents can be effective in reveals of dermatophytosis pathogenesis in human which some people are sensitive and some other are resistance to it. Amino acid changes may be a risk factor for infection with dermatophytes in mammals. In the framework of a survey on the comparative changes of free amino acids in stratum corneum in 60 patient with dermatophytosis in two site, one , near skin lesion and two, sole area and 60 healthy volunteers(normal subjects), at sole area were done Amino acid in stratum corneum analyzed by HPLC method and the identification of dermatophytosis was based on direct examination and culture. The results of research statistically were analyzed by software and comparison of mean by using the t-test. Achieved results between case and control in sole area showed that cases were significantly increased in amino acids: Aspartate - Tyrosine –Tryptophane - Phenylalanine and were significantly decreased in amino acids: Citrulline– Ornithine Similarly, in two sex male and female. In also people with dermatophytosis in two site near skin lesion and sole area distribution in associated were significantly increased in Glutamates - Asparagine - Histidine - Glutamine - Arginine - Citrulline - Threonine - Methionine - Leucine – Ornithine and were significantly decreased only in Glycine. Our research shows that due to the concentration, amino acids can effect stimulation or inhibition of dermatophytes growth in stratum corneum.

Key Words:- Dermatophyte - Stratum corneum, Free amino acids .

INTRODUCTION

Dermatophytes are a group of closely related fungi that have keratinase and can therefore cause infections in keratinized human and animal tissues (skin, hair and nails), leading to a disease known as dermatophytosis. The etiologic agents of the dermatophytosis (ringworm) are classified in three anamorphic (asexual or imperfect) genera, Epidermorphyton, Microsporum and Trichophyton.

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Physical and chemical agents can be effective in reveals of dermatophytosis pathogenesis in human which some people are sensitive and some other are resistance and might be dermatophytes also shown difference susceptible against of this agent. Several chemical factors such as amino acids can be effective in dermatophytes growth. Amino acid changes may be a risk factor for infection with dermatophytes in mammals (Rippon, 1988; Weitzman and Summerbell, 1995; Vermout *et al*., 2008; Achterman and White, 2012; Hashemi and Sarasgani, 2004).

Some investigations revealed inhibitory effect of some amino acids on growth of some dermatophytes for example L-cysteine hydrochloride, L-cysteine, L-aspartic acid, Lglutamic acid and DL-tryptophan and L-tyrosine have the most inhibitory effect on the studied dermatophytes, while arginine L-lysine and L-methionine have moderate effect and the rest of amino acids have less inhibitory, or even stimulatory effect on the growth of dermatophytes. Some data indicates that sulfur-containing amino acids and acidic amino acids have greater inhibitory effect against *M. canis* and *T. schoenleinii* (Sarasgani and Firoozraee, 2006; Sarasgani and Firoozrai, 2010; 2008).

Garachorlou and *et al* (2001-2012), revealed that aspargin and methionine causes decrease in *T. rubrum* and *T. verrucosum* growth, and inhibitory effect of valine on *T. mentagrophytes*. Histidine has inhibitory effect on *T. mentagrophytes* growth and the inhibitory effect of tryptophan on *T. verrucosum* and growth decreasing in *E. floccosum* were also reported (Gharachorlou and Gharachorlou, 2011a; 2011b; 2011c; 2012). Pandy showed acidic amino acids (acid aspartic) had inhibitory effect on *M. gypseum* and *T. mentagrophytes* growth. L-Cysteine hydrochloride exhibited absolute toxicity against both the test pathogens while DL-aspartic acid was found active against *M. gypseum* (Pandey *et al*., 1983).

Base on this finding we decided to determine the skin (stratum corneum) free amino acids concentration in patients with dermatophytosis and compare them with normal subjects for the first time in the world.

MATERIALS AND METHODS

Dermatophyte processing

From 370 persons with suspected dermatophytosis, 60 patients (females and males, 15-35 years old) were only with skin lesion (no hair or nail) were diagnosed by direct microscopic examination and culture method. The identification of dermatophytes was based on macroscopic and microscopic colony characteristics, sub culture on specific media and tests and PCR. In also 60 healthy volunteers (females and males, 15-35 years old) without clinical sign of skin disease or amino acids anomaly participated in the study.

