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The Effect of Topically Applied Boric Acid on Ephrin-Eph Pathway in Wound Treatment: An Experimental Study

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Abstract

Background: Wound healing has a vital importance for the organism and various agents are used to accelerate wound healing. Although the effect of boron on wound healing is known, its mechanisms are not completely clear yet. In this study, the effect of boron in the Ephrin /Eph pathway will be evaluated. **Methods:** Forty adult female rats were used in the study. A full-thickness excisional wound model was created in all groups divided as Control, Fito, Boron and Plu groups. After the applications performed twice a day and lasting 7 days, skin tissues obtained and evaluated histopathological (inflammatory cell infiltration, oedema, and fibroblast proliferation density) and immunohistochemical (TNF-α, EphrinA1, EphrinB1, EphrinB2 and EphB4). **Results:** Inflammatory cell infiltration score was found to be higher in the Fito group compared to Boron group ($p = .018$). Fibroblast proliferation density was higher in Plu group than Boron group ($p = .012$). While TNF-α was lower in boron group than Plu ($p = .027$) and Fito ($p = .016$) groups, EphrinA1 was higher in Boron group than Plu group ($p = .005$). EphrinB1 expression was higher in Boron group compared to Plu ($p = .015$) and Fito ($p = .015$) groups, and the same difference was also observed in EphrinB2 (p values .000). Similarly, EphB4 immunoreactivity was higher in the Boron group compared to Plu ($p = .000$) and Fito ($p = .002$). **Conclusion:** One of the mechanisms of action of boron in wound healing is to increase EphrinB1, EphrinB2 and EphB4. Low TNF-α and histopathological findings indicate that boron limits extensive wound healing.

Keywords

boron, Ephrin, Eph, wound healing, TNF-α

Introduction

Injuries occur on the skin as a result of the deterioration of the integrity of the skin for various reasons. After the breakdown of skin integrity, a complex process begins in the body, called wound healing, consisting of 5 main stages: homeostasis and inflammation, granulation tissue formation, neovascularization, re-epithelization, and remodelling.[1,2] In this process, which is regulated by many molecules, these events must occur sequentially in order for the wound to close effectively. Chemokines play important roles in regulating this sequence through the recruitment of inflammatory cells that secrete cytokines and growth factors to regulate angiogenesis and promote wound healing.[3]

Eph receptors and ephrin ligands are transmembrane proteins found in cells of many different organs. Ephrin's, and their receptors Ephs, constitute a large family of receptor tyrosine kinases. Mammals have 9 EphA receptors and 5 EphB receptors. 5 different Ephrin-A ligands can bind to these EphA receptors. There are 3 different transmembrane ephrin-B ligands that bind to EphB receptors.[4–6] Glycosylphosphatidylinositol anchor to the cell membrane is form of the A-class Ephrins. However, B-class Ephrins have a transcellular and cytoplasmic domain with a PSD-95/Dlg/ZO-1 (PDZ)-binding motif. The Eph receptors are transmembrane proteins with an extracellular domain. [7] In many cell types, these proteins play a role in the development of physiological and pathological events. It is known that they are effective in events such as cell adhesion,

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repulsion, proliferation, differentiation, shaping and migration, and in the control of bone remodelling.[4–6] Both receptors and ligands have intracellular and extracellular parts. The intracellular portions of the EphA and EphB receptors consist of the tyrosine kinase domain, sterile alpha motif domain (SAM), and PDZ binding domain. In the extracellular area, there is a fibronectin, cysteine-rich domain, and ligand binding domain. The intracellular domain of ephrin-A ligand consists of GPI binding, whereas that of ephrin-B ligand, unlike this, consists of phosphorylatable tyrosine and PDZ protein binding domain.[4,8,9]

Eph/ephrin connection is responsible for regulating events such as arteriovenous endothelial cell formation, stem cell differentiation, immune system response, intestinal epithelial cell migration, skeletal remodeling, angiogenesis, and axon guidance.[5] Also it is known that Eph / Ephrin proteins play an important role in wound healing.[10,11] It has been reported that Type A Ephrin-Eph proteins have angiogenic effects on VEGF (Vascular endothelial growth factor).[7] Ephrin-A1 expression is an important determinant of endothelial proliferation. In addition, studies have emphasized that Ephrin A1 and Eph A2 receptors have angiogenic effects through TNF-α (Tumor necrosis factor α).[12] Ephrin A1, A3 and A4 and Eph A1, A2 and A4 are known to exist in human skin tissue. It is emphasized that Ephrin B2 and Eph B4, which are reported to be involved in both physiological and tumor angiogenesis, are associated with inflammation.[12]

