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Research Article

A potential role for mycotoxins(Gliotoxin) in suppression of superantigens of *Streptococcus pyogenes*

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ABSTRACT

Although little research has been conducted on the role of Streptococcus pyogenes superantigens in psoriasis, exploring this area could lead to valuable insights and potential treatment options for individuals with psoriasis. This study aimed to assess the presence of superantigens, including SpeK, SpeL, SpeM, SpeC, and SmeZ, in plaque samples from Iranian medical centers and to examine any changes in their expression after gliotoxin treatment. Skin plaque samples were collected from 400 50-year-old patients using swabs. The presence of superantigens was determined using the multiplex PCR method. Streptococcus pyogenes strains were confirmed using a specific primer SPY1258, and gene expression after gliotoxin treatment was assessed using real-time PCR. According to our data, among 400 samples, 50 were found to contain Streptococcus pyogenes. The analysis further revealed that SpeK and SpeL were present in these samples with 50% and 8% prevalence, respectively. These were mostly found in samples collected from patients with hand lesions.. Additionally, there was a significant decrease in the expression of these two genes after gliotoxin treatment. However, there was no evidence of the presence of the other three genes. These findings suggest that microbial toxins, such as gliotoxin, can potentially be utilized to develop antimicrobial drugs for treating psoriasis. Therefore, further research should be conducted to explore the potential of gliotoxin as a treatment option for psoriasis

1. Introduction

Psoriasis is one of the most common autoimmune diseases characterized by thick red plaques and silver scales with a genetic nature, which leads to the occurrence of clinical lesions such as psoriasis of the head, nails, dropsy, erythroderma, reverse and plaque psoriasis through the disruption of the immune system, especially T immune cells. Microorganisms such as Streptococcus, Staphylococcus, Enterococcus, and Pseudomonas are involved in the occurrence and exacerbation of its clinical symptoms (Chen et al., 2020). Among them, Streptococcus pyogenes is a gram-positive bacterium without spores, which can cause mild skin and mucosal infections and severe systemic diseases. It generates and aggravates the condition by releasing inflammatory cytokines and superantigens. Superantigens and toxins secreted

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by this bacterium can induce the expression of receptors required by immune T cells by binding to the beta chain of T cell receptors. These superantigens produce skin lesions in people with psoriasis by polyclonal stimulation of T cells expressing the Vβ2 receptor through exotoxin C. Among the significant inflammatory cytokines in this disease are IL-12-23 and interferon-gamma (Proft and Fraser, 2022).

Previous studies have examined the importance of this bacterium in the occurrence of psoriasis and the examination of super antibodies and genes related to the disease (Allen et al., 2018); Sheikhabbasi et al., 2019) and the examination of the expression level of these genes after different treatments (Coleman et al., 2011; Tamiya et al., 2015; Siasi et al., 2017; Kim et al., 2023), along with measuring the levels of inflammatory cytokines and their relationship with the disease (Ramirez- Bosca et al., 2015).

Since antibiotics in treating psoriasis cause drug resistance, researchers have turned to using microbial secondary metabolites, including fungal toxins, in treating this disease. Gliotoxin is an essential pathogenic secondary metabolite of *Aspergillus fumigatus*, which can break the cell wall and disrupt the cell function of other microorganisms (Jendoubi et al., 2019).

Due to the accuracy of molecular methods and the importance of using secondary metabolites of microorganisms in the treatment of psoriasis, in this research, we dealt with 1) identifying and evaluating the superantigens of this bacterium (by multiplex PCR), 2) examining their expression after treatment with Gliotoxin (real-time PCR).

2. Materials and Methods

Sampling, preparation of direct smear, and culture were done using a sterile swab dipped in pure distilled water from psoriasis plaques on the skin of 400 patients with an average age of 50 years referring to Razi Skin Specialist Hospital in Tehran (with written consent) followed by gram staining.

After culture in blood agar (SBA), chocolate agar (chocolate agar), EMB, sabouraud dextrose agar and incubation in a candle jar for 24 hours, 50 streptococcus colonies were isolated from 400 samples, which were performed to definitively confirm group A streptococcus, PYR test and sensitivity to bacitracin.

