VAN TIP DERGİSİ Medical Journal

CLINICAL RESEARCH / KLİNİK ÇALIŞMA

Van Tıp Derg 30(1):60-65,2023
DOI: 10.5505/vtd.2023.15425

Efficacy of Topical Boric Acid in the Treatment of Experimental Pseudomonas Aeruginosa Keratitis in Rats

Sıçanlarda Deneysel Pseudomonas Aeruginosa Keratiti Tedavisinde Topikal Borik Asitin Etkinliği

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Abstract

Introduction: It was aimed to investigate the effect of topical boric acid on healing in the treatment of keratitis.

Materials and Methods: Keratitis was created by randomly 32 of 56 healthy rats with inoculating pseudomonas aeruginosa ($1x10^8$ colony-forming units/ml 0.05 ml of solution containing pseudomonas eruginosa-PA-ATC27853) by creating epithelial defects in the corneas and randomly distributed to 4 groups. The remaining 24 healthy rats were randomly divided into 3 groups, so that; group 1 (control group), group 2 (with pseudomonas keratitis), group 3 (with pseudomonas keratitis and 4% boric acid treatment), group 4 (with pseudomonas keratitis and 8% boric acid treatment), group 5 (with pseudomonas keratitis and vancomycin+ ceftazidime given), group 6 (non-keratitis and only 4% boric acid applied), group 7 (non-keratitis and only 8% boric acid applied). At the end of the study, the ocular tissues of the rats were removed and examined histopathologically.

Results: A clinically significant reduction in inflammation was detected in the groups using topical boric acid. Decreased TGF-β1 staining was detected in the groups in which keratitis + %4 and %8 boric acid was treated topically ($p<0,05$). Compared to the infected group (group 2), 4% and 8% boric acid administered groups revealed mild/moderate corneal edema and thickening, degeneration of the corneal epithelium, and mild/moderate inflammation in the interstitial.

Conclusion: Topical boric acid drops may be an important therapeutic factor in tissue healing and in the fight against inflammation in the treatment of keratitis.

Keywords: Boric acid; pseudomonas aeruginosa; keratitis.

Özet

Amaç: Bu çalışmada keratit tedavisinde topikal borik asitin iyileşmeye etkisinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: 56 sağlıklı rattan rastgele 32 tanesinin kornealarında epitel defekti oluşturulup psödomonas aeruginosa ($1x10^8$ koloni oluşturan birim/ml pseudomonas eruginosa (PA-ATC27853) içeren solüsyondan 0.05 ml) inoküle edilerek keratit oluşturulmuş ve rastgele 4 gruba eşit olarak dağıtılmıştır. Geriye kalan 24 sağlıklı rat rastgele 3 gruba eşit olarak dağıtılmıştır. Böylece; grup 1 (kontrol grubu), grup 2 (psödomonas keratitli), grup 3 (psödomonas keratitli ve %4 borik asit tedavisi uygulanan), grup 4 (psödomonas keratitli ve %8 borik asit tedavisi uygulanan), grup 5 (psödomonas keratitli ve vankomisin+seftazidim uygulanan), grup 6 (keratit olmayan ve sadece %4 borik asit uygulanan), grup 7 (keratit olmayan ve sadece %8 borik asit uygulanan) olarak oluşturulmuştur. Çalışmanın sonunda sıçanların oküler dokuları çıkarılarak histopatolojik olarak incelenmiştir.

Bulgular: Topikal borik asit (%4 ve %8) uygulanan gruplarda inflamasyonda anlamlı bir azalma saptanmıştır. Keratit + topikal %4 ve %8 borik asit uygulanan gruplarda TGF-β1 boyanmasında azalma saptanmıştır ($p<0,05$). Şiddetli inflamasyon ve dokularda şiddetli dejenerasyon içeren keratitli grupla (grup 2) karşılaştırıldığında, %4 ve %8 borik asit uygulanan gruplarda yapılan incelemede hafif/orta düzeyde kornea ödemi ve korneal kalınlaşma, hafif/orta düzeyde kornea epitelinde dejenerasyon ve intertisyel dokularda hafif/orta derecede dejenerasyon ve inflamasyon saptanmıştır.

Sonuç: Keratit tedavisinde topikal borik asit damlaları doku iyileşmesinde ve inflamasyonla mücadelede önemli bir terapötik faktör olabilir.