Boron, which atomic number is 5, is a semiconductor element, classified in the metalloid group, with metal-nonmetallic properties.[13,14] Boron is always 3-valent and bonds with many other elements to form trigonal planar structures such as orthoboric acid or tetrahedral structures such as anionic borate. Boron is widely found in soil and water and its concentration in soil is approximately 3 to 100 ppm.[15] In solutions with physiological pH and when not interacting with other biomolecules, boron exists in the form of uncharged boric acid ($B[OH]_3$).[16] Boron is found in nature as sodium and oxygen compounds such as borax, borates and boric acid. However, only organoborate complexes containing B-O or B-N bonds, such as orthoborates, are important in biological systems. These organoboron complexes are formed in plant, animal, and human tissues. Under normal physiological conditions in the organism, approximately 96% of boron is found in the form of boric acid $B(OH)_3$, and a small part is in the form of borate anion B(OH).[4–17,18] Both boric acid and borate interact strongly with biomolecules containing cis-hydroxyl groups, particularly riboflavin, adenosine monophosphate, pyridoxine, ascorbic acid, sugar molecules, and polysaccharides. Because the hydroxyl groups are next to each other and on the same side of the molecules, there is an interaction between boron and its ligands via ester bonds.[17]

Boric acid and borate are widely used in antiseptic, bactericide, cleaning agent such as soap and detergent, leather and wood preservatives, fire retardants, fertilizers, insecticides and herbicides, cosmetics. It also has uses for many industrial purposes, including glass manufacturing, fiberglass insulation, porcelain enamel, ceramic glazes, and metal alloys.[16,19] Healthy tissues contain appropriate amounts of boron in either boric acid or borate forms. The total amount of boron in the body is about 3 to 20 mg. The amount of boron contained in different tissues also varies. For example, bones and tissues containing keratin, such as skin and hair, are known as the tissues with the highest amount of boron. Boron concentration in body fluids is much lower than in tissues.[20]

Boric acid is effective in many metabolic events, including DNA damage repair mechanism and regulation of oxidative stress. When Boron is taken orally into the body, it is absorbed by 100% from the intestines and is metabolized in the liver into boric acid form. Boron, whose distribution in tissues is in the form of boric acid, is excreted from the kidney at a rate of 88 to 93% within the days following intake. [21]

Studies have shown that boron is effective in various areas such as prevention and treatment of various cancers, arthritis, neurodegenerative diseases, bone growth and bone healing, and tendon ruptures.[22] There are studies in the literature showing that topically applied 3% boric acid is effective in wound healing. [23–25] It is stated that it especially accelerates the production of extracellular matrix during the wound healing process.[26]

In the light of existing data, it is aimed to examine the effect of boric acid, which has been proven to be effective in the wound healing process, on the Eph-Ephrin mechanism and to reveal the pathways that play a role in the wound healing process.

Materials and Methods

Animals and Ethical Procedure

This study was supported by the Scientific Research Projects Coordination Unit of Çanakkale Onsekiz Mart University University with the project number TSA-2019-2831. Before starting the study, approval was obtained from the the Animal Experiments Local Ethics Committee of Çanakkale Onsekiz Mart University University (Ethical approval number: 2018/09-07). The current study was conducted in accordance with the recommendations of the World Medical Association's Declaration of Helsinki on animal studies. In the study, 40 adult female Wistar Albino rats weighing between 220 and 300 g were used, and the rats were obtained from Çanakkale Onsekiz Mart University University Experimental Research Centre. All steps of the study were carried out in the same centre. Rats were housed in standard rat cages at standard humidity and temperature (45% −50% humidity and 22 ± 2 ° C), 12 h dark, 12 h light period. The rats were fed ad libitum with

standard feed and tap water until 12 h before the performance of the study procedure. 12 h before the test procedure, the food was stopped and only water was given.

Experimental Protocol

In the experiment, forty adult female Wistar Albino rats were randomly divided into 4 groups. The groups are given below (Table 1).

The Wound Modelling and Preparation of Boric Acid Gel

Intraperitoneally 50 mg / kg of Ketamine hydrochloride (Ketalar®, Pfizer Pharmaceuticals Ltd Sti, Istanbul, Turkey) and 15 mg / kg Xylazine (Alfazyn 2%, Aegean Vet Ind. Trade, Izmir, Turkey) were given to the rats in all groups and after administration of anaesthesia, dorsal interscapular areas were shaved and cleaned with povidone iodine. The full-thickness excisional wound model was created as previously stated in the literature.[27] No.15 bistoury (lancet) was attached to a scaled handle and used for wound modelling. Approximately 6 mm diameter and 2 mm depth circler type skin were excised.

