

Sodium Pentaborate Pentahydrate and Pluronic Containing Hydrogel Increases Cutaneous Wound Healing In Vitro and In Vivo

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Abstract After a disruption of skin integrity, the body produces an immediate response followed by a functional and comparable regeneration period, referred to as wound healing. Although normal wounds do not need much attention during the healing period, chronic (non-healing) wounds are the major challenge of current dermatological applications. Therefore, developing new, safe, and effective wound healing drugs has always been an attractive area of international research. In the current study, sodium pentaborate pentahydrate (NaB), pluronics (Plu; F68 and F127), and their combinations were investigated for their wound healing activities, using in vitro and in vivo approaches. The results revealed that NaB significantly increased migration capacity and superoxide dismutase activity in primary human fibroblasts. Combinations of optimized concentrations for pluronic block co-polymers further increased cell migration, and the messenger RNA (mRNA) expression levels of important growth factor and cytokines (vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF- β), and tumor necrosis factor alpha (TNF- α)). NaB containing hydrogel co-formulated with pluronics was also investigated for their wound healing activities using a full thickness wound model in rats. Macroscopic and histopathological analysis

confirmed that wounds in combination gel-treated groups healed faster than those of control groups. NaB/Plu gel application was found to increase wound contraction and collagen deposition in the wound area. Therefore, our results suggest that NaB, and its pluronics combination, could be used in dermatological clinics and be a future solution for chronic wounds. However, further studies should be conducted to explore its exact action of mechanism and effects of this formulation on chronic wounds.

Keywords Boron · Pluronics · F127 · F68 · Wound healing

Introduction

After a disruption of skin integrity, the body produces an immediate response followed by a functional and comparable regeneration period, referred to as wound healing. The wound healing process consists of five main orderly but temporally overlapping phases; homeostasis and inflammation, granulation tissue formation, neovascularization, reepithelialization, and remodeling [1]. These phases are tightly regulated by a cascade of external and internal stimuli such as growth factors and cytokines in a well-orchestrated manner, resulting in regeneration and restoration of the damaged skin [2]. Although normal wounds do not need much attention during the healing period, chronic (non-healing) wounds, experienced by approximately 6.5 million patients in USA alone, are a major challenge of the current dermatological applications [3]. These wounds impose a heavy worldwide social, economic, and health burden due to a lack of efficient wound healing agents. Therefore, developing new, safe, and effective wound healing drugs has always been an attractive area of international research.

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Boron has been recognized as an important micronutrient in plant physiology for almost 100 years [4], while limited studies have reported its vital roles in animal and human systems without exploring its exact mode of action. It has been reported to be involved in embryogenesis [5], bone growth and maintenance [6], immune responses [7], hormone action [8], and brain and psychological functions [9] of animal and human metabolism. Besides, it has been shown to increase the wound healing rate in a few studies. Boric acid (3 % solution) treatment of deep wounds in an intensive care unit decreased hospitalization time by enhancing granulation tissue formation [10]. Although there are a few similar studies reflecting boron's positive effects on wound healing, the data supplied in the literature does not sufficiently explore its potential role for use in dermatological clinics.

Ploxamers, known as pluronics or kollophors, are non-ionic and amphipathic triblock copolymers consisting of a backbone of poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) (PEO-PPO-PEO), which can form micelles and hydrogels at above critical gel concentrations, under proper conditions [11]. These synthetic polymers have been used in a wide array of biomedical areas including medical, pharmaceutical, and the cosmetic industry [12]. They are mainly used in delivery of drugs such as therapeutic proteins, chemicals, cytokines, and antimicrobial agents [13]. Ploxamers have also been used for wound healing agent delivery studies [14, 15]. Other than being used as drug carriers, two important members of this family, F68 and F127, have been shown to be effective in wound healing themselves, by inhibiting inflammation and stimulating growth factor expression [16, 17].

In the present study, we evaluated the effects of a sodium pentaborate pentahydrate (NaB) containing carbopol-based gel composition, co-formulated with ploxamers (F68 and F127) on wound healing both *in vitro* and *in vivo* using a full thickness wound model in rats. This is the first study proved excisional wound healing properties of NaB-ploxamer containing hydrogel formulations.