Investigation of superantigens of Streptococcus pyogenes was done using multiplex PCR. The presence of genes of Streptococcus pyogenes super antigens isolated from plaques of psoriasis, including (SpeL, SpeK, SpeM, SpeC, SmeZ, and Spell) was done by designing primers and performing PCR using Cina Gen kit instructions (DNG-plus, No: EX6082). The primers designed in this section are presented below:

SpeL Forward: CCT GAG CCG TGA AAT TCC CA Reverse: ACA CCA GAA TTG TCG TTT GGT (657 bp), SpeK Forward: CCT TGT GTG TGT ATC GCT TGC Reverse: TTG CTG TCC CCC ATC AAA CT (68 bp), SpeM Forward: ATC GCT CAT CAA ACT TTT CCT Reverse: CCT TGT GTG TGT ATC GCT TGC (496 bp), SpeC Forward: GCC AAT TTC GAT TCT GCC GC Reverse: TGC AGG GTA AAT TTT TCA ACG ACA (405 bp), Smez Forward: TTT CTC GTC CTG TGT TTG GA Reverse: TTC CAA TCA AAT GGG ACG GAG AAC A (246 bp) Materials used in this reaction included H20 (1 μL), on and reverse primers (0.5 μL each), DNA (3 μL), and Master Mix (10 μL).

The reaction was initiated with initial denaturation at 95°C for three minutes. It was followed by denaturation at 95 degrees for 15 seconds, annealing at 60 degrees for 20 seconds, extension at 72 degrees for 2 minutes, and final extension at 72 degrees for 7 minutes. The resulting product was electrophoresed on a 1% agarose gel.

2.1. Determination of Gliotoxin concentration by HPLC

First, the Aspergillus fumigatus strain prepared from the mycology department of Tehran University Health Faculty was cultured on a medium of Sabourud dextrose agar. To investigate the production of Gliotoxin, using a sterile razor, pieces of mushrooms grown on potato dextrose agar medium to the 5 mm by 5 mm dimensions and approximately 5 mm in diameter in 100 ml potato dextrose broth liquid culture medium were cultured and kept statically in the dark and at room temperature. Gliotoxin was extracted by dissolving it in chloroform, with its concentration determined by HPLC 0.49 ng/ml.

2.2. Determination of Gliotoxin MIC

This research was done with the help of sterile 96-well flat-bottomed microplates as follows: the microbial stock was prepared in the wells with the serial dilution of the toxin. However, the positive control well contained only microbial stock and culture medium, and the negative control only had initial toxin stock and culture medium. The next step was to place the microplates on a shaker (30 seconds) and incubate them at 35℃ (18 hours). A well in which no bacterial growth was observed was considered MIC.

2.3. Investigating the expression of superantigens with real-time PCR

After treatment with gliotoxin concentration, RNA extraction and PCR were performed using the instructions of the RNA extraction kit (RNX-Plus, Cat, Number: Ex6101), with the RNA concentration extracted by a Nanodrop model device. To remove genomic DNA, 0.1-5 ng of RNA and 1 μL of random hexamer primer were poured into a DNase-RNase-free microtube, and the rest was filled up to 12 μL with nuclease-free water. First, it was incubated at 37℃ for 30 minutes and then by adding 1 μL, it was set at 65℃ for 10 minutes.

2.4. cDNA synthesis

To start this step, 0.1 ng of RNA, plus 1 μL of random hexamer primer, were poured into a DNase-RNase free microtube, where nuclease-free water was added to the rest of the microtube up to a volume of 12 μL. Then, it was incubated for 5 minutes at 65 ℃ to destroy the secondary structure of RNA. The materials required for cDNA strand synthesis are reported in Table 1.

Then, 5 minutes of incubation at 25 ℃ was done plus 60 minutes at 42 ℃ and enzyme inactivation at 70 ℃ for 5 minutes.

2.5. Real-time PCR

The reverse and forward primers (0.4 µl each) and cDNA (100 ng) were poured into the microtube (10.4 μL) SyBER Green PCR master mix + Day. It was filled with nuclease-free water to the final volume of 20 μL, mixed slowly, and placed in the thermocycler. The first step of the reaction (1 cycle) was done at 95 °C for 5 seconds, the second (1 cycle) at 95 °C for 10 seconds, and the third (40 cycles) at 60 °C for 30 seconds.

