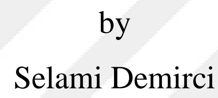
ROLES AND APPLICATIONS OF BORON COMPOUNDS IN CUTANEOUS ACUTE AND CHRONIC WOUND HEALING



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ROLES AND APPLICATIONS OF BORON COMPOUNDS IN CUTANEOUS ACUTE AND CHRONIC WOUND HEALING

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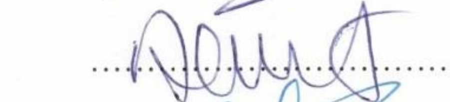

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ABSTRACT

ROLES AND APPLICATIONS OF BORON COMPOUNDS IN CUTANEOUS ACUTE AND CHRONIC WOUND HEALING

Acute wounds do not generally require professional treatment modalities and heal in a predictable fashion, but chronic wounds are mainly accompanied with infection and prolonged inflammation, leading to healing impairments and continuous tissue degradation. Therefore, life-threatening infections, extremity amputations or severely reduced health conditions due to these non-healing wounds are still the major challenges of the current technology. Although a vast amount of products have been being introduced to the market, claiming to provide a better optimization of local and systemic conditions of patients and an ideal wound healing environment, they do not meet the expectations due to being expensive and not easily accessible, requiring wound care facilities, having patient-specific response, low efficiency and severe side-effects. In this sense, developing new, safe, self-applicable, effective and cheap wound care products with broad-range antimicrobial activity is still an attractive area of international research. In the present work, boron derivatives (boric acid-BA and sodium pentaborate pentahydrate-NaB) were evaluated for their antimicrobial activity, proliferation, migratory, angiogenesis, gene and growth factor expression promoting effects on dermal cells *in vitro*. In addition, boron containing hydrogel formulation was examined for its wound healing promoting potential using full-thickness wound model in streptozotocin-induced diabetic rats. The results revealed that while both boron compounds significantly increased proliferation, migration, vital growth factor and gene expression levels of dermal cells along with displaying remarkable antimicrobial effects against bacteria, yeast and fungi, NaB displayed greater antimicrobial properties as well as gene and growth factor expression inductive effects. Animal studies proved that NaB containing gel formulation enhanced wound healing rate of diabetic animals and histopathological scores. Overall data suggest a potential promising therapeutic option for the management of chronic wounds but further studies are highly warranted to determine signaling pathways and target metabolisms which boron is involved to elucidate the limitations and extend its use in clinics.

ÖZET

BOR BİLEŞİKLERİNİN KUTANÖZ AKUT VE KRONİK YARA İYİLEŞMESİ ÜZERİNDEKİ ROLÜ VE UYGULAMALARI

Akut yaralar genellikle profesyonel tedavi yöntemlerine ihtiyaç duymadan tahmin edilebilir bir süreçle iyileşirler, ancak çoğunlukla enfeksiyon ve uzun süreli enflamasyonla beraber görülen kronik yaralarda, iyileşme bozukluğu ve kesintisiz doku parçalanması gözlenir. Bundan dolayı, bu yaralardan kaynaklanan ve yaşamı tehdit eden enfeksiyonlar, ekstremite amputasyonları veya sağlık koşullarındaki ciddi azalış mevcut teknoloji için ciddi bir zorluk oluşturmaktadır. Hastaların lokal ve sistemik koşullarının optimizasyonunu ve daha ideal yara iyileşme ortamını sağladığını iddia eden birçok yeni ürün markete sunulmasına rağmen, bu ürünler kolay ulaşılabilir olmamaları, pahalı olmaları, yara bakım merkezlerine ihtiyaç duymaları, hastaya özgü cevap vermeleri, düşük aktivite ve ciddi yan etkilere sahip olmaları gibi dezavantajlarından dolayı beklentilere cevap verememektedirler. Bu anlamda, yeni, güvenilir, kendi kendine uygulanabilir, etkin, ucuz ve geniş spektrumlu antimikrobiyal özellikleri olan yara bakım ürünleri uluslararası araştırma için halen cazip bir alandır. Bu çalışmada, bor türevlerinin (borik asit-BA ve sodyum pentaborat pentahidrat-NaB) *in vitro* koşullardaki antimikrobiyal etkilnlikleri, deri hücreleri üzerindeki proliferasyon, migrasyon, anjiyogenez, gen ve büyüme faktörü anlatım seviyelerini arttırıcı etkileri incelenmişlerdir. Ek olarak, bor içeren hidrojel formülasyonunun yara iyileşme hızını arttırıcı etkileri streptozotosin kullanılarak oluşturulmuş diyabetik sıçanlardaki tam katlı yara modeli kullanılarak araştırılmıştır. Sonuçlar, her iki bor bileşiğinin de bakteri, maya ve mantarlara karşı dikkate değer antimikrobiyal etkilerinin varlığının yanı sıra, deri hücrelerinin proliferasyonunu, migrasyonunu, önemli gen ve büyüme faktörü anlatım düzeyini arttırdığı göstermesine rağmen, NaB'nin antimikrobiyal etkinliğinin, gen ve büyüme faktörü anlatım düzeyini arttırıcı etkisinin daha fazla olduğu tespit edilmiştir. Hayvan çalışmaları, NaB içeren jel formülasyonunun diyabetik sıçanların yara iyileşme oranını ve histopatolojik skorları arttırdığını kanıtlanmıştır. Elde edilen veriler kronik yaraların tedavisi için potansiyel umut verici bir tedavi seçeneği sumaktadır, ancak borun klinikteki sınırlarını

belirlemek ve kullanım alanlarını genişletmek için, etkilediği sinyal yolaklarının ve hedef metabolizmalarının ek çalışmalarla belirlenmesine şiddetle ihtiyaç duyulmaktadır.

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LIST OF SYMBOLS/ABBREVIATIONS

ADSC	Adipose derived stem cells
AFMSC	Amniotic fluid derived mesenchymal stem cell
AGE	Advanced glycation end-products
AKT	Protein kinase B
AR	Androgen receptor
BA	Boric acid
BM-MSC	Bone marrow-derived mesenchymal stem cells
BMP	Bone morphogenic proteins
bNGF	Beta nerve growth factor
CFU	Colony forming unit
COX	Cyclooxygenase
CTGF	Connective tissue growth factor
DETCs	Dendritic epidermal T-cells
DFU	Diabetic foot ulcer
DHEA	Dehydroepiandrosterone
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EBM	Endothelial basal medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
eNOS	Endothelial nitric-oxide synthase
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
G-CSF	Granulocyte colony stimulating factor
GDNF	Glial cell line-derived neurotrophic factor
GM-CSF	Granulocyte-macrophage-colony stimulating factor
H&E	Hematoxylin and eosin
H_2O_2	Hydrogen peroxide

HA	Hyaluronic acid
HB-EGF	Heparin binding epidermal growth factor
HBO	Hyperbaric oxygen therapy
HF	Human fibroblast
HGF	Hepatocyte growth factor
$HOCl$	Hypochlorous acid
HUVEC	Human umbilical vein endothelial cells
IGF	Insulin like growth factor
IGFBP	Insulin-like growth factor-binding protein
IL	Interleukin
iNOS	Inducible nitride oxide synthesis
iPSC	Induced pluripotent stem cell
KGF	Keratinocyte growth factor
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MFC	Minimum fungicidal concentration
MIC	Minimum inhibition concentration
MIP1α	Inflammatory protein 1 alpha
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
MTS	3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium
NaB	Sodium pentaborate pentahydrate
NaBC1	Electrogenic Na^+-coupled borate transporter
NADPH	Nicotinamide dinucleotide phosphate oxidase
$NaOH$	Sodium hydroxide
NGF	Nerve growth factor
NO	Nitric oxide
HO	Hydroxyl radical
NSAID	Non-steroidal anti-inflammatory drugs
O^{2-}	Superoxide ion
PCL	Poly(ε-caprolactone)

PF4	Platelet factor 4
PI3K	Phosphoinositide 3-kinase
PLGA	Poly (lactic acid-*co*-glycolic acid)
PSA	Penicillin, streptomycin, and amphotericin B
ROS	Reactive oxygen species
SDF	Stromal-derived factor
STZ	Streptozotocin
TGF-β1	Transforming growth factor beta1
PDGF	Platelet-derived growth factor
TIMP	Tissue inhibitors of matrix metalloproteinase
TNF-α	Tumor necrosis factor alpha
UCB	Umbilical cord blood
VEGF	Vascular endothelial growth factor
VEGFR1/Flt1	Vascular endothelial growth factor receptor 1
VEGFR2/KDR/Flk1	Vascular endothelial growth factor receptor 2

1. INTRODUCTION

Wounds remain a serious problem for their early and late complications in clinical practice as they may result in high incidence of sickness and death [1]. In basic technology, new therapeutic agents and wound management strategies are being developed with the help of understanding the physiology and molecular mechanism of the wound healing process to decrease wound impairments and its complications [2]. The importance of wound management and new therapeutic agents is due to high incidence of chronic wounds especially among older populations. The prevalence of wounds, not only the acute one but also disease or abnormality associated chronic wounds, increases by the age. It has been reported that the frequency of chronic wounds among 45-65 years old people is 1.2% while it is about 8% in elderly population who are at the age of 75 years old or above [3].

A cutaneous wound defined as the disruption of the skin integrity, cellular function and anatomic structure results in partially or fully loss of function [4,5]. Wound can occur due to various reasons such as inflammation, infection, forceful trauma, metabolic dysfunction, hypoxia, extreme heat or cold [6,7]. The primary function of the skin is to protect the body against various environmental impacts and to provide hemostasis such as insulation and temperature regulation. Therefore, deformation in the skin can cause serious pathological situations including body fluid loss, infections, electrolyte imbalance [8]. In addition, large-scale damages in the skin caused by a disease or an injury can end up with serious dysfunctions or even death [9].

After the skin is torn, cut or punctured called as open wounds, or bruised by a traumatic force referred to as closed wounds, the body produces an immediate response (wound healing process) to the injury in milliseconds. This immediate response activates functional and comparable regeneration process [10]. Skin regeneration is, except fetal healing, not identical to a healthy skin. In general, wound healing consists of functionally and histologically distinct but transiently overlapping and well-orchestrated three main phases; (i) hemostasis and inflammation, (ii) epithelialization, angiogenesis and granulation tissue formation, and (iii) tissue remodeling [11]. Various cell types, growth factors, chemokines

and other proteins are linked to wound healing in a tightly controlled manner for functional and efficient tissue regeneration (Figure 1.1).

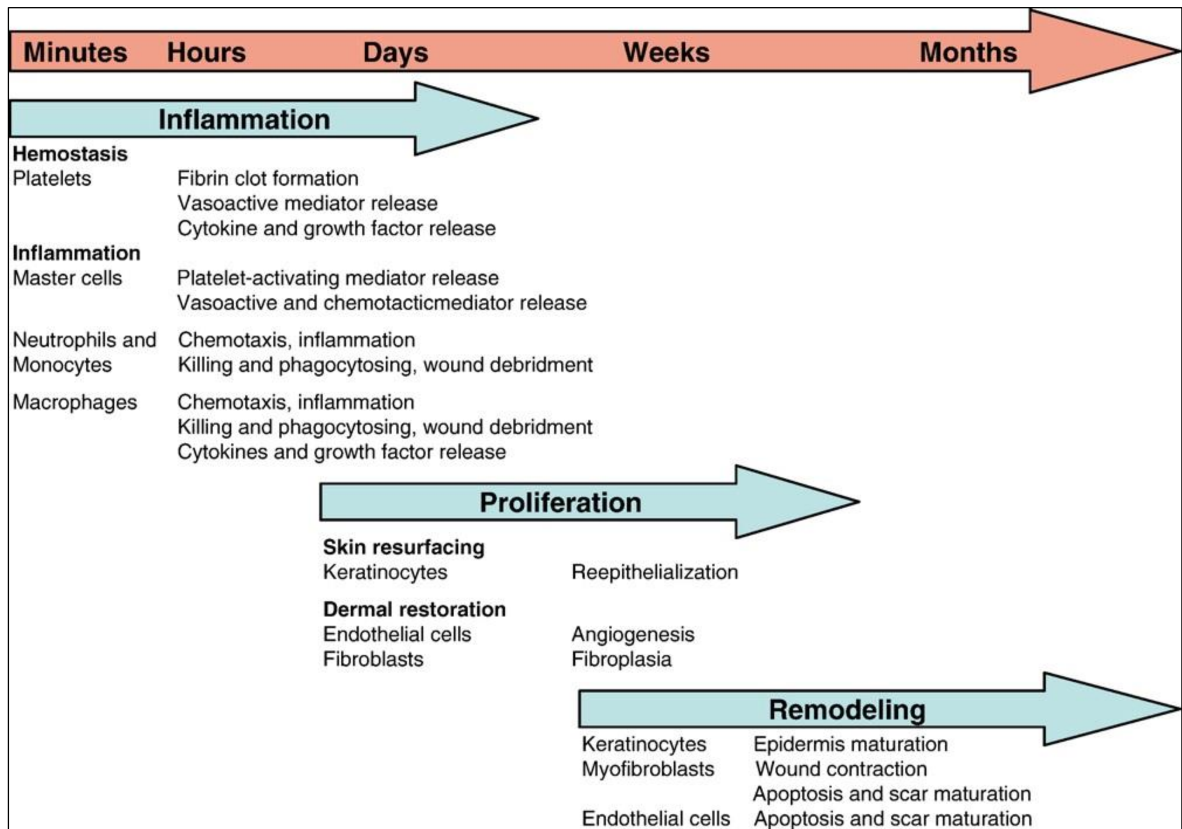


Figure 1.1. Major cells and steps in wound healing [10].

1.1. ANATOMY OF THE SKIN

Skin, the largest tissue in the body, is mainly composed of two connected layers; epidermis and dermis (Figure 1.2). These two layers are connected to each other by dermal-epidermal junctions. The outermost layer, epidermis, is stratified squamous epithelium that consists of melanocytes, Langerhans' cells, inflammatory cells, Merkel cells and primary keratinocytes with different differentiation stage [12]. Non-differentiated cells remain close to dermal layer while differentiating keratinocytes migrate towards outside. The main extracellular matrix (ECM) component in epidermal layer is hyaluronic acid (HA). The proliferation and loss of keratinocytes are equal so that epidermal layer renews its whole cell reservoir every 48 days [13]. Some components of epidermal layer reach and anchor deep dermal layer of dermis such as hair follicles, sweat glands and sebaceous glands [14]. On the other hand,

dermal layer includes several cell types such as fibroblast that produce ECM proteins (primarily collagen, elastin), endothelial cells that forms vascular network, dendritic cells, mast cells, neurons, hematopoietic originated macrophages and lymphocytes [15].

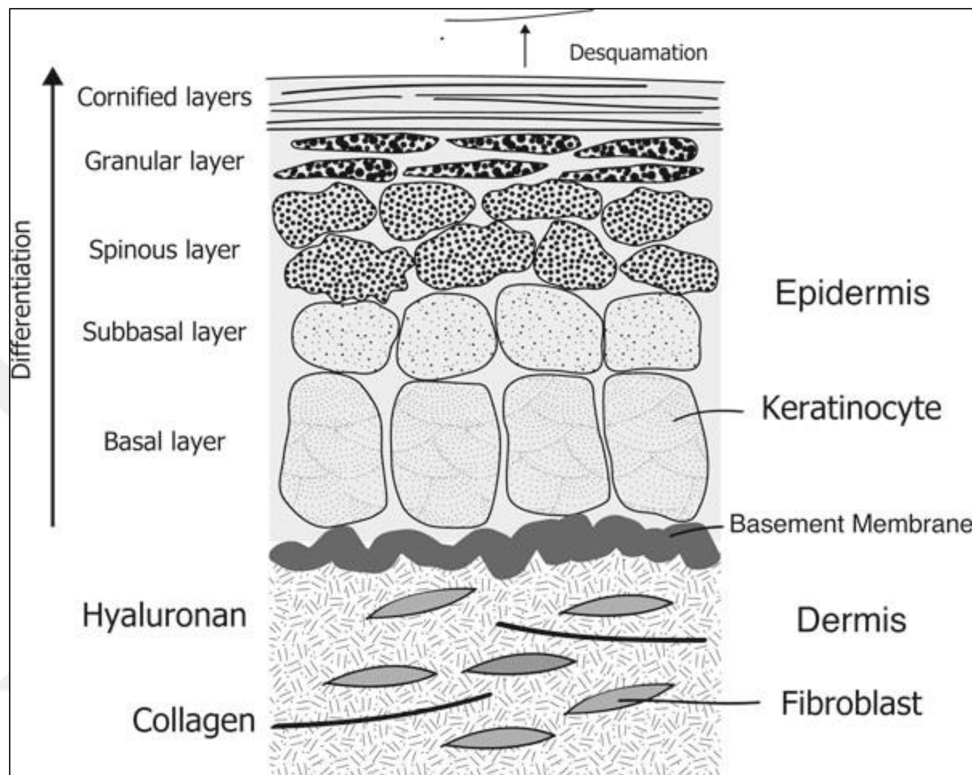


Figure 1.2. Basic structure of skin.

