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Antimicrobial, antifibrinolytic, enzyme inhibitory and wound healing properties of zinc borate

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ABSTRACT

Boron containing compounds (BCCs) have recently been used for pharmaceutical applications. Zinc, an essential element, is known to be one of the most promising biodegradable metals. The present study was conducted to determine the wound healing properties of zinc borate with its antimicrobial, antifibrinolytic and enzyme inhibitory characteristics. *In vitro* scratch wound healing assay revealed that zinc borate at 0.01 µg/mL concentration stimulated the proliferation of 3T3 fibroblast cells after 24 h of scar formation. The highest enzyme inhibition was observed against collagenase at 1 mg/mL (81.5%). Minimum inhibition concentration (MIC) values were determined as 1 mg/mL and 0.5 mg/mL against *Candida albicans* and *Staphylococcus aureus*, respectively. Zinc borate did not have antifibrinolytic activity at 1, 0.5 and 0.1 mg/mL concentrations. It can be suggested that zinc borate can be used effectively to improve the wound healing process and to prevent the possible wound infections.

1. Introduction

Loss of the integrity of the skin tissue can lead to uncontrolled bleeding, lesions or diseases that can lead to death [1]. While wound is one of the biggest health burdens [2], the delay in wound healing is one of the important therapeutic and economic issues in medicine. Therefore, an effective treatment is needed to reduce costs and mortality.

While healing occurs in four phases in acute wounds, normal progress is impaired in chronic wounds and wound healing may be slow or absent [3]. In addition to the damage and loss of tissue, many internal and external factors affect the success and duration of this repair process [4].

Infection is one of the most important factors that negatively affect the wound healing process. Bacteria of endogenous or exogenous origin can be found in all wounds. However, wound infection occurs with a bacterial localization that exceeds the increase in colonization and immunological reactions, which also develops due to the risk of contamination. As a result, wound healing is also interrupted [5].

Every open wound must be considered contaminated with microorganisms. There is an acute, subacute, or chronic infection in the open wound. In any case, the infection increases protease, collagenase, esterase, proteins are broken down in the infection medium, cell growth and collagen synthesis are inhibited. Bacteria secrete enzymes that inhibit the healing of the wound. As a result of the overgrowth of bacteria on the wound area, oxygen and nutrititonal substances in the environment are depleted; so the wound healing process is seriously impaired.

The biological roles of boron, an essential element, in the human and animal body have not been fully elucidated. However, it is known that boron plays a very important role in calcium metabolism, bone growth, hormone metabolism, immune system, antioxidant defense systems and wound healing [6,7]. It has been reported that boron affects the synthesis and transformation of the extracellular matrix (ECM), which plays asignificant role in wound repair by increasing the secretion of collagen, proteoglycans, proteins and TNFα [8]. In addition, boron has antimicrobial activity [9] and low toxicity to mammalian cells [10].

Zinc (Zn) ions, which are found as a trace element in the body, have positive effects in stimulating collagen deposition to accelerate wound healing [11]. In addition to its properties to produce fibroblasts,

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stimulate epithelial formation and increased migration of keratinocytes, it also has extraordinary antibacterial and anti-inflammatory abilities [12,13]. Zn can prevent bacterial growth by destroying the cell membrane and disrupting the bacterial biofilm [14].

BCCs (boron containing compounds) can be found in soil and in the plant cell wall in trace amounts [15]. BCCs have been recently used to produce useful products, such as pharmaceuticals [16]. Lately, anticancer products that contain BCCs have also been introduced to the market [17].

The use of BCCs provides new opportunities for discovering new wound healing agents and antimicrobials. To the best of our knowledge, there is no study about the biological characteristics of zinc borate. The aim of the study was to research the wound healing properties of zinc borate with its enzyme inhibition capacity and antifibrinolytic and antimicrobial activities.