Anahtar Kelimeler: Borik asit; psödomonas aeruginosa; keratit.

Introduction

Boric acid is an element found in nature as compounds. Boric acid, a water-soluble, inorganic acid with antibacterial properties, was discovered by Wilhelm Holmberg (1,2). Boron and boron

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Received: 24.10.2022, Accepted: 16.12.2022

products are used in many fields such as communication tools, agriculture, glass, ceramics, construction, energy and pharmaceutical industries. In medicine, it is used in allergic diseases, dental, bone and joint diseases, menopause and cancer treatment and psychiatric patients (3,4). Boric acid significantly inhibits the replication of bacteria for up to 48 hours at room temperature, has a bacteriostatic effect, and was first used as an antiseptic in 1875. Thanks to this feature, it is used as an antibiotic and antiseptic in sterilization processes and antibacterial creams (2,5). On the other hand, normal cellular organelles exposed to boric acid remain largely intact (3,4). Pseudomonas aeruginosa (P.aeruginosa) is one of the most important causes of treatment-resistant keratitis (6-8). Therefore, the development of new additional or alternative therapies is essential in its treatment. We think that boric acid may become one of the treatment modalities with its antiseptic, bacteriostatic, and inflammation-reducing effects in the fight against antibiotic resistance, which can also be encountered in the treatment of P. Aeruginosa keratitis, a significant cause of ocular morbidity in the clinic. In our study, a rat model of P. Aeruginosa keratitis was created, aiming to compare the efficacy of 4% and 8% topical boric acid application with the topical Vancomycin + Ceftazidime treated group and the control group, based on histopathological and immunohistochemical parameters.

Material and Method

The study was conducted in between October 2021-January 2022 and included 56 healthy female Sprague-Dawley rats (200–400 g). 2x2 mm central corneal epithelial defect was created with the help of a microkeratome under intraperitoneal ketamine + xylazine and topical 5% proparacaine anesthesia in 32 randomly selected rats from 56 healthy rats. Each of the 32 rats was inoculated with 0.05 ml of a solution containing 1 x 10^8 Colony Forming Units (CFU)/ml of P. aeruginosa (PA-ATC 27853). At the end of the 3rd day, keratitis was observed in the corneas of the rats. 32 keratitis rats were randomly divided into 4 groups, and the remaining 24 healthy rats were randomly divided into 3 groups: Group 1 (n=8) Control group (C). Group 2 (n=8) Pseudomonas keratitis + no topical treatment for 10 days. Group 3 (n=8) Pseudomonas keratitis (+) + 4% boric acid drop treatment 2x1 for 10 days. Group 4 (n=8) Pseudomonas keratitis (+) + 8% boric acid drop treatment 2x1 for 10 days. Group 5 (n=8) Pseudomonas keratitis (+) + Vancomycin + Ceftazidime drop treatment 5x1 for 10 days. Group 6 (n=8) healthy rats 4% boric acid drop treatment 2x1=Toxicity group 1. Group 7 (n=8) healthy rats 8% boric acid drop therapy 2x1=Toxicity group 2. On the 14th day, which was the end of the study, the rats were sacrificed, orbital structures of rats were removed as a whole, stored in 10% formaldehyde, and the sacrificed rats were examined microscopically by Hematoxylin-Eosin and Immunohistochemical staining by a pathologist who was unaware of the groups, and the results were evaluated.

Histopathological examination: Tissues taken for histopathological evaluation as a result of necrosis were fixed in 10% formalin solution for 48 hours and then washed in running tap water for 24 hours. After going through routine tissue follow-up procedures, paraffin blocks were embedded. Incisions of 4 mm thickness were taken from each block and preparates were prepared. Prepared samples for histopathological examination were stained with Hematoxylin-Eosin and examined under a light microscope. The incisions examined under the light microscope were evaluated as absent (-), mild (+), moderate (++) and severe (+++) according to the lesions, and pictures were taken. All incisions taken on slides with adhesive (poly-L-Lysin) for immunoperoxidase examination were passed through the xylol and alcohol series. After washing the incisions with phosphate buffered saline (PBS), they were incubated in 3% H_2O_2 for 10 minutes and endogenous peroxidase inactivation was achieved. In order to reveal the antigen in the tissues, they were treated with antigen retrieval solution for 2x5 minutes at 500 watts in a microwave oven and then left to cool. Tissues washed with PBS were then incubated at 37° C for 30 minutes with Transforming Growth Factor Beta1 (TGF-β1) Antibody (Catalog no: sc-130348, dilution 1/50; SantaCruz, USA) for the detection of DNA damage. They were incubated with biotinized secondary antibody for 10 minutes at room temperature. Incisions washed again with PBS were incubated in Streptavidin - Peroxidase for 10 minutes, and then, they were washed with PBS in the same way. 3-3' Diaminobenzidine (DAB) was used as a chromogen. It was washed with distilled water. Counterstaining was applied with Hematoxylin (Mayer's) for 15-20 seconds. It was passed through xylol and alcohol series and covered with a coverslip with the help of Entellan. Incisions were evaluated as absent (-), mild (+), moderate (++) and severe (+++) according to their immune positivity.