Table 1. The volume of material used in cDNA synthesis

MATERIAL	VOLUME
5X REACTION BUFFER	**4 µL**
Ribolock RNase Inhibitor (20u/ µl)	**1 µL**
10 mM dNTP Mix	**2 µL**
RevertAid M-MuLV Reverse Transcriptase (200u/ µl)	**1 µL**
Total volume	**20 µL**

3. Results

This study examined 400 samples and found 50 isolates of *Streptococcus pyogenes*. Of these isolates, 18 were from female patients and 32 were from male patients, with an average age of 50.78. The frequency of these two groups was 36% and 64%, respectively. The location of the lesion included the chest (n=2), leg (n=18), and hand (n=30), and their frequency was 4%, 36%, and 60%, respectively.

3.1. The results of investigating the presence of superantigens with multiplex

pyogenes isolates exposed to detect the specific gene (*spy*1258) and two of the virulence factors were *SpeK* and *SpeL* genes by PCR technique and DNA sequencing analysis, but the three other genes (SmeZ, SpeC and SpeM) are not presented in the studied isolates. Examining the presence/absence of Streptococcus pyogenes superantigens in the samples collected from psoriasis patients revealed that the most significant presence was related to SepK genes.

The data indicated the presence of the SpeL gene in 4(8%) samples (with hand lesions). The presence of the SpeK gene in 25 samples (50%) included 1 case of chest infection, 11 cases of leg infection, and 13 cases of hand infection, claiming 4%, 44%, and 52% positive cases for Spek, respectively (Table 2).

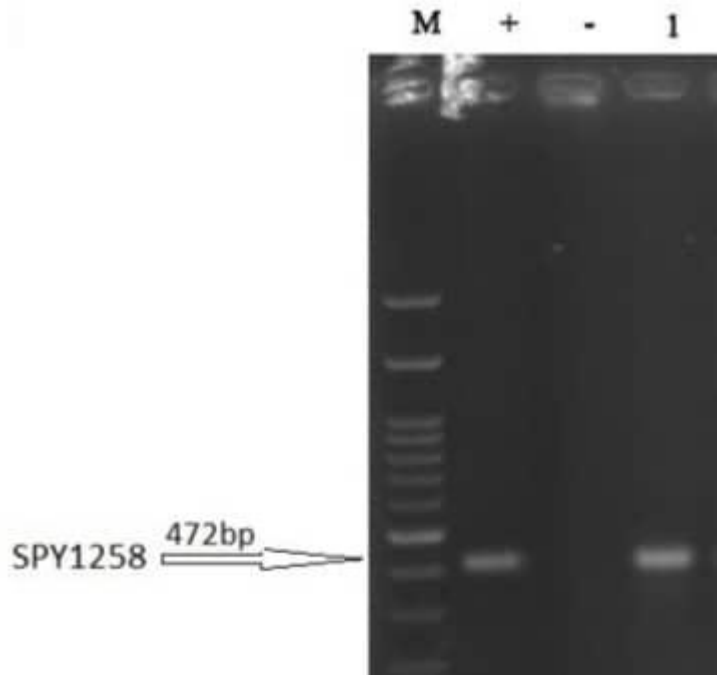

Figure 1. The results of PCR product electrophoresis to investigate the SPY1258 gene(specific gene to determine *Streptococcus pyogenes* strain) in 19 selected samples. Band formation in 472 bp.