For topical application of boric acid, a gel was used which consist of 3% boric acid as described in literature previously.27 Carbopol hydrogel has been prepared by distributing 1% (w/v) polymer in distilled water. The pH of the hydrogel was adjusted between 6 and 7 by 1 M NaOH solution. The hydrogel without any active ingredient was used as a vehicle. Pluronic gel F127 (Sigma-Aldrich, Germany, Lot no: BCBW5376) was added in vehicle at a final concentration of 3% (w/v) and applied in Group 4 (PLU group). Then, NaB (Merck KGaA, Darmstadt-Germany Product No: B6768), and Pluronic gel F127 were mixed in a 30 × 11 mm sterile petri dish at a final concentration of 3% (w/v). The gel formulation was prepared before the first use every morning and stored at + 4 °C for 8 h to dissolve.

Table 1. Groups and Their Applications.

Group 1 (Control)	Group of endogenous wound healing processes. After the wound modelling, there was not any application.
Group 2 (Fito)	Group of active control. After the wound modelling, Fito cream (Ethyleneglycol ether monefenil + triticum vulgare aqueous extract, Tripharma Pharmaceutical Co., Turkey) was applied twice a day for 7 days.
Group 3 (Boron)	Group of boric acid. After the wound modelling 3% boric acid-pluronic gel mixture was applied twice a day for 7 days.
Group 4 (Plu)	Group of vehicle control. After the wound modelling, Pluronic gel F127 standard form was applied twice a day for 7 days.

Sample Collection

After 7 days of wound treatment, wound biopsy was taken from the animals on the eighth day. The animals were prevented from accessing water and food 12 h before the procedure. Then, 50 mg/kg of Ketamine hydrochloride and 15 mg/kg Xylazine was given intraperitoneally, and the wound area all were excised. Tissue samples taken from animals were placed in 10% neutral buffered formalin.

Histopathological Evaluation

At the end of the study, collected tissues followed the 48-h fixation period, a manual routine tissue processing protocol was applied. After tissue processing, 5 µm thickness sections from the tissues embedded in paraffin blocks were taken with a Leica RM2125 RTS brand microtome. Sections were stained with the routine Haematoxylin-Eosin (H&E) staining protocol. Stained sections were evaluated under an Olympus CX43 camera attachment light microscope. Scoring was made in terms of inflammatory cell infiltration, oedema, and fibroblast proliferation density criteria for microscopic evaluation of wound healing. [27,28]

Inflammatory cell infiltration, oedema and fibroblast proliferation density was scored as indicated below:

1. No
2. Mild
3. Moderate
4. Severe.

Immunohistochemical Evolution

For immunohistochemical evolutions, 4 µm thickness sections were taken from paraffin blocks and stained after antigen retrieval method by using the routine immunohistochemistry protocol according to the manufacturer's data sheets of Anti-rat TNF-α (Biorbyt Inc., Cambridgeshire, UK. Cat No: orb11495), Anti-rat Ephrin A1 (Boster Bio, Pleasanton, CA. Cat No: PA1573), Anti-rabbit Ephrin B1 (Biorbyt Inc., Cambridgeshire, UK. Cat No: orb101544), Anti-rabbit Ephrin B2 (Cell Signalling, Danvers, USA. Cat No: #83029) and Anti-rabbit EphB4 (Cell Signalling, Danvers, USA. Cat No: 84029) antibodies. Light microscopic (Olympus CX43) evaluation of immunohistochemically stained sections was scored as described previously in the literature as 0, no staining; 1, weak but detectable staining; 2, moderate staining; and 3, strong staining. [29]

Statistical Analysis

Data analysis was performed using SPSS Packet Program 20.0 version (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). In determining the statistical

Table 2. Means and standard deviations of histopathological and immunohistochemical evolution scores.

	Inflammatory Cell Infiltration	Oedema	Fibroblast Proliferation Density	TNF- α	EphrinA1	EphrinB1	EphrinB2	EphB4
Control	2.2 ± .6	2.0 ± .5	2.6 ± .5	2.5 ± .5	2.1 ± .7	.5 ± .5	.6 ± .5	.5 ± .5
Fito	2.8 ± .4	2.4 ± .5	2.7 ± .5	2.6 ± .5	2.6 ± .5	1.3 ± .5	.5 ± .5	.8 ± .8
Boron	2.0 ± .8	2.0 ± .8	2.5 ± .5	1.7 ± .8	2.1 ± .9	2.0 ± .8	2.2 ± .6	2.2 ± .6
Plu	3.0 ± 0	2.0 ± .5	3.0 ± 0	2.5 ± .5	3.0 ± 0	1.3 ± .5	.8 ± .4	.5 ± .5

significance of the difference between the control variable in the study and the average values of other variables at 95% confidence interval, Independent Two-Samples -Test was used, and values below .05 were considered statistically significant.

Results

The mean scores and standard deviations of the histopathological and immunohistochemical examinations performed at the end of the study are given in Table 2.