Preparation of topical agents: 4% and 8% boric acid drops were prepared by diluting 0.4g and 0.8g boric acid powder (Galenik, Turkey) in 10 ml of preservative-free artificial tears, respectively. Vancomycin - Ceftazidime fortification drops were prepared by diluting 1g Vancomycin and 1g Ceftazidime vials with 10 cc dilution ratio of 2/5.

Statistical analysis: All statistical analyses were performed using SPSS for Windows, v. 18.0 software package, and a p value of <0.05 was accepted as statistically significant. Histopathological findings were analyzed with SPSS v. 20.00. The differences between the groups were determined using the Kruskal-Wallis test, a non-parametric method, and the Mann Whitney U test was conducted to identify the group that created the significant difference ($p < 0.05$).

Ethical consent: In our study, written consent was obtained from all the cases participating in our study, in accordance with the Declaration of Helsinki. Ethics Committee approval was obtained from Erzurum Atatürk University Animal Experiments Local Ethics Committee with its decision dated 27.12.2018 and numbered 233.

Table 1: Scoring of histopathological in cornea tissue.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Corneal edema and thickening	-	+++	++	+	+	-	-
Corneal epithelial damage	-	+++	++	+	+	-	-
Inflammation in the cornea	-	+++	++	+	+	-	-

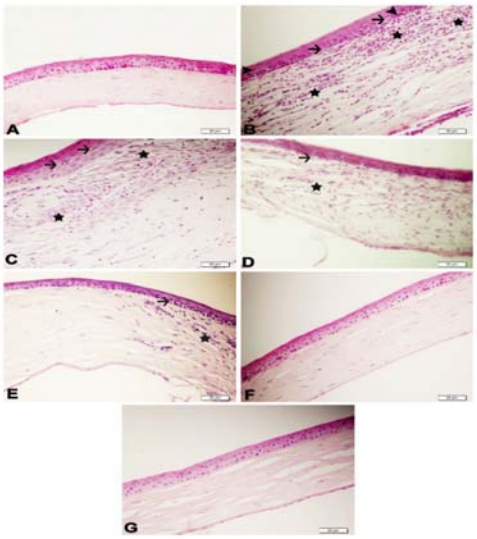

Figure 1: Cornea tissues. Control group, normal histological appearance (A). Infected group, severe corneal edema, thickening and inflammation (aterisks), severe corneal epithelial damage (arrows) (B). Infected + topical 4% boric acid group, moderate corneal edema, thickening and inflammation (aterisks), moderate corneal epithelial damage (arrows) (C). Infected + topical 8% boric acid group, mild corneal edema, thickening and inflammation (aterisk), mild corneal epithelial damage (arrow) (D). Infected + topical Vancomycin + Ceftazidime group, mild corneal edema, thickening and inflammation (aterisk), mild corneal epithelial damage (arrow) (E). Healthy + topical 4% boric acid group and healthy + topical 8% boric acid groups, normal histological appearance (F-G), H&E, Bar:50µm.