1.2. HEMOSTASIS AND INFLAMMATINON

In the case of vascular network injury upon the dermis layer during wound formation, bleeding occurs towards wound bed. In a normal body, bleeding is immediately stopped by means of physical and functional interventions, called as hemostasis consisting of two main processes; fibrin clot formation and coagulation [16]. A healthy hemostasis progress prevents exsanguinations so that the body can keep other organs unaffected except the injury site [3]. The second important function of the hemostasis is directly connected with the functional healing process. Bleeding triggers ECM protein synthesis such as collagen, fibronectin, and vitronectin that provide a suitable environment for cellular invasion into the wound bed [17,18]. Blood platelets, the first appeared cell type in the wound site, generate platelet plug in parallel with coagulation to stop bleeding. At the place of injury, cells starts

to secrete vasoconstrictors thromboxane A2 and prostaglandin 2-α that initiates vasodilatation to allow an increase in cellular trafficking [19]. After the emergence of coagulation by dense vasoconstriction followed by vasodilatation, there is an increase in capillary permeability leading to a leakage of matrix proteins [7]. Of those, soluble fibrinogen is converted into insoluble fibrin matrix fibers for a stable environment in which $\alpha_{IIb}\beta_3$ receptor provides the binding of activated platelets to fibrin clot [20]. This complex and filamentous structure not only forms a shield against microbial invasion but also remains as storage of growth factors required for cell activation and protein expression. The activation of platelets comes up with various types of growth factor and cytokine release including platelet factor 4 (PF4), transforming growth factor β1 (TGF-β1) and platelet derived growth factor (PDGF) which take place in different phases of wound healing such as cell adhesion and proliferation, extracellular matrix protein deposition, and angiogenesis [21,22].

As the hemostasis carries on activating cells, inflammatory phase is been initiated to fight with any microbial invaders and direct wound healing key players to the injury site. There are two separate inflammatory response during this phase; early and late responses [23]. In the last period of coagulation formation, early inflammatory response starts with launching recruitment of leukocytes to the wound bed [19]. Resident immune cells are activated by tissue damage associated pro-inflammatory molecules including cytokines, interferons, and lipopolysaccharides and hypoxic condition, which in turn increases levels of inflammatory signal molecules [24]. These molecules further provide direction of neighbor immune cells into the injury site. Resident platelet cells and macrophages secrete vital signaling molecules such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, TGF-β, and PF4 that attract neutrophils [19,25]. They enter wound bed within the first 24 hours and carry out phagocytosis of microbial invaders and debris by means of reactive oxygen species (ROS) and proteolytic enzymes [11]. ROS produced by leukocytes can be in different forms such as superoxide (O_2^-), hydrogen peroxide, (H_2O_2), hypochlorous acid (HOCl), nitric oxide (NO), peroxynitrite (OONO), hydroxyl radical (OH^-), chloramine and derivatives (R-NH2CI), and thiyl radicals [24]. In the very early step of inflammation, neutrophils are predominant cell type until they are replaced with monocytes. Microorganisms may enter the body through a break in the skin and produce some chemical molecules and microbial protein byproducts such as formylmethyonil peptides which also activate more neutrophils [26,27].

Struggling with bacteria and debris cause a definite change in the surface proteins of neutrophils and provide a sticky behavior which keeps neutrophils in wound area [23]. After finishing the task, neutrophils' function decreases dramatically. In order to prevent further tissue damage in the context of producing more ROS and strengthening of inflammatory response, neutrophils are taken away from the damaged tissue [28]. They are exported to the wound surface where they undergo apoptosis. Residual of the cells and debris are then phagocytosed by resident and invading macrophages [3].

After the body is secured from any possible infection by primary soldiers (neutrophils), various growth factors, chemokines such as PDGF, nerve growth factor (NGF), vascular endothelial growth factor (VEGF), TGF-β, leukotriene B_4, PF4, inflammatory protein (MIP)-1α and monocyte chemoattractant protein (MCP)-1 along with elastin and collagen byproducts attract circulating monocytes into wound site [29,7]. Monocytes attach to the fibrin clot via their integrin receptors and turn into matured macrophages. Macrophages are the most important intermediates of the inflammatory phases so that an efficient proliferative phase is not possible without macrophages infiltration. Studies have proven that lack of macrophages in wounded tissue results in healing impairments in the way of ineffective collagen synthesis, angiogenesis, granulation tissue formation and fibrosis [30,31]. After gaining activated phenotype, macrophages go on clearing cell debris and bacterial residuals. During this processes, macrophages secrete myriad of chemoattractants such as IL-1, IL-6, IL-12, TNF-α and inducible nitride oxide synthesis (iNOS) to induce further macrophage infiltration as well as enhancing dermal cell proliferation, ECM synthesis and angiogenesis [32]. As a specific example, macrophages have been shown to secrete excess amount of VEGF which is responsible for almost half of the pro-angiogenic effect in a wounded tissue [33]. The final participant of the inflammation step is lymphocytes activated by IL-1, complement factors and IgG by products [28]. Although the exact role of the T cells in wound healing is not well elucidated yet, one possible scenario is that the direct interaction between platelets, macrophages, keratinocytes, fibroblasts, endothelial cells and lymphocytes regulates chemoattractant secretion [34]. It has been proven that T-helper cells improve healing efficiency, while T suppressor cells inhibit wound healing [35,36]. Besides, dendritic epidermal T-cells (DETCs) provide efficient growth factor and cytokine secretion required for a healthy inflammation by cell-cell direct connection.

At the end of the inflammatory phase, the inflammatory response should be suppressed in order to shift toward the second stage of the wound healing process. As platelet plug and fibrin matrix are successfully organized and no more bacterial and tissue residue remains in the wounded section, macrophages change their pro-inflammatory status to anti-inflammatory status called as alternative phenotype. They starts to secrete IL-10, IL-1 receptor antagonist, and decoy IL-1 receptor type II to decrease inflammatory effect and TGF-β, VEGF, and insulin like growth factor (IGF)-1 to induce cell proliferation and granulation tissue formation [37].

1.3. PROLIFERATIVE PHASE

There are no certain lines between wound healing phases. Duration of the phases is strongly dependent on the degree of the wound, bacterial infection and the amount of debris in the wounded area. However, as soon as the inflammatory symptoms start to decline, proliferative phase begins. The proliferative phase is turned on by growth factors and cytokines of resident macrophages and active platelets [38]**.** In the proliferative phase, the main function is to restore lost or damaged skin layers roughly. This phase can be divided into four main subgroups; re-epithelialization, re-vascularization (angiogenesis), granulation, and contraction.

1.3.1. Re-epithelialization

The epidermal progenitor cells, at the end of the inflammatory phase, start to migrate and cover the denuded area in order to build a second barrier [39]. In brief, re-epithelialization can be summarized as migration, proliferation and differentiation of keratinocytes at the edge of the wound tissue. The mechanism of re-epithelialization and cell proliferation differ in epidermal (partial) and full thickness (both dermal and epidermal) wound. In epidermally damaged tissue, keratinocytes found in the edge of the injured tissue is first activated by the “free edge” effect that triggers keratinocyte migration towards wound bed [17]. The cell-cell interactions keep them silent, but as soon as the connection between them is broken by a cutaneous wound, the migration and motility of the cells in the adjacent tissue is activated [39]. The centripetal migration is highly important that only cell migration from the neighbor tissue would be enough to cover the damaged area without the necessity for proliferation

under certain wound size [40]. First action of the basal and suprabasal keratinocyte migration is losing attachment and being free to travel through provisional matrix. Additionally, keratinocytes secrete proteases for the breakdown of matrix proteins and change their adhesion characteristics [41,42]. During and after the migration of keratinocytes, they are induced to proliferate by various type of growth factors, mainly epidermal growth factor (EGF), TGF-α, IL-1α, TNF-α, keratinocyte growth factor (KGF) and IL-6 [39]. Although the exact explanation of why migration stops is not available yet, one possible explanation is the action of contact inhibition. In partial injury, keratinocytes move and proliferate in order to regenerate the tissue lost without synthesis and deposition of extracellular matrix proteins. However, in full thickness wounds, fibroblasts, keratinocytes and endothelial cells found in the intact skin move and proliferate along with functional protein synthesis, granulation and angiogenesis for a complete healing. Although the mechanism of cell migration is quite complicated, at macroscopic scale, the membrane profiles and phenotypes of the cells are changed via stimuli coming from adjacent cells for a migration and proliferation active status [43]. Signaling molecules force cells to lose their desmosomes and hemidesmosomes which provide the connection with keratin cytoskeletal network and basement membrane [14]. Suprabasal layer cells of the epidermis are mainly induced by newly exposed ECM and start to express keratin-16, Ki67 (the proliferation marker), matrix metalloproteinase (MMP)-2 and MMP-9 [44,45]. They attach to fibronectin, vitronectin and collagen via expressing newly synthesized integrins. Grose and colleagues have shown the deletion of β1 integrins (responsible for attachment of keratinocyte to fibronectin and collagen) detrimentally effects wound healing *in* vivo [46]. After the closure of the entire wound surface by migrated cells, the cells stimulated by KGF, EGF, fibroblast growth factor (FGF)-2, heparin binding EGF (HB-EGF), FGF -10, PDGF-β and granulocyte-macrophage-colony stimulating factor (GM-CSF), TGF-β to divide vertically to produce newly formed basement [32,14]. Moreover, these growth factors (especially FGF-7 and FGF-10) induce scavenging of ROS which prevent ROS activated apoptosis in wound [47]. However, the role of TGF-β1 is not well understood yet. The results from *in vitro* and *in vivo* studies are contradictory. Although TGF-β1 expression was found to increase the migratory integrin expression [48,49], it has also been proven to have inhibitory effect on the proliferation of keratinocytes in re-epithelialization [50]. Another conflicting result was published about increase in re-epithelialization via exogenous TGF-β application in porcine cutaneous wound [32]. However, Tredget and coworkers have proven that TGF-β increases re-

epithelialization in partial wounds, but inhibits in full thickness wounds [51]. In accordance with these data, it can be concluded that TGF-β may have migratory effect on keratinocyte and effective in partial wounds. Further studies are still required for a better understanding of growth factors' role on wound epithelialization. After the re-epithelialization completed in wound bed, proteinase expression is turned off and cell attachment to the basal lamina is provided by hemidesmosomes formation.

1.3.2. Granulation Tissue Formation

As epidermal layer of the wounded tissue is almost closed via keratinocyte migration and organized as a zipper-like shape, phenotypes and expression profiles of keratinocytes turn back to their standard conditions [52]. Fibroblasts found in the edge of the intact dermal layer start to migrate to wound inward as soon as keratinocytes form a safe barrier in the re-epithelialization step. According to an old general belief, all migrated fibroblast arises form dermal progenitor cells and resident fibroblasts. However, a recent study has proven a small part of fibroblast society originated from bone marrow derived stem cells that are stimulated to migrate to the injury site [53]. Migrating fibroblasts' main function is synthesizing and modeling extracellular matrix proteins (mainly collagen) referred to as granulation tissue. In granulation tissue formation phase, fibroblast travel towards wound center, proliferate and produce new extracellular matrix proteins. Growth factors (mainly PDGF, TGF-β and connective tissue growth factor (CTGF) secreted by platelets, macrophages, resident fibroblasts induce the proliferation and collagen deposition of fibroblast [54-56]. These growth factors orientate mainly ECM deposition such as collagen, fibronectin, laminin, proteoglycans and elastin [10]. ECM deposition is a vital factor in wound healing process as the matrix provides a rigid scaffold as stimulator reservoir and a safe path for cell attachment and migration. One of these stimulators, PDGF, primarily activates fibroblasts in both autocrine and paracrine manner. In addition to PDGF, there is an excess amount of inactive TGF-β (as pro-TGF-β) in the fibrin clot at beginning of granulation tissue formation step. Resident cells secrete proteinases which activate TGF-β. TGF-β triggers fibroblast to produce more ECM components, tissue inhibitors of matrix metalloproteinase (TIMP), and integrin receptors [19]. TGF-β deficiency was shown to inhibit granulation tissue formation *in vivo* [57]. Apart from growth factors, acidic and anaerobic conditions in the wound center

also direct cells to migrate directly towards to the center [19]. As new vessel formation starts, the wound bed is turned to oxygenic conditions so that the activation of fibroblast decreases.

1.3.3. Angiogenesis

New blood vessel mesh should be reorganized to provide nutrients and oxygen for migrated cells, and replacement of metabolic waste from the injured area in full thickness wounds. Angiogenesis, referred to as new blood vessel formation from preexisting ones adjacent to the wound, takes place in parallel with fibroblastic activation. Resident cells (macrophages, keratinocytes and fibroblast cells) secrete various type of pro-angiogenic factors. Activators of angiogenesis are primarily VEGF, PDGF, FGF-2 (also known as basic (b)FGF), IL-1, TNF-α, TGF-β, FGF-10, PF-4, angiogenin, angiotropin, angiopoietin, IL-8, serine protease thrombin, hypoxic environment, lactic acid and nitride oxide [19,58]. Activated endothelial cells migrate to and proliferate in the wound bed, forms tubular structure, and finally provide initial blood flow [59]. Endothelial secrete proteases that degrade basal lamina so that new blood vessels can go into wound center (called as "sprouting"). Although the level of VEGF, the main stimulator for angiogenesis, in intact wound is quite low, it suddenly increases at the time of injury as a result of low oxygen tensions [60]. VEGF activate endothelial cells trough two specific receptors; VEGF receptor 1 (VEGFR1/Flt1) and VEGF receptor 2 (VEGFR2/KDR/Flk1) [61-63]. As the endothelial cells activated for migration and proliferation, their motility is strictly dependent on a well-organized ECM [62,63]. Of those ECM proteins, laminin was shown to be indispensable for proliferation and tubule formation of human dermal microvascular endothelial cells [64]. Endothelial cells start to express specific integrin receptors which are not expressed ordinarily to migrate and adhere to specific ECM protein [65]. $\alpha_v\beta_3$ integrin receptor has been proven to be expressed only during wound healing process, and to have regulatory role in angiogenesis as well as MMP-2 deposition on endothelial cell surface [8].

1.3.4. Wound Contraction

In granulation tissue formation and angiogenesis phases, sufficient oxygen, nutrient and waste trafficking are provided and adequate number of cells are generated. After the granulation tissue formation, wound fibroblasts undergo myofibroblast differentiation by

producing actin microfilaments [66]. It is the main part of the wound closure as it has been proven that contraction is responsible for about 90% decrease in wound size in mice and 50% in human [67,14]. After differentiation of myofibroblasts, they start to express α-smooth muscle actin, myosin and desmin [10]. Transformed myofibroblasts change their integrin receptor profiles and bind to specific ECM components and each other. This complex network allows the transmission of myofibroblast contraction to whole wound and edges of the tissue [68]. Protrusions of cell edges widen and bind to ECM with its specific receptors which in turn cause retraction of the wounded tissue sides into the center [10]. In order to provide a proper wound contraction and myofibroblast differentiation, however, growth factor stimulation is compulsory. Two main growth factors, TGF-β and PDGF, have been indicated as key regulatory elements in myofibroblast differentiation and wound contraction [69]. *In vitro* and *in vivo* studies have shown that efficient myofibroblast differentiation and smooth muscle expression in fibroblast are provided by proper TGF-β stimulation [70,71]. Furthermore, IL-8 have been linked to inhibition and retardation of wound contraction which may be a possible explanation for why long lasting inflammatory phase prevent a complete wound healing and regeneration [7]. In conclusion, the proliferative phase of the wound healing is quite complex, masterfully orchestrated and consisting of several overlapping functions in which myriad of growth factors, cytokines, proteins and cells are included. After an untroubled epithelization, contraction and angiogenesis, inappropriate ECM proteins should be rearranged to provide high tensile strength.

1.4. REMODELING

During the proliferative phase, newly exposed ECM components are arranged randomly and should be organized in a correct manner in remodeling phase. This phase can take several weeks, even years depends on wound type, place and environmental conditions. Randomly arranged collagen fibrils and proteoglycans are remodeled in this step to provide an adequate tensile strength and mechanical force as in intact skin before wounding [72]. Collagen type III, predominant granulation tissue component at the beginning of remodeling phase, is replaced by collagen type I [73]. Apart from collagen, fibronectin and hyaluronic acid are degraded in the scar tissue via MMPs secreted by resident fibroblast endothelial cells and

macrophages [74]. Different forms of collagen are constantly produced and degraded under the control of MMPs and TIMPs.

