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Investigation of *In Vitro* Efficacy of Boric Acid on *Pseudomonas aeruginosa* Strains Isolated from Diabetic Foot Infections

Diyabetik Ayak Enfeksiyonlarından İzole Edilen *Pseudomonas aeruginosa* Suşları Üzerinde Borik Asidin *İn Vitro* Etkinliğinin İncelenmesi

Yavuz PİRHAN[1], Mustafa CİHANGİROĞLU[2]

[1]Amasya University Sabuncuoğlu Şerefeddin Training and Research Hospital, Department of General Surgery, Amasya, Turkey
[2]Amasya University Sabuncuoğlu Şerefeddin Training and Research Hospital, Department of Infectious Diseases and Clinical Microbiology, Amasya, Turkey

Abstract

Introduction: The aim of this study is to investigate the efficacy of boric acid as an antiseptic agent for use in wound care against *Pseudomonas aeruginosa* (*P. aeruginosa*) strains isolated from diabetic foot infections.
Materials and Methods: A total of 25 *P. aeruginosa* strains isolated from diabetic foot infections were included in the study between January 2010 and June 2015. The susceptibility of these strains to various antibiotics was determined. Dilutions of various concentrations were prepared from boric acid to test these strains' growth at different concentrations. Our study was conducted in accordance with the Helsinki Declaration.
Results: At the end of the incubation period, growth was observed in all isolates to which 1.6 mg/l boric acid solution was exposed. No growth was observed in any of the 25 wells to which 25 mg/l, 50 mg/l, and 100 mg/l boric acid solution was added. A significant difference was observed between the lowest concentration without reproduction (25 mg/l boric acid) and other concentrations where reproduction was detected. The minimum inhibitory concentration of boric acid for *P. aeruginosa* was 25 mg/l. High antibiotic resistance was noteworthy in *P. aeruginosa* strains, which reproduced at low boric acid concentrations.
Conclusion: Boric acid has an *in vitro* inhibitory effect on *P. aeruginosa* strains isolated from diabetic foot infections. Low-cost boric acid may be a suitable option for the local treatment of diabetic foot infections caused by *P. aeruginosa*.
Keywords: Antibiotic resistance, diabetic foot infection, *P. aeruginosa*, boric acid

Öz

Giriş: Bu çalışmada diyabetik ayak enfeksiyonlarından izole edilen *Pseudomonas aeruginosa (P. aeruginosa)* suşlarına karşı yara bakımında kullanılan bir antiseptik ajan olan borik asitin etkinliğinin belirlenmesi amaçlanmaktadır.
Gereç ve Yöntem: Çalışmaya Ocak 2010 - Haziran 2015 tarihleri arasında diyabetik ayak enfeksiyonlarından izole edilen toplam 25 adet *P. aeruginosa* suşu alındı. Bu suşların çeşitli antibiyotiklere duyarlılıkları belirlendi. Borik asitten çeşitli konsantrasyonlarda dilüsyonlar hazırlanarak bu suşların farklı konsantrasyonlarda üremesi test edildi. Çalışmamız Helsinki Deklarasyonu uyarınca yapıldı.
Bulgular: İnkübasyon süresi sonunda 1,6 mg/l borik asit çözeltisinin maruz bırakıldığı tüm izolatlarda üreme gözlendi. 25 mg/l, 50 mg/lve 100 mg/l borik asit çözeltilerinin eklendiği 25 kuyucuğun hiçbirinde üreme gözlenmedi. Üremenin olmadığı en düşük konsantrasyon olan 25 mg/l borik asit konsantrasyonu ile üremenin tespit edildiği diğer konsantrasyonlar arasında anlamlı bir fark gözlenmiştir. Borik asitin *P. aeruginosa* için minimum

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Address for Correspondence/Yazışma Adresi: Yavuz Pirhan MD, Amasya University Sabuncuoğlu Şerefeddin Training and Research Hospital, Department of General Surgery, Amasya, Turkey
Phone: +90 358 218 40 00 **E-mail:** ypirhan@gmail.com
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inhibitör konsantrasyonu 25 mg/l olarak bulundu. Düşük borik asit konsantrasyonlarında üreyen *P. aeruginosa* suşlarında yüksek antibiyotik direnci dikkat çekiciydi.
Sonuç: Borik asit, diyabetik ayak enfeksiyonlarından izole edilen *P. aeruginosa* suşlarına karşı *in vitro* etkinlik göstermektedir. Maliyeti düşük olan borik asitin *P. aeruginosa*'nın neden olduğu diyabetik ayak enfeksiyonlarında lokal tedavide uygun bir seçenek olabileceğine inanıyoruz.
Anaktar Kelimeler: Antibiyotik direnci, diyabetik ayak enfeksiyonu, *P. aeruginosa*, borik asit