2. Materials and Methods

2.1. Materials

Bovine hyaluronidase, *Clostridium histolyticum* collagenase, N-(3-[2-Furyl]-acryloyl)-Leu-Gly-Pro-Ala (FALGPA), porcine pancreatic elastase, sodium hyaluronate, epigallocatechin gallate (EGCG) and N-Succinyl-Ala-Ala-Ala-p-nitroanilide, tricine buffer, acetate buffer, Tris HCl buffer, trypsin inhibitor from soybean, sodium borate, tannic acid, fibrinogen and zinc borate were purchased from Sigma-Aldrich, USA. Sodium hydroxide, calcium chloride, other chemicals and solvents were purchased from Merck Chemical Co., Germany. Dulbecco′s modified Eagle's Medium (DMEM), fetal bovine serum, antibiotic-antimycotic solution and Dulbecco′s phosphate buffer saline (dPBS) were purchased from PAN BIOTECH, Germany. Culture mediums were purchased from Merck, Germany and Difco, USA.

2.2. Cell Line

NIH-3T3 is a fibroblast cell line that was provided by American Type Culture Collection (ATCC) were delivered in DMEM (Dulbecco′s modified Eagle's Medium) supplemented with fetal bovine serum and antibiotic-antimycotic solution at 37°C and in a moistened atmosphere containing 5% CO_2.

2.3. In Vitro Scratch Wound Assay

Fibroblasts were seeded into cell culture dishes to a final density of $75x10^4$ cells/dish and cultured for approximately 48 hours before a wound was created on the cell layer with a micropipette tip. To remove the cellular debris, dishes were washed with Dulbecco′s phosphate buffer saline (dPBS). Then, fibroblasts were treated with fresh medium containing zinc borate at final concentrations of 0.01 µg/mL. Control group was prepared with basal medium. The scratched areas from each cell culture dish were photographed to evaluate the distance between adjacent layers of cells.

2.4. Enzyme Inhibitory Activity

Enzyme inhibitory activity of zinc borate was evaluated using three enzymes; elastase, collagenase and hyaluronidase.

Elastase inhibitory activity was detected in accordance with Lee et al. [18]. Briefly, 25 µL of porcine pancreatic elastase enzyme, 50 µL of Tris-HCl buffer and 50 µL of zinc borate (at 1 mg/mL and 500 µg/mL concentrations, w/v dH_2O) were mixed and incubated at 25ºC for 20 min. Thereafter, 125 µL of elastase substrate N-Succinyl-Ala-Ala-Ala-p-nitroanilide was delivered to the mix and again maintained at 25ºC for 20 min. After, soybean trypsin inhibitor was added to the mixture and the amount of released p-nitroanilide was determined at 410 nm. Epigallocatechin gallate was used as a reference.

For collagenase inhibitory activity, *Clostridium histolyticum* collagenase was mixed with 25 µL of tricine buffer and 25 µL of zinc borate (at 1 mg/mL and 500 µg/mL concentrations, w/v dH_2O). The mix was pre-incubated for 20 min. Then, N-(3-[2-Furyl]-acryloyl)-Leu-Gly-Pro-Ala was delivered to the reaction, the absorbance was measured immediately at 335 nm for 20 min. The measurement was continued at every minute interval [19]. Epigallocatechin gallate was used as a reference in the study.

In hyaluronidase inhibition, 100 µL of zinc borate at different concentrations (at 1 mg/mL and 500 µg/mL concentrations, w/v dH_2O) was added to bovine hyaluronidase dissolved in acetate buffer and maintained at 37ºC for 20 min. After, 100 µL of $CaCl_2$ was inoculated and incubated for 20 min. Then, sodium hyaluronate was added and incubated for 40 min at 37ºC. The mixture was treated with NaOH and sodium borate and, maintained in a hot water bath for 3 min. Thereafter, the mix had cooled to room temperature, 1.5 mL of p-dimethyl aminobenzaldehyde was inoculated and incubated for another 20 min at 37ºC. Tannic acid was used as a reference and absorbance was taken at 585 nm [18].