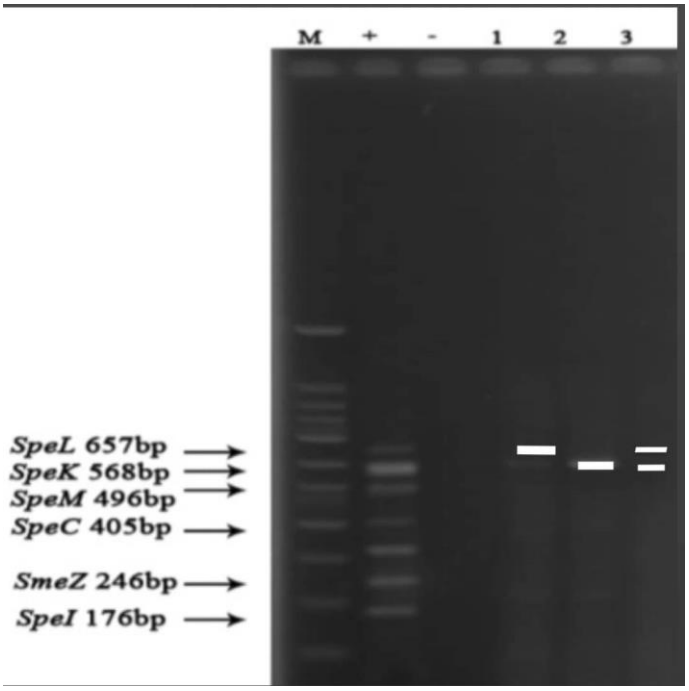

Figure 2. Results of multiplex PCR product electrophoresis. The ladder used in this method was bp 100. a, b, c, d PCR results of 50 patient samples, 8% contained SpeL genes on the bp 657 band, 25% had Spek on the bp 568 band, and the other investigated genes did not form a band.

Table 2. Comparison of the number and frequency of SpeK and Spel genes in the samples taken from the lesion site

		Number	Percent
Gender	Female	18	36.0
	Male	32	64.0
Lesion	Chest	2	4.0
	Foot	18	36.0
	Hand	30	60.0
SpeL	Negative	46	92.0
	Positive	4	8.0
SpeK	Negative	25	50.0
	Positive	25	50.0

3.2. MIC results

According to visual turbidity measurement, the minimum inhibitory concentration (MIC) of Gliotoxin for *Streptococcus pyogenes* bacteria with superantigen genes (Spek, SpeL) was reported to be 512 µg/ml (Fig 3).

According to the MIC concentration, the bacteria present at a concentration of 256 µg//ml (Sub MIC) were used as bacteria treated by Gliotoxin to check the lack of gene expression by Real-Time PCR molecular reaction.

3.3. Real-Time PCR reaction results

The study of SpeK gene expression change was carried out using the molecular method. The Real-time PCR product's electrophoresis indicated that this gene's expression diminished significantly after treatment with Gliotoxin.

3.4. Investigating the effect of Gliotoxin on the SpeL gene

The data revealed that treatment with Gliotoxin reduced the expression of this gene (Figure 5). Data analysis using Shapiro Wilk and T-Test methods indicated that the fold change of the SpeK gene was 1.000000 and dropped to 0.652432 after the treatment. This level of SpeL decreased from 1.000000 to 0.437544.