Histopathological Findings

In the evaluation made in terms of inflammatory cell infiltration; it was noticed that values of Plu and Fito groups were significantly higher when compared to the Control group (p values .002 and .025 respectively). The same significance was not observed between the Control and Boron groups (p = .564). Similarly, there was no significant difference between Plu and Fito groups in terms of inflammatory cell infiltration (p = .146), but when Plu and Boron groups were compared, it was found that the inflammatory cell infiltration score of the Plu group was statistically significantly higher (p = .002). When the Fito and Boron groups were compared, it was observed that the inflammatory cell infiltration score was statistically significantly higher in the Fito group (p = .018). Photographs of inflammatory cells and the presence of oedema are presented in Figure 1.

No statistically significant difference was found between the Control and other groups in the evaluation in terms of oedema (p = 1.000 for Plu; p = .090 for Fito; p = 1.000 for Boron). Similarly, there was no statistical significance in terms of oedema between the Plu group and the Fito and Boron groups (p values of .900, 1.000 respectively) or between the Fito and Boron groups (p = .246).

In the evaluations made in terms of fibroblast proliferation density; Plu group showed significantly higher fibroblast proliferation density compared to the control group (p = .029). There was no statistically significant difference between the Control group and the Fito and Boron groups (p values .648 and .661, respectively). Similarly, there was no significant difference between Plu and Fito groups (p = .067), but when the Plu and Boron groups were examined, it was noted that fibroblast proliferation density was statistically significantly higher in the Plu group compared to the Boron group (p = .012).

Immunohistochemical Findings

In the statistical analyses regarding the evaluations made with the TNF-α primary antibody, no significance was found between the Control group and the Plu and Fito groups (p values 1.000 and .661 respectively). In the comparison made with the Control group and the Boron group, it was observed that TNF-α showed more immunoreactivity in the Control group (p = .027). It was noted that TNF-α showed a statistically significant positivity in the Plu group in the analyses performed with the Plu and Boron groups (p = .027), while there was no statistically significant difference between Plu and Fito groups (p = .661). Similarly, in the analyses between the Fito and Boron groups, it was observed that the Boron group showed less immunoreactivity than Fito group (p = .016). TNF-α immunohistochemical staining patterns are shown in Figure 2.

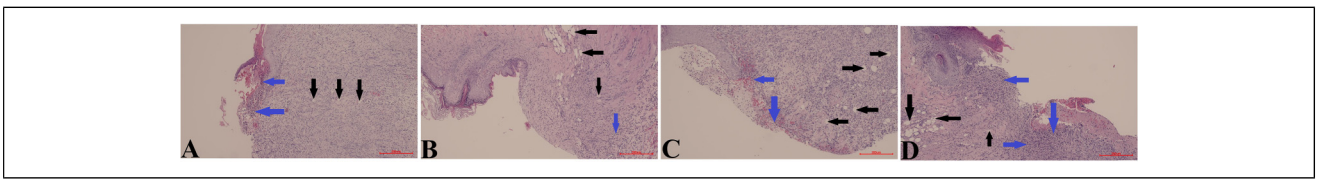

Figure 1. Light microscopic images of H&E stained sections of experimental groups. Microscopic photographs of Control, Fito, Boron and Plu groups are given in A, B, C and D, respectively. Blue arrows in the sections indicate areas of inflammatory cell infiltration, while black arrows indicate areas of edema.

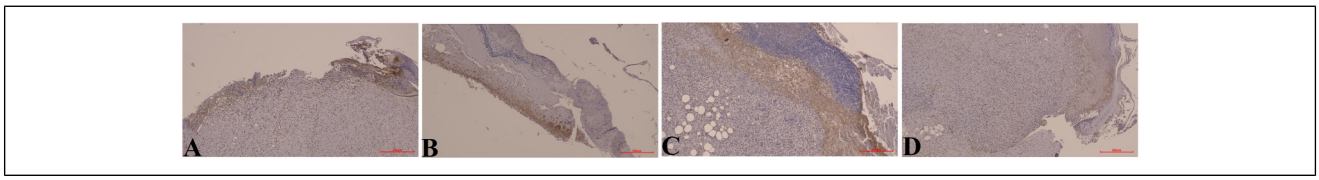

Figure 2. Light microscopic images of histological sections of experimental groups and sections stained immunohistochemically for TNF-α. Microscopic photographs of Control, Fito, Boron and Plu groups are given in A, B, C and D respectively.

In the statistical analysis of the scores of the staining for the Ephrin A1 antibody demonstrated in Figure 3, there was no significant difference between the Control group and the Fito and Boron groups (p values were .112 and .968 respectively). A statistically significant increase in positivity was found in the Control group compared to the Plu group (p = .002). When the Plu and Boron groups were compared, a statistically significant increase in immunoreactivity was noticeable in the Boron group (p = .005), while the same significance was not observed between the Fito and Boron groups (p = .185).