Elastase, collagenase and hyaluronidase enzyme inhibitory experiments were as mean ± Standard Deviation (S.D.) of three parallel measurements (n=3). The data was entered into a Microsoft Excel database.

2.5. Antimicrobial Efficacy

2.5.1. Microorganisms

Staphylococcus aureus ATCC25923 and *Candida albicans* ATCC1023 strains were used to determine the antimicrobial properties of the zinc borate. Nutrient

Broth (NB) and Sabouraud Dextrose Broth (SDB) were used as cultivation mediums for *S. aureus* and *C. albicans*, respectively.

2.5.2. Tube dilution method

Two-fold serial dilutions (20 mg/mL-0.125 µg/mL) of zinc borate (in 1% acetic acid) were added to glass tubes containing Mueller-Hinton Broth (MHB). Then, freshly prepared inoculums were delivered to glass tubes and incubated at 37°C for 24-48 h for *S. aureus*, and 24-48 h at 30°C for *C. albicans*. The minimum inhibition concentration (MIC) was defined as the lowest concentration of zinc borate where no visible growth was observed after 24 h. 1% acetic acid was used as negative control.

For evaluating the minimum lethal concentrations (MLC), 100 µl from each negative test tube were sub-cultured onto Mueller-Hinton Agar (MHA) plate and Sabouroud Dextrose Agar (SDA) plate for *S. aureus* and *C. albicans*, respectively. All experiments were performed in triplicates.

2.6. Antifibrinolytic Activity

The antifibrinolytic activity of the zinc borate was detected using a plasma clot lysis test. A fibrinogen solution was prepared by adding 2 mL of water to 0.04 g fibrinogen. Then, 10 µL of calcium chloride (1 M), 0.5 mL of commercial plasma and 25 µL of fibrinogen solution were transferred to the test tubes. The mixture in the tubes was incubated for 90 min at 37°C and the liquid part was discarded. The clot was washed 3 times with dH_2O and 100 µL of zinc borate (0.1, 0.5 and 1 mg/mL, in 1% acetic acid) was added into the tubes. Streptokinase (30.000 and 15.000 U) and 1% acetic acid were used as positive and negative controls, respectively. After incubation at 37°C for 90 min, the liquid part in the tubes was discarded and the clots were weighed to calculate the percentage of lysis. All experiments were performed in triplicates.

3. Results and Discussion

In vitro scratch assay is a well-developed method to evaluate the migration potential of cells across an artificial wound. Therefore, it is also a tool to assess the cytotoxicity of the compounds [20,21]. The wound healing activity of the zinc borate was determined for changing the migration rate of 3T3 cells and compared with the control group which was only treated with basal medium. The scratched area formed on 3T3 cells was examined using an inverted microscope at 0, 24, 36 and 48 hours (Figure 1). It was observed that zinc borate at 0.01 µg/mL concentration enhanced the wound closure rate after 24h. Similar to the control group, the scratched area was completely healed after 48 hours. The results indicated that zinc borate can be safely used to improve the wound healing and had a marked effect on cell proliferation and migration.