Introduction

Foot infections are among the serious and frequent complications of diabetes[1] and are a significant cause of morbidity and mortality. About one-fourth of all individuals with diabetes develop foot infections. These infections are a contributing cause of hospital admissions[2]. Diabetic foot infections are generally associated with a more prolonged hospital stay than the other complications of diabetes, and are a major cause of non-traumatic lower extremity amputations[3]. Infections in the diabetic foot cause prolongation of the disease and increase treatment costs[4]. Despite some geographical differences, recent studies found a predominance of resistant Gram-negative bacteria in diabetic foot infections than staphylococci. In this regard, *P. aeruginosa* is a gram-negative pathogen commonly isolated in many diabetic foot wound studies[5,6]. The follow-up and treatment of diabetic foot infections require a multidisciplinary approach. Wound care is an integral part of treatment.

While boric acid has been used for centuries as a topical antiseptic agent to treat wound infections, the effectiveness of boric acid has been neglected. Its effectiveness has not been extensively investigated with the introduction of antibiotics for wound treatment[7]. Boric acid (H_3BO_3) is a compound with a pH of approximately five and is found in trace amounts in the human body. Its molecular weight is 61.84 g/mol and is moderately water soluble[8]. Boric acid is a compound with defined antioxidant, anti-inflammatory, anti-cancer, anti-candidal, and antimicrobial properties. It is used as an antiseptic or buffering agent, and it is not absorbed from the skin's surface[9]. For this reason, this study was designed using boric acid to treat diabetic foot wounds with *P. aeruginosa* infection.

Materials and Methods

Twenty-five *P. aeruginosa* strains isolated from diabetic foot wounds between January 2010 and June 2015 were included in the study. A single clinical example from each patient was included in the study. Identification of isolated strains was made using the VITEK 2 automated system (bioMérieux, France) after detecting *P. aeruginosa*'s typical findings by conventional methods (typical aromatic odor, colony morphology, and oxidase test). Isolates antibiotic resistance status was determined by the VITEK 2 system and E-test (AB Biodisk, Sweden) method and evaluated according to Clinical and Laboratory Standards Institute criteria. Isolates were stored at -70 °C in 150 mg/l glycerol agar. During the study, they were inoculated on eosin methylene blue (EMB) agar and incubated for 24 hours at 37 °C. In this study, a 200 mg/l liquid solution was prepared using powder boric acid between 40 °C and 50 °C to form the boric acid concentrations. Following this first step, 100 µl of this solution was added to the well of the microplate. Next, 100 µl of McFarland 0.5 turbid bacterial solution was added to the well. Finally, 100 mg/l concentration of the boric acid solution was completed in the first well. The dilution was performed to achieve boric acid concentrations of 50 mg/l, 25 mg/l, 12.5 mg/l, 6 mg/l, 3.2 mg/l, and 1.6 mg/l, and kept at 37 °C for 12 hours. The pH of the wells varied between 6 and 6.5. For each isolate, a control inoculation was performed with sterile physiological saline. After incubation at 37 °C for 12 hours, they were transferred onto EMB agar for bacterial growth determination. Comparing the isolates antibiotic susceptibility and their growth levels in different boric acid concentrations were conducted. For this study, ethics committee approval was received from the Non-invasive Clinical Research Ethics Committee of the University of Amasya (decision number: 2021/01, date: 07.01.2021).

Statistical Analysis

All analyses were conducted using the Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS, Chicago, IL, USA). The chi-square test was used. P values of <0.05 were considered statistically significant.

