



Research Article

The Significance of Measuring Human Beta Defensin-2 in Patients with Diabetic Foot Ulcer

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Abstract

Background: Approximately one out of every four diabetic patients will acquire a diabetic foot ulcer (DFU) in their lifetime. Human beta-defensin (HBD) promotes wound healing. **Objective:** To find the correlation between HBD-2 and ulcer grade, diabetic foot infection, and the type of bacterial isolates recovered from bacteriological culture. **Methods:** We included forty-nine patients with DFU and obtained blood samples and wound swabs from each participant between October 2023 and December 2023. We measure HbA1c using ARCHITECT c4000 system, and HBD-2 using the ELISA technique. Classification of DFU was done based on Wagner's method. Swabs from foot ulcers are used for isolation and preliminary identification of bacteria based on standard guidelines. The VITEK® 2 system confirmed the diagnosis. **Results:** The patients' mean age was 57.31 years, and the male/female ratio was 1.57. Grade 3 was the most common type (57.1%). We observed the highest significant level of HBD-2 in grade one, non-infected DFU patients, and ulcers infected with gram-positive bacteria. Patients infected with *Staphylococcus aureus* showed the highest HBD-2 level according to the type of isolate, while patients infected with *Proteus mirabilis* showed the lowest level. **Conclusions:** HBD-2 levels might reflect the impaired or dysregulated immune response in patients with type 2 diabetes mellitus (T2DM) and have a negative impact on wound healing. The type of bacteria influenced this level, with *Staphylococcus aureus* infections reporting the highest level.

Keywords: DFU, Diabetic foot infection, HBD-2, Ulcer Grade.

أهمية بيتا ديفينسين-2 البشري في المرضى الذين يعانون من قرحة القدم السكرية

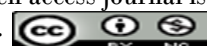
الخلاصة

الخلفية: ما يقرب واحد من كل أربعة مرضى سكري سوف يصابون بـ DFU في حياتهم. يعزز بيتا ديفينسين البشري (HBD) التئام الجروح. **الهدف:** إيجاد العلاقة بين HBD-2 ودرجة القرحة وعدوى القدم السكرية ونوع العزلات البكتيرية المستردة من المزرعة البكتريولوجية. **الطرق:** تم تضمين تسعة وأربعين مريضاً يعانون من DFU. وتم جمع عينات الدم ومسحات الجروح من كل مريض بين أكتوبر 2023 وديسمبر 2023. تم قياس HbA1c تلقائياً باستخدام نظام ARCHITECT c4000 و HBD-2 باستخدام تقنية ELISA وتم تصنيف DFU بناءً على طريقة فاغنر. **النتائج:** من بين 49 مريضاً من مرضى DFU، كانت نسبة الذكور إلى الإناث (1.57). وكان متوسط عمر المرضى 57.31 سنة. وكان المستوى الثالث هو الأكثر شيوعاً (57.1%). أظهر HBD-2 أعلى مستوى متوسط ملحوظ في المستوى الأول، في مرضى DFU غير المصابين، وفي المرضى الذين يعانون من قرحة مصابة بالبكتيريا إيجابية الجرام. ولوحظ أعلى متوسط لـ HBD-2 في المرضى المصابين بالمكورات العنقودية الذهبية، تليها الإشريكية القولونية، وكان أدنى مستوى في المرضى المصابين بالمتقلبة الرائحة. **الاستنتاجات:** قد تعكس مستويات HBD-2 ضعف الاستجابة المناعية أو خلل التنظيم لدى المرضى الذين يعانون من داء السكري من النوع الثاني ويكون لها تأثير سلبي على التئام الجروح. وقد أثر نوع البكتيريا على هذا المستوى، حيث سجلت عدوى المكورات العنقودية الذهبية أعلى مستوى.

