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Human Amniotic Fluid: a New Challenge for the Control of Seborrheic Dermatitis

Mohadeseh Nasiri[1], Leila Fozouni[1]*

[1]Department of Biology, Gorgan Branch, Islamic Azad University, Gorgan, Iran

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***Correspondence**
Email: lili_kia@yahoo.com
Tel: +98 9111518674
Fax: +98 1133329496

ABSTRACT

Introduction: Seborrheic dermatitis is a skin condition that has become widespread in the recent decades. This study aimed to investigate the antimicrobial effect of amniotic fluid of pregnant women on the growth of ketoconazole-resistant *Malassezia* species isolated from patients with seborrheic dermatitis. **Methods:** We obtained human amniotic fluid from 20 pregnant women during amniocentesis and cesarean delivery at hospitals of Gonbad-e Kavus, northeastern Iran. *Malassezia* isolates were collected from 120 patients suspected of seborrheic dermatitis and were identified using species-specific biochemical tests. Antibiotic susceptibility and minimum inhibitory concentration (MIC) of *Malassezia* isolates against ketoconazole were determined by the broth microdilution method. Also, the antifungal effect of various concentrations of amniotic fluid on ketoconazole-resistant *Malassezia* isolates was assayed using the disk diffusion method. **Results:** The mean MIC of ketoconazole against *Malassezia* isolates was 0.3 µg/mL, with the most changes observed at 0.5 µg and 1 µg/mL. Ketoconazole inhibited the growth of *Malassezia* isolates completely, at a concentration of 8 µg/mL. Moreover, amniotic fluid showed inhibitory effects on 60% of ketoconazole-resistant *Malassezia furfur* isolates and 45% of *Malassezia globosa* isolates. There was a significant correlation between amniotic fluid concentration and the diameter of the growth inhibition zone (P<0.01). **Conclusion:** Our findings indicated that amniotic fluid could exhibit favorable dose-dependent antifungal activity against ketoconazole-resistant *Malassezia* isolates from patients with seborrheic dermatitis. Further studies are required to confirm the efficiency of amniotic fluid for the treatment of such infections.

INTRODUCTION

Seborrheic dermatitis is a chronic skin disorder of unknown etiology that affects both infants and adults. This condition is characterized by the formation of scaly patches, red skin, and dandruff in sebum-rich areas, such as the scalp, central areas of the face, eyebrows, armpit, groin and upper chest [1]. This disease has a global distribution, and in Iran, it is more widespread in hot and humid southern regions [2]. Seborrheic dermatitis links to *Malassezia*, a genus of lipophilic fungi that are natural flora of humans and other warm-blooded vertebrates' skin. Increased sebaceous gland activity, acquired immunodeficiency syndrome, antibiotic use, steroids, and pregnancy are associated with an increased prevalence of *Malassezia* species and aggravation of skin disorders. For many years, the damage, especially skin disorders such as dandruff, seborrheic dermatitis, and pityriasis versicolor was attributed only to *Malassezia furfur*. Several studies have reported the association of other *Malassezia* species and seborrheic dermatitis [3, 4]. Other important species in the genus *Malassezia* are *M. pachydermatis*, *M. globosa*, *M. restricta*, *M. sympodialis*, *M. nana*, *M. japonica*, and *M. slooffiae*. These fungi can aggravate skin lesions by stimulating the production of cytokines, especially interleukin-2 via the Toll-like receptor-2-dependent pathway and infiltration of inflammatory cells into the skin. Moreover, they can induce surface protein expression and pro-inflammatory cytokine production in keratinocytes [5].

Azoles, such as ketoconazole, are the most commonly used antibiotics for the treatment of seborrheic dermatitis. These drugs compromise fungal cell membrane permeability by inhibiting the production of ergosterol, a vital component of the cell membrane. Besides, they inhibit the activity of oxidative and peroxidative enzymes, causing toxic accumulation of hydrogen peroxide within the cell, which may ultimately result in cell necrosis [6]. Despite the efficacy of these antifungals in seborrheic dermatitis treatment, the increasing rate of resistance to these agents signifies the need to evaluate the effects of natural biological compounds or antimicrobials, such as human amniotic fluid.

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Some studies indicated the inhibitory effect of amniotic fluid against common bacterial pathogens [7, 8]. During pregnancy, various natural compounds with favorable antimicrobial effects are produced in the amniotic fluid that later accumulates in the placenta, endometrium, and fetal membrane. The innate immunity of the uterus is mainly due to antimicrobial peptides called *defensins*. Also, *secretory leukocyte protease inhibitors,* as part of the innate immunity, protect the amniotic membrane from infectious agents [9, 10].