Results

At the end of the incubation period, growth was observed in all the isolates exposed to 1.6 mg/l boric acid solution. Growth was observed in 22 of 25 wells exposed to 3.2 mg/l boric acid solution, 12 of 25 wells exposed to 6 mg/l boric acid solution, and five of 25 wells exposed to 12.5 mg/l. There was no bacterial growth in any isolate exposed to a boric acid solution at concentrations of 25 mg/l or greater (Table 1, Figure 1). Growth was observed in all control groups. A significant difference was observed between 25 mg/l boric acid concentration, which is the lowest concentration where reproduction is absent, and other concentrations where reproduction was detected. Bacterial growth was not observed at concentrations of 25 mg/l, 50 mg/l, and 100 mg/l. In contrast, bacterial growth was observed at concentrations of 1.6 mg/l, 3.2 mg/l, 6 mg/l, and 12.5 mg/l boric

acid. The lowest minimum inhibitory concentration value for *P. aeruginosa* was 25 mg/l boric acid concentration. Antibiogram susceptibilities of *P. aeruginosa* isolates are presented in Table 2. Five isolates that can grow in 12.5 mg/l boric acid solution were resistant to cefepime, ceftazidime, ciprofloxacin, amikacin, and netilmicin. In addition, one of these isolates was resistant to all antibiotics. Our results suggest a linear correlation between the concentrations of boric acid and the resistance of *P. aeruginosa* to antibiotics. Therefore, as the concentrations of boric acid increase, the resistance of *P. aeruginosa* against antibiotics also increases.

Discussion

As emphasized by the International Diabetic Foot Working Group, diabetic foot management requires a multidisciplinary approach, including relieving pressure, ensuring skin perfusion, managing infection, controlling metabolism, and providing local wound care[8]. Dressings play a key role in the continuity of wound care. Previous studies established the efficacy of wet wound care models, which led to the introduction of different wound care products (hydrogel, hydrocolloid dressing materials)[9]. An ideal dressing material should prevent drying, absorb exudative secretions, allow gas exchange, and provide a barrier between the wound and contaminated environments[1]. Antiseptic agents, such as hydrogen peroxide, povidone-iodine, acetic acid, and others, have toxic properties. Therefore, their role in diabetic wound care is generally discouraged[10]. Removal of keratotic skin at wound margins and the facilitation of superficial epithelization are significant measures for wound care[8,10]. For this reason, we chose boric acid as it has little or no irritant effect

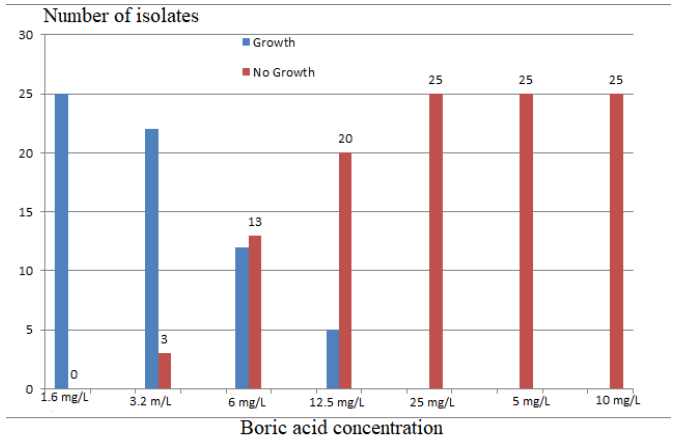

Figure 1. Relationship between boric acid concentrations and bacterial counts

Table 1. The total number of bacterial growth in different boric acid concentrations

Boric acid concentrations (mg/l)	Bacteria growth, n/N (%)	p value
1.6 mg/l	25/25 (100)	p=incalculable
3.2 mg/l	22/25 (88)	p=0.074
6 mg/l	12/25 (48)	p=0.000071
12.5 mg/l	5/25 (20)	p<0.0001
25 mg/l	0/25 (0)	p<0.0001
50 mg/l	0/25 (0)	p<0.0001
100 mg/l	0/25 (0)	p<0.0001

n: The number of *P. aeruginosa* isolates that reproduce after acid boric is applied.

N: The number of *P. aeruginosa* isolates.

Table 2. Antibiotic susceptibility of isolated *P. aeruginosa* strains (n=25)

Antibiotics	S		I		R		CLSI limit values[24]
	n	%	n	%	n	%	
Cefepime	7	28	3	12	15	60	≤8 ¬ ≥32
Ceftazidime	6	24	4	16	15	60	≤8 ¬ ≥32
Amikacin	15	60	3	12	7	28	≤16 ¬ ≥64
Netilmicin	16	64	4	12	5	20	≤8 ¬ ≥32
Imipenem	22	88	2	8	1	4	≤4 ¬ ≥16
Meropenem	22	88	2	8	1	4	≤4 ¬ ≥16
Ciprofloxacin	16	64	3	12	6	24	≤1 ¬ ≥4
Cefoperazone/sulbactam	22	88	2	8	1	4	≤ 16 ¬ ≥64
Piperacillin/tazobactam	23	92	1	4	1	4	≤64\4 ¬ ≥128\4