Ephrin B1 immunohistochemical staining images are presented in Figure 4. When the Ephrin B1 immunoexpressing was evaluated, it is observed that the expression in the Control group was statistically significantly lower than the Plu, Fito and Boron groups (p values .005, .005 and .001 respectively). While the same significance was not observed in the analyses performed between Plu and Fito groups (p = 1.000), it was found that Ephrin B1 expression in Boron group was statistically significantly higher than Plu and Fito groups (p values .015 and .015 respectively).

In the evaluation results of Ephrin B2 stained immunohistochemical sections, when the Control group and Plu (p = .342) and Fito (p = .661) groups were compared, no statistically significant difference was observed, while statistically significantly lower positivity was found compared to the Boron group (p = .000). When boron group and Plu (p = .000) and Fito (p = .000) groups were compared, it was observed that the immunoreactivity observed in Boron group was higher than both groups. Immunohistochemical staining patterns are shown in Figure 5.

In the comparison of Eph B4 primary antibody scores between the groups, no significant difference was observed between the Control group and Plu (p = 1.000) and Fito (p = .403) groups, while a statistically significantly increased positivity was found in the Boron group compared to the Control group (p = .000). In the analyses performed with the boron group, it was noted that Eph B4 immunoreactivity was higher compared to Plu and Fito groups (p value .000 and .002, respectively). Eph B4 immunopositivity is demonstrated in Figure 6.

Discussion

The disruption of the integrity of the skin, which is the most important barrier between higher organisms and the outside

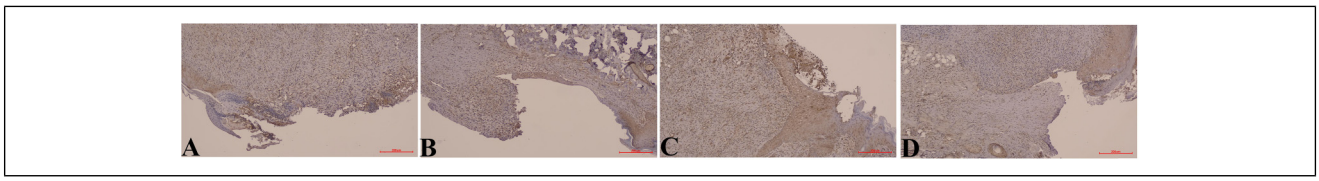

Figure 3. Light microscopic images of the histological sections belonging to the experimental groups and sections stained immunohistochemically for Ephrin A1. Microscopic photographs of Control, Fito, Boron and Plu groups are given in A, B, C and D respectively.

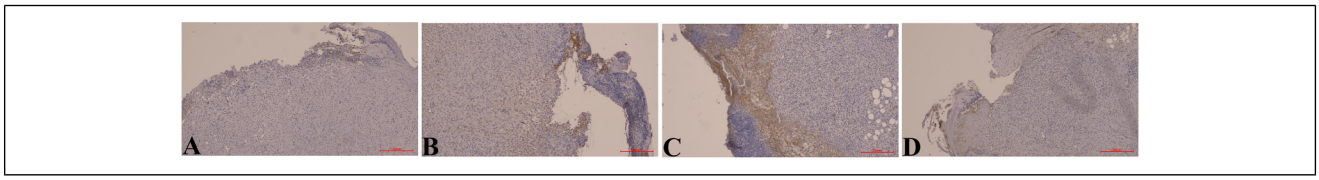

Figure 4. Light microscopic images of the histological sections belonging to the experimental groups, immunohistochemically stained for Ephrin B1. Microscopic photographs of Control, Fito, Boron and Plu groups are given in A, B, C and D respectively.

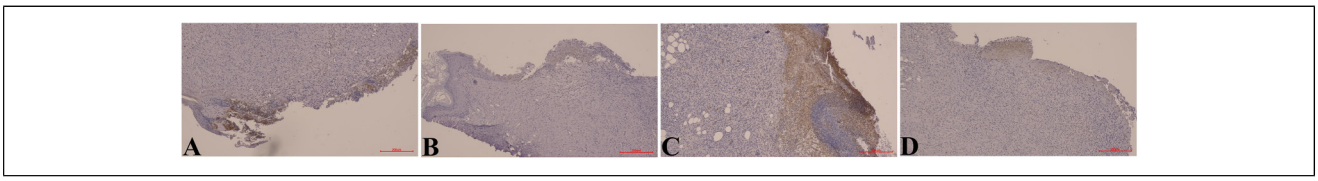

Figure 5. Light microscopic views of the histological sections belonging to the experimental groups and sections stained immunohistochemically for Ephrin B2. Microscopic photographs of Control, Fito, Boron and Plu groups are given in A, B, C and D respectively.