Materials and Methods

Cell Lines and Culture Conditions

Primary human dermal fibroblasts (HF) were isolated from neonatal foreskin as described before [18], after the informed consent of patients and ethics committee approval of Kocaeli University were taken. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) at 37 °C and 5 % CO₂ in a humidified incubator. Cells were trypsinized after they reach 80 % confluence. HF cells were characterized by collagen type I (ab292, Abcam, Cambridge, MA) immunostaining according to the protocol

described before [19]. All experiments were repeated at least three times.

Cell Viability Assay

NaB was kindly obtained from National Boron Research Institute-BOREN (Ankara, Turkey) and prepared in a culture medium at a stock concentration of 0.1 g/ml. Following filtration using a 0.2- μ m filter (Sartorius AG, Göttingen, Germany), consecutive dilutions were prepared in DMEM. Pluronic block co-polymers (F68 and F127) were purchased from BASF Corporation (Badische Anilin und Soda-Fabrik, Ludwigshafen-am-Rhein, Germany), and stock solutions were prepared as described previously [20]. A total of 10 mg/ml concentration of each block copolymer were dissolved in PBS incubating on ice. The stock solution was filtered through a 0.2- μ m filter and subsequently diluted to 1 mg/ml in DMEM. Five different concentrations of NaB (10, 15, 20, 75, and 100 μ g/ml) and three different concentrations (5, 10, and 20 μ g/ml) of pluronics were prepared in DMEM. Effects of pluronics and NaB were tested on the cell viability of HF. Briefly, cells were seeded onto 96-well plates (TPP, Switzerland) at a concentration of 5×10^3 cells/well, and freshly prepared reagents were added. Cells were maintained at 37 °C and 5 % CO₂ in a humidified incubator for different time intervals (24, 48, and 72 h), and the viability was measured using the MTS assay (CellTiter96 Aqueous One Solution; Promega, Southampton, UK) according to the manufacturer's instructions.

Scratch Assay

Scratch assays were conducted according to the protocol previously described [21]. The cells were used for scratch wound healing assay to test the effects of pluronics (10 μ g/ml for each), NaB (15 μ g/ml), and their combinations on the migratory potential of the cells. Cells were seeded onto 12-well plates (TPP, Switzerland) to a final cell density of 1×10^5 cells/well and incubated in a humidified incubator overnight at 37 °C and 5 % CO₂. Adherent cells were scratched with a sterile 1,000- μ l tip, and the medium was immediately changed with fresh medium containing specified concentrations of the reagents. Cells were observed under an inverted microscope (Nikon Eclipse TE200, Nikon, Tokyo, Japan), and pictures were taken at different time intervals (0, 12, and 24 h).

Real-Time (RT) PCR Assay

SYBR Green Real-Time PCR method was used to determine the gene expression levels of selected growth factors and cytokines. Briefly, total RNAs were isolated using High Pure RNA isolation kit (Roche, USA) according to the

manufacturer's instructions. High Fidelity complementary DNA (cDNA) synthesis kit (Roche, USA) was used to synthesize cDNAs. Specific primers designed by using Primer BLAST online software of The National Center for Biotechnology (NCBI) against vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNF- α), transforming growth factor beta (TGF- β), and fibroblast growth factor 7 (FGF7) genes were mixed with cDNAs and Maxima™ SYBR Green qPCR Master Mix (2 \times) (Fermentas, USA) in a final volume of 20 μ l. GAPDH gene was used as the housekeeping gene for normalization of the data. Primer sequences are shown in Table 1. All RT-PCR experiments were performed using an iCycler RT-PCR (Bio-Rad, Hercules, CA, USA, icycler iQ Optical Module) detection system.

Antioxidant Enzyme Activity

Superoxide dismutase (SOD) activity was measured using a commercially available spectrophotometric-based SOD Assay Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions.

In Vivo Studies

Gel Preparation

Carbopol hydrogels were prepared by dispersing 1 % (w/v) polymer (Carbopol Ultrez-21, Lubrizol, USA) in distilled water. A total of 1 M sodium hydroxide solution (defined quantity) used as the neutralizing agent for the gelation of the polymer was added to adjust the pH of hydrogels to be between 6 and 7. The hydrogel without any active ingredient was used as a vehicle in the animal experiments and referred to as hydrogel. Then, NaB, F68, and F127 were mixed into a blank hydrogel at a final concentration of 3 % (w/v), 2 % (w/v), and 2 % (w/v), respectively. The gel formulation was stored at 4 °C until it completely dissolved (approximately 24 h). The NaB, F68, and F127 containing hydrogel was used in the

animal experiments as an active formulation and is referred to as gel combination (NaB/Plu) group.

