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Research Article

In Vitro Effect of Boron Compounds in Combination with Photobiomodulation Therapy by 905 nm on the Viability of Human Gingival Fibroblasts

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Abstract

This study aimed to evaluate the effect of Low-level laser therapy (LLLT) and Boric acid (BA) or Borax decahydrate (BD) on the cell survival of gingival fibroblasts. Fibroblast planted plates were divided into 10 groups: Control group, BD 100 µg/mL (BD 100), BD 200 µg/mL (BD200), BA 100 µg/mL (BA100), BA 200 µg/mL (BA200), LLLT, LLLT+BD100 µg/mL (LLLT+BD100), LLLT+BD200 µg/mL (LLLT+BD200), LLLT+BA100 µg/mL (LLLT+BA100) and LLLT+BA 200 µg/mL (LLLT+BA200) groups. LLLT was performed at a dose of 4 J/cm^2 for 160 sec. Following LLLT, MTT analysis was performed after the cells were kept in the incubator for 24 h. The LLLT+BA 200 group exhibited the highest cell density. In MTT analysis, significantly higher cell numbers were observed in the BA200, LLLT+BA100, and LLLT+BA200 groups compared to the control group ($P<0.05$). Then, the 3-(4,5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide assay and 8-hydroxy-2'-deoxyguanosine and Bcl-2 Associated X-Protein were analyzed. The 8-hydroxy-2'-deoxyguanosine and Bcl-2 Associated X-Protein levels were decreased in the LLLT+BA200 group compared to the control group ($P<0.05$). This study suggests that BA200, LLLT+BA100, and LLLT+BA200 increase the survival of fibroblast cells.

Keywords: Borax decahydrate, Boric acid, MTT, Photobiomodulation, Photobiostimulation

Introduction

Boric acid (BA) and borax decahydrate (BD) are recognized for their diverse biological effects, which include antimicrobial [1], anti-inflammatory [2], and antioxidant properties [3]. These compounds have garnered attention across various disciplines, particularly in periodontology, for their potential therapeutic applications. Notably, boron, present in both BA and BD, is frequently used in dentistry due to its antimicrobial and immunomodulating properties [4,5].

Low-level laser therapy (LLLT), also known as photobiomodulation, has emerged as a promising therapeutic avenue for various medical conditions, including wound healing, fracture healing, and pain management [6-11]. Despite its demonstrated efficacy in diverse applications, the combination of LLLT with BA remains relatively unexplored. A previous study [6] employing a combination of laser and a chemical compound concluded that concurrent application of the compound and laser accelerates bone formation.

Fibroblasts, integral to wound healing and tissue repair, play a crucial role in extracellular matrix production and maintenance [12,13]. Gingival fibroblasts, specifically, are pivotal for gum wound healing and exhibit rapid adaptability to the oral cavity environment [14,15]. Apoptosis, regulated by a balance of antiapoptotic and proapoptotic proteins, is fundamental to cellular homeostasis. Bcl-2 family proteins, including BAX, serve as key regulators of apoptosis, with BAX predominance promoting apoptosis [16,17]. Additionally, oxidative stress, characterized by elevated reactive oxygen species levels, can induce DNA damage, as evidenced by the marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) [18,19].

This study aims to investigate 8-OHdG and BAX positivity in gingival fibroblasts treated with BA and BD alongside LLLT, examining their relationship and potential effects on fibroblast viability. We hypothesize that BA and BD in combination with LLLT will enhance fibroblast survival.

Material and Methods

Ethical Approval

The Atatürk University Animal Experiments Local Ethics Committee approved the study protocol of this study (Decision No: 17/2022).

Chemical and Reagent

BA and BD were obtained from Eti Boron Mining Institute. The Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 (DMEM-F12), Fetal Bovine Serum (FBS), and Antibiotic (penicillin, and streptomycin) were obtained from Sigma Aldrich (Missouri, USA). The flask 25 cm^2 and plates were purchased from Corning (Arizona, USA).