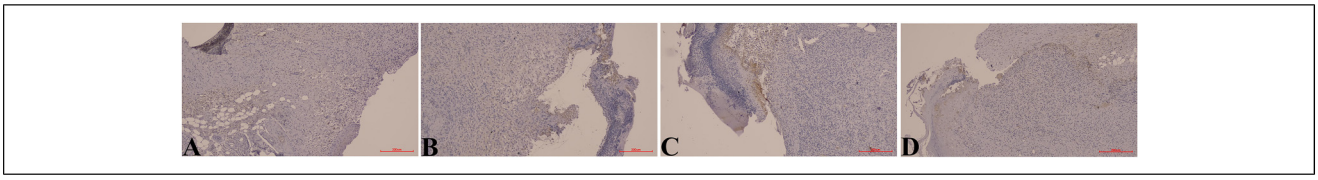

Figure 6. Light microscopic images of the histological sections belonging to the experimental groups and sections stained immunohistochemically for Eph B4. Microscopic photographs of Control, Fito, Boron and Plu groups are given in A, B, C and D respectively.

world, is called a wound. The response of the organism to wound formation, which can occur due to many reasons such as abrasion, incision, and burns, is through a process called wound healing. Since the skin protects the important tissues of the body from damage caused by mechanical force, infection, ultraviolet radiation, and excessive heat. It is quite important to repair the wounds that may occur on the skin for any reason. Wound healing, which occurs after skin disintegration, is a highly dynamic complex event involving inflammation, keratinocyte proliferation, epithelial cell and fibroblast activation and cell differentiation. [30] Wound healing, which starts with inflammation, continues with proliferation, and ends with regeneration, is a regular and programmed sequence of events. Factors such as oxygenation, infection, age and sex hormones, stress, diabetes, obesity, drugs, alcoholism, smoking and nutrition can affect one or more phases of this process and cause inaccurate or impaired wound healing. [31] The effects of the wound healing process and the therapeutics used in the wound healing process should be well documented in order to have smooth and / or rapid wound healing and to reduce / eliminate the effects of the factors that adversely affect wound healing. Formulas containing boric acid have been previously used in the literature to accelerate the wound healing process and its effectiveness has been demonstrated.[23,27,31]

Boron is a non-metallic element with diverse and vitally important role for metabolism. Also, it is stated that boron may have played an essential role in the prebiotic origins of genetic material. [32] Boron with high affinity for oxygen; forms compounds containing adjacent hydroxyl groups in cis position, such as sugars, their derivatives and riboflavin in organisms, and these complexes bind calcium ions. [22] In higher organisms, boron plays a role in the metabolism of many nutrients, especially vitamin D, calcium, and many hormones. [33] Boron, which has an effect on the production of inflammatory cells as well as enzymes such as elastase and collagenase; It accelerates extracellular matrix production and affects TNF-α release from fibroblasts. [26,32,33] Boron participates in hydroxylation reactions and in addition to its use in musculoskeletal diseases and cardiovascular diseases, it acts as an anti-inflammatory and antioxidant agent in cancer, reducing genotoxicity and modulating mitochondrial membrane activity. [1,32,34,35] When wound healing is examined, topically applied boric acid is effective in wound healing; It has even been reported that 3% boric acid solution reduces the duration of stay in intensive care units by 2/3 in deep wounds. [24,31,36] In this study, boron did not show a statistically significant difference with the Control group in terms of inflammatory cell infiltration ($p = .564$), while it was found that inflammatory cell infiltration score in the Boron group was statistically significantly lower than the Plu and Fito groups (p values, respectively, .002, .018). While no statistically significant difference was observed between the groups in terms of oedema ($p > .05$), it was noted that fibroblast proliferation density was statistically significantly lower in the Boron group compared to the Plu group ($p = .012$). Considering these histopathological findings, it is thought that the positive effects of boron on wound healing may be related to the limitation of inflammation and extensive fibrosis.

Boron is an element that is taken orally in the normal diet. At a physiological pH, borate salts are completely

converted to boric acid. From this point of view, it was stated that boric acid and borate salts have similar toxicological properties. Average daily boron intakes for adults are expressed as 1.17, .96, 1.47 and 1.29 mg/day for men, women, vegetarian men, and women, respectively.[37] The acceptable safe range for boron intake is between 1 and 13 mg/day. Data cited in reports from poisonings indicate that the lethal dose of boric acid is 3 to 6 g for infants and 15 to 20 g for adults.[38] Minimal and insignificant amounts of boric acid were absorbed by the intact skin, demonstrating its effectiveness as a barrier to the percutaneous absorption of this agent. Urinary boron excretion levels after topical boric acid application in open wounds with impaired epithelial integrity were slightly higher than urinary boron levels observed in animals receiving 200 mg/kg orally of boric acid. However, it is stated that there is no toxic amount at this level of intake.[39]