Animals

Healthy adult male Sprague-Dawley rats (200–250 g, $n=24$) were housed in standard laboratory conditions. The rats were subjected to 12-h light/dark cycle at a constant temperature of 23 °C and fed with food and water ad libitum. An approval for the use of animals and the protocol was obtained from Yeditepe University Ethics Committee of Experimental Animal Use and the Research Scientific Committee at the same institution.

Excisional Wound Creation

The rats anesthetized intraperitoneally (i.p.) with pentobarbitone sodium (40 mg/kg) were divided into three groups ($n=8$). Dorsal skin of the rats was shaved and cleaned with 70 % ethanol and iodine solution. Full thickness excisional wounds (approx. 6 mm in diameter, 2 mm in depth) were created, and the animals were housed individually in disinfected cages after recovery from anesthesia. Animals were left untreated (group 1) or treated with vehicle hydrogel (group 2) or NaB (3 % w/v), F68 (2 % w/v), and F127 (2 % w/v) containing hydrogel (group 3). Respective gel formulations were applied topically twice a day for 7 days. Photographs of each wound with an internal scale were taken at day 0, 2, 4, 6, and 7 to calculate wound contraction. Wound surface areas were calculated using Image J software (NIH).

Histopathology

The animals were sacrificed at day 7 with an overdose of diethyl ether. The skin samples from each animal were immersed into 10 % neutral formaldehyde. Furthermore, the skin was processed in ascending concentrations of ethanol, cleared with xylene (Leica tissue processor, TP1020; Leica, Germany), and embedded in paraffin (Leica embedding station, EG1160; Leica, Germany). The paraffin-embedded tissue blocks were cut with a Microm HM 325 (Microm,

Table 1 The sequences of primers used in RT-PCR assay

Marker	Sense (5'-3')	Antisense (5'-3')	Product size
TGF- β	GGCTTTCGCCTTAGGCCCA	TTGGTGTCCAGGGCTCGGC	322 bp
TNF- α	TGGCCAATGGCGTGGAGCTG	TAGGAGACGGCGATGCGGC	151 bp
VEGF	TTGCCTTGCTGCTCTACCTC	GCTGCGCTGATAGACATCC	116 bp
FGF7	CCAGCCCTGAGCGACACACAA	GCCACAATTCCAAGTCCACTGTC	187 bp
GAPDH	GGTATCGTGAAGGACTCA	GCAGGGATGATGTTCTGGA	122 bp

TGF- β transforming growth factor beta, TNF- α tumor necrosis factor alpha, VEGF vascular endothelial growth factor, FGF7 fibroblast growth factor 7, GAPDH glyceraldehyde 3-phosphate dehydrogenase

Germany) microtome. For each animal, four randomly taken tissue sections (5 μm) were processed for hematoxylin and eosin (H&E) and Masson trichrome stain using standardized programme in Leica autostainer XL (Leica, Germany). All tissue sections were examined under the Leica DM 4000 microscopy system by experienced histologists, who were uninformed of the treatments (Ü.U and A.C.). The scoring was performed according to article [22], and each slide was scored by a histological criteria ranging from 0 to 2 (see Fig. 4b). In case that some criteria may alter in different groups, six individual parameters, epidermal regeneration, thickness of epidermis, thickness of granulation tissue, inflammatory cell infiltration, proliferation density of fibroblasts, and collagen deposition were scored separately.

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey *post hoc* test was performed for multiple comparisons of data using GraphPad Prism statistical software 5.0 (GraphPad Software, La Jolla, CA, USA). The values of $P < 0.05$ were considered statistically significant.

Results

Cell Viability

Cell viability results were obtained by using isolated and characterized HF cells (Fig. 1a, b). NaB was found to increase cell viability at concentrations of 15, 20, and 100 $\mu\text{g/ml}$ at the end of the first day, compared to growth medium-treated group (control) (Fig. 1c). Although there were no significant

differences between groups at day 2 and 3, 15 $\mu\text{g/ml}$ still increased the viability relatively more efficiently than other groups. Moreover, HF proliferation was significantly enhanced upon stimulation with 10 $\mu\text{g/ml}$ of both F68 and F127 for 24 h compared to the control groups (Fig. 1d, e). As fibroblast proliferation is a critical step in wound healing process, 15 $\mu\text{g/ml}$ for NaB, 10 $\mu\text{g/ml}$ for F68 and F127 were chosen for further in vitro experiments.