In the remodeling phase, the cellular and vascular components of the granulation tissue is reduced for an effective skin pattern. The cells, not essential anymore such as most of neutrophils, macrophages, and myofibroblasts, undergo apoptosis or pass towards neighboring tissue leaving a few cells behind [75,76]. Moreover, revascularization occurs at this time to arrange blood vessel network. As angiogenic activity decreases due to oxygenic condition and blood flow diminishes, the color of wounded area turn from red to pink as a result of reduction in capillary density [8]. Having limited data in the literature, exact molecular mechanisms of remodeling is not well elucidated. There is a still huge requirement for *in vivo* and *in vitro* studies in this subject. Fully organized intact skin as before wounding can never be regenerated even in an functional and effective healing process [77]. Hair follicles or sweat glands, normally found in skin, cannot be recovered after a significant injury [58]. In addition, an important dermal-epidermal tight connection mediator, rete pegs, found in the connective tissue, is not reconstructed after the damage [2]. Thus, tensile strength would not be as in normal intact skin. It was reported that one week after the injuries the wound can recover only 3%; 30% in three weeks and an almost 80% of normal skin strength after three months [78].

New findings in wound healing studies contribute to and extend our understanding of the cascade day by day. As the mechanism gets unraveled, it becomes more complex to elucidate. Despite the fact that wound healing process is quite puzzling, it is more than that in chronic wounds. The standard wound healing mechanism is not applicable to chronic wounds because of having variable phase durations depend on the chronicity.

1.5. CHRONIC WOUNDS

Acute wound healing, as mentioned above, is a routine processes in which several types of cells, growth factors, proteins, and cytokines are involved in a well-organized manner. Any scarcity or redundancy of growth factor secretion or disruption of the functional queue can cause prolonged phases (mainly inflammatory phase) and wound impairment. Most of these situations result in severe pathological conditions called as chronic (non-healing) wounds.

The major types of chronic wounds are pressure ulcers, diabetic lower extremity ulcers, and venous stasis ulcers [79]. From a scientific point of view, chronic wound is a quite hot topic in dermatology as it displays a major health burden and its incidence is very high in especially aged and/or diabetic populations. It is estimated that 15% of diabetic patients experienced chronic wounds and 600.000 limb amputation, with a life expectancy of 5 years (69%) after the surgery, per year in US [80,81]. Any wounds can pass into chronic stage when inflammatory response prolongs, cytokine and growth factor profile changes, secretion schedule of proteinases and TIMPs alters or proliferation of wound cells is inhibited via senescence [39]. General characteristic of chronic wounds is primarily extended inflammation phase. Unlike healing wounds, chronic dermal ulcers have been shown to display sustained pro-inflammatory cytokines and growth factors, less fibroblast and keratinocyte proliferation activity [72]. Excessive infiltration of inflammatory cells such as neutrophil and macrophage result in high expression levels of pro-inflammatory cytokines including IL-1, IL-6 and TNF-α, ROS, and MMPs (MMP-2, MMP-9, and MMP-13) as well as low levels of TIMPs [72]. In addition, elevated MMP activation causes degradation of vital growth factors including PDGF which has been found insufficient in chronic dermal ulcers [82]. Apart from MMPs, ROS produced by immune cells causes tissue and newly exposed extracellular matrix degradation [83]. Therefore, inflammatory response should be controlled to improve chronic ulcers as a first line management strategy. In order to heal chronic ulcers, the reason for chronicity should be removed. Factors that affect wound healing should be carefully examined and determined in clinical care units to control chronic wounds.

1.6. FACTORS AFFECTING WOUND HEALING

The complexity of wound healing process makes injured area susceptible to systemic and local problems. Although systemic factors are strictly connected with the health status of the individuals, local factors have a straight relationship with wound itself [84]. As these factors can influence the normal scheme of wound healing, it may delay tissue regeneration and even cause wound healing impairments. The next section is introduced to clarify main factors involved in chronic wound formation from an experimental and clinical perspective.

1.6.1. Local Factors

The normal scheme of the wound healing processes can be detrimentally affected and delayed by local factors such as ischemia, bacterial infection, dehydration, necrosis, edema and pressure.

1.6.1.1. Ischemia

Oxygenation is a vital process in a healthy healing process as it is involved in almost all phases of wound healing. It is required for bacterial infection prevention, wound cell activation, migration and proliferation, wound contraction and remodeling [85]. In microscopic scale, oxygen is essential for the production of ATP required for sustainability of cytokine production, cell proliferation and migration in metabolically active wounds [86]. Wound cells produce ATP via glycolysis for metabolic functions in earlier phases of wound healing [24]. On the other hand, much more ATP is needed for cell proliferation, migration and protein synthesis at later stages. Therefore, cells should switch their energy producing status to oxidative phosphorylation. In addition, oxygen is required for the ROS production during the inflammatory phase as it is expected. ROS production is induced by not only hyperoxia but also hypoxia. Therefore, prolonged hypoxic conditions originated from systemic dysfunctions such as diabetes and vascular inadequacy may cause excess ROS formation which in turn result in further tissue breakdown [87]. Although hypoxia acts as a stimulator for cytokine secretion, angiogenesis, cell activation, proliferation and migration in early phases of the wound healing, elevated hypoxic conditions lead to chronic wound formation [88]. Transcutaneous measurements and *in vivo* studies have proven that impaired wounds are more hypoxic than normal healing wounds [89].

The reason for an ischemic wound is mostly systemic such as capillary density at the skin, peripheral vascular diseases (main symptom of diabetes), atherosclerosis, anemia or heart failure [86]. These complications should be addressed by therapeutic interventions and medications. Apart from systemic problems, prolonged pressure as in bedridden patients can cause decrease in blood flow at the area under pressure. This eventually causes oxygen inadequacy and hypoxia around injured tissue leading to ischemic tissue and subsequently chronic wound.

1.6.1.2. Microbial Infection

As wound is formed, microorganisms found in the dermal flora gain access to get in underlying tissues. As explained in previous sections, inflammatory phase takes care of microbial invasion via neutrophils, macrophages and their products such as proteases and ROS in normal healing conditions. Microorganisms in wounded tissue are characterized as contamination (nonreplicative microorganism), colonization (biofilm producing and limited number of microorganism), critical colonization/local infection (only granulation tissue infecting microorganism) and invasive infection (additional surrounding tissue infecting microorganism) [90]. Microbial function in the wound is dependent on host response, inflammatory cell activity, number, pathogenicity and virulence of microorganism [91]. In general, healthy intact skin carries 10^5 CFU/gr tissue and higher than this number account for a wound infection [92]. However, some microbial species such as β-hemolytic *Streptococci* spp. cause detrimental wound infections under 10^5 CFU/gr tissue because of having high virulence factor [93]. Therefore, determining microbial number in the wound site is not always a good way for infection diagnosis. Polymicrobial contaminations can also result in severe pathological conditions because less virulent microorganisms can affect synergistically each other. Apart from β-hemolytic *Streptococci* spp., *Pseudomonas* spp. and *Staphylococcus* spp. are predominant in both chronic and acute wound tissues [84]. *Pseudomonas* spp. communicates each other and produce biofilm layer containing extrapolymeric substances composed of polysaccharides, nucleic acid, protein, and cellular debris [94]. This biofilm resist to current therapies and act as a shield for the bacteria. Prolonged microbial survival in the wound bed causes excessive protease and endotoxin secretion leading to degradation of proliferating cell and ECM proteins. Besides, proliferating microorganisms stimulate resident cells to produce pro-inflammatory cytokines and growth factors such as IL-1 and TNF-α that promotes chronic inflammatory phase [95]. These elevated response also causes constant expression of MMPs and sudden decrease in TIMPs that ends up with continuous surrounding tissue degradation [96].

In order to improve chronic wound healing arisen from infection, inflammatory response should be suppressed and microbial contamination should be removed from wound area. Antibacterial formulations such as silver containing products and antibiotics are used in current clinical practices, but antibiotic resistance and severe side effects are generally experienced during treatments. Chronic silver toxicity has been reported in patients exposed

to topical silver sulfadiazine during the treatment of burn wound infections [97]. Bacterial species are primary sources of the wound infections, but they are not alone. Especially in burn wounds, fungal infections results in severe morbidity and mortality. It has been published that controlling bacterial infections with topical antimicrobial therapy and systemic antimicrobial agents is much more effective compared to fungal infections [98]. These fungal infections remain stable due to the limited number of available antifungal agents and toxicity of antifungal agents which have already been used [99]. Therefore, new, safe, antimicrobial (antibacterial, anticandidal and antifungal) agent should be introduced to be used in infected wound treatments. However, as chronicity originates from several independent factors, treating infection may not be a complete solution alone for chronic wounds. Therapeutic agents should be antimicrobial along with promoting other factors which is insufficient in chronic wounds such as fibroblast proliferation.

1.6.1.3. Pressure

Although some patients do not have any systemic problems, they develop chronic wounds due to sustained and/or excessive pressure exposure. Increased pressure, both internal (compartment syndrome) or external (extrinsic force) leads to hypoxia as a result of decrease in capillary blood flow [100]. If this undesirable situation elevates due to mandatory such as bed disability, tissue undergo necrosis referred to as pressure ulcers [19].

1.6.2. Systemic Factors

Some systemic disabilities can obstruct wound healing although they are not directly related with wound itself and its location. Age, gender, sex hormones, alcoholism and smoking, nutritional status, chronic diseases (such as diabetes, obesity, uremia, rheumatoid arthritis and chronic osteomyelitis), medications, immunosuppression and radiation are the most important systemic factors that have profound effects on wound healing.

1.6.2.1. Age and Gender

Wound healing gets harder and delayed as people ages. Even healthy older people (above 60 years of age) without any systemic disorders can experience delay in wound healing [101]. In contrast, young people have more active cells with high proliferation rate, more ECM proteins production capacity that improve skin tensile, and inflammatory response

[102]. Although, there isn't any other systemic differences between young and old mice, it has been reported that old mice have experienced elevated inflammatory cell infiltration and angiogenesis, decreased ECM synthesis, poor skin tensile, insufficient growth factor release, cell activation and proliferation rate compared to younger mice [103]. Moreover, gender and sex hormones play crucial roles in aged skin wound healing. Estrogens, androgens and their steroid precursor dehydroepiandrosterone (DHEA) have vital influences on wound healing process [104]. Aged males have been reported to experience more healing impairments in contrast with aged females [84]. Administrations of estrogen (topically or systemically) have increased wound healing *in vivo* via decreasing inflammatory response [105] while androgen application detrimentally decreases wound healing by promoting inflammation [106]. Therefore, estrogen application for wound healing is less effective in aged males than aged women probably due to androgen level sustainability in male blood. Although, exact mechanism of sex hormones' contribution to wound healing remains unclear, accumulating evidences suggest that males are more susceptible to have chronic wounds due to their androgenic status. Apart from being older, *in vivo* studies have also shown that absence of estrogen in ovariectomized young female rodents have displayed delayed cutaneous wound healing [107]. The result has been confirmed by restoring healing impairment by exogenous estrogen treatment. In general, estrogen directly affects wound healing associated growth factors and cytokine secretion profile. Pro-inflammatory cytokines such as TNF-α reduce while resident cell activating and wound contraction enhancing cytokines such as PDGF increase during estrogen administration [108,109]. Besides, estrogen has been found to promote re-epithelization by increasing keratinocyte proliferation and migration, angiogenesis by enhancing endothelial cells binding to ECM proteins, granulation tissue formation by augmenting TGF-β secretion, and wound contraction by fibroblast activation [107,104,110,111]. On the other hand, male sex hormone is detrimental to dermal wound healing. Androgens have been found to be an important stimulator for macrophage infiltration, TNF-α and IL-6 production which initiate and reinforce inflammatory response [104]. These results strongly correlate with observations in castrated rodent cutaneous wound healing. Castration provides a sudden decrease in inflammatory response and growth factor production. Presence of the androgen receptor (AR) on the surface of inflammatory cells is another explanation of androgens' role in inflammation regulation. Using AR antagonist, flutamide, balance inflammation response and heal is accelerated [106]. However, aging is not the only problem in older ages. Comorbidity such as diabetes, venous

insufficiency, heart failure is strongly associated with aging. Therefore, chronic diseases and aging together affect wound healing even worse.

1.6.2.2. Chronic Diseases

Chronic venous insufficiency, diabetes mellitus, obesity and cancer are major chronic diseases that have negative effects on wound healing processes. The primary reason for lower extremity ulcers is chronic venous insufficiency [112]. Microcirculation at lower limbs is disturbed by mainly venous structure deformation and dysfunction in calf muscles [113]. Vascular system of the lower limbs consists of superficial veins, perforator veins, and the deep veins. The blood pressure and flow direction are tightly controlled in these systems. Any disability in valves that control blood flow through one direction among these veins can cause blood overload in deep veins which should normally be maintained in low blood pressure [114]. When the blood circulation is disturbed as a consequence of such a systemic dysfunction and/or calf muscle disability or any other factors, chronic venous hypertension, primary reason of venous ulcers, occurs. There are some possible theories for mechanism of venous ulcer formation. It was suggested that fibrinogen leakage occurs in hypertension trough widen capillary pores [115]. The leakage forms an outer layer outside the capillaries after fibrinogen polymerization. Thus, gas and nutrient exchange is diminished, ischemia, necrosis and ulceration develop. Another accepted theory is accumulation of leukocytes after blood flow dysfunction and hypertension, generates high levels of proteases and inflammatory cytokines such as TNF-α [116]. Constant production of TNF-α improves inflammation response and induces ulceration as discussed in detail in earlier sections. Falanga and Eaglestein have hypothesized a different mechanism for venous ulcer formation [117]. According to their hypothesis, growth factors such as TGB-β are trapped by macromolecule aggregations (e.g. fibrinogen). Growth factor deficiency affect all phases of classical wound healing including macrophage and neutrophil infiltration, fibroblast, keratinocyte and endothelial cell proliferation and migration. All those events are basic symptoms of venous ulcers [114].

Apart from venous insufficiency, diabetes mellitus, regardless of type, is another important systemic factor which detrimentally affects wound healing and cause ulcers. There are nearly 170 million diabetic people in the world, and 15% of this patients experience diabetic foot ulcers (DFU) in their lifetime [118]. Diabetes mellitus is not the only reason for a DFU

formation. There are always one or more accompanying pathological conditions that contribute to DFU formation. Several extrinsic factors such as callus formation, infection, pressure and trauma and intrinsic factors such as neuropathy, vasculopathy, endothelial cell abnormalities and other complications arisen from diabetes result in DFU [119]. In diabetic patients, capillary size reduction and basement membrane thinning result in microvascular blood circulation defect. The contribution of vascular dysfunction to ulcer formation was explained above in detail. Moreover, hyperglycemia and related ROS production have been suggested to cause cellular and tissue damage, though the exact molecular mechanism is unknown [120]. Hyperglycemia activates some enzymes such as protein kinase C and lead to production of advanced glycation end-products (AGE). AGEs can generate improper ECM formation that inhibits cell binding, migration and collagen formation via binding to AGE receptors of wound fibroblasts [121]. In addition, increased protein kinase C activity promotes diabetic ulcer formation through nicotinamide dinucleotide phosphate oxidase (NADPH) activation which produce superoxide anion [122]. Therefore, excessive ROS particles are produced compared to antioxidant capacity of the body resulting tissue and cellular damage. Although ROS production is necessary for early wound healing phase, overabundant amount of ROS causes detrimental effects on tissue rigidity and wound healing. Another important antioxidant mechanism, glutathione scavenging activity has been found to be lower in hyperglycemic conditions [123]. Reduced level of NADPH or rate of NAPH/$NADP^+$ directly interfere with catalase, which carry out scavenging of hydrogen peroxide, activity [122]. Besides, as diabetic patients have elevated free fatty acid through acetyl-CoA utilization, metabolism of fatty acid increases ROS content [124]. Other than producing oxidative stress in wounded areas, hyperglycemia can also contribute bacterial invasion through diabetic ulcers. Hyperglycemia negatively affects the function of macrophages and neutrophils and makes wound more susceptible to infections [125]. Microbial degradation along with diabetic inflammatory response synergistically destroy wounded tissue. Another major comorbidity of diabetes is neuropathy. Neuropeptides such as NGF take critical roles in wound healing via inducing chemotaxis and cell activation [126]. Additionally, sensory nerves have been shown to participate in direction of inflammatory cells to the injured area [127]. Indeed, management of diabetic ulcers arisen from oxidative stress is complicated as several factors should be controlled in order to determine intervention strategy.