This study investigates the in vitro antifungal effect of amniotic fluid on drug-resistant *Malassezia* species isolated from patients with seborrheic dermatitis.

MATERIAL AND METHODS

Patients and fungal isolation. The study population comprised of 120 patients (60 men and 60 women) from Gonbad-e Kavus City, northeast of Iran with suspected seborrheic dermatitis presenting symptoms such as scaling and sometimes itching. Patients with a history of steroids or antibiotic use were excluded from the study. After obtaining written consent, specimens were taken from the skin of the neck, face, arm, abdomen, shoulder, and scapula using sterile surgical scalpels. The specimens were stained with methylene blue and 10% KOH, cultured in Dixon's agar (Merck, Germany) supplemented with chloramphenicol and cycloheximide, and finally incubated at 32°C for 7-10 days. Yeast cells were first examined by eye for colony morphology and then microscopically following calcofluor white staining. In this investigation, 1-2 single spores were identified as positive 1 (+1), and 2 - 6 integrated spores or 3-12 single spores were defined as positive 2 (+2) [11]. The *Malassezia* species were identified by growth on Sabouraud dextrose agar (Merck, Germany), catalase test, esculin hydrolysis, and Tween test [5].

Antibiotic susceptibility. The *Malassezia* isolates susceptibility to ketoconazole (0.03-8 µg/mL) was determined by using the broth microdilution method, according to CLSI-M27-A3 [12]. A ketoconazole stock solution was prepared by mixing ketoconazole powder (Gibco Co., Germany) with dimethyl sulfoxide. Then 100µL of yeast suspension (1×10^3 cells/mL) was seeded onto a 96-well ELISA microplate containing 100µL Dixon broth (Merck, Germany) and 100 µL various concentrations of ketoconazole. The plate was placed in an incubator shaker at 32°C for 72 h. The minimum inhibitory concentration (MIC) was determined by the lowest concentration of ketoconazole that inhibited the growth of fungal isolates. The first well of the microplate with no visible growth was reported as the MIC. According to the CLSI guidelines [11], MICs values of ≥1, 0.25-0.5, and ≤0.125 µg/mL are considered as resistant, intermediate, and sensitive to ketoconazole. In addition, minimum fungicidal concentration (MFC) was investigated in the same range. For this purpose, 100 µL from wells with a concentration higher than the specified MIC and 100 µL from the positive control well were separately transferred onto Dixon agar medium and incubated at 32°C for 3 to 5 days [12]. The minimum fungicidal concentration was defined as the lowest concentration of ketoconazole at which no growth occurred. In this study, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as controls.

Collection and antifungal effect of amniotic fluid. In this study, amniotic fluid was taken from 20 pregnant women aged 33 ± 9 years. The women had undergone amniocentesis for karyotyping during the first trimester of pregnancy and had the cesarean delivery in gynecology wards of Gonbad-e Kavus hospitals, Iran, from Oct. 2018 to Jun. 2019. They had no history of antibiotic use and were free of systemic diseases, urinary tract infection, acquired immunodeficiency syndrome, hepatitis B, syphilis and toxoplasmosis, and cytomegalovirus infection. The samples were collected in a sterile container using a sterile syringe and then transferred within an hour to the microbiology laboratory at Islamic Azad University of Gorgan, Iran. The samples were centrifuged at 300 g for 5 min at 5°C, and the recovered supernatants were sterilized by a 0.2 µm membrane filter. Six mL of the supernatants were mixed with 2 mL of physiological saline to obtain a concentration of 3000 mg/mL. The mixture was diluted to 750 mg/mL and maintained at -20°C until used. According to the Kirby-Bauer disk diffusion susceptibility test, several dry and sterile blank disks (PadtanTeb Co., Iran) were placed in tubes containing various concentrations of the above mixture and then dried in a dry incubator for 3-4 h [13]. Then, 100 µL of the fungal cell suspension (1×10^3 cells/mL) were uniformly cultured on the Dixon agar medium using a sterile swab. The prepared disks were placed on the medium, and the plate was incubated at 32°C for 3-5 days. Blank paper disks were used as the negative control. Finally, the diameter of the inhibition zone around the disks was measured. A diameter ≥14 mm indicated susceptibility, and ≤10 mm or absence of inhibition zone indicated resistance to amniotic fluid [10].