Scratch Assay

The results revealed that a slight increase in migration was observed in NaB-treated cells (58.8 ± 7.3 %) compared to growth medium-treated control cells (40.7 ± 9.7 %). Although F68, F127, and pluronic combination (F68-F127) treatment did not remarkably affect the migration rate of fibroblast cells (46.7 ± 3.5 , 46.5 ± 3.1 and 55.3 ± 3.8 , respectively) with respect to the control group, combination of these pluronics with NaB significantly promoted cell migration (72.3 ± 8.4 %), indicating synergistic activity between pluronics and boron (Fig. 2a).

Antioxidant Enzyme Activity

The results confirmed that while NaB-treated cells exerted the highest enzyme activity, the combination group displayed approximately 2-fold increase in SOD activity compared to growth medium, F68, F127, and pluronic combination (F68-F127)-treated groups (Fig. 2b).

RT-PCR Assay

While no obvious disparity in expression levels of messenger RNAs (mRNAs) between F68, F127, NaB, and pluronic

Fig. 1 **a** Cell morphology (Scale bar 400 μm) and **b** collagen type I immunostaining (Scale bar 100 μm) of human fibroblasts (HF) from neonatal foreskin at passage number 2. The viability of HF cells treated with different concentrations of **c** NaB, **d** F68, and **e** F127 for 24, 48, and 72 h. Data represent the mean values \pm S.D. Comparisons were performed by ANOVA (Tukey *post hoc*). * $P < 0.05$, ** $P < 0.01$: comparison with control group. NaB sodium pentaborate pentahydrate, NC growth medium-treated HF cells

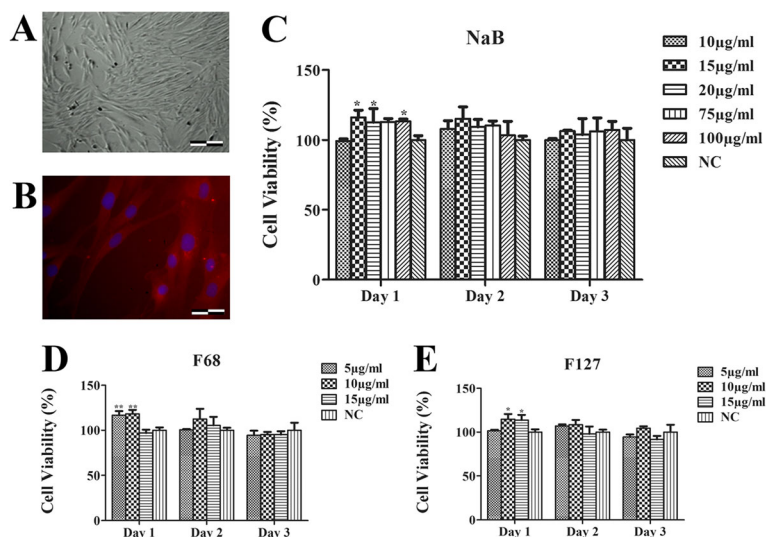
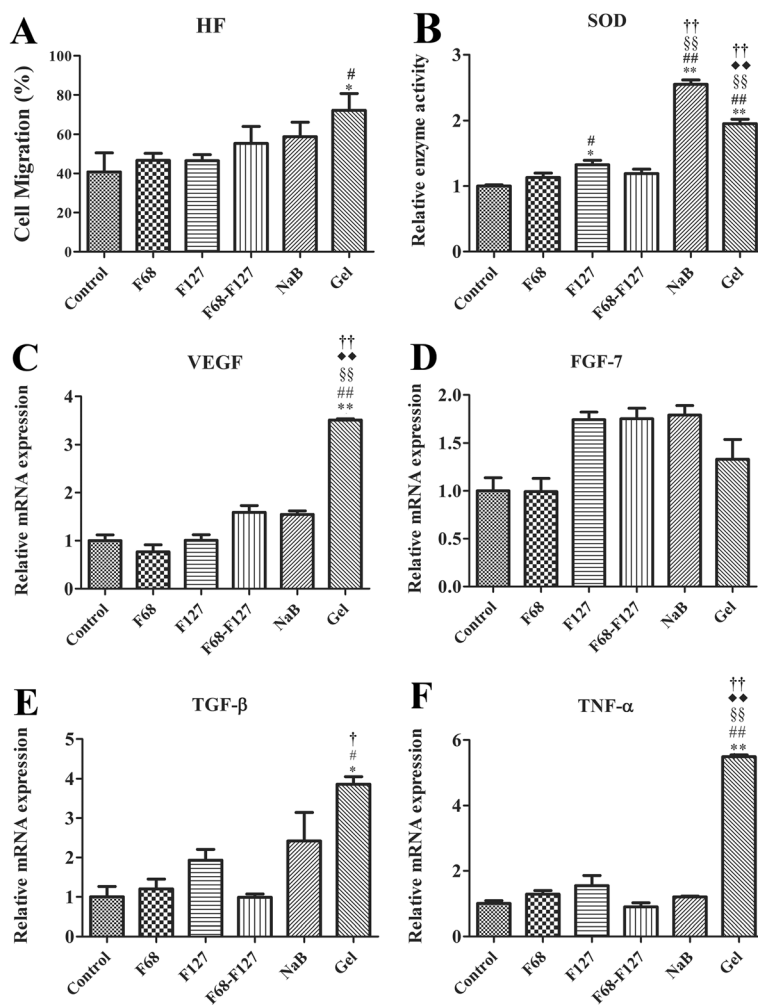