Results

Histopathological Findings: Control group: It was determined that the cornea tissue was in normal histological appearance (Fig. 1A). Infected group (group 2): Examination of the cornea tissue revealed severe corneal edema and thickening, severe degeneration and necrosis of the corneal epithelium, and severe mononuclear cell infiltrations in the stroma (Fig. 1B). Infected + topical 4% boric acid group (group 3): Examination of the cornea tissue revealed moderate corneal edema and thickening, moderate degeneration of the corneal epithelium (Fig 1C). Infected + topical 8% boric acid group (group 4): Examination of the cornea tissue revealed mild corneal edema and thickening, as well as mild inflammation (Fig. 1D). There was no statistically significant difference in the findings from the infected + topical Vancomycin + Ceftazidime group (group 5). A statistically significant difference was found when compared with the infected group (group 2) ($p<0.05$). Infected + topical Vancomycin + Ceftazidime group (group 5): Examination of the cornea tissue revealed mild corneal edema and thickening, and mild degeneration of the corneal epithelium (Fig. 1E). When compared with the infected + topical 8% boric acid group (group 6), no statistically significant difference was found. The findings were statistically significantly different from the

infected group (group 2) ($p<0.05$). Healthy + topical 4% boric acid group (group 6): It was determined that the cornea tissue was in normal histological appearance. (Fig. 1F). Healthy + topical 8% boric acid group (group 7): Examination of the cornea tissue revealed normal histological appearance (Fig. 1G) (Table 1).

Immunohistochemical findings: Control group: Immunohistochemical analysis revealed negative TGF-β1 expression. Infected group revealed severe TGF-β1 expression in the interstitial spaces, in inflammatory cells and around the vascular structures. Infected + topical 4% boric acid group moderate TGF-β1 expression was detected in the interstitial spaces, in inflammatory cells, and around the vascular structures. Infected + topical 8% boric acid group revealed mild TGF-β1 expression in the interstitial spaces, inflammatory cells, and around the vascular structures. No significant difference in the findings from the infected + topical Vancomycin + Ceftazidime group.

Table 2: TGF-β1 expressions of groups

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Expression of TGF-B	-	+++	++	+	+	-	-

A statistically significant difference was found when compared with the infected group ($p<0.05$). Infected + topical Vancomycin + Ceftazidime group revealed mild TGF-β1 expression in the interstitial spaces, inflammatory cells, and around the vascular structures .When compared with the infected + topical 8% boric acid group, no statistically significant difference was found. The findings were statistically significantly different from the infected group ($p<0.05$). Healthy + topical 4% boric acid group revealed negative TGF-β1 expression. Healthy + topical 8% boric acid group revealed negative TGF-β1 expression (Table 2).

Discussion

P.aeruginosa is one of the most important causes of treatment-resistant keratitis (6-8). Therefore, the development of new additional or alternative therapies is essential in its treatment. In severe keratitis that progresses to the stroma, the transparency of the cornea may be permanently impaired and may cause vision loss that affects daily life. P. Aeruginosa keratitis, especially seen in contact lens wearers, has an aggressive course and can cause serious ocular problems such as leukoma and corneal perforation. The standard treatment for P. Aeruginosa keratitis is topical antibiotics (6). Since the discovery of the first antibiotic by Alexander Fleming, various bacteria have developed resistance to different antibiotics, one of which is P. Aeruginosa (9). In recent years, different antimicrobial agent resistance mechanisms have emerged in microorganisms such as P. Aeruginosa, and therefore, new health problems such as hospital-acquired infections have begun to be seen more frequently. Consequently, it is essential to apply new treatments in addition to or as an alternative to antibiotics in the treatment of resistant bacterial keratitis. Boric acid, which exerts an antibacterial effect by causing mitochondrial dysfunction in microorganisms is found as an antiseptic in lens solutions, eye drops, and in the composition of many medical products (2,5,10,11). Studies on the development of new antibacterials against various bacteria continue intensively (12). Some researchers have conducted studies to determine the antibacterial activity of boric acid. Haesebrouck et al. (1) reported that 1/2 and 1/4 dilutions of a solution of 2% boric acid and 2% acetic acid in equal amounts inactivated $5X10^7$ CFU/ml of S. Pseudintermedius in 30 minutes and achieved effective results. Another study reported that boric acid solution applied to the mouth had a strong antibacterial effect on Enterococcus Faecalis (4). MIC (minimal inhibitory concentration) and MBC (minimum bactericidal concentration) values of boric acid against S. Aureus, Acinetobacter Septicus, Escherichia Coli, and P. Aeruginosa have been reported as 3.80 mg/ml, 3.80 mg/ml, 7.60 mg/ml, and 7.60 mg/ml, respectively (2). For this reason, our study was carried out as a preliminary study to determine the antibacterial activity of boric acid, a by-product of boron mine, against P. Aeruginosa in a model of keratitis in rats. In our study, the examination of the cornea tissue in the infected + topical 8% boric acid group revealed mild corneal edema and thickening, mild inflammation, mild degeneration of the epithelium, and mild inflammation in the interstitial spaces. No significant difference in the findings from the infected + topical Vancomycin + Ceftazidime group. A statistically significant difference was found when compared with the infected group