Wound cell activation is critical part for all healing phases. Chronic inflammation [128], low levels of growth factor and cytokine production [129], impairments in angiogenesis and VEGF production [130], decreased epidermal and dermal cell activity [131,132], inefficient granulation tissue and collagen formation [133] have been reported in diabetic patients and *in vivo* animal models. Without any stimulators and correct signaling cascade, cells are not able to proliferate and migrate. In normal cutaneous wound healing process, fibroblast and keratinocytes are highly active. However, diabetic ulcer fibroblasts have been reported to barely proliferate and migrate compared to diabetic but not ulcer fibroblasts [134]. In addition, diabetic keratinocytes are found to express c-myc and β-catenin that inhibit migration of keratinocyte towards wound center and promotes the formation of callus at the edge of wounded tissue [135]. As indicated above, angiogenesis impairment is experienced in DFU due to low level secretion of VEGF [136]. Besides, essential growth factors including GM-CSF, EGF, and TGF-β has been detected in low levels in keratinocytes isolated from diabetic callus edges [129]. In the same study, it has been also shown that down regulation of PDGF, IL-8, IL-10, IGF-1, iNOS has been detected in both diabetic ulcer keratinocyte and endothelial cells. This profound effect can be as a result of low growth factor production as well as high proteinase production. As another molecular mechanism might be low levels of ECM because ECM protein degradation is not even comparable with synthesis in diabetic foot ulcers. It has been reported that average MMP levels has been 60 times higher in diabetic wound fluid in comparison with acute wound fluid [84]. Apart from producing high MMP levels, decreased TIMP levels have also been observed in diabetic ulcers. Bone marrow derived epidermal precursor cells contribution to wound healing is also prevented via decreased endothelial nitric-oxide synthase (eNOS) production in diabetic wounds [118]. All these situations stuck wound healing in a chronic inflammatory phase. As inflammatory phase gets strengthen, its response gets amplified that may cause irreversible cellular and tissue damage.

Another major chronic wound forming comorbidity is obesity. Prevalence of obesity, the disease of modern era, increases day by day. According to Organization for Economic Co-operation and Development statistics, the average incidence of obesity is about 14.1% in the whole world and 12% in Turkey (http://www.oecd.org/). Obesity enhances risk of many vital diseases such as heart failures, hypertension, diabetes, cancer as well as wound healing impairments [84]. Severe wound complications have been linked to obesity in several

reports. Accumulating evidences have suggested that infections are the major complication connected to obesity [137-139]. As skin begins to fold with the increasing body weight, moisture in these folds rises which may support microbial growth and threaten tissue damage. Cytokines released from adipose tissue (referred as adipokines such as adiponectin) interfere with inflammatory cell activation in which infection takes place easily [140]. Other complications linked with obesity are dehiscence, hematoma and seroma formation, pressure and venous ulcers. These problems are generally related with vascular dysfunction, local ischemia and hypertension [141]. Anoxygenic conditions under the subcutaneous adipose tissue cause inefficient oxygen supply, neutrophil and macrophage infiltration. Moreover, systemic antibiotic applications may fail due to poor vascularity. Increased tension at injured site sometimes generates dehiscence as well as preventing sufficient nutrient supply [142].

Cancer can also have connection with wound impairments. Mutations involved in cancer progression can occur during proliferation phase of wound healing process. Thus, wound cells can degenerate into cancer cells. Tumors such as melanoma and squamous cell carcinoma can form chronic wounds, or treatment of chronic wounds can lead to cancer formation [143]. All these chronic diseases and others that are not mentioned (e.g. rheumatoid arthritis) prolong one or more wound healing phase, mainly inflammatory phase, that ends up with non-healing wound formation. In the management of these chronic diseases, especially cancer, medications may lead to insufficient growth factor synthesis, cell deactivation and immune suppression.

1.6.2.3. Medication and Immunosuppression

Stress, chronic or acute disease and medication can decrease immune system function. Suppression of the immune system is associated with several complications including wound healing impairments. Reduced inflammatory response can ended up with microbial infection and chronic wound formation [144]. Steroids, non-steroidal anti-inflammatory drugs, chemotherapeutic agents, antimalarials, anticoagulants and vasoconstrictors are main drug groups that have detrimental effects on wound healing. As cancer development and wound healing pathways resemble each other, chemotherapeutic agents may also delay wound healing. Based on their molecular mechanism, drugs can impede healing in different ways. As a specific example, bevacizumab, an angiogenesis inhibitor drug, also impairs wound healing angiogenesis via VEGF depletion [145]. Besides, they can negatively affect

inflammatory response due to the immunosuppression, lack of fibroblast activation, collagen synthesis and wound contraction [146].

Glucocorticoid steroids are the other important medication known to suppress inflammatory cells along with decreasing fibroblast activation, collagen synthesis, and wound contraction [146,84]. In microscopic scale, steroids have been proven to reduce and change cytokine expression profiles of dermal cells. In a steroid-induced wound impairment study, TGF-β and IGF-1 administration partially contributes to healing process [147]. Non-steroidal anti-inflammatory drugs (NSAID) are generally used for pain regulation in inflammatory diseases such as rheumatoid arthritis via mainly cyclooxygenase-2 (COX-2) inhibition [148]. These drugs may also be responsible for an open wound infection due to inflammation suppression. Moreover, reduced re-epithelization, angiogenesis, fibroblast activation, wound strength and contraction have been observed in NSAID treated rodents [149]. Apart from anti-inflammatory drugs and immunosuppressants, antiplatelet drugs such as aspirin, anticoagulants, and vasoconstrictors such as nicotine and cocaine unfavorably affect wound healing and may cause chronic ulcers [150]. Therefore, appropriate prescription is urgently required to enhance wound healing especially in patients with chronic diseases such as diabetes. In the condition of obligatory usage of these drugs, wound management should be done carefully and additional precautions (e.g. special diet or antibiotics) should be implemented.

1.7. CURRENT TECHNOLOGIES AND STRATEGIES IN WOUND HEALING

Wound healing process should not be oversimplified and management of wounds (especially chronic wounds) should be cared professionally. Although normal healing wound does not require much attention, substantial researches have been conducted in development of wound healing strategy because non-healing wounds or delayed wound healing problems cause an important health and life burden. Recent molecular and cellular advances in wound healing cascade have brought proper understanding in our mind that lead to new developments and strategies in clinical practice.

1.7.1. Devices and Procedures

Wound healing devices are generally used along with conventional wound healing modalities. Devices used in wound healing practice are mainly low level laser light, active pulsed electro-magnetic field, bio-electrical stimulation therapy and ultrasound-guided foam sclerotherapy [151]. The principle of low level laser therapy depends on producing laser (via different inert gases such as helium neon, ruby, argon, and krypton) and applying power for a specific irradiation time [152]. This technique has been proven to change cellular behavior, increase collagen synthesis and cell proliferation [153]. In active pulsed electro-magnetic field application, a magnetic field is formed at the center of the wound. Essential wound resident cells including fibroblasts and keratinocytes are forced to migrate towards magnetic field, proliferate and contribute to wound healing both *in vitro* and *in vivo* studies [154,155]. It is known that lower tissue layers remain negatively charged in comparison with the skin. This allows skin to have positive potential. Therefore, applying direct low dose current to wounded are will stimulate skin cells. In the literature, it has been shown that an appropriate dosage and application time of electrical stimulation to wounds (even non-healing) activates fibroblast, angiogenesis and ATP production [151]. As a different treatment strategy, ultrasound is used to direct sclerosant under control of foam. As sclerosant is injected into vein lumen, foam increases the availability of sclerosant. This technique has been introduced to treat superficial venous insufficiency, incompetent perforator veins and venous hypertension [156,157]. As it is reliable and used for the treatment of venous problems, it is effective in the treatment of venous ulcers [158].

Another technique presented by Wake Forest University in early 90s is negative pressure wound (referred as vacuum-assisted closure) [159]. The application is mainly based on dressing wound with a medical-grade, open-cell and polyurethane ether foam, and applying subatmospheric pressure (100-125 mmHg). This process provides the removal of edema leading to improved cell migration and blood flow, and disposal of excess inflammatory growth factors, cytokines, and bacterial contamination generally found in chronic wounds [160]. In several laboratory experiments and case studies, usage of negative pressure on chronic wounds has been presented as a remarkable stimulator for granulation formation, epithelization and contraction [161,162]. However, there are some complications about

using negative pressure such as lack of comfort and bleeding due to granulation tissue integration into foam dressing.

Hyperbaric oxygen therapy (HBO) in wound healing was used in the treatment of gas gangrene in 1961 [160]. As discussed in the “factors affecting wound healing” section, local hypoxia is one of the major reason for chronic wound formation. The technique is based on providing 100% pressurized (1.5 and 3 times of atmospheric pressure) for one or two hour in several sections [163]. The basis of the idea is providing enough oxygen to the wound area to promote wound healing phases. As oxygen is compulsory for enough ATP production, oxygenation of wounds mostly results in enhanced healing. Apart from ATP, oxygen is also necessary for ECM organization, cell proliferation, and angiogenesis [164]. In several case studies, HBO therapy has been used for the treatment of chronic ulcers along with a control group, and it is proven that HBO increase the rate of healing individuals and reduce limb amputation incidences [165,166]. On the other hand, although side effects of HBO are seldom, they can also cause life-threatening side-effects including reactive airway disease, barotraumatic otitis, hyperoxic seizures, and pneumothorax, neurologic oxygen toxicity [167]. All these mentioned devices and processes have been proven to improve healing capacity, but they reach partial success in specific wounds. Additionally, they possess critical risks that may ended up with death. Therefore, these techniques should be advanced or new strategies should be established for future clinical practice.

1.7.2. Dressings and Topicals

Wound dressing has been a common practice in clinical applications since ancient times. Cotton, wool or synthetic bandages and gauzes have been utilized in both acute and chronic wound managements to prevent microbial invasion, and to keep wounds moist. In the modern science, however, new dressing applications have been introduced to provide optimum conditions to enhance ulcer healing cascade by supplying enough oxygen circulation, moisture environment, and antimicrobial surfaces [4]. Wound dressing materials are classified according to physical structure, function and raw material. Dressing might be developed from synthetic or natural polymers such as collagen, alginate or hydrocolloids in the forms ointment, gel, foam and film [168,169]. Hydrocolloid dressings are composed of gelling agent; gelatin or carboxymethylcellulose and other polymers are used for their

desirable rigidity and porous structure [170]. Gelling agent produces a barrier for wounds as well as moisture environment. In several *in vivo* studies and case reports, these dressings have been found to be superior compared to standard tulle gauze dressings in the way of reducing inflammatory response, augmenting granulation formation, encapsulating and stabilizing bacteria inside gelling agents [171,172]. Another important dressing type is hydrogels. Hydrogels' raw materials are water and swellable polymers. Hydrogels are applied in the amorphous gel form or film to provide elasticity or rigidity. If they are applied in gel forms, a secondary dressing such as bandages should be used in order to stabilize dressing. On the other hand, hydrogel films are sustainable on wounds and provide water permeability as well as moisture [173]. It has been reported that hydrogels are good stimulators for all wound phases and suitable dressing for all kinds of wounds except infected wounds [174]. A dressing should also be capable of absorbing wound fluid to prevent microbial infection and tissue maceration. Foam dressing, therefore, is used to absorb wound exudates, satisfy thermal balance and avoid microbial invasion [4]. There are myriad of introduced dressing types having different properties. They are reported to have promoting effect on acute and chronic wound healing in case studies and *in vivo* experiments. However, their promoting mechanism consist of only providing suitable environment for wounds and removing undesired substance from injured area. Hence, scientists are still trying to find more inductive proteins and chemicals that can be incorporated into these dressings.

1.7.3. Tissue Engineering

Tissue engineering is a developing area in wound management in correlation with scientific inventions. The first attempt to recover healing is skin transfer in tissue engineering practice. However, there are some critical limitations that full thickness skin transfer can only be applied in up to 2% total skin surface area lost patients [175]. Therefore, it is not suitable for patients with deep burn (50-60% of total skin surface area or more). Besides, in partially or full-thickness skin transfer applications, several abnormal healing problems have been reported [176]. Therefore, tissue engineering aims to develop skin like constructions that can enhance functional healing capacity in all types of wounds in a controlled manner. In this point of view, scaffolds are in great interest due to having compatibility with skin cells. A well-designed scaffold (either natural or synthetic polymer) system is generally used to close

open wound either alone or inoculated with allogenic skin cells as a promising tissue engineering strategy. However, using allogenic skin cells is time consuming and producing new wounds in patients body [177]. Several biocompatible types of scaffold systems composed of natural polymers including collagen [178], hyaluronic acid [179], gelatin [180], silk fibroin [181] and chitosan [182] have been constructed to enhance wound healing in skin tissue engineering. During construction, pore size is critically arranged to provide a suitable environment for cell migration, proliferation and angiogenesis. Apart from natural products, synthetic polymers such as poly (lactic acid-*co*-glycolic acid) (PLGA) [183], poly(ε-caprolactone) (PCL) [184] or their combination with natural polymers [185] have been utilized for skin tissue engineering. Accumulating evidences report these polymers' positive effect on all phases of wound healing although they vary in hydrophilicity, biocompatibility, and biodegradability. They have some limitations due to their physical and chemical structure (e.g. being hydrophobic and less rigid), but this undesired situation is being currently investigated by combining two or more polymers in one scaffold system. They only provide a suitable environment for wound cell that they can migrate, proliferate and produce newly formed skin network. A further advance in scaffold technology is to incorporate a chemical or biomolecules that may protect wound from infection, decrease inflammatory response, activate cell migration and proliferation, enhance angiogenesis, and improve wound contraction or remodeling.

1.7.4. Scaffolds/Dressings for Drug, Growth Factor Release and Gene Delivery

Using wound healing stimulators in topical formulations such as hydrogels, ointments, or foams requires repeated process. Additionally, cells are exposed to high concentration of the active molecules at one time. Therefore, technology of sustained and controlled release of activators has partially overcome these disadvantages. The idea of incorporation of active ingredients into dressing or scaffold systems has been tried for many different compounds including antibacterial agents, growth factors, cytokines and supplements.

1.7.4.1. Antimicrobials

Antimicrobial agents incorporated dressings and scaffold systems are generally used for diabetic foot ulcers, burn wounds and venous ulcers where immunity is not enough to fight against microbial invasion. Some of these approaches are gentamycin releasing collagen

scaffold [186], minocycline incorporated chitosan sheet [187], Nano silver embedded β-chitin scaffold [188], and vancomycin releasing polyurethane scaffold [189]. These systems have been shown to inhibit microbial growth including *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* in wounds. Additionally, as they prevent microbial infection and chronic inflammatory response, they are found to accelerate granulation tissue formation and angiogenesis. Local antibiotic application is superior to systemic administration due to high dosage necessity, organ and cellular toxicity, reduced bacterial resistance. However, local antibiotic administration may cause, albeit at low levels, sensitization, superinfection, resistant bacteria selection in repeated antibiotic implementation [190].

1.7.4.2. Growth factors

Growth factors are essential elements in wound healing so that different growth factors play distinct roles in every healing stages. They stimulate each other and cells in a tightly controlled and ordered manner. Any growth factor deficiency due to ineffective formation or excessive degradation disturbs the cascade and results in abnormal wound healing or chronic wound. Therefore, supplying missing growth factors or growth factor that takes role in several processes is a quite popular research area among scientist. Although, several reports suggest potential therapeutic roles of growth factor application in wound healing, their availability is limited due to lack of delivery methods. PDGF-BB in gel form has the only FDA approval for chronic DFU patients [191]. Though several papers have reported its efficacy in both acute and non-healing wounds [192,193], some patients do not respond to PDGF gel because of rapid degradation rate [194]. Taking into account this, growth factor encapsulated/incorporated scaffold systems and dressing become an area of much interest in order to prevent growth factor from degradation in harsh wound conditions, and provide sustained and constant stimulation.

Nontoxic, safe, biocompatible, biodegradable natural or synthetic polymers are embedded with different growth factors and found to be effective in different healing process. FGF family consist of 23 members, of those FGF-1, -2, -7, -10, and -22 are activated in the wounded skin [32]. After being released into wound area, they bind to their specific receptors and activate Ras/MAPK and PI3K/Akt pathways [9]. One important member of this family, FGF-1 is integrated into collagen scaffold and shown to promote angiogenesis and re-

epithelization in rabbit acute wounds [195]. Another member of fibroblast growth factor family, FGF-2, has been impregnated into chitosan film [196]. Released FGF-2 has been found to be active and enhance re-epithelization, granulation tissue formation and wound contraction rate. FGF-7 (also known as KGF-1) is conjugated into fibrin scaffold which has significantly augmented re-epithelization rate compared to control group [197].

TGF-β family has nearly more than 30 members but only TGF-β1, TGF-β3, bone morphogenic proteins (BMPs) and activin have been linked to the wound healing process [198]. As discussed earlier, TGF-β applications has been claimed to exhibit contradictory results in wound healing. However, TGF-β impregnated collagen scaffold has enhanced re-epithelization and wound contraction in a rabbit acute wound model [195]. EGF family consisting of EGF, HB-EGF, and TGF-α has been shown to be effective in the healing processes [7]. *In vivo* studies have exhibited that, EGF containing gelatin based dressings [199] and collagen gel dressing [200] have increased granulation tissue formation and re-epithelization rates. In addition, VEGF conjugated with alginate microspheres has displayed remarkable vascularization levels *in vivo* [201].