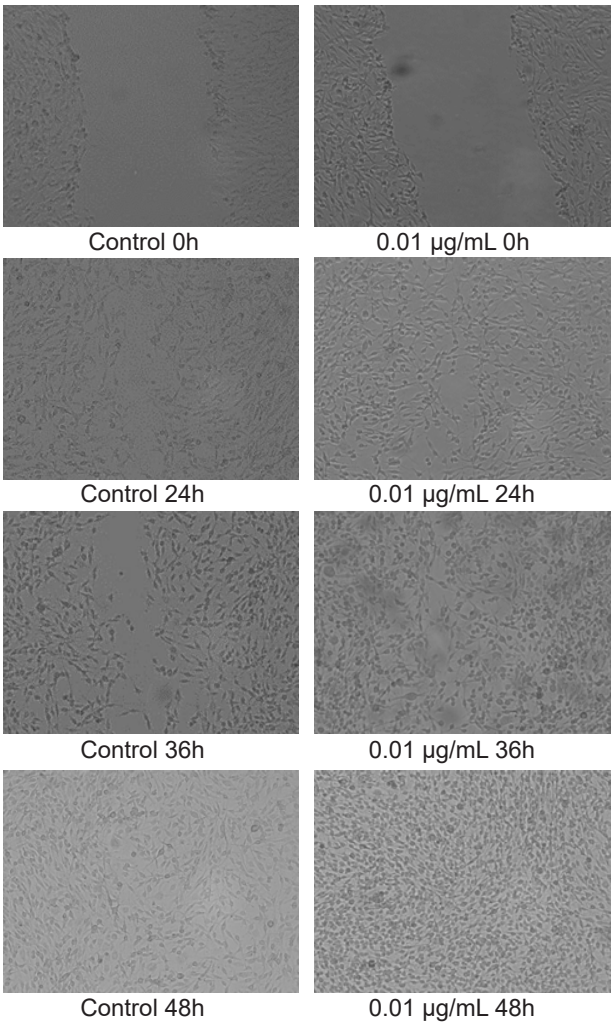

Figure 1. Images of *in vitro* scratch assay for 0-48 hours.

Demirci et al. [6] who studied the effect of sodium pentaborate pentahydrate (NaB) on migratory behavior of mouse embriyonic fibroblast cells (NIH-3T3) concluded that the scratches closed remarkably faster in the presence of NaB than in control or gel combination medium (NaB+pluronic). In another study of Demirci et al. [22], they revealed that NaB significantly increased the migration capacity in primary human dermal fibroblasts. Gündoğdu et al. [23] evaluated the effects of boronophenylalanine (BFA) and zinc (Zn) on *in vitro* wound healing model using human dermal fibroblast cells. Similar to the present study, they reported that BFA, Zn, and their combinations increased the proliferation of the fibroblasts after 24 hours of incubation and showed no cytotoxic effect.

Zinc borate showed good elastase and collagenase inhibition at 1 mg/mL concentration. Zinc borate showed elastase inhibition of 43.4±7.09% at 1 mg/mL concentration and 38.6±2.98% at 500 µg/mL concentration, while epigallocatechin gallate used as control showed 47±7.94% enzyme inhibition at 100 µg/mL concentration. Again, zinc borate showed 81.5±4.82% collagenase inhibition at 1 mg/mL concentration, while this inhibition was 16.9±5.51% at 500 µg/mL concentration. In the study, hyaluronidase

Table 1. Inhibitions of elastase, collagenase and hyaluronidase of zinc borate and positive controls.

Test samples	Concentration	Elastase inhibitions %	Collagenase inhibitions %	Hyaluronidase inhibitions %
Zinc borate	1 mg/mL	43.4±7.09	81.5±4.82	17.6±8.49
	500 µg/mL	38.6±2.98	16.9±5.51	11.3±4.16
Epigallocatechin gallate	100 µg/mL	47±7.94	20.4±3.09	NT*
Tannic acid	100 µg/mL	NT*	NT*	30.8±1.39

*NT: Not Tested.

inhibition of zinc borate was determined as 17.6±8.49% for 1 mg/mL and 11.3±4.16% for 500 µg/mL (Table 1).

Zn is an essential element in humans and an essential nutrient required for numerous biological activities. Its deficiency can cause many system dysfunctions, as well as delay wound healing. It has been determined that many biochemical and molecular events in wound repair can be accelerated by the addition of zinc ions [24]. Similarly, boron is an element that is effective in the wound healing process [25]. Boron compounds are known to remarkably enhance proliferation, migration, gene expression levels and growth factor in dermal cells [25,26].