Fig. 2 **a** Migration rates, **b** superoxide dismutase (SOD) activity and relative mRNA levels of **c** vascular endothelial growth factor (VEGF), **d** fibroblast growth factor 7 (FGF7), **e** transforming growth factor beta (TGF- β), and **f** tumor necrosis factor alpha (TNF- α) in human fibroblast (HF) cells treated with sodium pentaborate pentahydrate (NaB, 15 $\mu\text{g/ml}$), F68 (10 $\mu\text{g/ml}$), F127 (10 $\mu\text{g/ml}$), F68-F127 (10 $\mu\text{g/ml}$ for each one), and their combinations (Gel). Data represent the mean values \pm S.D. Comparisons were performed by ANOVA (Tukey *post hoc*). * $P < 0.05$, ** $P < 0.01$: comparison with control group; # $P < 0.05$, ## $P < 0.01$: comparison with F68; §§ $P < 0.01$: comparison with F127; † $P < 0.05$, †† $P < 0.01$: comparison with F68-F127; ** $P < 0.01$: comparison with NaB. Control: growth medium-treated HF cells



combination-treated groups was noted, VEGF, TNF- α , and TGF- β mRNA levels were found to be significantly higher in the combination (NaB, F68, and F127)-treated group compared to other groups (Fig. 2c, e, and f). However, although a modest increase in FGF7 mRNA levels for F127, NaB, and combination groups was observed, there was no statistically significant difference between all groups (Fig. 2d).

In Vivo Wound Healing Assays

As there were no significant effects of F68 and F127 (alone or in combination) on cell migration, gene expression and antioxidant enzyme activity assays, they were not tested in in vivo experiments. Vehicle hydrogel without any active ingredient and NaB/Plu gel formulation containing NaB, F68, and F127 were investigated for their healing activities using full thickness excisional wound model. No adverse effects of hydrogel treatments on body weight, behavior, or skin such as irritation were observed (data not shown). Effects of gel formulations on wound contraction were evaluated

macroscopically by measuring open wound surface area at different time intervals (0, 2, 4, 6, and 7 days). Figure 3 depicts that the wound closure rates of NaB/Plu gel-treated animals ($82.46 \pm 3.65\%$) were significantly higher than untreated and blank hydrogel treated animals (63.91 ± 6.95 and $73.66 \pm 6.67\%$, respectively) at the end of 7 days of treatment. Although there was no statistical difference in wound contraction rates between vehicle hydrogel and NaB/Plu gel in the beginning of healing process (0–4 days), wounds in NaB/Plu group healed faster between days 4 and 7 over that of vehicle hydrogel-treated group.