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder currently known as a global health emergency in the 21st century [1]. Diabetes caused about 5 million deaths worldwide in 2017 among people aged 20 to 99 years [2]. In 2019, the projected direct healthcare cost of DM was 760 billion US dollars globally [3] and carries high rates of morbidity and mortality [4]. The prevalence and incidence of T2DM, which account for more than 90% of all DM cases, are increasing rapidly throughout the world [5]. Diabetic foot ulcers (DFU) are critical complications of T2DM and are usually combined and associated with major morbidity, mortality, costs, and reduced quality of life [6,7]. Diabetic patients with a DFU showed a higher mortality rate in comparison to those without a DFU, with a mortality rate of 99.9 per 1000 person-years in diabetic individuals with DFU compared to 41.6 per 1000 in the population with diabetes alone [8]. The diabetic foot's pathological base includes tissue ischemia, peripheral neuropathy, and infection, and the development of infection is directly associated with the risk of amputation [9]. Around one in four people with diabetes will develop DFU in their lifetime [10]. Other reports showed that 11% to 14% of diabetic patients will develop foot ulcers [11]. Low-grade inflammation is a feature of T2DM [12], and the microenvironment with high blood sugar may slow down diabetic wound healing by affecting the functions of keratinocytes [13]. Antimicrobial peptides (AMPs), which are also called host defense peptides, are short polypeptides or oligopeptides with 20 to 60 amino acid residues that are both cationic and amphipathic [14]. AMPs are part of the innate immune system and have two functions: first, they can protect the host against many pathogens by killing them directly; second, they may change the immune system by changing both innate and adaptive immune responses [15]. Defensins are a class of cationic AMPs made up of six cysteine residues that form three disulfide linkages based on their distribution, defensins are divided into three subfamilies: α -defensins, β -defensins, and θ -defensins [16]. Human beta-defensin (HBD) enhances wound healing [17]. On the other hand, Han *et al.* show that the HBDs have been shown to be effective against a few nosocomial pathogens, including *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*, which are considered the most prevalent in diabetic wounds [18]. Various studies have demonstrated the importance of HBD-2 in DFU patients. However, little is known about the relationship between HBD-2 serum level, ulcer grade, diabetic foot infection, and the type of isolated bacteria. The study's goal is to find a link between HBD-2 and ulcer grade, diabetic foot infection, and the type of bacterial isolate found in a bacteriological culture.

METHODS

Study design and setting

This is a prospective cross-sectional study of 49 patients with DFU. We collected samples from AL-Kafeel Hospital at Kerbala and Imam Al-Hassan Center for Endocrinology and Diabetes over a period of 2 months, from October 2023 to December 2023.

Ethical consideration

The study protocol was approved by the Ethical Committee at the College of Applied Medical Sciences, University of Kerbala (Certificate Number 259 on September 24, 2023). All patients included in this study were informed and verbal agreement was obtained prior to sample collection.

Sample Selection

This study included confirmed T2DM patients with diabetic foot ulcers from both sexes, ranging in age from 18 to 80 years. Exclusion criteria include: age less than 18 years; other types of diabetes mellitus; pregnant or lactating women; patients with malignancy, autoimmune diseases or a history of steroid treatment; patients with foot ulcers without T2DM; and cancerous ulcer wounds.

Data collection and outcome measurements

We documented demographic data such as age and sex, along with other details such as the ulcer's grade, the presence of a diabetic foot infection, and the use of superoxide to treat the ulcer. Blood and diabetic foot ulcer swabs were collected from each patient. We used blood samples to measure both HBA1c and HBD-2 using the Architect Abbott C4000 (Germany) and ELISA techniques (Elabscience, USA), respectively. The swabs were taken after wound cleansing using microcin and gauze and the removal of necrotic tissue or foreign material. We did not introduce any antimicrobial agent or antiseptic into the wound until five days after specimen collection. Each swab was cultured aerobically within 30 minutes on blood and MacCkonky agar for preliminary identification of microorganisms and then diagnosis was confirmed using the VITEK® 2 system (BioMérieux, Marcy l'Etoile, France).

Statistical analysis

The Statistical Package for the Social Sciences (SPSS), version 22 software (IBM Corp., NY, USA), was used to analyze the data. Frequency, mean, standard deviation, and cross-tabulation were determined using descriptive statistics. The Analysis of Variance (ANOVA) test was used to compare means. The statistical significance level was established at $p < 0.05$.

RESULTS

Forty-nine DFU patients were included in this study during the period between October 2023 and January 2024 in Kerbala Governorate, Iraq. Thirty patients (61.2%) were male and 19 (38.8%) were female, as

shown in Table 1. In terms of age, the mean age of patients was 57.31 ± 10.27 years. More than half of the patients (59.2%) were within the age range of 51–69 years, as shown in Table 1. The mean onset age of T2DM was 41.22 ± 11.07 years for DFU patients, while the mean disease duration of T2DM was 16.16 ± 6.78 years (Table 1).