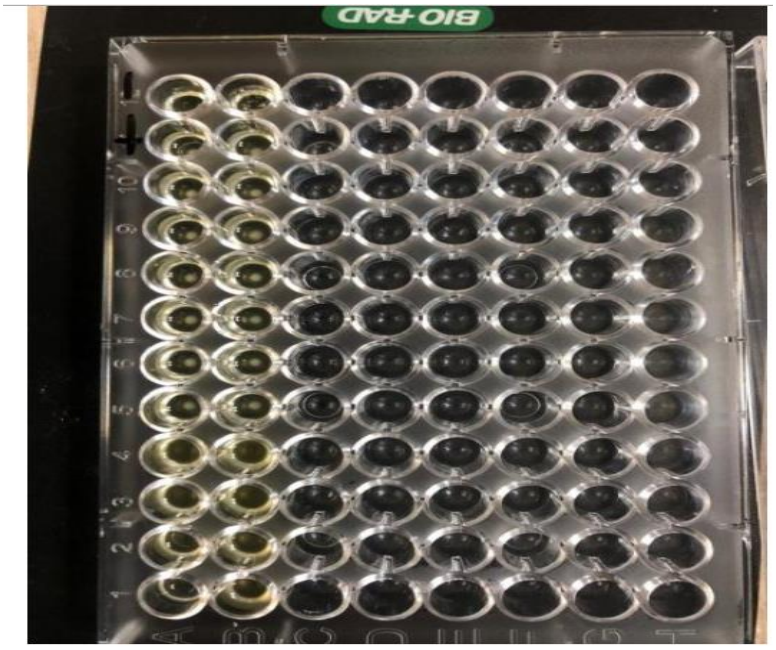

Fig 3. MIC results after exposure to gliotoxin effect

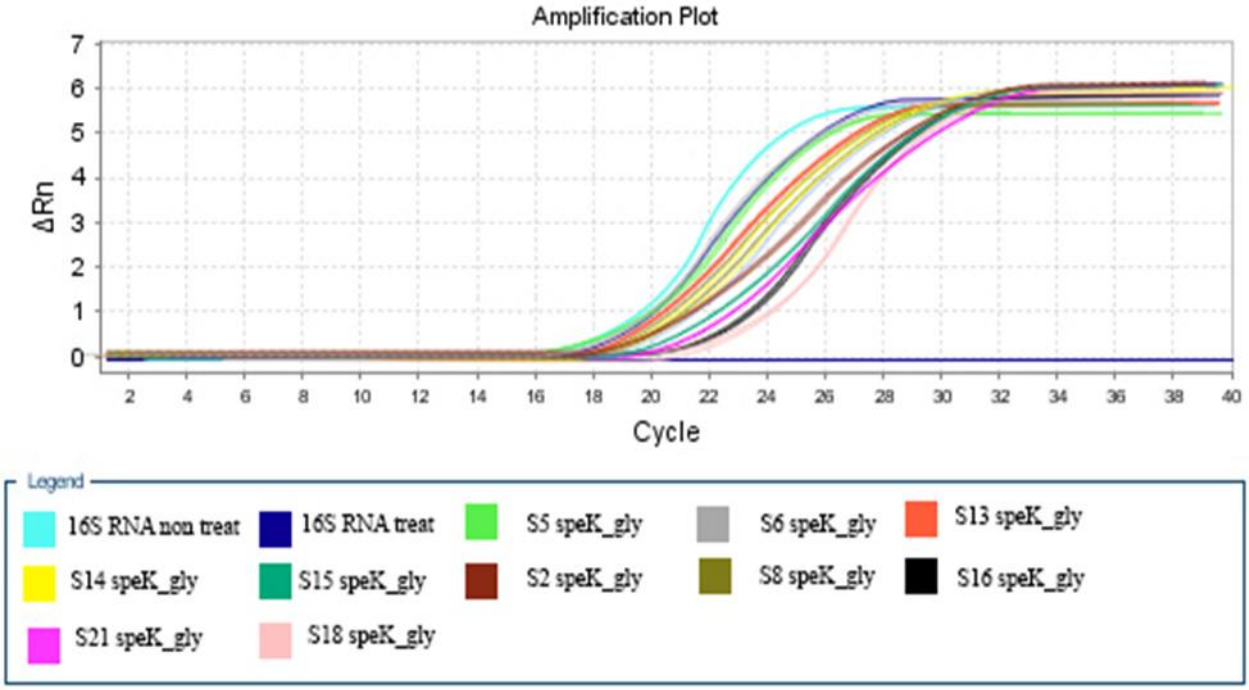

Figure 4. Diagram of real-time PCR results of SPeK gene under the influence of Gliotoxin