Biochemical processing

Stratum corneum samples

The 60 patients skin specimens (scale) with dermatophytosis in two site near skin lesion and sole area and 60 healthy volunteers(normal subjects), at sole area were scraped off with a *sterile surgical* scalpel *Blade No.21* and the scales were collected directly onto a tube. The samples were prepared with added 100 µ double-distilled water (DDW) and 100 µ pure methanol for extracted free amino acids and added 200 µ acetonitrile for remove protein and centrifuge in 2000 rpm 5 min at 10ºC and supernatant were stored at –20°C until used . To standardize the concentration number of cells Concentration factor of each sample was found the samples were diluted with normal saline and shaken in a vortex shaker and stratum corneum cells were counted using a haemocytometer (Takahashi and Tezuka, 2004).

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a powerful method for assaying physiological amino acid concentrations in biological fluids. Four pre-column derivatization methods, with *o*-phthaldialdehyde (OPA), 9-fluoronylmethyl chloroformate (FMOC-Cl), phenyl isothiocyanate (PITC) and 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl-Cl) were assessed with respect to their applicability in biological research (Fürst *et al*., 1990; Liu and Worthen, 1992; Mark and Harding, 2013). Of this reaction were resolved on a high-performance liquid chromatography (HPLC) reversed-phase column (Younglin Acme 9000). The amino acid-OPA derivatives were separated on reverse phase 5 µm C18 ultrasphere column Aglient (250 × 4.6 mm I.D.) kept at 30°C. OPA reagent: mixture was prepared by mixing 200 µl of K-borate buffer pH 8.5 and 40 µl of OPA dissolved in methanol (5 mg ml^{-1}).

Solvent A was a mixture of 50 mm sodium acetate adjusted to pH 7 with acetic acid plus 1% (v/v) tetrahydrofuran. Solvent B was pure methanol. A mixture include of 25 µ l of supernatant and 25 µl internal standard (100 µm/l) and 50 µl methanol vortex 30" and centrifuge 10.000 rpm for 5 min.50 µl of supernatant was derivatized with OPA reagent (50 µl) for 5 min and 10 µl of the mixture were injected and eluted at a flow rate of 1 ml min^{-1} at 30°C in gradient condition wavelength 34o nm emission 450 nm (Di Martino *et al*., 2003).

Statistical analysis

The samples were grouped by donor group and analyzed using t test paired two samples for mean. *P* values <0.05 were considered significant. Infections and aminoacids were compared using an independent variable *t*-test. Analysis for trends by sex group used the mean, standard deviation and measure of variance.

Quality control

For quality control and calibration and precision and accuracy we used Levey Jenning and Westgard multirole (Westgard and Barry, 1981).

Younglin Acme 9000 for all case and control and test for accuracy we test HPLC Agilent 1200 infinity series for 10% of case and control for precision.

Standard that we used: SIGMA AZ161
Control that we used: Recipe Lot 722 level 2

For remove all errors we take a decision to measure qualitative and compare between patients and normal control and we analyze each amino acids independently and we analyzed the results of each amino acid independently.

RESULTS

Dermatophyte results

T. interdigital was the most common isolate (41.7%) followed by *T. rubrum* (31.7%), *E. floccosum* (16.7%), *M. canis* (3.4%), and *M. gypseum* (1.7%). Other species (4.8%) in male and female.

Biochemical results

About 80% of free amino acids in stratum corneum is: Serine - Glycine - Alanine - Ornithine - Threonine - Histidine - Valine and it is same and common in man and woman in normal objects.