Table 1: Demographic Data of the DFU Patients

Variables	DFU (n=49)
Sex	
Male	30(61.2)
Female	19(38.8)
Age (year)	57.31 ± 10.27
Age groups (year)	
≤ 50 years	11 (22.4)
51-69 years	29 (59.2)
>69 years	9 (18.4)
T2DM onset (year)	41.22 ± 11.07
T2DM Duration (year)	16.16 ± 6.78

Values were expressed as frequency, percentage, and mean±SD.

The cases of DFUs were classified according to the Meggitt-Wagner system into grade one 8 (16.3%), grade two 11 (22.4%), grade three 28 (57.1%), and grade four 2 (4.1%) cases, as shown in Figure 1.

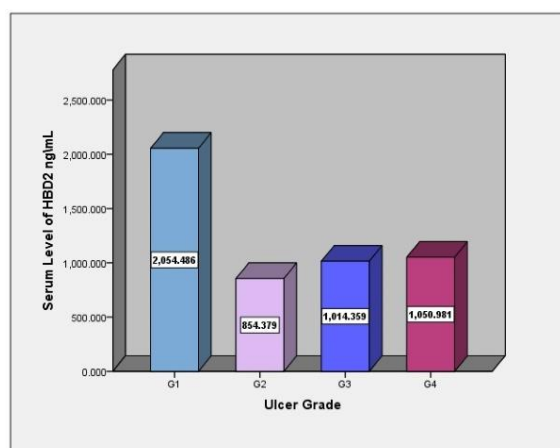


Figure 1: Human beta defensin-2 (HBD-2) concentration according to ulcer grade.

Only eighteen (36.7%) of patients were treated with superoxide water during the management of their wound, as shown in Table 2.

Table 2: Characterization of diabetic foot ulcer

Variables	DFU patients (n=49)
Ulcer grades (Meggitt-Wagner system)	
Grade 1	8(16.3)
Grade 2	11(22.4)
Grade 3	28(57.1)
Grade 4	2(4.1)
Superoxide use	
Yes	18(36.7)
No	31(63.3)

Values were expressed as frequency and percentage.

This study showed significant differences in the mean level of HBD-2 with ulcer grade (the highest mean was seen in grade one), as shown in Figure 1, with diabetic foot infection (non-infected DFU patients had the highest mean level), as shown in Figure 2.

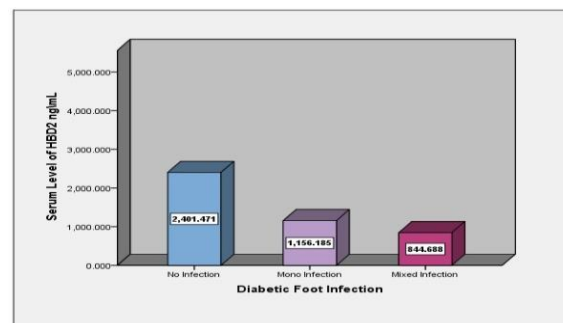


Figure 2: Human beta defensin-2 (HBD-2) concentration according to diabetic foot infection.

with gram reaction (higher mean was seen in ulcers infected with gram-positive bacteria), as shown in Figure 3.

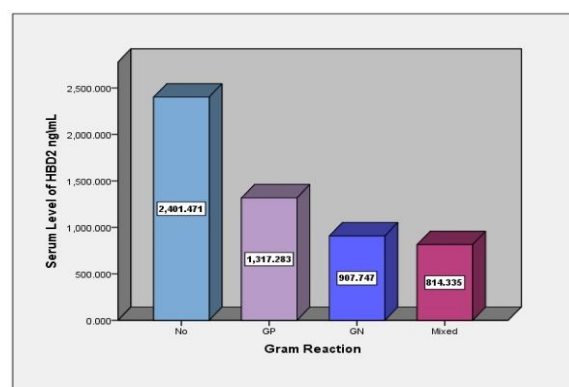


Figure 3: Human beta defensin-2 (HBD-2) concentration according to Gram stain reaction.

With Superoxide (Microcin) usage (DFU patients whom they didn't use superoxide water had higher mean level of HBD-2), as shown in Figure 4. In order to investigate the effect of bacterial isolates on the mean level of serum HBD-2, the mean level of DFU patients infected with certain types of bacterial isolates was compared to the mean level of uninfected DFU patients. Significant differences were observed between uninfected patients and patients infected by *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*.