Cell Cultures

Primary Gingival Fibroblast Normal; Human, Adult (HGF) was purchased at ATCC (CRL-2014, Virginia, USA). The cell was resuspended by fresh medium Dulbecco's Modified Eagle's Medium nutrient mixture F-12 (DMEM-F12), 10% Fetal Bovine Serum, and antibiotic 1% (penicillin, amphotericin B, and streptomycin) (Corning, USA). After the cells reached 80% coverage, they were removed with the help of 0.25% trypsin-0.02% ethylenediaminetetraacetic acid and inoculated on 96 and 24-well plates with the use of new medium. The planted plates were kept in the incubator (5% CO_2 and 37°C) until the start of the experiment.

Experimental Design

Fibroblast planted plates were divided into 10 groups: Control group (Neither treated with BD/BA nor LLLT), BD 100 µg/mL (BD 100), BD 200 µg/mL (BD200), BA 100 µg/mL (BA100), BA 200 µg/mL (BA200), LLLT, LLLT+BD100 µg/mL (LLLT+BD100), LLLT+BD200 µg/mL (LLLT+BD200), and LLLT+BA100 µg/mL (LLLT+BA100) and LLLT+BA 200 µg/mL (LLLT+BA200) groups.

Laser Irradiation

A GaAs (gallium arsenide) laser device (Lasermed 2200, Eme Phsio, Italy) was set at λ = 905 µm, 10000 Hz, 25 mW, and peak power 25 W in continuous mode used for laser therapy. The device was calibrated before the experiments. LLLT was applied by concentric circular motions of the optical fiber at a distance of 10 mm from the surface of the cell culture to irradiate the entire well-containing cells. By inserting the probe from the bottom through a hole in a specific shelf that was made especially for this purpose, culture plates in the research groups were subjected to laser treatment. To provide an even distribution of pulse energy across the whole surface area of the plate where the culture plate was exposed to radiation, a distance of 3 cm between the cell culture plate and probe lens (divergent lens component) was adjusted. It was done at ambient temperature and in the dark. Laser treatment (power/beam field) was calculated according to the formula (power/beam field)×time= J/cm^2. 30 min after drug addition, laser application was performed at 4 J/cm^2 for 160 sec. All LLLT procedures were carried out under aseptic conditions in a Biological Safety Cabinet (ESCO, Esco Micro Pte. Ltd, Singapore).

MTT Assay

Following the LLLT, the cells were kept in the incubator for 24 h. Then, 10 µL (5 mg/mL) of MTT solution was added to the wells and 100 µL of DMSO solution was added to each well to dissolve the purple-colored formazan crystals formed after 4 h. The absorbance of the purple color formed was measured with an ELISA plate reader at 570 nm (µQuant, Bad Friedrichshall, Biotek), and the values obtained were expressed as % cell viability versus control.

The 8-hydroxy-2'-deoxyguanosine (8-OHdG) and Bcl-2 Associated X-Protein (BAX) Analysis

Cultured cells were incubated in a paraformaldehyde solution for 30 min. The cells were then incubated in 3% H_2O_2 for 5 min. 0.1% Triton-X solution was dripped onto the cells washed with PBS and left for 15 min. After the incubation period, protein blocks were dripped onto the cells and kept in the dark for 5 min. Then, the primary antibody (8-OHdG Cat no: sc-66036, Dilution Ratio:1/100 US) was dropped and incubated according to the instructions for use. An immunofluorescence secondary antibody was used as a secondary marker (FITC Cat No: ab6785 Diluent Ratio: 1/500, UK) and kept in the dark for 45 min. Then, the second primary antibody (BAX Cat No: 7480, Dilution Ratio: 1/100, US) was