Achieved results between case and control in sole area have shown that cases were significantly increased in case in amino acids: Aspartate - Tyrosine – Phenylalanine - Tryptophane and were significantly decreased in case in amino acids: Citrulline – Ornithine. Similarly, in two sex male and female result. and have not any significant different in other amino acids in stratum corneum (Figure 1). Achieved results have shown that people with dermatophytosis in two site near skin lesion and sole area distribution in associated were significantly increased in near skin lesion in amino acids: Glutamates - Asparagine - Histidine - Glutamine - Arginine - Citrulline - Threonine - Methionine - Leucine – Ornithine and were significantly decreased in near skin lesion in only amino acid : Glycine and have not any significant different in other amino acids in stratum corneum (Figure 2).

Figure 1. Comparative in mean concentration of free amino acids (µm/l in 10^9 stratum corneum cells) in control (normal skin) and cases (with dermatophyte) in sole area with significant in male and female

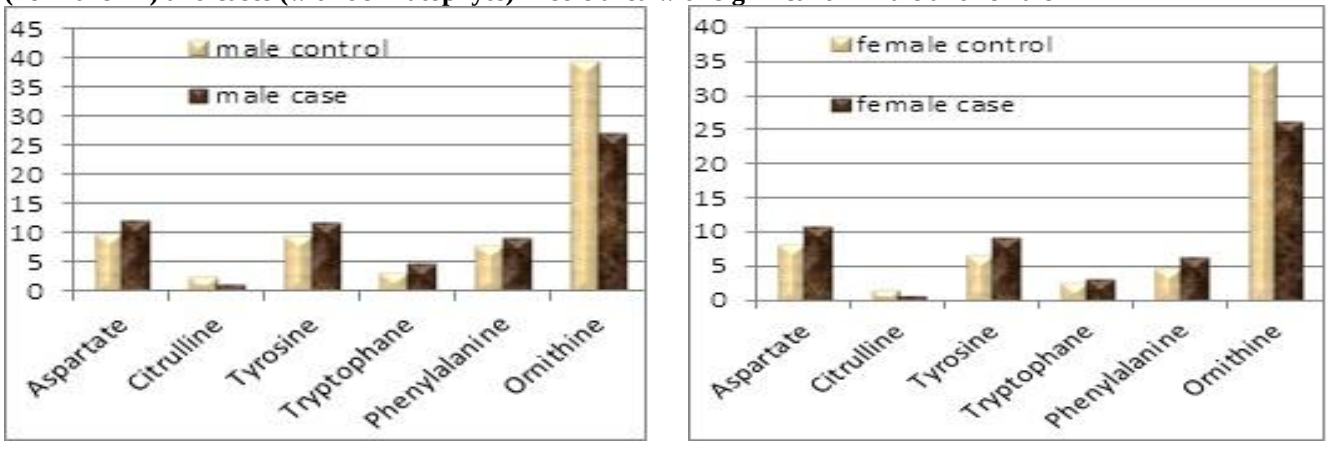
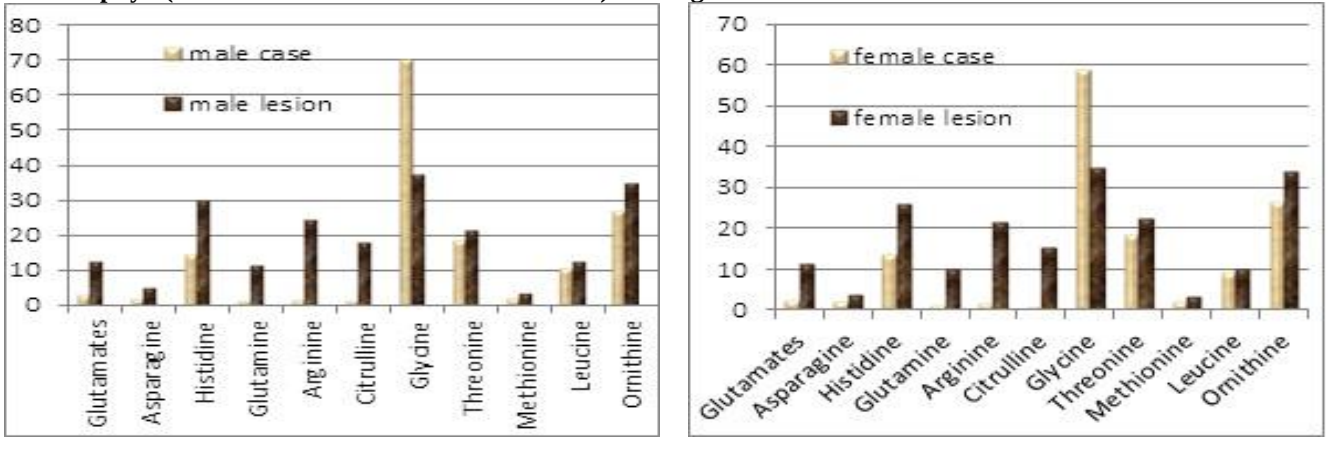