Ethical considerations. We obtained the written consent of all participants. This study was performed under the Declaration of Helsinki statement for medical research involving human subjects, and the Ethics Committee of Islamic Azad University, AliAbad Branch Golestan, Iran approved the study (code No. 1398007).

Data analysis. All statistical analyses were performed with SPSS software (v. 23.0). The normality of the data was checked by the Kolmogorov-Smirnov test. The age and pregnancy month mean were compared between pregnant women using the ANOVA and Kruskal-Wallis tests. *P* values were considered significant at 0.05 levels.

RESULTS

Frequency of Malassezia spp. Isolates. Out of 120 specimens collected from seborrheic dermatitis patients, 92 (76.6%) isolates showed *Malassezia* infection. Out of these, 72 (78.3%) were *M. globosa*, and 20 (21.7%) were *M. furfur* species. There was no difference between the frequency of species isolated from men and women. The frequency of *Malassezia* isolates from patients with seborrheic dermatitis was identical in men and women (50%). The frequency of *Malassezia* isolates was highest in individuals aged 10-20 years (83%) and lowest in those over 30 years old (2%).

Minimum Inhibitory Concentration. Based on the results, 55% of the *Malassezia* isolates had a MIC value of

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≥1 µg/mL and defined as ketoconazole resistant. The mean MIC of ketoconazole against *Malassezia* isolates was 0.3 µg/mL. No growth occurred at a concentration of 8 µg/mL. Ketoconazole was able to inhibit the growth of 50% of *Malassezia* isolates (MIC_{50}) in a dose-dependent manner and at concentrations ≥1 µg/mL so that 58% of *M. globosa* and 40% of *M. furfur* isolates were in this range. The MFC of ketoconazole against *M. globosa* and *M. furfur* isolates was 4-8 µg/mL and 2-4 µg/mL, respectively.

The MFC of ketoconazole against *Malassezia* isolates ranged from 2 and 8 µg/mL. The MFC of ketoconazole was 4 µg/mL, and more than 4 µg/mL in 32.5% and 43.5% of *Malassezia* isolates, respectively. At concentrations of 4 µg/mL, 2 µg/mL and 1 µg/mL, ketoconazole eliminated 17%, 4.5% and 2% of the isolates, respectively. This indicates that this antifungal had a desirable effect on *Malassezia* isolates at a concentration of 1-8 µg/mL. In comparison, the highest fungicidal activity was observed at concentrations of ≥4 µg/mL (Table 1).

Antifungal effects of the amniotic fluid. Overall, the amniotic fluid showed a relatively good inhibitory effect on *Malassezia* isolates. About 60% and 45% of ketoconazole-resistant *M. furfur* and *M. globosa* isolates were susceptible to the amniotic fluid, respectively. Also, none of the ketoconazole-resistant isolates was able to grow at concentrations ≥3,000 mg/mL of amniotic fluid (Fig. 1). There was a significant correlation between the concentration of amniotic fluid and the diameter of the inhibition zone (P<0.01, Table 2). It is worth noting that 73% of useful amniotic fluid samples belonged to multigravida women. There was no significant relationship between the women's age and the antifungal effects of amniotic fluid, but the antimicrobial effect of amniotic fluid increased with months of pregnancy.

Table 1. MIC and MFC values of ketoconazole against *Malassezia* isolates

***Malassezia* species**	**Concentration (µg/mL)**	**MIC_{50} (µg/mL)**	**MFC (µg/mL)**
M. globosa	0.06, 8.00	0.13, 0.50	4.00, 8.00
M. furfur	0.06, 8.00	0.06, 0.13	2.00, 4.00

MIC, Minimum Inhibitory Concentration; MFC, Minimum Fongicide Concentration

Table 2. Effect of various concentrations of amniotic fluid on the mean diameter of inhibition zone around ketoconazole-resistant *Malassezia* isolates

Mean (mm) ± SD	Concentration of amniotic fluid (mg/mL)
3,000	18.00± 3.8
1,500	10.00±0.3
750	8.00± 0