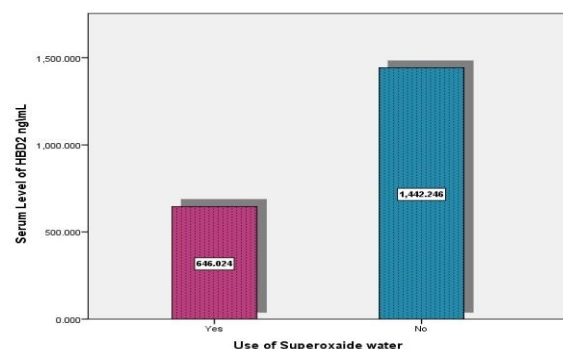


Figure 4: Human beta defensin-2 (HBD-2) concentration according to the use of superoxide water.

The highest mean HBD-2 was observed in patients infected with *Staphylococcus aureus*, followed by *Escherichia coli*, and the lowest level was seen in patients infected with *Proteus*, as shown in Table 3.

Table 3: Differences in HBD-2 concentration according to the type of bacterial isolates

Infection type	n	HBD-2 Level	p-value
<i>S. aureus</i>			
Infected	16	1104.78±182.73	0.020
Uninfected	5	2401.47±741.71	
<i>E. coli</i>			
Infected	11	998.23±186.96	0.024
Uninfected	5	2401.47±741.71	
<i>P. aeruginosa</i>			
Infected	6	857.98±195.25	0.056
Uninfected	5	2401.47±741.71	
<i>K. Pneumoniae</i>			
Infected	6	801.35±202.44	0.050
Uninfected	5	2401.47±741.71	
<i>P. mirabilis</i>			
Infected	9	781.31±142.25	0.015
Uninfected	5	2401.47±741.71	

Values were expressed as mean±SE.

DISCUSSION

Defensins are known for their excellent antibacterial activity [19]. Sanapalli *et al.* [17] showed that defensins play an essential role in the complex pathophysiological changes of diabetic wounds. This study aims to find the correlation between HBD-2 and ulcer grade, diabetic foot infection, and the type of bacterial isolate recovered from bacteriological culture. This is, to the best of our knowledge, the first study to address this goal. This study showed that the male/female ratio was 1.57 (Table 1). Similar findings were reported in previous studies, in which male patients were more frequent than female patients [20]. Anderson *et al.* [21] reported a higher frequency of DFU among males (55.7%), and Mineoka *et al.* [22] found a higher frequency of DFU among males (56.2%). This might be because the population of men is more exposed to hard work than females, with a larger risk of trauma in their workplace [23]. Most of the patients were in the 5th and 6th decades (Table 1). This might be because today's professional activities and lifestyle cause the foot to withstand higher pressure, especially in this age decade [24]; thus, DFU was higher in this age group. A similar finding was reported by Singh *et al.* [25]. However, a lower mean age (52.76±11.31) in DFU patients was recorded by Younis *et al.* [26]. Previous studies reported that most patients suffering diabetic ulcers were >55 years old because age poses a risk of reduced self-care capability due to poor vision and limited movement [27]. The mean age of diabetes onset among DFU patients was 41.22±11.07, and the mean T2DM duration was 16.16±6.78 years (Table 1). Qadir *et al.* [24] reported a comparable mean DM duration (14.6±7.9) years. However, a lower mean of DM duration (9.96±6.35) was reported by Younis *et al.* [26]. Hamilton *et al.* [28] reported that the median age of diabetes duration was 17.1 [10.0–22.8], and other previous studies documented that the mean duration of DM among DFU individuals was 11.68±4.8 [23]. Among DFU patients, grade 3 was the most prevalent grade, followed by grade 2. A similar finding was reported by Malepati *et al.* [23]. Hadi *et al.* [29] and Jasem and Abdul-Razaq [30] found that grade 4 is the most predominant grade type. The differences in the distribution of patients with ulcer grades could be