Figure 2. Comparative in mean concentration of free amino acids(µm/l in 10^9 stratum corneum cells in cases with dermatophyte(two site near skin lesion and sole area) with significant in male and female



DISCUSSION

The stratum corneum is the first defense layer which stands in the face of pathogenic microorganisms. Identification of the components in stratum corneum can provide plenty of information on its resistance against superficial infections.

One of these compounds is filaggrin. That is histidine-rich .In adition to filaggrin's role in the assembly of keratin bundles during terminal differentiation; its hydrolysis is carefully regulated to generate free amino acids that contribute to the water-holding properties of the stratum corneum. The conversion of filaggrin to amino acids is preceded by the dephosphorylation of a large precursor molecule (profilaggrin) that is then susceptible to proteolytic degradation into lower-molecular-weight poly peptides. Proteolysis occurs within the stratum corneum, liberating hygroscopic free amino acids.A blockade of this sequence leads to a build-up of filaggrin and amino acids in the stratum corneum. Without these molecules, the amount of water retained in the stratum corneum is decreased. Because water. Has a profound effect on the plasticity and elasticity of the stratum corneum, its absence is associated with inflexibility, cracking, scaling, and flaking of the skin (Scott and Harding, 1982; Jackson and Williams, 1993).

A disturbance in the degradation of profilaggrin, the principal component of keratohyalin granules, may be responsible for a variety of stratum corneum abnormalities (Mendez-Tovar, 2010).

Production of any dermatophyte proteases is repressed by small molecules such as carbohydrates and amino acids. Arthroconidia from infected material are stimulated to germinate by components of the urea cycle and by certain amino acids. Leucine, for example, stimulates the germination of *T. mentagrophytes* arthroconidia. Carbohydrates do not stimulate germination of conidia of this species and during log-phase growth most of the proteolytic enzymes of *T. rubrum* are repressible in vitro by small molecules such as amino acids (Kunert, 2000; Peres *et al*., 2010).

We have any report and investigation about stratum corneum amino acids in patient with dermatophytosis but in this way there are some invitro research reveal coloration between amino acids and dermatophyte.

For example some investigation have reported that L-lusin were elicited to growth inhibition of *M. gypseum* and Argenine also in concentration of 1 and 0.1 gr/dl have inhibitory effects but were not causes complete growth inhibition even in concentration of 1 gr/dl. Methionine also has no effect on *E. floccosum* and was shown mildly effect on *M. gypseum*. The results showed that L-cysteine hydrochloride, L-cysteine, L-aspartic acid, Lglutamic acid and DL-tryptophan and L-tyrosine had the most inhibitory effects on the studied dermatophytes, while Arginine L-lysine and L-methionine had moderate effects and the rest of amino acids had less inhibitory or even stimulatory effects on the growth of the dermatophytes. *M. canis* and *T. schoenleinii* has a different sensitivity to amino acids. This data indicates that sulfur-containing amino acids and acetic amino acids have greater inhibitory effect against these two dermatophytes (Sarasgani and Firoozrai, 2006; 2008; 2010;).