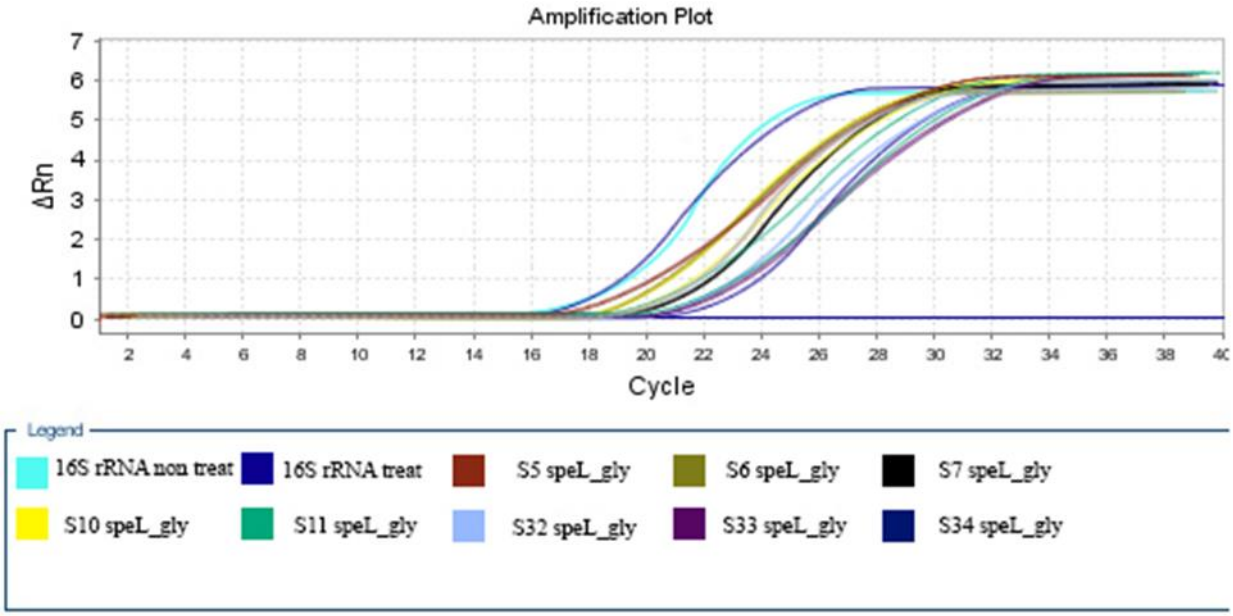

Figure 5. Graph of real-time results of SpeL gene under the influence of Gliotoxin.

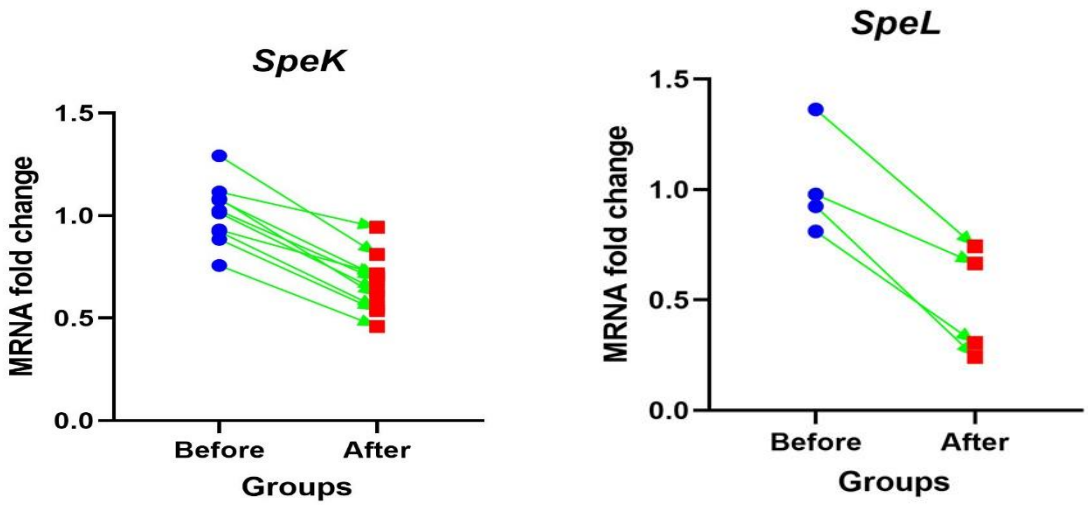

Figure 6. Comparison of the gene expression (SpeL and SpeK) after treatment with Gliotoxin.

4. Discussion

Psoriasis is a skin disease with an unknown genetic and immune pathogenesis. However, T-cell activation is considered a crucial factor in its development. Environmental factors, such as infections caused by microorganisms, also play a role in the spread of psoriasis. Streptococcus, Staphylococcus, Enterococcus, Malassezia, and Candida are among the microorganisms that contribute to the disease (Neema et al., 2019). Among the various factors that cause psoriasis, Streptococcus pyogenes bacteria is one of the most significant reasons. The proliferation of superantigens, including viral or bacterial proteins, can bind directly to the major histocompatibility complex (MHC) class II and Vβ component of T-cell receptors, causing T-cell hyperactivation and psoriasis. Superantigens are present in two forms of the disease (guttate and secondary infections) and are considered one of the influential factors in the progression of the disease (Staberg et al., 1983).