Different biopolymers incorporated with several other growth factors including stromal-derived factor-1 (SDF-1) [202], GM-CSF [203], and TGF-α [57] have been constructed and proven to be effective in acute and chronic wound. However, as growth factor and cytokine signaling is a quite complex process, applying one growth factor may sometimes be ineffective. There have also been some approaches to deliver multiple growth factors simultaneously. Angiogenesis in FGF-2 and VEGF containing collagen–heparin scaffolds group has been reported to be more efficient in comparison with individually treated groups [204]. Moreover, PDGF and VEGF encapsulated PLGA microspheres have stimulated angiogenic activity efficiently [205]. Although applying growth factors to wounded area is a potential therapeutic way, their efficacy in clinical trial hasn't been proven and approved globally. Moreover, growth factors in scaffold network and dressing system are still susceptible to environmental and wound harsh conditions. Most of the time, a secondary dressing is necessary especially in ECM based scaffolds [198]. One growth factor can induce a specific phase in wound healing while it can inhibit others as in TGF-β stimulations [50]. Besides, growth factor treatment commonly results in overhealing and scar formation.

Therefore, more controlled gene and stem cell therapy may be a solution for imbalance between non-healing and overhealing problems.

1.7.4.3. Gene Delivery

Gene therapy referred to as delivering genes into host cells in order to improve congenital defect or metabolic dysfunction. In theory, gene therapy is transcendent over exogenous growth factor administration due to having long lasting and stable production as well as having "on/off" criteria. The wound conditions, as in chronic or infected one, are physically and biochemically complex. As discussed earlier, growth factor administration can fail because of rapid degradation or metabolic inhibition. Therefore, a strictly controlled and locally restricted protein expression via targeted gene delivery is advanced to cope with topical growth factor linked problems [206]. There are mainly two basic strategies in gene therapy; integrating gene into the host chromosome for a stable expression and transient expression by using episomal vectors [207]. Gene transformation is processed either *in vivo* or *ex vivo*. *In vivo* gene transfer requires direct administration of genes to the host organism, while *ex vivo* needs isolation and expansion of appropriate cells and transfection *in vitro*, then after transplantation of cells with a proper methods [208]. Whereas using viral vectors for gene therapy is highly stable, it consists of some critical risks including mutagenesis, carcinogenesis and immune responses [209]. Non-viral vectors (episomal vectors) are generally preferred in clinical trials. Although viral vector transfection does not require much attention due to its high infectivity, non-viral based transfection should be paid more attention because of low efficiency. The naked cDNA construct can be delivered via direct injection to the targeted tissue but overall accumulating results indicate that the transfection efficiency and vector penetration to deep layers would not be in desired levels [210]. Therefore, different strategies are developed for the delivery of gene construct to enhance transfection rate.

Liposomal transfection is one of the technique that increase the gene transfer efficiency compared to direct injection. However, it is not without its limitations such as lack of direct targeting gene delivery and efficacy compared to other methods [206]. 3-D scaffold with porous structure has been shown to contribute to gene transfer as it provides enough place that cells and gene can interact [211]. A vast amount of natural (collagen type I, alginate, chitosan, and hyaluronan) and synthetic (PLGA, carboxymethylcellulose and PLG)

polymers have been introduced for growth factor gene delivery in wound healing experiments [206,212]. In most of the papers, a gene of interest has been immobilized onto scaffold network for targeted gene delivery so that only migrating cells are transfected [213]. Therefore, it is possible to increase migration of tissue cells into scaffold by forcing scaffold cells to express appropriate growth factor.

Another important advantage of gene delivery systems is to control the level and location of delivered gene expression. Tissue specific promoters (such as FGF-inducible response element) control the location of protein expression while inducible promoter (active in the presence of activator such as tetracycline for tetracycline inducible promoter) controls the time and level of expression [214,215]. These developments are all encouraging but their efficiency and safety is not well established enough to be used in dermatological clinics. Many other studies are required to optimize timing and targeting of gene expression in the light of wound healing molecular mechanisms.

1.7.5. Stem Cell Therapy

Stem cells have self-renewing and proliferating capacity that they can differentiate into several cell types. Theoretically, stem cells are good candidate for tissue regeneration as they may replace lost or damaged tissue along with contributing cellular function via paracrine signaling. Chen and colleagues have indicated that signaling molecules presented in the conditioned medium of stem cell culture are the main activators of wound healing [148]. Although there are some other researches presenting the direct application of stem cells to wound area [216], stem cells combined with scaffold system provide more effective regeneration rate [217]. Normally, stem cell therapy prefers autologous transplantation instead of allogenic transplantation due to immune response and rejection problems. However, autologous stem cell therapy requires *in vitro* culturing and expansion of cells, lasting for several weeks. Different stem cell types including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adult stem cells (mainly mesenchymal stem cells (MSCs)) have been stated as alternative strategies for wound healing and skin regeneration.

ESCs derived from inner cell mass exhibit pluripotent characteristics even after several passages *in vitro* [218]. It has been hypothesized that ESCs may increase wound healing rate

not only by paracrine signaling but also differentiating into epidermal and dermal cell linages. In an *in vitro* study, mouse ECSs have been successfully differentiated into epidermal cell precursors and transformed cells have efficiently expressed epidermal development markers including keratin-8, keratin-14, keratin-18 and eratin-19 [219]. In addition, a fully differentiated skin by using embryonic stem cells has been generated in *in vitro* conditions [220]. In that promising study, keratinocytes derived from ESCs has been cultured on ECM scaffold. Stratified epithelium formation, fibroblast specific protein and basement membrane protein expressions have been detected after culturing scaffold in the presence of ascorbate or BMP-4. However, there are important problems with using ECSs for tissue regeneration. ECSs can cause teratomas and carcinogenicity due to uncontrolled proliferation and differentiation [221]. Besides, having ethical and legal controversies, ECSs are not suitable for clinical trials. A recently emerged promising therapeutic alternative, iPSCs, have been presented in order to overcome ethical and lack of stem cell sources problems. Takahashi and Yamanaka have constructed iPSCs that have pluripotent stem characteristics similar to ECSs via transfecting fully differentiated somatic cells with c-Myc, Klf4, Oct3/4, and Sox2 transcription factors [222]. Although iPSCs are good alternatives for ECSs, their reprogramming capacity is relatively low. Furthermore, they cause carcinogenicity and teratomas even in higher rate than ECSs regardless of the transplantation place [223]. Therefore, MSCs harvested from adult tissues are the safest therapeutic application in tissue regeneration applications without having any ethical and legal considerations.

Multipotent MSCs are obtained from many sources including bone marrow [224], adipose tissue [225], cord blood [226], amniotic fluid [227], dental pulp [228] and synovial membrane [229] that can differentiate into mesodermal, endodermal and neuroectodermal cell types. These cells display immuno-suppressive properties and are not rejected by the host system [230]. Therefore, the use of MSCs in the treatment of acute and chronic wounds has been of particular interest. *In vitro* studies have proven spectacular skin cell differentiation capacity of MCSs [231]. Hypoxic environment of wound attracts MSCs to produce more potent growth factors that may reduce inflammation and induce angiogenesis, re-epithelization and cell migration [232,233]. After a direct transplantation of MSCs into wound place, they remarkably enhance wound healing rate by several distinct pathways. Bone marrow-derived stem cells (BM-MSCs) transplanted into both normal and diabetic

wounds promoted wound healing rate [234]. It is suggested that BM-MSCs secrete pro-angiogenic factors including VEGF and angiopoietin-1 leading to considerable increase in angiogenic response. In a different study, autologous MSCs transplantation has positive effect on chronic wounds via increasing blood vessel formation, and wound contraction in both human and animal wounds [235]. BM-MSCs and adipose derived stem cells (ADCSs) have increased vascularization, re-epithelization, and wound contraction via expressing key growth factors including FGF-2, VEGF and HGF along with direct integration into dermal layers via specific differentiation into epithelial and endothelial cell types [236,234]. An alternative MSCs source, umbilical cord blood (UCB) cells has also been tested in dermal tissue healing studies. Effective differentiation of UCB cells into keratinocytes has been proven in the wound site [237]. A recent study has compared wound healing stimulatory capacity of UCB- and ESC-derived endothelial progenitor cells [238]. Findings of the study suggest that although ECS derived progenitor cells exhibit the superior results, UCB derived cells are more effective in chronic wound healing compared to control group. Amniotic fluid derived mesenchymal stem cells (AFMSCs) are other important mesenchymal stem cells with their high growth factor production and differentiation capacities into several cell linage. AFMSCs have been reported to secrete high amount of pro-angiogenic factors including IGF-1, EGF and IL-8 compared to ADSCs and promote re-epithelization and cellularity in diabetic NOD/SCID mice [239].

All these encouraging findings have been extended to chronic wounds by transplanting MCSs inoculated scaffold systems. In order to maximize MSCs benefits, scaffold/dressing system should be designed in a way of promoting proliferation, adhesion and migration [240]. Collagen gel solution [241], basement membrane matrix gel [148] and fibrin matrix [235] are a few examples of scaffold materials used in cell delivery into the wound area. However, all these methods and therapeutic interventions are not completed their clinical trials, even preclinical examinations. Besides, most of the methods and activators are not cost-effective, host compatible, easily applicable and accessible. Especially in growth factor delivery systems, their safety and efficacy are not proven globally. Therefore, there is a huge demand for developing novel, effective in almost every phase of scarless wound healing, safe, cost-effective and easily accessible activators and systems in skin tissue engineering.

1.8. BORON

Boron [242] is a semiconductor semimetal with an anomic number of 5. The physical properties of boron are between metals and nonmetals. Boron is found in the nature as in two isotopes ^{11}B and ^{10}B with a ratio of 80/20 [242]. Boron does not exist in cation form in the nature unlike similar elements in trivalent oxidation state (+3). It is highly reactive with oxygen and always forms B-O bounds in natural conditions [243]. The main forms of boron in nature are boric acid (H_3BO_3 or $B(OH)_3$), borates and borosilicate minerals [244]. At neutral pH, boron is found in biological fluids as boric acid with a small quantity of borate. Boric acid, a weak monobasic acid, can easily dissolve in water based on the equation 1.1 [245];

$$B(OH)3 + H2O \Leftrightarrow B(OH)4^{-} + H^{+} \quad (1.1)$$

Boron has been determined as an essential element for plants as it contributes to plant cell wall structure and function [246]. Other than that, boron requirement has been proven for higher organisms including trout, frog, zebrafish, buffalo and mouse [247,248]. Although the exact mechanism of action is not well elucidated, there are several lines of evidence suggesting the importance of boron in human nutrition and metabolism. Boron action on mammalian cells was believed to be through passive diffusion till the discovery of highly specific transportation of boron through electrogenic Na^{+}-Coupled Borate Transporter (NaBC1) in 2004 [249]. The first observations on boron and human health were associated with bone growth. Boron deficiency in diet significantly has reduced the growth of rat and pig bone [250]. Additionally, systemic boron supplementation has attenuated alveolar bone loss and periodontal inflammation in experimental periodontitis [251]. Three independent studies have demonstrated molecular mechanism of boron on bone mineralization. It was reported that boron (as boric acid) has significantly increased the level of mineralization and bone associated protein expression in osteoblast [252], bone marrow stromal cells [253] and human tooth germ stem cells [254]. In another study, boron has been incorporated into dexamethasone releasing bioactive glass scaffold for bone tissue engineering [255]. Both dexamethasone and boron have enhanced the proliferation, mineralization and differentiation of osteoblasts. Boron has also been indicated as augmentative for brain function as deprivation of boron has negatively affected motor speed, skills, psychomotor

action and short-term memory [256]. However, these findings are not satisfying enough as in bone growth and should be confirmed by several clinical studies.

After the discovery contradictory relationship between boron and prostate cancer, scientists have tested boric acid exposure on prostate cancer cell lines. The results have postulated that boric acid treatment (1mmol/L) have significantly inhibited DU-145, PC-3 and LNCaP cell proliferation compared to healthy prostate cell lines, PWR-1E and RWPE-1 [257]. Apart from prostate cancers, breast, lung and cervical cancers have been reported to be inhibited by boron treatment [258-260]. The mechanism of action still remains unknown except finding of significant increase in apoptotic markers.

Scientists have paid little attention to wound healing effect of boron although there are some encouraging findings in the literature. Topical boron application and thermal water boron are strongly associated with augmented wound healing rate [261]. Administration of 3% boric acids on deep wounds has remarkably contributed to healing cascade and decreased hospitalization time in intensive care units [262,263]. Although almost nothing is being known about the mechanism of boron on wound healing, limited studies have reported some molecular changes in cellular activities after boron treatment *in vitro*. One possible mechanism has been proposed by Benderdour and colleagues that boric acid treatment have increased the secretion of ECM proteins (proteoglycans and collagen), and TNF-α [264]. In addition, four different boron analogues (triethanolamine borate; N-diethyl-phosphoramidate-propylboronique acid; 2,2-dimethylhexyl-l,3-propanediol-aminopropylboronate and 1,2-propanediol-aminopropyl-boronat) have been reported to increase ECM proteins more than boric acid did, but they have exhibited higher toxicity levels compared to boric acid [265]. VEGF, the main stimulator for angiogenesis, is also found in connection with boron exposure. High VEGF expression has been determined in both RNA and protein level after boron application in a cell free transcription and translation system [266]. Sodium borate, sodium salt of boric acid, has been indicated to have no proliferating but migration stimulatory effect on human keratinocytes [267]. The same group have reported boron induced MMP-2 and MMP-9 expression that are involved in cell migration and remodeling phase [268]. Recently, we have proven that sodium pentaborate pentahydrate combined with pluronic block co-polymers has increased migration, superoxide dismutase activity and vital wound healing-associated gene expressions of

primary human fibroblast cells *in vitro* along with augmenting collagen deposition and wound contraction *in vivo* [269]. In conclusion part of all these articles, authors mentioned the sentences of “Further researches should be conducted in order to understand the exact molecular mechanism of boron on wound healing”.

There are also several indirect studies investigating activity of boron on living organisms that can be associated with wound healing processes. The most important issue of these subjects is inflammation. Boron supplementation in diet has been effective in arthritis (inflammation in a joint that causes pain, swelling and stiffness) [270,271]. These observations have been confirmed by animal and *in vitro* studies. High boron dieted rats, injected with *Mycobacterium butyricum* to induce arthritis, have exhibited low arthritis symptoms compared to low boron fed rats [272]. A recent *in vitro* study has declared the effect of boron, as in the form of calcium fructoborate, on inflammatory mediator secretion from lipopolysaccharide (LPS)-stimulated murine macrophage RAW 264.7 [273]. Boron has significantly reduced NO, IL-1β and IL-6, increased TNF-α while not changed the mRNA level of COX-2.

Apart from anti-inflammatory effect of boron supplementation, immunity and oxidative stress regulatory roles have been mentioned in the literature. Natural killer cell circulation has decreased with increasing boron supplementation in diet [272]. Furthermore, antioxidant enzyme activity has been significantly augmented via low dose boron (boric acid, borax, calcium borate and sodium-calcium borate) application [274]. The other important property of boron derivatives is having biocidal effect on microorganisms. Existing researches indicate the antimicrobial characteristics of some boron derivatives. Boric acid displays a wide range of antibacterial activity against several bacterial species including *E. coli*, *P. aeruginosa*, *Acinetobacter calcoaceticus*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Proteus* spp., *Morganella* spp., *Salmonella* spp. *Staphylococcus* spp., *Streptococcus* spp. and anticandidal effect against *Candida albicans* in a dose dependent manner [275]. Apart from boric acids, boron containing compounds have been tested on gram negative bacteria, *E. coli*, and found bactericidal [276]. Antifungal effect of potassium tetraborate (0.1% w/v) has been reported on *Penicillium expansium* [277]. In addition, a plant pathogen *Botrytis cinerea* (primary reason of gray mold disease) has been controlled by potassium tetraborate application [278]. These findings suggest the possible role of boron containing

compounds on management of wounds with chronic inflammation and infection. Although there are limited reports that indicate the potential role of boron on wound healing, further experiments should be conducted to extent boron's potential role in chronic wound healing and understand the exact mechanism of action in all separate healing phases.

1.9. THE AIM OF THE STUDY

Ample evidence obtained from clinical and experimental studies suggest that boron and its derivatives may have positive inductions on wound healing. Limited studies have reported only a minor part of action mechanism as several pieces of the missing picture are waiting for the discovery. Although, all boron compounds are thought to be effective due to their boric acid contents, our previous studies have shown that every boron compound has distinct effect on different cell types even in the condition of boron equivalency.

The current study will be held to; (i) figure out boron compounds' effect on different primary cells and cells lines *in vitro* in the context of antimicrobial activity, growth factor and cytokine profile, proliferation, migration, and angiogenesis; (ii) and investigate wound healing potential of boron derivatives on non-healing wounds of streptozotocin induced diabetic rat by means of macroscopic and histopathological examinations.

2. MATERIALS AND METHODS

2.1. *IN VITRO* STUDIES

2.1.1. Preparation of Boron Solutions

Sodium pentaborate pentahydrate (NaB), provided by National Boron Research Institute-BOREN (Ankara, Turkey), and boric acid (BA) (#10043-35-3, Bio Basic Inc., Canada) were dissolved in Dulbecco's modified Eagle's medium (DMEM) (#41966-029, Invitrogen, UK) at a concentration of 10mg/ml. The solutions were sterilized using 0.45µm filter (#99745, TPP, Switzerland) and subsequently diluted in DMEM for further *in vitro* experiments.