In the other study that was done by Garachorlou *et al* reveled that Aspargin and Methionine amino acids causes decrease in the *T. rubrum* and *T. verrucosum* growth and the inhibitory effect of valine on *T. mentagrophytes* were assessed and shown that concentration of 0.1% valine causes maximum decrease in trichophyton mentagrophytes growth. In one other study by Garachorlou *et al* revealed that Histidine has inhibitory effect on *T. mentagrophytes* Growth and the inhibitory effect of study the inhibitory effect of Tryptophan on *T. verrucosum* were assessed and shown that concentration of 1% Tryptophan causes maximum decrease in *T. verrucosum* growth and that Tryptophan causes growth decreasing in *E. floccosum* (Gharachorlou and Gharachorlou, 2011a,b,c)

Acidic amino acids also either was shown inhibitory effect on two dermatophytes that the Aspartate (aspartic acid) inhibitory effects on *M. gypseum* growth were determined in pandy study. Some of amino acids were assayed at 1% concentration for their toxicity against the mycelial growth of two dermatophytes viz., *M. gypseum* and *T. mentagrophytes*. L-Cysteine hydrochloride exhibited absolute toxicity against both the test pathogens while DL-Aspatate (aspartic acid) was found active against *M. gypseum* only. The minimum inhibitory concentrations of L-cysteine hydrochloride was found to be 0.5 and 0.4% against *M. gypseum* and *T. mentagrophytes*, respectively, at which it showed mycostatic nature. However, the amino acid exhibited mycocidal activity at 0.9 and 0.8% against *M. gypseum*, *T. mentagrophytes*, respectively (Pandey *et al*., 1983).

We analyzed the results of each amino acid independently. Most amino acids in stratum corneum in normal skin are common and same in man and woman: Serine-Glycine-Alanine-Ornithine about 80% of free amino acida in stratum corneum. Other amino acid Included essential: Histidine – Isoleucine – Leucine – Lysine – Methionine -Threonine –Tyrosine – Valine – Phenylalanine and non-essenttial: Arginine – Asparagine – Citrulline -Glutamic acid – Glutamine – Taurine –

Carnosine that have lowest concentrations in the skin and it is natural that smaller amounts of essential amino acids are present in the skin. Because the stratum corneum of the skin and whatever secreted surface of skin Includes waste that are often non-reuptake.

Our results for normal skin (control) are also in very good agreement with the most recent experimental results (Pratzel and Fries, 1977; Koyama *et al*., 1984; Horii *et al*., 1989; Denda *et al*., 1992; Sylvestre *et al*., 2010; Visscher *et al*., 2011; Rawlings and Voegeli, 2013).

We have any report reserch about stratum corneum amino acids in skin in patients with dermatophytes for comparison whit our data. Patterns of tests results are very different between people with dermatophytosis in two site near skin lesion and sole area distribution and also between case and control in sole area. Our research shows that due to the concentration, amino acids can effect stimulation or inhibition of dermatophytes growth in statum corneum. In current study this appears case and control in sole area had significantly increasion in Aspartate - Tyrosine – Phenylalanine - Tryptophane and significantly decreasion in Citrulline– Ornithine in case in compare to control.Due to concentration have probably causes Prevent the spread of the disease in people against dermatophytosis because this results (due to concentration) is agreement with invitro research.

People with dermatophytosis in two site near skin lesion and sole area increased in amino acids near lesion compare with case in sole area: Glutamates - Asparagine - Histidine - Glutamine - Arginine - Citrulline - Threonine - Methionine - Leucine – Ornithine. Probably stimulated the dermatophytes growth.This results is agreement with previous invitro research. Because arthroconidia are stimulated to germinate by components of the urea cycle (Arginine – Ornithine – Citrulline) and by certain amino acids Leucine, for example, stimulates the germination of *T. mentagrophytes* arthroconidia. Amino acids (Glutamates - Glutamine - Arginine – Ornithine – Citrulline) best of nitrogen source and Asparagine - Histidine good nitrogen source for fungus.

This study has led to an understanding of mechanisms, and variation and virulence factor. We need more research for confirm or refute the suggestion that these changes in amino acids are primary or secondary otherwise active or passive, or a way to fight against the further spread within the body or to invade region is limited or stimulation factor depended on concentration.

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