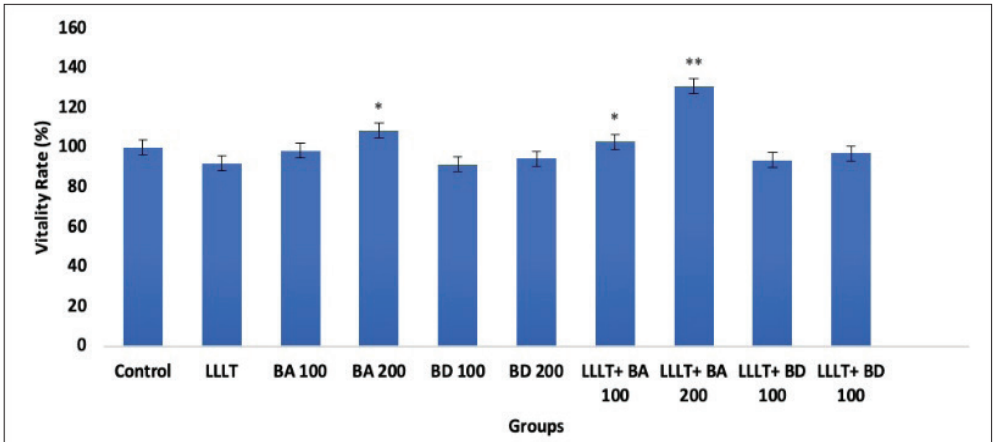

Fig 1. Cell viability was measured with an MTT assay (n = 6). Effects of Boric acid (BA) and Borax decahydrate (BD) with and without Low Level Laser Therapy (LLLT) on vitality rate (%) of gingival fibroblasts for 24 h. The results are presented as the average of three separate experiments. Statistical significance: *P<0.05; **P<0.001

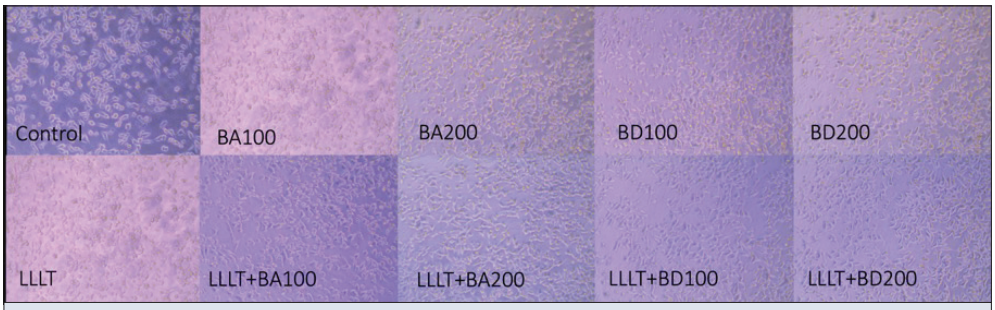

Fig 2. Light microscope images (×40) of gingival fibroblasts cells treated with different concentrations of boric acid (BA), borax decahydrate (BD) and Low Level Laser Therapy (LLLT) (4 J/cm^2) for 24 h

dripped onto the tissues and incubated by the instructions for use. An immunofluorescence secondary antibody was used as a secondary marker (Texas Red Cat No: ab6787 Diluent Ratio: 1/1000 UK) and kept in the dark for 45 min. Afterward, DAPI with mounting medium (Cat no: D1306 Dilution Ratio: 1/200 UK) was dripped onto the sections and kept in the dark for 5 min, and the sections were closed with a coverslip. In order to determine the intensity of positive staining from the pictures obtained as a result of immunofluorescent staining; 5 random areas were selected from each image and evaluated in the ZEISS Zen Imaging Software program.

Statistical Analysis

Statistical comparisons between groups in MTT analysis were calculated using one-way ANOVA and Tukey's HSD method. One-way ANOVA followed by Tukey's test was also performed to compare positive immunoreactive cells and immunopositive stained areas with healthy controls. All calculations were performed using SPSS 20 software for statistical analysis, and a P<0.05 was considered a statistically significant difference in all tests. Data were presented as mean and standard deviation (mean ± SD).