It is important to maintain a balance between decomposition and ECM synthesis in wound healing. Extreme degradation of the newly formed ECM has been found to be associated with non-healing wounds. Components with anti-hyaluronidase, anti-collagenase and anti-elastase properties can increase the amount of hyaluronan, elastin and collagen in the extracellular matrix (ECM) by preventing matrix degradation [27]. Although the effect of zinc borate on these enzymes has not been determined before, peptitic boric acid compounds from tetrahedral borates are known to bind covalently to the active sites of serine proteases such as elastase [28]. In a study of Nzietchueng et al. [8], it was reported that boron directly inhibited elastase and alkaline phosphatase activities of human fibroblasts but did not have a direct effect on trypsin-like and collagenase activities. They concluded that, part of the boron effect on wound healing may be via synthesis of cytokines involved in wound healing, or via generation of free radicals (or other compounds) and activation of transcription factors.

Table 2. Minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) of zinc borate.

	S. aureus **ATCC25923**	***C. albicans*** **ATCC1023**
MIC	0.5 mg/mL	1 mg/mL
MLC	10 mg/mL	>10 mg/mL

MIC values of zinc borate were determined by tube dilution method. MIC value of zinc borate against *C. albicans* was determined as 1 mg/mL, it was determined as 0.5 mg/mL against *S. aureus* (Table 2). MLC of zinc borate against *S. aureus* was determined as 10 mg/mL while this value was >10 mg/mL against *C. albicans* (Table 2). There are some studies about the antimicrobial potential of boron compounds in the literature [29-31]. Yılmaz [29] reported the MICs of boric acid as 7.60 mg/mL, 7.60 mg/ mL and 3.80 mg/mL; against *Escherichia coli*, *Pseudomonas aeruginosa* and *S. aureus*, respectively. Argın et al. [32] indicated that biodegradable gelatin films incorporated with disodium octaborate can be used as an antimicrobial packaging material. Dembitsky and Srebnik [33] reported that carboxyboranes have hypolipidemic, anticancer and antifungal activities. Jabbour et al. [34] who investigated the antibacterial activities of oxazaborolidines resulted that these compounds have remarkable antibacterial activity against *Streptococcus mutans*. Ugur et al. [35] who studied the antioxidative and mutagenic properties of zinc borate reported that zinc borate had moderate total antioxidant and hydroxyl (H_2O_2) scavenging activities besides having moderate antimutagenic potential.

It was determined that zinc borate did not have any antifibrinolytic activity at 1, 0.5 and 0.1 mg/mL concentrations (Table 3). The antifibrinolytic agents prevent the breakdown of fibrin, the main protein in blood clots. They can be used to help prevent or control bleeding during or after surgery or after a traumatic injury. They are also useful for preventing clot disruption in areas rich in fibrinolytic activity, including the nasal cavity, oral cavity, and female reproductive system.

Table 3. Antifibrinolytic activity of of zinc borate.

Test samples	Concentration	Antifibrinolytic activity
Streptokinase	15000 U	52.8
	30000 U	84.3
Zinc borate	0.1 mg/mL	NT
	0.5 mg/mL	
	1 mg/mL	
Acetic acid (1%)	-	NT

NT: Not Tested.

4. Conclusions

The present study was conducted to evaluate the antimicrobial, antifibrinolytic, enzyme inhbitory and wound healing properties of zinc borate. Similar to most of the boron containing compounds, it was revealed that zinc borate has antimicrobial properties

against pathogenic microorganisms. Among the tested concentrations, the highest enzyme inhibition capacity of zinc borate was determined against collagenase, an enzyme which plays a significant role in the wound repair process. Besides, zinc borate has the ability to stimulate the normal fibroblast at low doses. On the other hand, zinc borate did not have antifibrinolytic activity. The results of the study indicated that zinc borate can promote the wound healing process by accelerating the migration and proliferation of fibroblasts, inhibiting the enzymes related to wound healing and prevent wound infections.

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