It has been noted in in vitro studies that the effects of boron on wound healing are versatile. The production of extracellular matrix via fibroblasts and its effects on elastase, collagenase, trypsin-like enzymes in fibroblasts indicate that boron regulates collagen and extracellular matrix relations.[26,40] In addition, a study published in 2010 found that boron regulates many extracellular matrix proteins by messenger RNA regulation, suggesting that it may play a role in many events other than wound healing.[41] One of the important factors in wound healing is keratinocyte proliferation and migration. In vitro studies have reported that wound closure in keratinocytes incubated with boron salts is faster than in control medium. In the same study, it was suggested that this effect of boron salts is related to keratinocyte migration rather than keratinocyte proliferation.[42] In another study performed in vitro in wound healing, two molecules related to keratinocyte migration and granulation tissue formation were examined. In this study, which examined the expression of Matrix metalloproteinase (MMP) −2 and MMP-9 in human keratinocyte culture by immunohistochemical and western blot method, it was noted that boron increased MMP-9 expression. [43] In an in vitro + experimental study on burns, one of the most serious wound types, it was reported that boron contributes not only to fibroblastic activity but also to wound healing with increased vascularization. In addition, migration, angiogenesis, and contraction-related protein expressions including collagen, α-smooth muscle actin, transforming growth factor-β1, vimentin, and vascular endothelial growth factor have also been reported to increase in the boron application group.[24] However, there is no information about the effects of boric acid on Ephrins, which are a type of tyrosine kinase family, and their receptors Ephs.

Eph receptors were first identified in carcinomas in which they overexpressed.[44] The Eph receptor family gets its name from its expression in an erythropoietin-producing hepatocellular (EPH) carcinoma cell line, and Ephrins are membrane-bound ligands of the Eph receptor family (Eph family Receptor Interacting proteins).[6] Ephrins receptors are members of the tyrosine kinase family that play a role in developmental processes, cell adhesion, motility, proliferation, and differentiation. As a result of studies related to Eph / Ephrin, it was understood that Eph proteins allow short-range cell-cell communication by binding Ephrin ligands attached to the membrane. The activation of signalling pathways affecting the cytoskeleton first causes cell separation and sometimes cell adhesion. So Ephrin-Eph signals are required for rapid changes in cellular mobility and / or morphology.[45] In other words, while the ligands of other receptor tyrosine kinase families are generally soluble, the cell surface location of Ephrins causes the signalling through receptors to be dependent on cell-cell contact. With this feature that makes Eph receptors unique, cells become aware of their own microenvironment. Thus, they play an important role in normal physiological processes such as the formation of embryonic tissue borders and the orientation of developing axons. [46] In adult tissues, they help wound healing and preservation of intestinal cell populations in certain parts.[46,47]

Ephrin receptors are divided into EphA or EphB subfamilies according to their binding status to Ephrin ligands called membrane-anchored Ephrin-A's and transmembrane Ephrin-B's.[48] In the light of the data obtained as a result of the studies conducted to define the functions of Eph / Ephrin family members, it is thought that the Eph-Ephrin mechanism affects wound healing, especially through EphB2 and Ephrin-B1/2. Upregulation of EphB2 and Ephrin-B1/2 and the resulting interactions cause actin stress fibres to break up and their junction to rupture when the wound is formed. As a result, the epithelial layer loosens, and the wound is closed by providing the cells with the environment it needs to migrate collectively. In addition, the in vitro coupling of EphB1 and Ephrin-B1 leads to increased cell adhesion through α1β5 integrin activation, an effect that is dependent on the surface density of Ephrin-B1 expression. [49] Increased cell adhesion is also a process that accelerates wound healing by increasing connection complexes between keratinocytes. Ephrin B2 acts through its receptor, EphB4, and cell-cell and cell-extracellular matrix adhesion increases as a result of EphrinB2 / EphB4 interaction. Cell migration and proliferation are also induced.[50] In this way, it is thought to contribute to the acceleration of wound healing, as well as to strengthen the scar tissue by increasing cell-cell and cell-extracellular matrix adhesion. In the current study, it was observed that Ephrin B1 increased in all three experimental groups when compared with the Control group. However, when Boron group was compared with Plu and Fito groups, a significant increase was observed in Boron group (p values .015, .015 respectively). This indicates

that Boric acid accelerates cell adhesion by increasing the expression of Ephrin B1, thus contributing to the wound healing process. In addition, a significant increase was observed in Ephrin B2 immunoexpression in the Boron group compared to the Control group ($p = .000$), while the positivity in the Boron group was also significantly higher in the analysis between the Boron group and the Plu and Fito groups (p values were .000 respectively). This result reveals that the increase in Ephrin B2 expression also plays a role in the positive effects of boric acid on wound healing. In addition, Eph B4, which is the receptor of Ephrin B2, showed a statistically significant increase in the Boron group compared to the Control ($p = .000$), Plu ($p = .000$) and Fito ($p = .002$) groups. This observation suggests that the Ephrin B2 - EphB4 interaction is a mechanism by which boron can promote wound healing.