Results

MTT Assay

The MTT assay results are shown in *Fig. 1.* No significant difference was observed between the control and LLLT, BA100, BD100, BD200, LLLT+BD100, and LLLT+BD200 groups in regards to MTT analysis (P>0.05). A significantly higher cell count was observed in the BA200, LLLT+BA100 and LLLT+BA200 groups than in the control group (P<0.05). There was a proliferative peak in the LLLT+BA 200 group (P<0.001). Light microscope results are shown in *Fig. 2.* The microscope and MTT results show a positive correlation. The highest cell density was seen in the LLLT+BA 200 group.

The 8-hydroxy-2'-deoxyguanosine (8-OHdG) and Bcl-2 Associated X-Protein (BAX) Analysis

The 8-hydroxy-2'-deoxyguanosine (8-OHdG) and Bcl-2 Associated X-Protein (BAX) expressions in cell lines were very mild in the LLLT+BA 200 group, mild in the BA200, LLLT+BA100, LLLT+BD100, LLLT+BD200 groups, moderate in the control, BA100, BD100, and

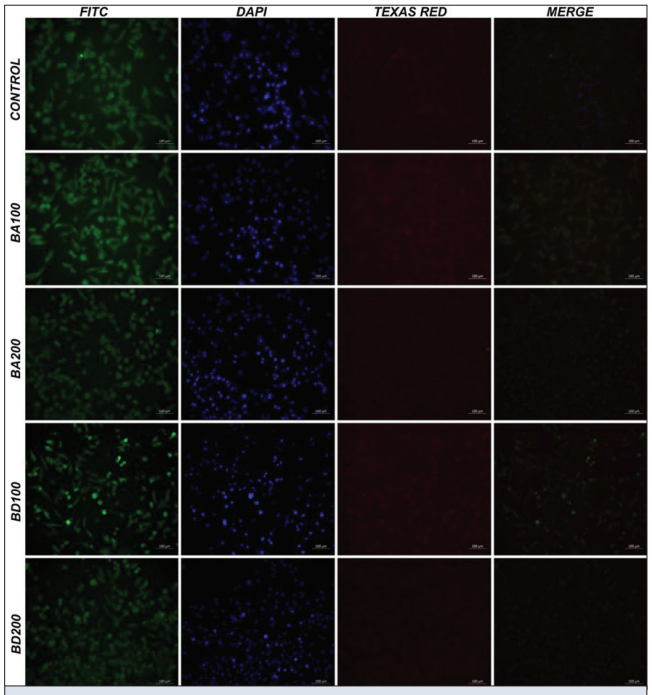

Fig 3. Fibroblast line, 8-OHdG expressions (FITC), BAX expressions (Texas Red), IF, Bar: 100 µm

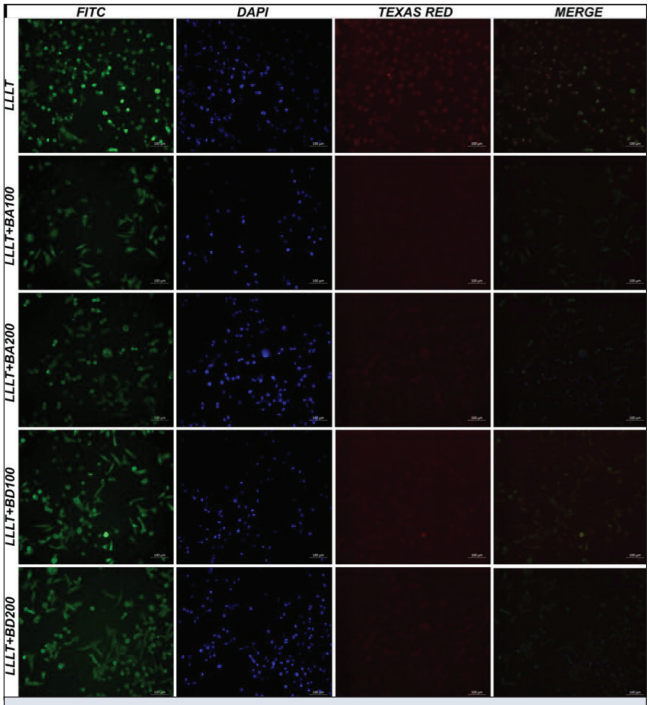

Fig 4. Fibroblast line, 8-OHdG expressions (FITC), BAX expressions (Texas Red), IF, Bar: 100 µm