Histopathological Examinations

Histopathological outcomes of routine H&E and Masson trichrome stains (TCM) of sections from control, vehicle, and NaB/Plu hydrogel-treated animals at 7 days post excisional wound formation are shown in Fig. 4. Histopathological evaluation of wound tissue after combination gel treatment revealed significant proliferation of

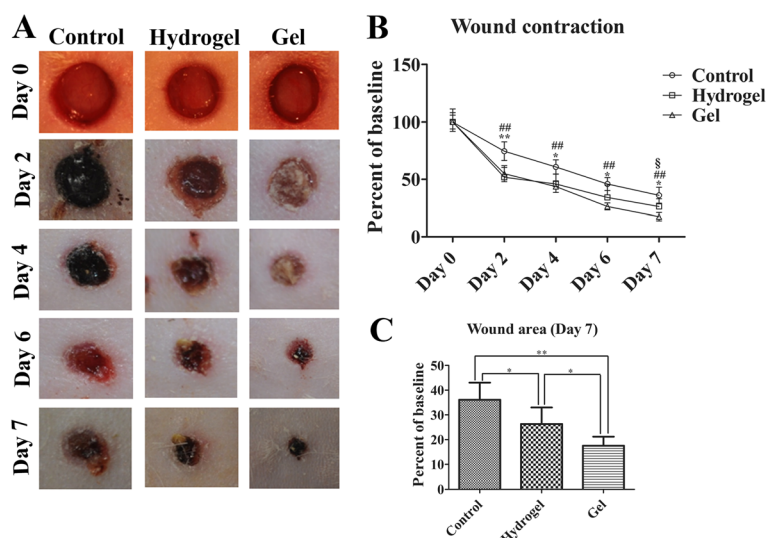


Fig. 3 **a** Photographic representation of wound left untreated (control) or treated with vehicle (hydrogel) and combination hydrogel (Gel). **b** Wound contraction rates were expressed as percentages of baseline ($n=8$), $*P<0.05$, $**P<0.01$: control versus hydrogel, $##P<0.01$: control versus gel, $§P<0.05$: hydrogel versus gel. **c** Wound area

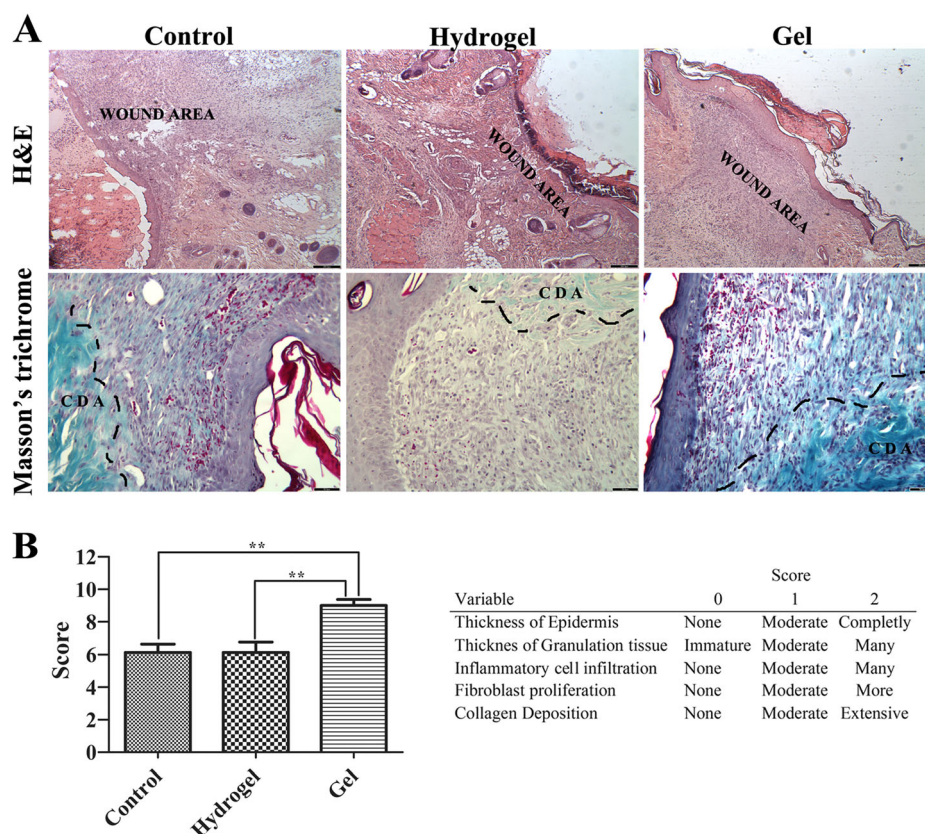
was expressed as percentages of baseline ($n=8$), $*P<0.05$, $**P<0.01$. Hydrogel: vehicle carbopol-based hydrogel, gel: 3 % (w/v) sodium pentaborate pentahydrate, 2 % (w/v) F68 and 2 % (w/v) F127 containing hydrogels. Data represent the mean values \pm S.D. Comparisons were performed by ANOVA (Tukey *post hoc*)

fibroblasts and noticeable collagenization at the wound area compared to control groups. Although epithelization was evident in all groups, obvious difference was noticed in the NaB/Plu group.