Ephrin A's are known to suppress cell adhesion genes, especially integrins, while inducing epidermal differentiation markers. In addition, Ephrin A1 has been shown to increase collagen production in the skin.[51] Ephrin A1 becomes upregulated in endothelial cells and skin with TNF-α stimulation.[7,37] The angiogenic effects of Type A Ephrin-Eph group on VEGF are reported.[7] Ephrin-A1 expression is a critical determinant of endothelial proliferation. According to current knowledge, Ephrin-A1 is notably involved in basic processes of endothelial migration such as cellular polarization, migration direction and velocity. These data support the idea that Ephrin-A1 plays an important role in the basal mechanisms of re-endothelialisation.[7] In addition, it has been emphasized in various publications that Ephrin A1 and Eph A2 receptors have angiogenic effects on TNF-α. Ephrin A1 has been shown to play an important role in the adhesion of TNF-α mediated monocytes to endothelial cells.[12] In the present study, TNF-α expression was found to be statistically significantly lower in the Boron group compared to the Control ($p = .027$), Plu ($p = .027$) and Fito ($p = .016$) groups. According to previous studies in the literature, the increase in TNF-α is expected to induce Ephrin A1. However, in the study, it was noted that although TNF-α value in Boron group showed lower immunoreactivity compared to all other groups, Ephrin A1 had higher activity in Boron group than Plu group ($p = .005$).

Fito cream (Tripharma Pharmaceutical Co., Turkey) is a pharmaceutical cream containing 15% (w/w) Triticum Vulgare aqueous extract. Studies show the beneficial effect of Triticum Vulgare extract on wound healing.[52–54] It shows this effect by inducing fibronectin synthesis and also increasing cell migration.[53] It also acts by modulating AKT, p65, and MMP9 protein expression.[54] In clinical practice, Fito cream is used in the treatment of surgical wounds, open wounds and burns. In our current study, Fito cream was applied to the therapeutic control group and compared with the Boric acid group. When evaluated in terms of immunoreactivity, a lower positivity rate was observed in the Boron group. This reveals that boron is more effective than Fito cream, especially in terms of suppressing the inflammatory response. In terms of Ephrin A1, there was no significant difference between Boron and Fito groups, while the expression of Ephrin B1, Ephrin B2 and EphB4 was significantly increased in Boron group compared to the Fito group. In this respect, it is seen that boron application is more effective in wound healing in terms of induction of Ephrin-Eph pathway.

Pluronic F-127 gel is used as a vehicle for drug administration in many studies.[55–57] Among the topical application examples, it is seen that it is preferred as a vehicle especially in dermal applications of boron and its derivatives.[21,23,24,27] In this study, Pluronic F127 gel is preferred for boric acid application, as previously used in the literature. However, in order to consider the effects of this gel alone, it was applied to a group (Plu group) alone. In group comparisons, it was seen that the results of Plu group were similar to Control group. In this way, it was understood that the effects seen in the Boron group were not the effect of the Pluronic F-127 gel, but the direct effect of boron.

When these results are analysed together, it suggests that TNF-α values and Ephrin A1 values may have increased in the first days of wound healing when inflammation was high, but their expression tended to decrease in the 7-day healing period to limit wound healing. Similarly, in an in vitro study, boric acid was reported to reduce TNF-α secretion by a thiol-dependent mechanism.[58] When evaluated together with the histopathological findings in the current study, the low inflammatory cell infiltration and fibroblast proliferation density scores observed in the Boron group compared to the other groups indicate that boron prevents excessive wound healing in addition to its accelerating effect on wound healing.

Conclusion

In many studies, Boron used in wound healing since the 1990s; has been shown to act in different pathways. The Ephrin / Eph system is not included among the known effect pathways in wound healing. In the present study, it has been determined that boric acid may increase wound healing by Ephrin / Eph system leading an increase in Ephrin B1 resulting with increased cell adhesion, increasing the expression of Ephrin B2 and its receptor EphB4 and accelerating wound healing while histologically limiting wound healing by reducing TNF-α expression as well as inflammation and fibroblastic activity (Graphical abstract). In the light of this information, it is thought that boric acid prevents extensive wound healing in addition to its wound healing accelerating properties.

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Ethical Approval

Before starting the study, approval was obtained from the Animal Experiments Local Ethics Committee of Çanakkale Onsekiz Mart University (Ethical approval number: 2018 / 09-07).

Consent to Participate

The authors declare their consent to participate.

Authors' Contributions

BB, YA and HAE made the study design. BB, CA and HAE collected data and made interpretation. BB, CA, and YA made the analysis and wrote manuscript. BB and YA made the manuscript revisions. All authors read and approved the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Competing Interests

Authors declare no competing interest.

Availability of the Data and Materials

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Author Contributions

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Supplemental Material

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