2.1.2. Cell Viability

Primary dermal human fibroblast (HF) cells were isolated from foreskin according to the protocol described in the literature [279] after obtaining ethical approval from Kocaeli University (Kocaeli, Turkey) and informed consent of the patients. Effects of NaB and BA on skin cells including HF, human umbilical vein endothelial cells (HUVEC, ATCC-CRL 1730), mouse fibroblast cell line (L-929, ATCC-CCL 1) and human keratinocyte cell line (HaCaT) (CLS 300493, DKFZ, Heidelberg) were investigated using 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS) assay (#G3582, CellTiter96 AqueousOne Solution; Promega, Southampton, UK) according to the manufacturer's instructions. Eleven separate concentrations ranging from 1 to 2000µg/ml (1, 2, 5, 10, 20, 50, 100, 200, 500, 1000 and 2000µg/ml) for NaB and BA were prepared in DMEM containing 10% fetal bovine serum (FBS) (#10500-064, Invitrogen, UK), and 1% of penicillin, streptomycin, and amphotericin B (PSA) (#15240-062, Invitrogen, UK) from the main stock solutions. Cells were seeded onto 96-well plates (#CLS6509, Corning Plasticware, Corning, NY) at a concentration of 5×10^3 cells/well and cultured overnight in a humidified chamber at 37 °C and 5% CO_2. The next day, medium was exchanged with fresh medium containing aforementioned concentrations of NaB and BA. As a toxic positive control, 20% of dimethyl sulfoxide (DMSO, #D4540, Sigma-Aldrich, USA) was used. Cell viability at different time intervals (24, 48 and 72h) was

measured by adding 10% MTS reagent containing complete growth medium into each wells. After a 2h incubation period at 37 °C, the absorbance was measured at 490nm using an ELISA plate reader (Biotek, Winooski, VT).

2.1.3. Scratch Assay

As an *in vitro* wound healing model, scratch assay was performed for the investigation of boron compounds' effects on cell migration as described before [280]. Briefly, cells were seeded at a concentration of 1×10^5 cells/wells onto a 24-well plate (#CLS3527, Corning Plasticware, Corning, NY) and cultured at 37 °C and 5% CO_2 for 24h to provide cell attachment. Cells were scratched using a sterile 200µl micropipette tip and the medium was immediately changed with fresh complete medium containing specified concentrations of boron derivatives. Photographs of cell scratches at 0, 12 and 24h were taken using an inverted microscope (Zeiss PrimoVert light) equipped with AxioCam ERc5s camera and Zen 2011 software (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) and wound closure rate was measured using image analysis software (ImageJ, NIH, Bethesda, MD). Wound closure rates were calculated using the equation 2.1 given below;

$$\%\ \text{Wound closure} = [1 - (A\text{i} - A\text{f})/Ai] \times 100 \tag{2.1}$$

where A_i represents initial length between edges of scratched cell layers and A_f represents final length between edges of scratched cell layers at the end of incubation period.

2.1.4. Quantitative Real-time PCR (RT-PCR) Assay

Target gene primers were designed using Primer-BLAST online software of the National Center for Biotechnology (NCBI, Bethesda, MD) and synthesized by Macrogen (Seoul, Korea). β-actin gene was used as a housekeeping gene for the normalization of results. The sequences for primers and conditions used in RT-PCR assay is given in **Table 2.1 and Table 2.2**, respectively. Total RNAs from boron treated cells were isolated using RNAeasy plus mini kit (#74136, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using High Fidelity cDNA synthesis kit (#05081955001, Roche, USA) following company's recommendations. Primers (10pmol for

each), cDNA (800ng), dH_2O and Maxima™ SYBR Green qPCR Master Mix (2×) (#K0221, Fermentas, USA) were mixed in a final volume of 20µl. All RT-PCR experiments were conducted using iCycler RT-PCR system (Bio-Rad, Hercules, CA). Data were calculated by normalization of results against respective β-actin values and represented as fold change of control.

2.1.5. Nitrite Oxide (NO) Assay

To evaluate anti-inflammatory effect of NaB and BA, inhibitory effect of boron compounds on NO release from boron treated mouse macrophage cells (Raw264.7, ATCC-TIB 71) was determined as described previously with minor modifications (Park et al., 2005). Raw264.7 cells were seeded at a density of 1×10^5 cells/wells onto 48-well plate (#92096, TPP, Switzerland) and incubated overnight at 37 °C and 5% CO_2. Cells were stimulated with 1µg/ml LPS (#L4391, Sigma-aldrich, USA) for 1h, followed by addition of indicated concentration of boron derivatives into respective wells. After a 16h incubation period, NO amount in cell culture medium was measured using Griess reagent system (#G2930, Promega, USA). Briefly, 50µl of conditioned medium and 50µl of the Sulfanilamide solution (provided with the kit) were added to wells of a 96-well plate in triplicate. The plate was incubated 10 min in a dark environment. Then, 50µl of the NED Solution (provided with the kit) was added to all wells and incubated for further 10 min in dark. The absorbance was measured at 525nm using an ELISA plate reader.

2.1.6. Matrigel Tube-formation Assay

Effects of NaB and BA on tube-like structure formation capacity of HUVECs cultured on Matrigel (#354234, BD Biosciences, Bedford, MA) were determined as described in the literature [281]. In short, pre-chilled 48-well plates were coated with Matrigel (100µl) at 4 °C and incubated at 37 °C for 30 min to allow polymerization. HUVECs were seeded at a density of 2×10^4 cells/well onto Matrigel coated wells, together with or without specified concentrations of boron derivatives and incubated at 37 °C in a humidified chamber with 5% CO_2. Morphogenesis of capillary tube was visualized after 7h under an inverted Microscope (Nikon Eclipse TS100, Nikon, Tokyo, Japan) and capillary formation at randomly selected five areas were assessed.

Table 2.1. Primers used in RT-PCR assays

Gene	Species	Sequence	Product length
Akt	Human	F 5’ GGGACCTGAAGCTGGAGAA 3’ R 5’ CCTGGTTGTAGAAGGGCAGG 3’	240bp
Akt	Mouse	F 5’ GAAGCTGCTGGGCAAGGGGCA 3’ R 5’ GTGGGCCACCTCGTCCTTGG 3’	124bp
Fibronectin	Human	F 5’ AGCCTGGGAGCTCTATTCCA 3’ R 5’ CTTGGTCGTACACCCAGCTT 3’	109bp
Fibronectin	Mouse	F 5’ GCAAATCGTGCAGCCTCAATC 3’ R 5’ GGGCGCTCATAAGTGTCACC 3’	243bp
Laminin	Human	F 5’ CACATGTCCGTCACAGTGGA 3’ R 5’ TAGAGGCTGACCACCTCCTC 3’	218bp
Laminin	Mouse	F 5’ GACCACAGCGTGTGTTTGAC 3’ R 5’ TTCCTGTGGGACGGAGAGAT 3’	250bp
MMP2	Human	F 5’ TTTCCATTCCGCTTCCAGGGCAC 3’ R 5’ TCGCACACCACATCTTTCCGTCACT 3’	253bp
MMP2	Mouse	F 5’ GTTCAACGGTCGGGAATACA 3’ R 5’ GCCATACTTGCCATCCTTCT 3’	103bp
MMP9	Human	F 5’ GACGCAGACATCGTCATCCAGTTT 3’ R 5’ GCCGCGCCATCTGCGTTT 3’	200bp
MMP9	Mouse	F 5’ AAACCTCCAACCTCACGGAC 3’ R 5’ TTGGAATCGACCCACGTCTG 3’	221bp

Akt: Protein kinase B, MMP2: matrix metallopeptidase 2 (gelatinase A), MMP9: matrix metallopeptidase 9 (gelatinase B).

2.1.7. Rat Aortic Ring Assay

Pre-chilled 48-well plates were coated with 75µl growth factor reduced Matrigel (#354230 Becton-Dickinson, Bedford, MA) at 4 °C and kept at 37 °C for 30 min to provide polymerization. Thoracic aortas were excised from 6-8 week old Wistar rats (250-300g) and

fibroadipose tissue was carefully removed. Aortas were sliced (≈1mm) and placed onto polymerized Matrigel. Additional 75µl of Matrigel was poured onto aorta slices and incubated 37 °C for 30 min. EGM-2 medium (250µl) containing EBM-2 (#CC-3156, Endothelial basal medium, Lonza, USA) and endothelial growth factors supplied with EGM-2 bullet kit (#CC-3202, Lonza, USA) added into each well and incubated for 24h. The next day, medium was changed with EBM-2 containing 2% FBS, 1% PSA and indicated concentrations of boron derivatives. Vessel spouting from thoracic aortas was monitored at day 7 and pictures were taken using an inverted microscope with AxioCam ERc5s camera.

Table 2.2. Real-time PCR conditions

Cycle	Repeats	Step	Dwell time	Set point
Initial Denaturation	1	1	3 min	93 °C
Denaturation	36	1	30 sec	93 °C
Annealing		2	40 sec	61 °C
Extension		3	45 sec	72° C
Final extension	1	1	10 min	72 °C
Melt curve	110	1	12 sec	-0.5 °C/cycle
Hold	1	1	-	4°C

2.1.8. Growth Factor Array Assay

Growth factors secreted from HF and HaCaT cells, cultured in the presence of boron compounds, were evaluated using Human Growth Factor Antibody Array C1 (#AAH-GF-1, RayBiotech, Inc., GA) according to the manufacturer's instructions. Briefly, cells were treated with 100µg/ml of BA and NaB for 24h at 37 °C in a humidified incubator with 5% CO_2. Cells were collected by trypsinization (0.25% trypsin-EDTA, #25200-056, Gibco, UK) for 3 min at 37 °C. Total proteins were isolated using RIPA buffer (#sc-24948, Santa cruz, USA) and BCA Protein Assay Kit (#23227, Pierce, Rockford, USA) was used to estimate protein concentrations according to the supplier's recommendations. Antibody printed ready-made membranes were incubated with blocking buffer for 30 min. Protein samples (100µg) diluted in 1ml of blocking buffer were added onto the membranes and incubated

overnight at 4 °C. After a three times washing step, membranes were incubated with 1ml of Biotinylated Antibody Cocktail overnight at 4 °C. After washing steps, membranes were incubated with HRP-Streptavidin solution overnight at 4 °C. After an additional three times washing step, membranes were visualized using a luminometer system (ChemicDoc XRS, Biorad, USA). Average spot intensities of each growth factors were calculated using ImageJ software and normalized to respective control samples. Fold changes of 0.77< and >1.3 were designated as the definition of down-regulation and upregulation, respectively, as described in the literature [282].

2.1.9. Micro-well Dilution Assay

Minimum inhibition concentrations (MICs) for the microbial strains tested were determined by micro-well dilution assay as described previously [283]. In short, microbial inoculum (McFarland 0.5) prepared from fresh cultures were spread onto tryptic soy agar (#22091, Sigma-Aldrich, USA), sabouraud dextrose agar (#CM0041, Oxoid, UK), potato dextrose agar (#110130, Merck, Darmstadt, Germany) for bacteria, candida and fungal species, respectively. NaB and BA were dissolved in tryptic soy broth (#CM0129, Oxoid, UK) and sabouraud dextrose broth (#LAB033, LABM, UK) at a concentration of 30mg/ml for bacteria and fungi, respectively, and sterilized using 0.45µm filters. Two-fold dilutions were prepared in sterile test tubes. First seven wells of each column of 96-well plate were filled with 95µl of respective broth and 5µl of inoculum. 100µl of serially diluted NaB and BA solutions were added into each consecutive wells. The last well of each column containing 195µl of respective medium and 5µl of inoculum was used as positive control. The plate were covered with a sealer and shaken at 300 rpm for 20 seconds. Inoculated 96-well plates were incubated at 36±1 ºC for 24h for bacteria, 48h for candida and at 27±1 ºC for 72h for fungal species. At the ends of incubation periods, microbial growth was detected by reading the absorbance at 600nm and 530nm for bacterial and fungal isolates, respectively.

2.1.10. Minimum Bactericidal (MBC) and Fungicidal (MFC) Concentration

MBC/MFC values of NaB and BA for each microorganism tested were evaluated by transferring of 5µl of each well content prepared for micro-well dilution assay to tryptic soy agar, sabouraud dextrose agar and potato dextrose agar for bacteria, candida and fungi,

respectively, at the end of incubation periods. Inoculated plates were incubated for at 36±1 ºC for 24h for bacteria, 48 h for candida and at 27±1 ºC for 72h for fungal species. MBC/MFC values were determined as the lowest concentration of boron derivatives at which no microbial growth was observed.

2.2. *IN VIVO* STUDIES

2.2.1. Animals

Male Wistar rats (n=24) weighing 300±20 g were used in wound healing experiments. The animals were housed at a constant temperature (23±1 °C) and humidity (60±10%), and subjected to an artificial 12-h light/dark cycle and fed with food and water *ad libitum*. Animal handling and surgical procedures were approved with an ethical permission obtained from the Yeditepe University Ethics Committee of Experimental Animal Use and the Research Scientific Committee at the same institution.

2.2.2. Gel Preparation

Carbopol based hydrogels were prepared by dispersing 1% (w/v) polymer (Carbopol Ultrez-21, Lubrizol, USA) in ddH_2O. 1M sodium hydroxide (NaOH) solution (1.6g for 1L carbopol-ddH_2O suspension, # 221465, Sigma-Aldrich, USA) was used as the neutralizing agent for the gelation of the polymer. Then, NaB was mixed at a final concentration of 3% (w/v) and the pH was adjusted to 6.5 using 1M NaOH. The prepared formulation was stored at 4 °C until the day of experiment. Carbopol based hydrogel without NaB additive was used a control.

2.2.3. Induction of Type 1 Diabetes Mellitus

Animals were fasted for 16h and subjected to a single intraperitoneal streptozotocin (STZ, 65mg/kg) injection (#sc-200719, Santa Cruz Biotechnology Inc., Santa Cruz, CA), freshly prepared in ice cold sodium citrate buffer (pH 4.5) as described before [284]. Non-diabetic rats (control group) received same volume of citrate buffer (500µl). At day seven of STZ injection, fasting blood glucose level of blood sample taken by a distal tail small injury was

measured using a commercially available glucometer (Accu-chek, Roche, Germany). Diabetes status of rats was defined as fasting blood glucose levels greater than 300mg/dl. The animals with blood glucose levels lover than 300mg/dl for diabetic groups were excluded from the study. Diabetic animals were kept for 21 days to stabilize their diabetic state before starting wound healing experiment.

2.2.4. Full-thickness Skin Wounding and Treatment

Wounding protocol was performed under general anesthesia (ketamine (100 mg/kg)/xylazine (10mg/kg)). Rats were divided into 3 groups (n=8). Dorsal part of the rats were shaved and cleansed 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. Diabetic rats were left untreated (group 1), treated with vehicle hydrogel (group 2) or treated with NaB (3 % w/v) containing hydrogel (group 3). Gel formulations were applied once a day for seven days. Photographs of each wound with an internal scale were taken every two days throughout the study to calculate wound contraction rate. Wound surface areas were calculated using Image J software.

2.2.5. Estimation of Wound Healing (Closure) Rate

Wound healing rate was calculated according to the equation 2.2 below using respective initial and final wound areas;

$$\text{\% of wound contraction} = (\mathrm{Ai} - \mathrm{At})/\mathrm{A0} \times 100 \quad (2.2)$$

where A_i is the initial area of wounded area and A_t is the wound area at day 2, 4, 6 and 8, accordingly.

2.2.6. Histopathological Examinations

The animals were sacrificed at day 8 by decapitation under moderate ether anesthesia, and skin samples from each animal were collected in 10% neutral formaldehyde at room

temperature for 48h. The samples were dehydrated in a graded series of alcohol (70%, 90%, 96% and 100%) and cleared with toluene. Tissues were embedded in paraffin blocks and cut by a cryostat microtome (Slee, Mainz, Germany). Skin sections from each animal (4µm) were processed for hematoxylin and eosin (H&E) and Masson's trichrome stain to evaluate the general skin morphology as described previously [285]. The sections were deparaffinized using toluene and rehydrated using decreasing concentrations of alcohol (70%, 90%, 96% and 100%) followed by staining with hematoxylin for 15 min. The sections were rinsed under running tap water for 10 min and stained with eosin for 8 min subsequently. The samples were dehydrated using graded series of alcohol and cleared with toluene.

The cross sections to be stained with Masson's trichrome were deparaffinized with toluene and rehydrated in decreasing alcohol series (100%, 96%, 90% and 70%). The sections were immersed in dH_2O and stained with Masson tricromica kit (#04 – 010802, Bio-Optica, Milan, Italy) according to the manufacturer's instructions. Then, the sections were dehydrated in increasing concentrations of alcohol and cleared in toluene. Photomicrographs were taken using Nikon Eclipse Ni-U microscope and a Nikon Digital DS-Fi1-U3 camera with corresponding software (Nikon, Tokyo, Japan).