In this research, the collected samples revealed that among the 400 psoriasis plaque samples, 50 isolates were *Streptococcus pyogenosa*. SpeK and SpeL genes (superantigens of *Streptococcus pyogenes*) were identified and determined using Multiplex PCR. Then, Gliotoxin was extracted from the Aspergillus fumigatus fungus by the solubility method in chloroform, and its concentration was determined by HPLC to be 0.49 ng/ml. After deciding on the MIC (526 μg/g/ml), the concentration of 256 μg/g/ml (Sub MIC) was used to investigate the expression of the genes of the identified superantigens. It was also found that gene expression (Spel = 0.03320 ρ) and SpeK (ρ = 0.00005) showed a significant reduction post-treatment.

Studies have been conducted to investigate the effect of Streptococcus pyogenosa superantigens. According to the report of Bartengio et al. (2000) on 40 patients (64%), psoriasis infection was caused by two groups of group A streptococci and staphylococci, among which the share of streptococci was reported as 82.6%. Eva Marcus et al. and Allen showed that group A streptococcus was found in 31% and 75% of the examined patient samples, respectively (Allen et al., 2018).

Saisi and colleagues (2016) revealed that among the 60 examined samples, Streptococcus pyogenes superantigens, speA gene in 24 samples (84.3%), speC gene in 16 samples (33.3%), and speB gene in all models (100%) were obtained. It was reported that there is a significant relationship between the presence of speA, speB, plus speC genes and the development of psoriasis. Also, the results of Sheikh Abbasi et al.'s research (2019) showed that out of 60 skin samples of psoriasis patients in Tehran medical centers, (25%) of samples carried SmeZ, (8.3%) SpeL and (8.3%) SpeH

superantigen genes. Similar to this research, the present study revealed that out of 400 samples prepared, 50 were Streptococcus pyogenes. However, the genes identified in the present study differed, including SpeL superantigens in 4 people (8%) and SpeK in 25 people (50%), and two isolates carried both genes (4%).

Studies have been done on the effect of microbial toxins on superantigen gene expression. By inspecting the impact of bacteriocin Nisin on strains containing SmeZ by real-time PCR method, Sheikh Abbasi et al. (2019) showed that the expression level of this gene was reduced after treatment. In the present study, after treatment with Gliotoxin, the expression level of SpeL and SpeK genes diminished significantly ($\rho<0.0005$). Fold change analysis of the genes studied in this research showed a significant decrease after treatment with Gliotoxin (Table 3).

One of the most common problems among psoriasis patients is dermatophytosis infection, which represents a significant defence mechanism against fungal invasion (Lintges et al., 2010). In some studies, the prevalence of dermatophytosis in psoriasis patients was very high (Marcus et al., 2011), while in others, it was shallow (Vander-Velden et al., 2013). In this research, 50 samples were prepared from different parts of the patient's body, including hands, chest, and feet. Most of the samples belonged to hand lesions (60%) of people who worked with detergents. Probably, since the pH of detergents is variable and they have high alkalinity, they can damage the skin of people's hands and increase the incidence of eczema, psoriasis, and sometimes dermatophytosis (Vander-Velden et al., 2013). Also, after analyzing the results of this study, it was found that the expression of two SpeL and SpeK genes in the examined samples from patients was very high (100% and 52%, respectively), which diminished after treatment with Gliotoxin. By comparing the results of this research with other studies that have been done regarding the relationship between psoriasis disease and microbial factors, it seems that the bacterial exotoxins isolated from Streptococcus pyogenes can act as superantigens and help increase the penetration power of T cells and monocytes in people living with psoriasis and be effective in causing the disease. The role of pathogenic genes and bacterial super antigens' coding is more critical (Vander-Velden et al., 2013

Conclusion

Our study found that SpeK and SpeL were present in the samples, with 50% and 8% respectively, mainly in samples collected from patients with hand lesions. After treatment with Gliotoxin, there was a significant reduction in the expression of these two genes. This suggests that microbial toxins like Gliotoxin could be used to develop antimicrobial drugs for treating psoriasis.

Refereces

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