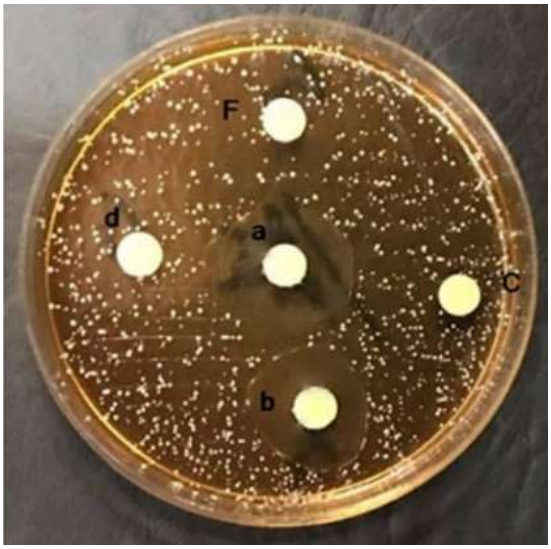

Fig. 1. The antifungal effects of amniotic fluid on the growth of ketoconazole-resistant Malassezia isolates. a: Undiluted amniotic fluid, b: 3000 mg/mL, c: 1500 mg/mL, d: 750 mg/mL, f: negative control

DISCUSSION

In this study, we investigated the effects of various dilutions of amniotic fluid from pregnant women against ketoconazole-resistant *Malassezia* isolates from patients with suspected seborrheic dermatitis. According to the findings, the frequency of *Malassezia* isolates was 76.6% among samples collected from patients suspected with seborrheic dermatitis. In previous studies in northern Iran, the frequency of *Malassezia* isolates among patients with seborrheic dermatitis were reported to be 36% and 70% [14, 15].

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In the present study, the *Malassezia* species were *M. globosa* (78%) and *M. furfur* (22%). In previous studies, the most common *Malassezia* species were *M. furfur* (34%) and *M. pachydermatis* (4.5%) in Iran [16], *M. furfur* (35%) and *M. globosa* (22%) in Japan [17], *M. furfur* (25.7%) and *M. globosa* (22.7%) in Brazil [18], *M. globosa* (40%) and *M. furfur* (7%) in Argentina [19], *M. furfur* (32.86%) and *M. globosa* (14.28%) in India [20], and *M. globosa* (16%) and *M. furfur* (2%) in Bosnia and Herzegovina [21]. These differences might be due to the difference in time of the study, the geographical characteristics of the studied areas, and ethnic diversity of patients.

Our results showed that 55% of the isolates were resistant to ketoconazole. In a previous study, the rate of ketoconazole resistance in *Malassezia* isolates was 61%, which is higher than the rate observed in our study [14]. In our study, the MIC of ketoconazole against *Malassezia* isolates ranged from 0.06 to 0.5 µg/mL. In Iran, the MIC of ketoconazole against *Malassezia* species was 0.06-0.12 µg/mL in 2006 [21], 0.03-1 µg/mL in 2008 [22] and 0.032-0.5 µg/mL in 2009 [23].

In our study, MFC of ketoconazole against *Malassezia* isolates was within the range of 1 to 8 µg/mL, which is higher than the values reported in other studies [21-24]. Resistance to antifungal agents is increasing worldwide at an alarming rate across all groups of fungi [8]. According to our results, the rate of ketoconazole resistance was higher in *M. globosa* isolates (58%) than in *M. furfur* isolates (40%). In a similar study, only 10% of *M. furfur* isolates were resistant to ketoconazole [25]. In the present study, the amniotic fluid exhibited favorable antimicrobial effects against *Malassezia* isolates from patients with seborrheic dermatitis. Besides, amniotic fluid could inhibit 50% of ketoconazole-resistant *Malassezia* isolates. Given that the antimicrobial effect of amniotic fluid increases with increasing the month of pregnancy, we collected amniotic fluid samples from pregnant women in the final weeks of pregnancy (weeks 15 and 16). Recently, two studies demonstrated that human amniotic fluid was of high antimicrobial activity against chronic wound pathogens *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus pneumonia* [26, 27].

A study in 2017 on the antimicrobial effects of amniotic fluid and chorionic membrane on several microbial species showed that the effect of the chorionic membrane was greater than that of amniotic fluid [28].

As shown in previous studies, the uterus and uterine fluids have antimicrobial properties, which are probably due to the presence of peptides such as defensins and elafin [8]. Therefore, verifying the antifungal properties of amniotic fluid may denote its dominance on drug-resistant fungal infections. Given the favorable inhibitory properties of amniotic fluid against antifungal-resistance isolates, it can be used as an alternative or complementary therapy for the treatment of antifungal-resistant infections. Since the immune function varies significantly between individuals, further studies are required to determine the factors involved in the antimicrobial effects of amniotic fluid.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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