2.2.7. Histological Grading

The tissue samples stained with H&E were examined in terms of general morphology and epithelization, and the samples stained with Masson's trichrome were evaluated in terms of collagen deposition, fibroblast density and inflammatory cell migration. All sections from each groups were scored from 1 to 12 based on criteria published previously [286] as below;

- **1-3:** No or weak cellular accumulation. No granulation or epithelial migration.
- **4-6:** Immature granulation where majority of cells are inflammatory cells. Weak collagen deposition, capillary formation and few fibroblast cells. Minimal epithelial migration.
- **7-9:** Moderate granulation; from being dominated inflammatory cells to dense collagen deposition and a great number of fibroblast cells. Intense neovascularization. Minimal or average epithelial migration.

- **10-12:** Rigorous vascular granulation tissue formation. Majority of cells are fibroblasts. From partially to completely recovered epithelium.

2.3. STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was conducted 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.

3. RESULTS

3.1. CELL PROLIFERATION

The effects of boron derivatives on dermal cells proliferation (HF, L-929, HUVEC and HaCaT) were evaluated measuring mitochondrial activity by MTS assay. The results revealed that concentrations between 5 and 1000µg/ml for BA and NaB significantly increased the proliferation rate of HF cells on day 1 and 2 ($P<0.05$). Interestingly, low dose boron treatment for both BA and NaB diminished viable HF cell number compared to growth medium treated control cells after a 3-day incubation period. BA and NaB treatments for 24h resulted in an approximately 30% and 40% increase, respectively, in cell number for the concentrations ranging from 5 to 1000µg/ml. BA and NaB administrations were also found to increase viable cell numbers at day 2. Almost an 80% increase in cell proliferation was noted for particular concentrations (100 and 200µg/ml) of BA and NaB at day 2, indicating the proliferative effect of boron compounds on HF cells (Figure 3.1). However, there was not any statistically significant difference between any experimental groups at day 3 ($P<0.05$).

HUVEC cells displayed quite similar pattern as HF cells when they were exposed to BA or NaB. Endothelial cells treated with BA at the concentrations between 5 and 1000µg/ml led to an average increase of 15% in cell mass at day 1. Similarly, 20-1000µg/ml of NaB administration caused an average increase of 20% in cell number compared to control group after 24h. Although proliferative effects of BA and NaB treatment were detected with an increase up to 80% and 60%, respectively, in comparison with growth medium treated HUVEC cells at day 2, the number of boron treated cells was not significantly different from control cells at day 3 ($P<0.05$), showing repressing effect of prolonged boron treatment on proliferative activity. Moreover, low doses, 1 and 2µg/ml, of both BA and NaB treatments were found to reduce viable HUVEC cells as it was in HF cells (Figure 3.2).

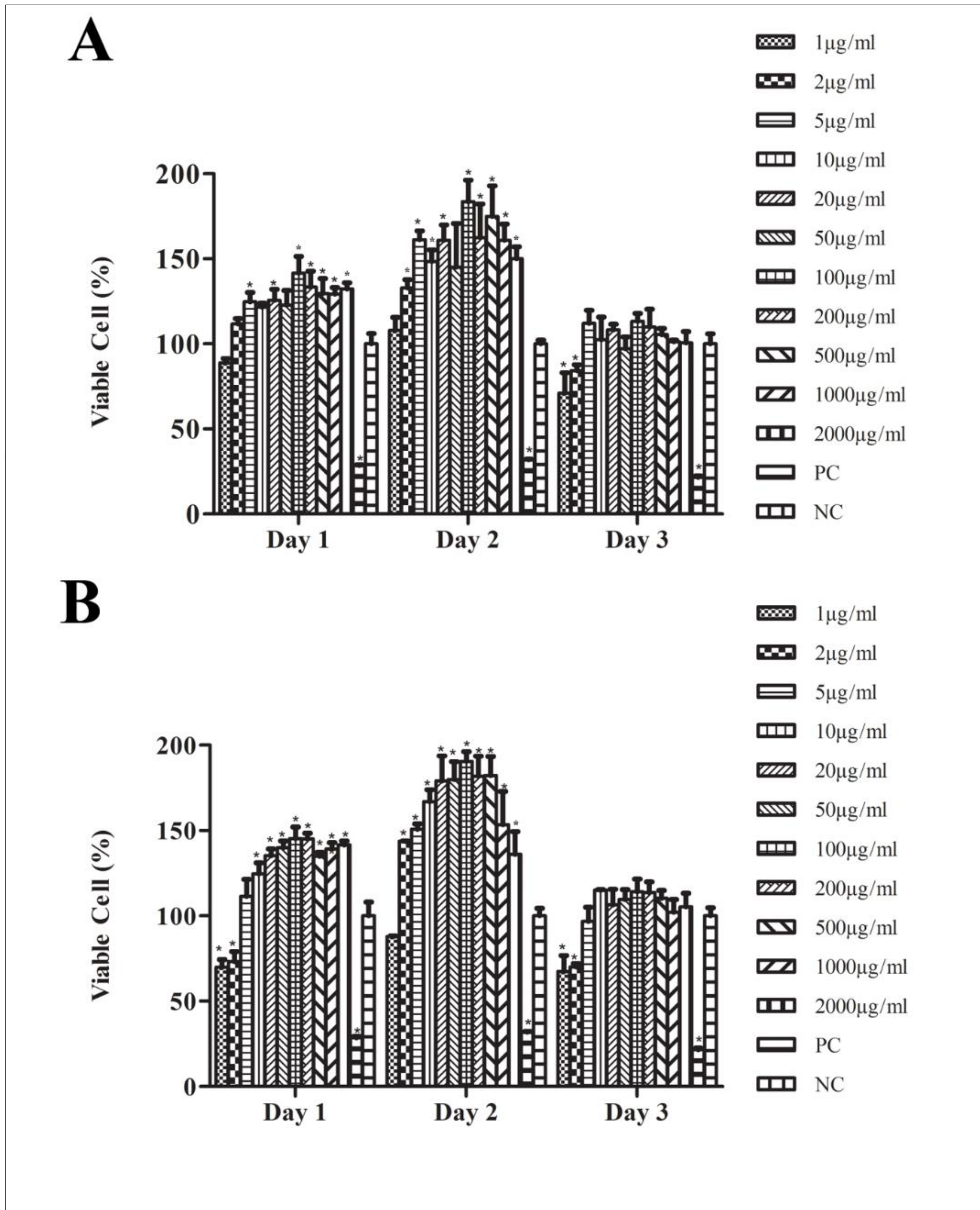


Figure 3.1. Effect of various concentrations of boric acid (A) and sodium pentaborate pentahydrate (B) concentrations on proliferation of human fibroblast (HF) cells. Notes: NC: Negative Control (complete growth medium), PC: Positive Control (20% DMSO containing growth medium) *$P<0.05$. NC was accepted as 100% for each day.

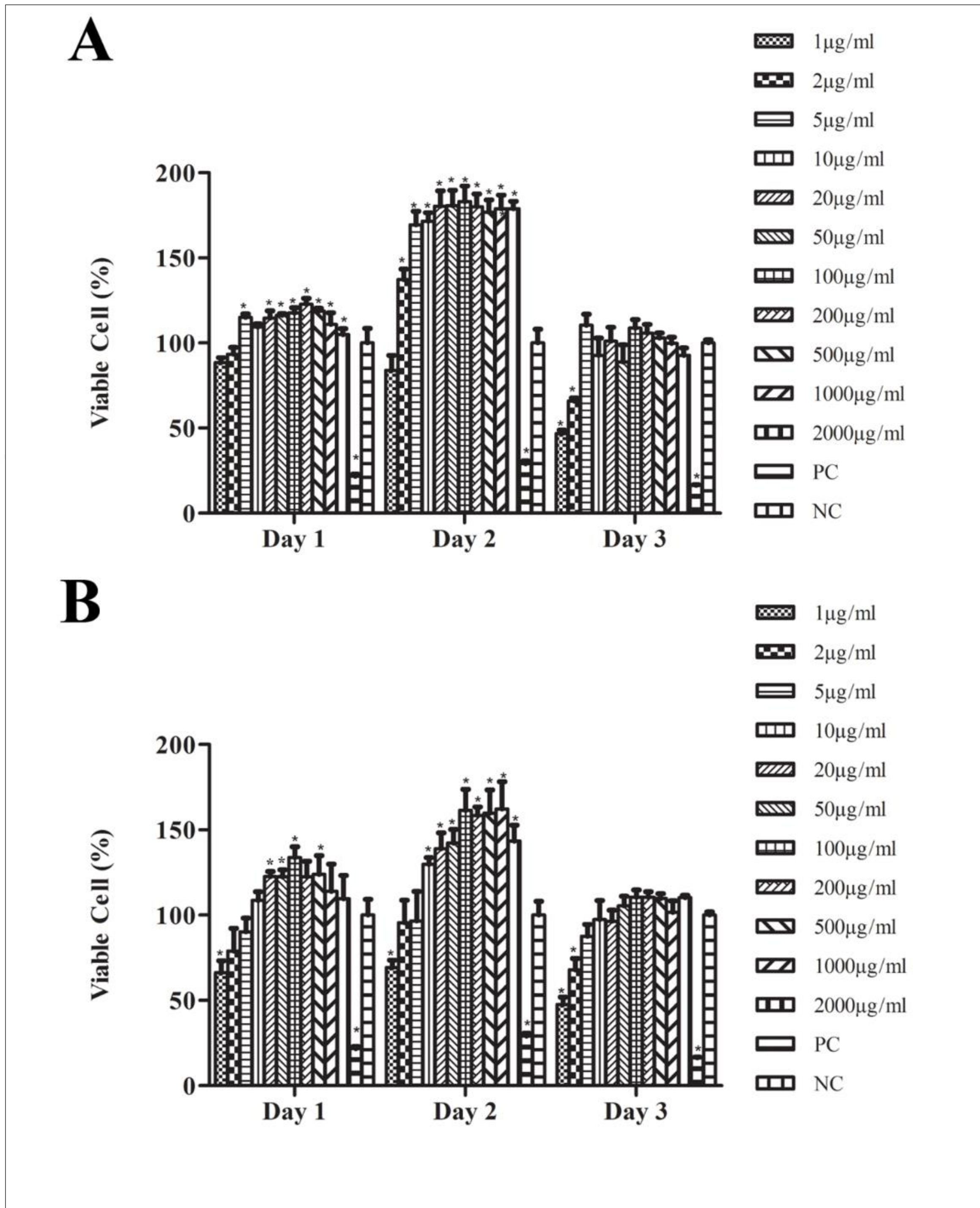


Figure 3.2. Effect of various concentrations of boric acid (A) and sodium pentaborate pentahydrate (B) on proliferation of Human umbilical vein endothelial cells (HUVEC). Notes: NC: Negative Control (complete growth medium), PC: Positive Control (20% DMSO containing growth medium), **P*<0.05. NC was accepted as 100% for each day.

Proliferation of HaCaT cells was triggered by boron application at the concentrations ranging from 1 to 200µg/ml of BA and NaB at day 1 and 2. An average increase of 30% in viable cell ratio was obtained at day 1 for both BA and NaB. Boron treatment displayed similar effect with an average 25% increase in cell proliferation rate at day 2 for all concentrations from 1 to 200µg/ml of BA and NaB (Figure 3.3). Just as in boron treated HF and HUVEC cells, no statistical difference in terms of cell viability was detected between experimental groups at day 3. High dose boron applications (500, 1000 and 2000µg/ml), however, exhibited significant levels of cytotoxicity on HaCaT cells in a time- and dose-dependent manner.

To evaluate possible species-specific roles of boron, mouse fibroblast cells (L-929) were treated with indicated concentrations of BA and NaB for 3 days. The results illustrated that both boron derivatives significantly increased the viability of mouse fibroblast cells on day 1. BA and NaB application led to an approximately 30% and 40% increase in viable cell ratio, respectively, compared to growth medium treated control group at day 1 for the concentrations between 5 and 1000µg/ml of BA and NaB. On the other hand, proliferative effect of boron treatment disappeared on day 2 and 3. In addition, low concentrations of boron, 1 and 2µg/ml, decreased viable cell ratio compared to control groups at day 2 and 3 (Figure 3.4).

According to the *in vitro* toxicity analysis, boron treatment (both BA and NaB) was generally found to increase cell proliferation for two-days incubation period at intermediate concentrations tested. Up to 200µg/ml (except 1 and 2µg/ml) of BA and NaB treatments were found to increase cell viability of all dermal cells tested (HF, L-929, HaCaT and HUVEC) at the end of 24h and 48h incubation periods, and did not exert any cytotoxic effect after 72h. Therefore, four separate concentrations of BA and NaB (15, 20, 100 and 200µg/ml) were selected for further *in vitro* experiments.

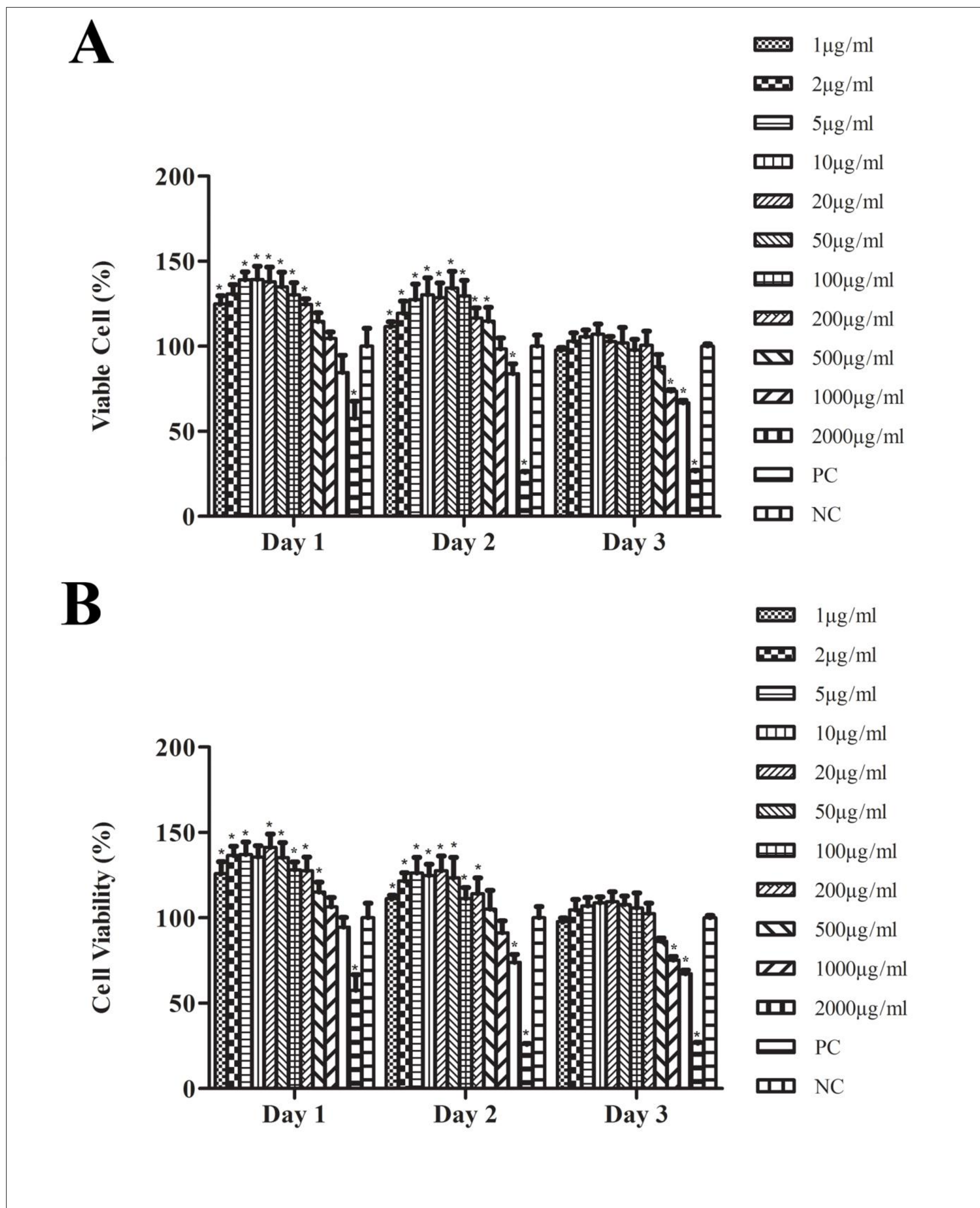


Figure 3.3. Effect of various concentrations of boric acid (A) and sodium pentaborate pentahydrate (B) on proliferation of HaCaT keratinocyte cells. Notes: NC: Negative Control (complete growth medium), PC: Positive Control (20% DMSO containing growth medium), *$P<0.05$. NC was accepted as 100% for each day.