S: Susceptible, I: Intermediate, R: Resistant

on human skin. *P. aeruginosa* is more common in developing countries with warm climates, especially in Asia and Africa. Patients exposing their ulcers to water or moist environments also increase their risk for *P. aeruginosa* infection[11]. *P. aeruginosa* forms biofilms more readily in the diabetic wound environment, which leads to increased resistance to antimicrobial agents. This could help explain why diabetic wounds are typically slower to heal and more difficult to treat than non-diabetic wounds[12]. In addition, increased drug resistance makes infection treatment caused by this pathogen difficult[13]. Boric acid is an inorganic substance ingested in low amounts daily with food and drinks, and the majority is excreted via the urine within 24 hours. Boric acid absorption through the skin is negligible unless the skin's integrity has been disrupted. It is also applied locally for the treatment of vulva-vaginal candidiasis[14]. Boric acid is involved in many enzymatic processes, the stability of the cell membrane, the metabolism of vitamin D and steroids, and the development of mental functions. Studies have found that even 3 to 5 grams of boric acid taken orally may lead to toxic effects. On the other hand, it may also augment phagocytic functions and may exert anti-bacterial and anti-candidal effects[15,16]. In wound care, generally, a 2% concentration of boric acid is applied[17]. Kujath and Hügelschäffer[18] were the first to state that boric acid at a 3% concentration was used against pseudomonal wound infection. They emphasized that such a dose can benefit treatment without any side effects. Also, Adarchenko et al.[19] studied and analyzed boric acid. They concluded that it could be more effective against isolates of *P. aeruginosa* when compared with other agents. However, due to its toxicity, it may be hard to handle safely. According to the research conducted, boric acid's bactericidal effect has more than one target in the bacteria cell. *P. aeruginosa* develops resistance to antibiotics by using an intrinsic defense mechanism, changing its membrane structure against antibiotics, producing enzymes inactivating antibiotics, and draining antibiotic agents from bacterial cells via efflux pumps. Boric acid acts as a bactericidal against *P. aeruginosa*[20]. Boric acid can help in the local treatment of wounds caused by *P. aeruginosa*. Further, there is evidence that the appearance of the biofilms in infections, such as chronic wound infection, chronic otitis media, chronic rhinosinusitis, urinary tract infections caused by catheter use, and keratitis caused by contact lenses, is linked to *P. aeruginosa*[21]. Youn et al.[22] found that a 4% boric acid concentration is a strong biofilm inhibitor. However, according to Youn et al.[22], boric acid had to remain in the same environment with bacteria for a long time (24-72 hours) to be effective at a concentration of 4%. Likewise, Saha et al.[17] tested the boric acid on *P. aeruginosa* and observed the anti-biofilm property of boric acid. This led them to conclude that boric acid application could be used as an alternative treatment method against *P. aeruginosa*. Based on this, they applied a 2% concentration of boric acid for wound care. In a study conducted by Kumara et al.[23], the researchers evaluated the effectiveness of ascorbic acid, acetic acid, and boric acid on several microorganisms. They reported that thirty minutes incubation of 0.5%, 1%, and 2.5% concentrations of boric acid was effective for inhibition of ten *P. aeruginosa* isolates. Similarly, there was no growth in the twenty-five *P. aeruginosa* isolates in the present study after 12 hours of incubation at 25 mg/l and higher concentrations. This research is subject to a limitation. It was difficult to collect *P. aeruginosa* strains in diabetic wound infections. Therefore, we recommend that future researchers focus on collecting more isolates in their studies.

Conclusion

Boric acid showed in vitro efficacy against *P. aeruginosa* strains isolated from diabetic foot infections. We suggest that the minimum of 25 mg/l concentration of boric acid could be a good option in wound care treatment since it provides cost-effective treatment for long-term care, reduces hospitalization periods, makes treatments at home possible, and enables local treatment rather than systemic treatment.

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Ethics

Ethics Committee Approval: Ethics committee approval was received from the Non-invasive Clinical Research Ethics Committee of the University of Amasya (decision number: 2021/01, date: 07.01.2021).

Informed Consent: Retrospective study.

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Authorship Contributions

Surgical and Medical Practices: Y.P., M.C., Concept: M.C., Design: Y.P., M.C., Data Collection or Processing: Y.P., M.C., Analysis or Interpretation: Y.P., M.C., Literature Search: Y.P., M.C., Writing: Y.P., M.C.

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