($p<0.05$). Therefore, in the 8% boric acid group that was not treated with antibiotics; statistically significant difference was observed when compared with the infected group. Furthermore in the 8% boric acid group obtaining similar results with the antibiotic-administered treatment group and the lack of statistically significant difference indicates the antibacterial effectiveness of boric acid. P. Aeruginosa keratitis, like other keratitis, is characterized by infiltration of inflammatory cells and tissue destruction (7,8). Therefore, in addition to fighting bacteria in keratitis, it is also necessary to reduce inflammation that causes ocular damage. However, steroids cannot be used to reduce inflammation during the active phase of keratitis, as they may exacerbate the course of keratitis (13). P. aeruginosa develops resistance to various antimicrobial agents, causing intense ocular inflammation, causing important health problems in the field of ophthalmology. As a result we aimed to reveal the anti-inflammatory effect of boric acid, which is a by-product of boron mine, as well as its antibacterial effect. TGF-β1 is the proinflammatory cytokine. It has been reported that in the presence of infectious agents or tissue damage, TGF-β1 increase is observed within 4-8 hours, reaching the highest level in 16-24 hours, and its release continues according to the continuity of the stimulus (14,15). In our study, a significant reduction in inflammation was observed in the examination of cornea in the infected + topical 8% boric acid group, and a statistically significant difference ($p<0.05$) was found when the inflammation level was compared with the infected group. At the same time, immunohistochemical examination in the infected + topical 8% boric acid group revealed mild TGF-β1 expression in the interstitial spaces, inflammatory cells, and around the vascular structures and a statistically significant difference ($p<0.05$) was detected when compared with the infected group. So, we think that, besides its antibacterial use, boric acid can also be used as an anti-inflammatory agent in keratitis. It has been shown in the literature that boron is also essential for maintaining the structure and function of cell membranes and normal cellular organelles exposed to boric acid remain largely intact (16).

Similarly, the immunohistochemical examination in the healthy + topical 8% boric acid group in our study revealed a normal histological appearance with negative TGF-β1 expression. Therefore, it has been observed that 8% boric acid has no toxicity when applied topically and does not increase TGF-β1. Furthermore, the advantages of boric acid powder are that it is easy to find, is inexpensive, and the drop form is easy to prepare. Nevertheless, this study has certain limitations. First of all, the experimental nature of the study and its application in rats is itself a limitation, since the results of the treatment in rats may be different in humans. Another limitation is that we have very little information regarding the use of boric acid in ophthalmology, especially in keratitis, and hence, the results cannot be compared due to the lack of similar studies. We think that boric acid may find wider use in ophthalmology like obstetrics, orthopedics, emergency medicine, nuclear medicine and dentistry with further studies. Moreover, we think that it can be used in other types of bacterial keratitis such as S. Aureus, where its antibacterial activity is shown (17).

Study limitations: Our study has some limitations. Although our study was conducted on a sufficient number of rats according to the g power analysis, more rats can be used. In addition, 4% and 8% doses of boric acid were used in our study, different doses of boric acids can be used.

Conclusion

As a result, it was seen that boric acid has an important potential in terms of antibacterial activity in P. aeruginosa keratitis, and even has a significant inflammation-reducing effect. For this reason, it was concluded that it would be beneficial to conduct more detailed in vitro and in vivo studies including different bacterial species.

Conflict of interest: All authors declare that they have no conflict of interest.

Financial disclosure: No financial support has been received for this study.

Ethical consent: In our study, written consent was obtained from all the cases participating in our study, in accordance with the Declaration of Helsinki. Ethics Committee approval was obtained from Erzurum Atatürk University Animal Experiments Local Ethics Committee with its decision dated 27.12.2018 and numbered 233.

Author contributions: Concept (OO, BU, KB,SY), Design (OO, BU, KB,SY), Data Collection and/or Processing (OO, BU, KB,SY), Analysis and/or Interpretation (OO, BU, KB,SY)

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