attributed to various factors such as the study design, the type of patients included, irregular or non-existent diabetes treatment, or improved patient awareness and effective disease control. During diabetic wound management, 18 (36.7%) patients were administered with superoxide water (Microcin), as shown in Table 2. This may be due to the fact that microcin is only available at Al-Kafeel Hospital in Kerbala, a private hospital, and not in public health settings. Furthermore, many DFU patients either have low incomes, cannot visit Al-Kafeel Hospital, or lack knowledge about the use of microcin in wound management. In addition to that, not all of the included patients in this study were residents of Kerbala Governorate; therefore, a lower percentage of patients were using the superoxide water (Microcin). In their study [31], Martínez-De Jesús *et al.* found that a neutral pH superoxide solution may be better than regular disinfectants at getting rid of infections, bad smells, and redness in diabetic foot infections. Regarding differences in HBD-2 concentration in DFU patients, previous studies reported that reduced expression of HBD is correlated with delayed wound healing [32,33] and that their effects may vary according to concentration. In this study, a higher mean level of HBD-2 was found in ulcers with grade 1 in comparison to other grades. This could be explained by the fact that inflammation is the initial stage of the healing process of wounds, characterized by platelet aggregation and migration of leukocytes which involve macrophages and neutrophils that release defensins and subsequently enhance wound repair [34]. In wounds related to diabetes, the number of neutrophils expands abnormally, and macrophage polarization is inhibited, resulting in increased inflammatory expression [35,36]. Persistent inflammation of the wound could lead to decreased expression of HBD [37,38], and this was proven by this study in which the mean level of HBD-2 was seen to be decreased with higher ulcer grades. This study also showed that non-infected wound patients had a higher mean level of HBD-2, followed by wounds infected with a single bacterial isolate. It has been found that HBD3 exhibits increased cytotoxic effects with increased concentration [39,40] and that human defensins are created to remove invading pathogens during the early phases of wound development [41]. Therefore, a decreased expression level of HBD may reflect and be associated with diabetic wound infection and impair wound healing. A significantly higher mean of HBD-2 was seen in patients infected with gram-positive bacteria (Figure 3). This could potentially be attributed to the prevalence of gram-positive bacteria (*S. aureus*) in the feet of DFU patients and various human infections. It was proven that human alpha-defensins protect leukocytes from neutralization by gram-positive pathogenic bacterial toxins [42]. A higher mean level of HBD-2 was found in DFU patients who didn't use superoxide water in DFU management (Figure 4). This may indicate that microcin has a negative impact on bacteria due to its antimicrobial activity, which may result in a reduction in bacterial load and, subsequently, a reduction in HBD-2 expression. Linn *et al.* [43] said that diabetic

angiopathy may also reduce blood flow in the mucosa, which makes it harder for the epithelial cells to fight off infections and can cause ulcers to form. Lan *et al.* concluded that the high-glucose-cultivated keratinocytes express lower levels of HBD-2 [44]. Regarding the differences in HBD-2 concentration according to the type of bacterial isolates, the current study demonstrates a significantly higher mean of HBD-2 in patients infected with *S. aureus*, followed by *E. coli*, and the lowest concentration was observed in patients infected with *Proteus*, as shown in Table 3. No previous study focused on the differences in HBD-2 serum levels according to the type of bacterial isolate. However, Bolatchiev *et al.* documented that the antimicrobial peptides showed considerable antibacterial activity against both MSSA and MRSA strains isolated from wound discharge of individuals with diabetic foot syndrome, and also reported that HBDs exhibit a high killing effect against common bacterial species of diabetics, such as *E. coli* and *S. aureus* [45]. Mathew and Nagaraj [46] reported that human beta-defensin-2 has broad antibacterial activity and penetrates the cell membrane of *E. coli*. Pachón-Ibáñez *et al.* [47] reviewed that the HBDs exhibit a strong ability to eradicate various types of pathogens, such as *Staphylococcus aureus* and *Escherichia coli*, which frequently infect chronic wounds.

Study limitations

The most significant limitation of this study is its small sample size and its lack of focus on antibiotic usage among DFU patients.

Conclusion

A significantly higher mean of HBD-2 was found in ulcers with grade 1 in comparison to higher grades, which may reflect a negative association of HBD-2 with ulcer grade. Moreover, a significantly lower mean of HBD-2 in infected wounds compared to non-infected wounds may confirm the dysregulated immune response of T2DM, making the diabetic wound more susceptible to infection and impairing wound healing. Furthermore, a significantly higher mean of HBD-2 was seen in patients infected with gram-positive bacteria and the highest mean level was seen in patients infected with *Staphylococcus aureus*. This may reflect that HBD-2 production is influenced by the type of bacteria.

Conflict of interests

No conflict of interests was declared by the authors.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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