Discussion

Impaired wound healing is the major challenge in dermatological clinics. Development of new formulations might

Fig. 4 **a** Histopathological examinations of wound tissue sections performed by H&E (Magnification=4 \times , Scale bars=260 μ m) and Masson's trichrome (Magnification=20 \times , Scale bars: 50 μ m) stainings. Dash lines sides show the histological evidence of collagen deposition area (CDA) along wounding area. **b** Histological scores in wounds ($n=7$) left untreated (control) or treated with vehicle (hydrogel) and combination hydrogel (gel). Hydrogel: vehicle carbopol-based hydrogel, gel: 3 % (w/v) sodium pentaborate pentahydrate, 2 % (w/v) F68 and 2 % (w/v) F127 containing hydrogels. Data represent the mean values \pm S.D. Comparisons were performed by ANOVA (Tukey *post hoc*)



provide an opportunity for treatment of hard-to-heal wounds. In the current study, we developed a new hydrogel formulation containing NaB (boron source) and pluronics (F68 and F127) for effective and functional wound healing. Although the exact action of mechanism has not been elucidated yet, boron has been claimed to increase wound healing activity [23]. In line with these findings, boron-enhanced migration of fibroblast and combining pluronic F68 and F127 with NaB further increased cell migration. In agreement with the in vitro findings, NaB and pluronics containing hydrogel formulations accelerated wound healing in full thickness rat excisional wounds.

TGF- β is a vital growth factor which mediates almost each phase of the wound healing process including inflammation, angiogenesis, cell proliferation, and extracellular matrix production [24]. Moreover, TGF- β has been proven to stimulate human fibroblast migration in vitro [25]. In this line, the migratory action of NaB/Plu gel on fibroblast cells could be explained by high TGF- β expression. TGF- β also regulates other key growth factors and cytokine production and release from wound resident cells. One of the TGF- β signaling regulated cytokines, TNF- α , is necessary for the initiation of an inflammatory response and macrophage infiltration [26]. Increased TNF- α levels in the combination (NaB, F68, and F127)-treated cells might be associated with pro-inflammatory actions of the gel formulation. Treating cells with gel combination also increased the expression of VEGF, a pro-angiogenic factor like TGF- β . Consistent with the current findings, another study found that NaB treatment increased VEGF and TGF- β expressions but not FGF1 and TNF- α expressions [27]. Moreover, combining F68 and F127 with NaB caused a significant increase in TGF- β , VEGF, and TNF- α expression levels. Pluronics have been reported to be used in drug and gene delivery applications, along with a diagnosis as a carrying agent of contrasting chemicals [28]. As pluronic block copolymers form micelles, dependent on temperature and concentration, they increase the activity and transport efficiency of the drug incorporated into the core of micelles by interacting targeted cell membrane and its surface proteins [29]. Therefore, the reason of the highest healing activity in combination gel-treated cells and wounds might be attributed to enhanced boron transportation due to pluronic micelle formation. A second possible explanation for the synergistic activity of boron and pluronics could be the direct healing effect of the pluronics. Pluronic F127 gel treatment has increased VEGF and TGF- β expressions, microvessel density, and wound contraction [24]. In addition, F127 treatment has been found to increase third degree burn wound healing in pigs [30].

Conclusion

The overall data suggest that a combination of boron and pluronics increases fibroblast migration, antioxidant enzyme activity, growth factor expression levels, and acute cutaneous wound healing. Although the results are encouraging, further studies are highly warranted to elucidate the exact mechanism of the synergistic activity between pluronics and boron. In addition, this formulation should be tested in chronic wounds such as trauma, diabetic, decubitus, and venous leg ulcers in order to explore its full potential in dermatological science. Finally, as the formulation provides a remarkable increase in fibroblast proliferation and collagen synthesis, potential use of the formulation for cosmetic purposes should be investigated.

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Conflict of Interest The authors deny any conflicts of interest.

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