Table 1. *Detection of 8-OHdG and BAX expression levels in gingival fibroblasts after treated with control, BA100, BA200, BD100, BD200 with and without Low Level Laser Therapy (LLLT)*

Group	8 OHdG	BAX
Control	42.19±2.18[a]	35.43±1.02[a]
BA100	39.15±3.85[a]	33.42±1.75[a]
BA200	28.13±2.57[b]	22.19±2.99[b]
BD100	35.42±2.74[a]	36.18±2.08[a]
BD200	39.74±1.94[a]	34.18±2.26[a]
LLLT	52.48±1.68[c]	51.67±1.23[c]
LLLT+BA100	18.43±2.06[b]	20.36±0.75[b]
LLLT+BA200	14.26±3.06[d]	16.43±1.07[d]
LLLT+BD100	20.12±2.18[b]	19.35±0.73[b]
LLLT+BD200	19.85±2.98[b]	19.52±1.68[b]

Different lowercase letters (a, b, c, d) in the same column represent a statistically significant difference
BA100: Boric acid 100 µg/mL, BA200: Boric acid 200 µg/mL, BD100: Borax decahydrate 100 µg/mL, BD200: Borax decahydrate 200 µg/mL, LLLT: low-level laser therapy

BD200 groups, and severe in the LLLT group *(Fig. 3, Fig. 4)*. 8-OHdG and BAX levels were decreased in the LLLT+BA200 group compared to the control group (P<0.05) *(Table 1)*.

Discussion

This *in-vitro* experimental study aimed to investigate the effects of administering BA and BD in combination with LLLT on gingival fibroblasts. In line with the experimental hypothesis, viability significantly increased in the LLLT+BA 200 group compared to the control group, while 8-OHdG and BAX levels decreased.

Co-administration of BA with LLLT in gingival fibroblasts resulted in biostimulation of cell cultures and increased cell viability. The molecular absorption of laser light is a prerequisite for cellular action. The effects of laser on cells are considered to be wavelength and dose-dependent [20]. Overall, LLLT exposure induced positive and negative effects on gingival fibroblasts depending on the range dose. Previous studies have shown that laser irradiation has stimulating effects at energy densities up to 4 J/cm^2, while at energy densities greater than 4 J/cm^2 it has inhibitory properties of fibroblast proliferation [20-22]. The laser dose was determined as 4.0 J/cm^2 by reference to a previous report [22], which suggests that the dose accelerates fibroblast proliferation.

In this study, we detect that boric acid (BA) has supportive effects when used with laser at doses of 100 and 200 µg/mL. The cell viability data of our study shows that 200 µg/mL BA applied with laser is greater than 100 µg/mL BA. There are many *in vitro* studies on boric acid. A study by Bunning et al.[23] found that 100 µg/mL boron caused no toxicity in normal cells and also eliminated cancer cells

with over 90% killing, demonstrating an outstanding therapeutic index. Ozansoy et al.[24] showed that both sodium borate decahydrate and boric acid had viability-promoting effects in the amyloid-beta toxicity model when used at doses of 200 µg/mL. Cell viability data in the same study showed that 10 µg/mL BA was more toxic than other concentrations (5-15-20-50-100-200 µg/mL). In this study, 100 and 200 µg/mL boron was applied to cell cultures. A similar dose of boron had previously been applied to cell cultures [23,24].

At all times of cellular life, the DNA molecule is subject to oxidative damage caused by reactive oxygen species. Cells provide control of DNA damage by endogenous antioxidant mechanisms and DNA repair systems. Moreover, if the rate of DNA damage exceeds the cell's repair capacity, the accumulation of errors can overwhelm the cell. These cumulative DNA damages can lead to mutations and potentially cancer [22,25]. To control the negative effects of DNA damage, cells present an apoptosis mechanism based on the detection of DNA damage by the p53 protein. Bcl-2 and BAX molecules belonging to the Bcl-2 family are key regulators of the caspase activation pathway initiated by mitochondria during apoptosis [26]. As an antiapoptotic gene, Bcl-2 maintains mitochondrial membrane integrity and prevents the release of internal calcium stores into the cytoplasm [27]. In contrast, as a proapoptotic gene, BAX moves from other cellular compartments to mitochondria in response to apoptotic stimulus and promotes cytochrome c release [28]. Subsequently, activation of the caspase family triggers a series of apoptosis-related events [29]. Apoptosis is triggered by the downregulation of Bcl-2 and overexpression of BAX c, which triggers the change in the mitochondrial membrane that carries cytochrome c from this organelle to the cytoplasm. An increase in cytochrome c levels can trigger the caspase apoptotic pathway, which includes a regulator protein CASP 8 and its executive proteins CASP3 and CASP1 [30]. Therefore, cellular boric acid exposure can trigger apoptotic events in fibroblast cells [31]. Therefore, in this study examined the effects of LLLT and BA on CASP modulation. The reason why 8 OHdG and BAX expressions were moderate in the control, BA100, BD100 and BD200 groups, and severe in the LLLT group was thought to be DNA damage and apoptosis.

Previous studies have shown that laser radiation has stimulatory effects at energy densities up to 4 J/cm^2 [22] while it has highly inhibitory properties at higher energy densities [22,32]. Therefore, a dose of 4 J/cm^2 was used in this study.

The limitation of the study is that different energy densities are not used combine with boron compounds in this study. Determining the optimum dose is extremely important, as the biological response to LLLT stimulation is dependent on wavelength, irradiance, time, pulse, light consistency, polarization, and many other parameters [22]. Therefore, it is extremely important to determine the best LLLT exposure dose that may have some beneficial effects in reversing the changes induced by fibroblast exposure to boron compounds.

The main limitation of this study is that the efficacy of different doses of LLLT on the proliferation of BA and BD was not evaluated. Different energy densities have been tested in most *in vitro* studies [20,33]. Previous studies have shown that an energy density of 2-4 J/cm^2 is most effective in improving cell growth [34,35]. Power density is also important in achieving improved cell proliferation [33].

In conclusion, our study has revealed that the combined application of boric acid and 905 nm LLLT at a dose of 4 J/cm^2 caused a statistically significant increase in the survival of gingival fibroblasts. Clinically, the combination of boric acid and LLLT may be useful in periodontal diseases, wound healing, and obtaining an optimal number of fibroblasts for soft tissue regeneration. These laboratory results will guide future studies on laser tissue irradiation, its effects on tissue healing *in vivo* and the clinical use of boron compounds and LLLT. Further studies are required to elucidate the effects of different power densities and different BA doses on gingival fibroblasts.

Declarations

Availability of Data and Materials: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Ethical Statement: The Atatürk University Animal Experiments Local Ethics Committee approved the study protocol of this study (Decision No: 17/2022).

Competing Interest: The authors declare that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The author declare that the article tables and figures were not written or created by AI and AI-assisted technologies.

Author's Contributions: FT: Literature search, study design, data collection and interpretation, manuscript preparation, LEY: Statistical analysis, data collection and interpretation, manuscript preparation, SY: Immunofluorescence analysis and data collection. AT and YY: MTT analysis and data collection, SO: Literature search and data collection and OTO: LLLT application to fibroblast. All authors read and approved the final manuscript.

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