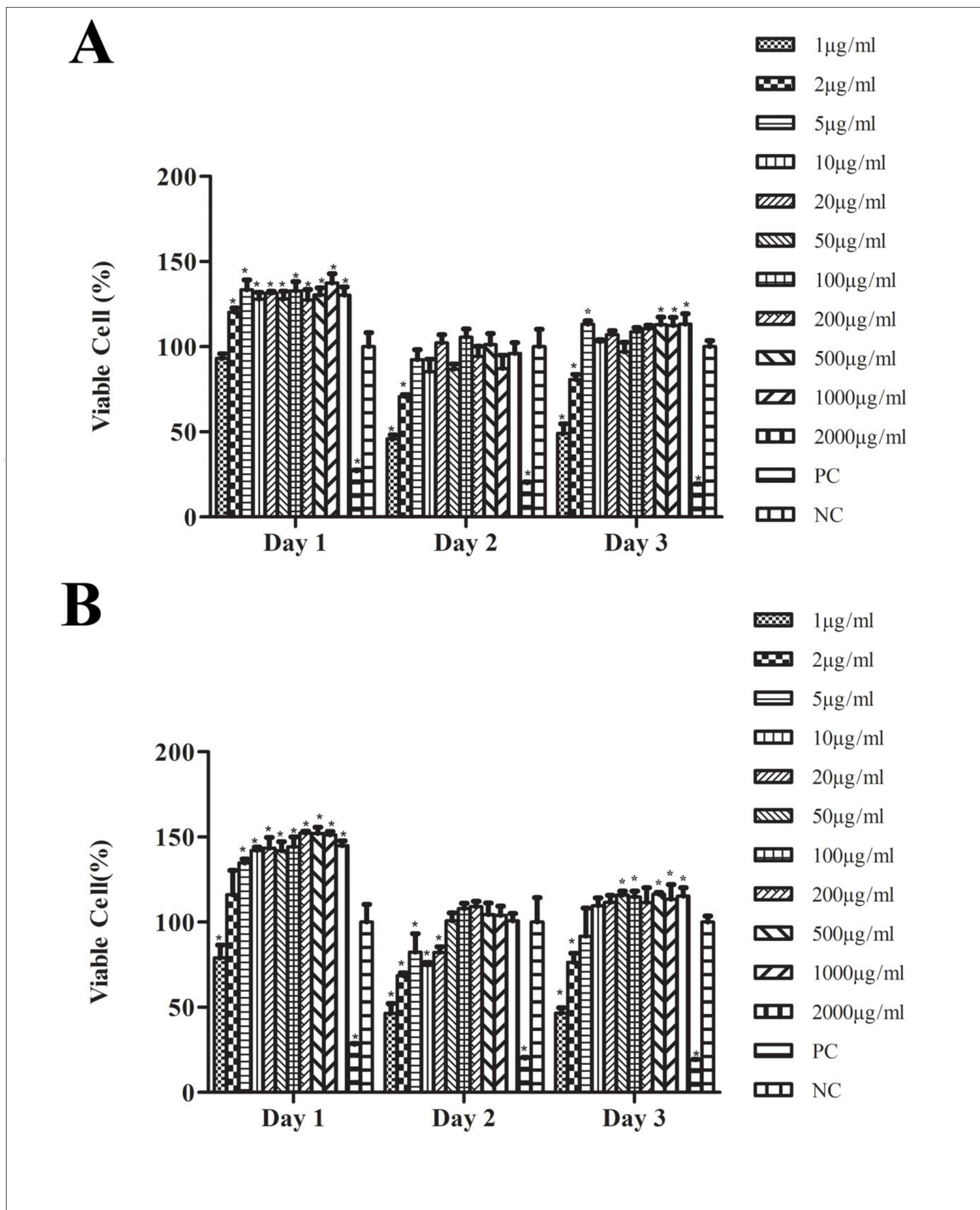


Figure 3.4. Effect of various concentrations of boric acid (A) and sodium pentaborate pentahydrate (B) on proliferation of mouse fibroblast (L-929) cells. Notes: NC: Negative Control (complete growth medium), PC: Positive Control (20% DMSO containing growth medium), *$P<0.05$. NC was accepted as 100% for each day.

3.2. CELL MIGRATION ANALYSIS (SCRATCH ASSAY)

To evaluate potential migration stimulatory or inhibitory effects of boron derivatives on dermal cells, scratch assay was conducted. The results showed that as HF cells treated with 15, 20, 100 and 200µg/ml of BA, 83%, 84%, 86% and 80% wound closure rates were obtained after 24h, respectively, while negative control cell wound closure rate was only 60%. All tested concentrations were found to be effective at the second half time period of the experiment (12-24h). Although NaB also significantly increased the cell migration ratio of HF cells, BA was detected to be more efficient compared to NaB. The concentrations of 15, 20, 100 and 200µg/ml for NaB induced HF cell migration to the denuded area in rates of 63%, 72%, 67% and 69%, respectively, indicating boron's migratory activity on HF cells along with its proliferative potential. In line with the effect of BA on HF migration, migratory activity of NaB was observed between 12 and 24h of treatment while there was not any significant difference between NaB treated (15, 100 and 200µg/ml) and untreated cells within the first twelve hours (Figure 3.5).

HUVEC cells was introduced to specified concentrations of both BA and NaB to evaluate the effects of boron on endothelial cell migration. According to the results, while cells in 15, 20 and 100µg/ml of BA treated and untreated cells migrated vigorously and completely closed the wounded area after 24h, 200µg/ml of BA treatment reduced the cell migration and provided an 85% wound closure rate on average. In consistent with BA treatment, HUVEC cells gave quite similar response to NaB treatment. Although 15µg/ml of NaB exposed and untreated control cells completely recovered the gap, 20, 100 and 200µg/ml of NaB slightly decreased (not significantly) the migration potential of HUVEC cells with the rates of 97%, 98% and 93%, respectively, showing potential migration inhibitory activity of boron compounds regardless of the formula of boron derivatives (Figure 3.6).

To evaluate the effect of boron derivatives on keratinocyte migration and re-epithelization, BA and NaB treated HaCaT cells, reference human keratinocyte cell line, were subjected to migration analysis. The results proved that HaCaT cells migrated faster to the cell-free area as they were treated with both BA and NaB. Figure 3.7 illustrates that 75%, 72%, 88% and 92% wound closure rates were noted for 15, 20, 100 and 200µg/ml of BA treated cells, respectively. Moreover, concentrations of 15, 100 and 200µg/ml for BA were found to

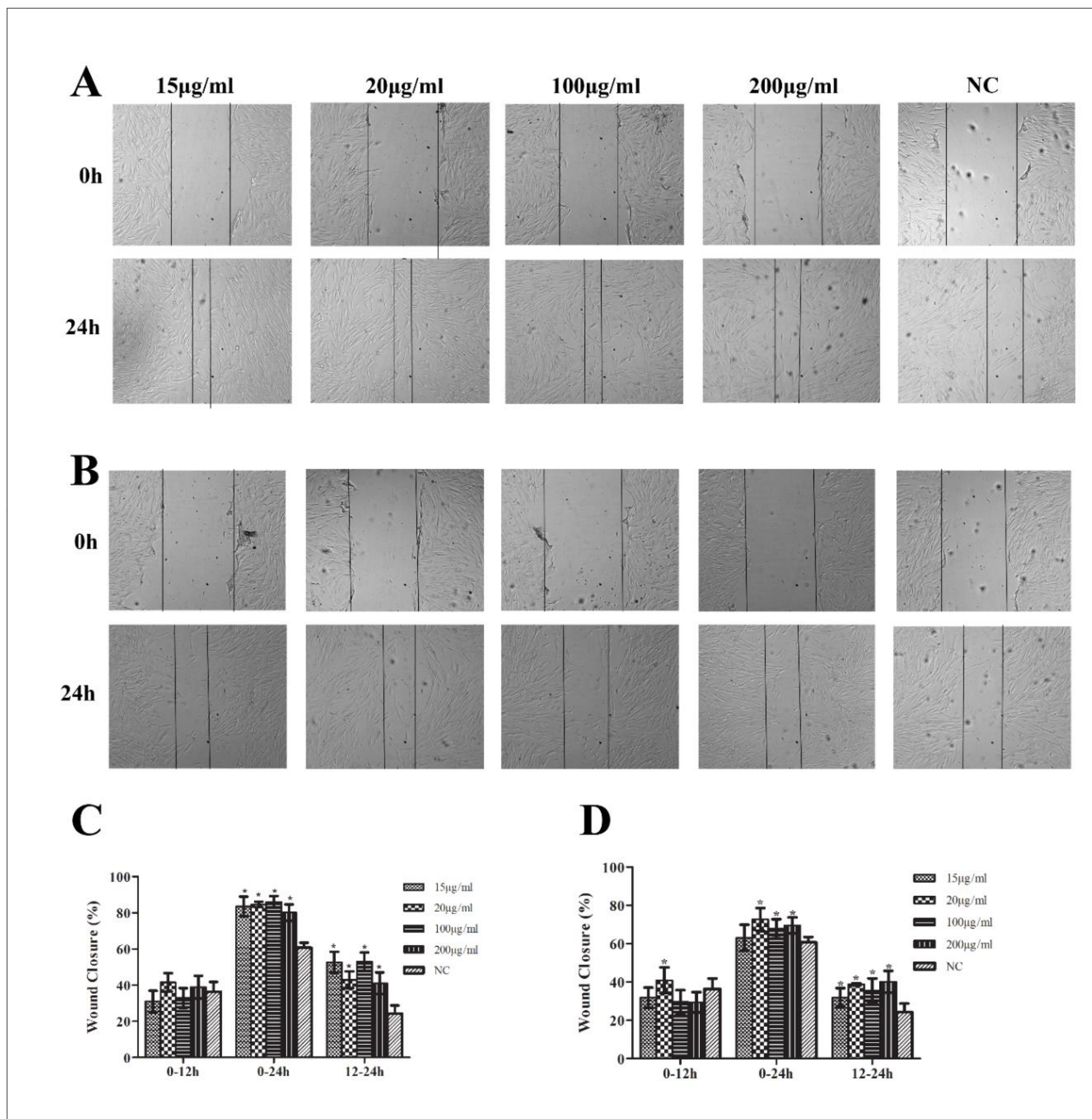


Figure 3.5. Scratch assay analysis of human fibroblast (HF) cells treated with various concentrations of boric acid (BA) and sodium pentaborate pentahydrate (NaB). (A) Representative images of BA treated HF scratches taken using inverted light microscope. (B) Representative images of NaB treated HF scratches taken using inverted light microscope. (C) Wound closure rates of HF cells after BA application. (D) Wound closure rates of HF cells after NaB application. NC: Growth medium treated cells, $^*P<0.05$, magnification: 40x.

significantly increase keratinocyte migration at first 12h, whereas the concentrations of 20 and 200µg/ml were effective at the second half (12-24h) of incubation period. Although both

boron compounds tested were found to be effective, NaB treated HaCaT cells migrated faster in comparison with BA treated cells. Cell migration percentages for 15, 20, 100 and 200μg/ml of NaB treated HaCaT cells were 80%, 90%, 93% and 92%, respectively, while negative control cell wound closure rate was only 67% (Figure 3.7).

L-929 cells were used as a model mouse fibroblast cell line in scratch assay to investigate potential species-specific migration induction or inhibitory activity of boron compounds. BA treatment at 20 and 100μg/ml concentrations enhanced fibroblast migration compared to growth medium treated control cells. While the scratch closure rate of control group was 61%, it was 78% and 84% for 20 and 100μg/ml of BA treatments, respectively. Migratory activity of BA on mouse fibroblasts was greater in the first 12h. Except the highest concentration, all concentrations of (15, 20 and 100μg/ml) of NaB promoted L-929 cell migration significantly at the end of 24h. In consistent with BA's activity, NaB treated cell motility was significantly faster at the first 12h period. An average rates of 70%, 71%, 70% and 60% scratch closure were measured for 15, 20, 100 and 200μg/ml of NaB, respectively (Figure 3.8).

3.3. RT-PCR ANALYSIS

To examine potential action of mechanism for proliferative and migratory activity of boron derivatives, HF, L-929 and HaCaT cells were treated with boron derivatives (NaB and BA) at aforementioned concentrations (15, 20, 100 and 200μg/ml) for 24h and wound healing associated gene expression levels were evaluated using RT-PCR analysis. Interestingly, it was found that AKT, laminin and fibronectin gene expressions were found to be higher in NaB treated HF cells in a dose-dependent manner, whereas BA treatment did not result in any change in mRNA expressions of these genes (Figure 3.9 and 3.10). Collagen type I gene levels were not significantly different in both boron treated and growth medium treated control cells. In addition, while BA treatment decreased mRNA levels of MMP2 and MMP9 in a dose-dependent manner, NaB application did not result in a significant change in the expression levels. An average of 10-fold decrease for MMP2 and MMP9 mRNA levels was observed for the BA treated cells. MMP2 expression level slightly reduced in high dose NaB applications but MMP9 mRNA levels remained constant.

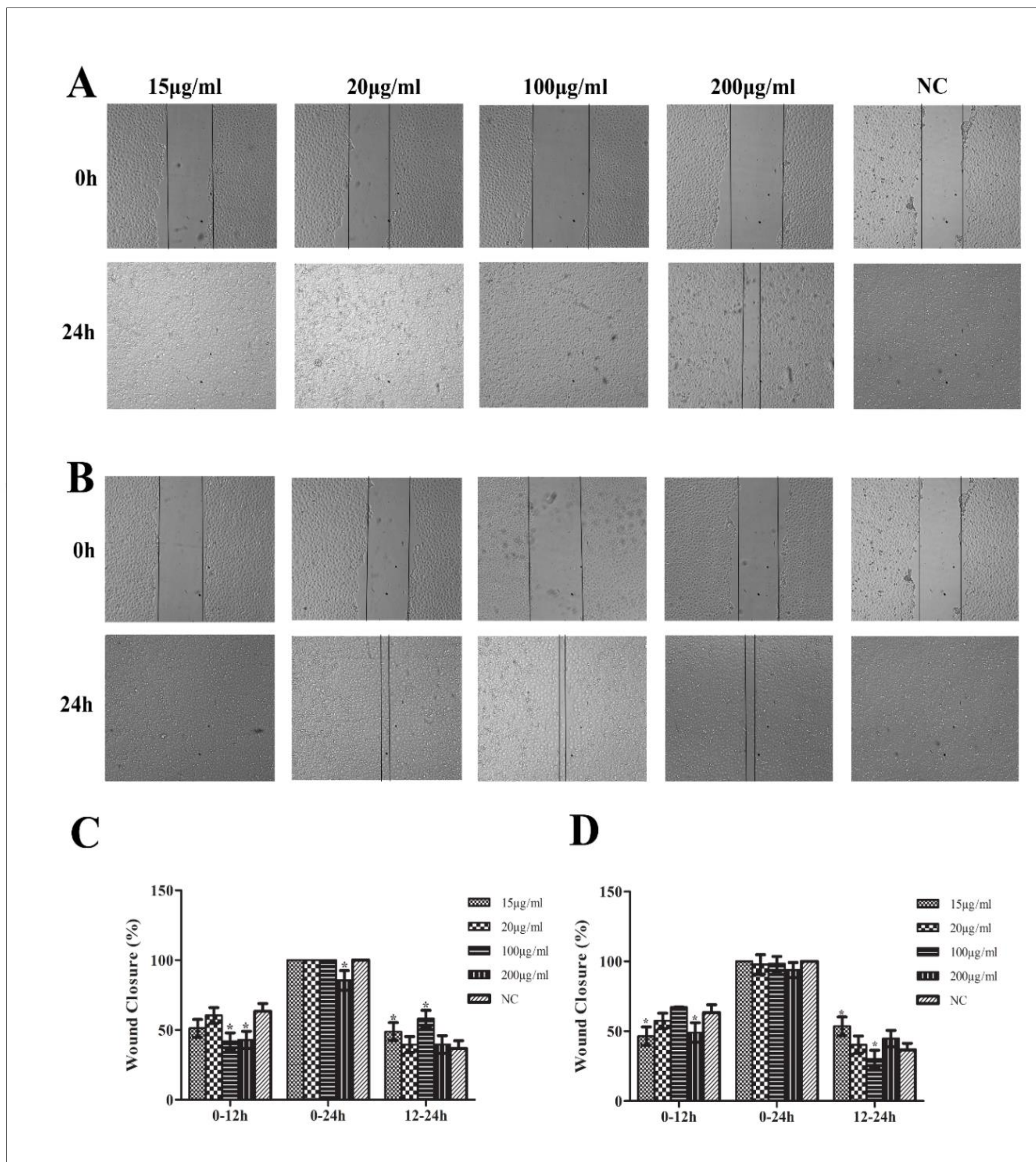


Figure 3.6. Scratch assay analysis of human umbilical vein endothelial cells (HUVECs) treated with various concentrations of boric acid (BA) and sodium pentaborate pentahydrate (NaB). (A) Representative images of BA treated HUVEC scratches taken using inverted light microscope. (B) Representative images of NaB treated HUVEC scratches taken using inverted light microscope. (C) Wound closure rates of HUVEC cells after BA application. (D) Wound closure rates of HUVEC cells after NaB application. NC: Growth medium treated cells, *$P<0.05$, magnification: 40x.

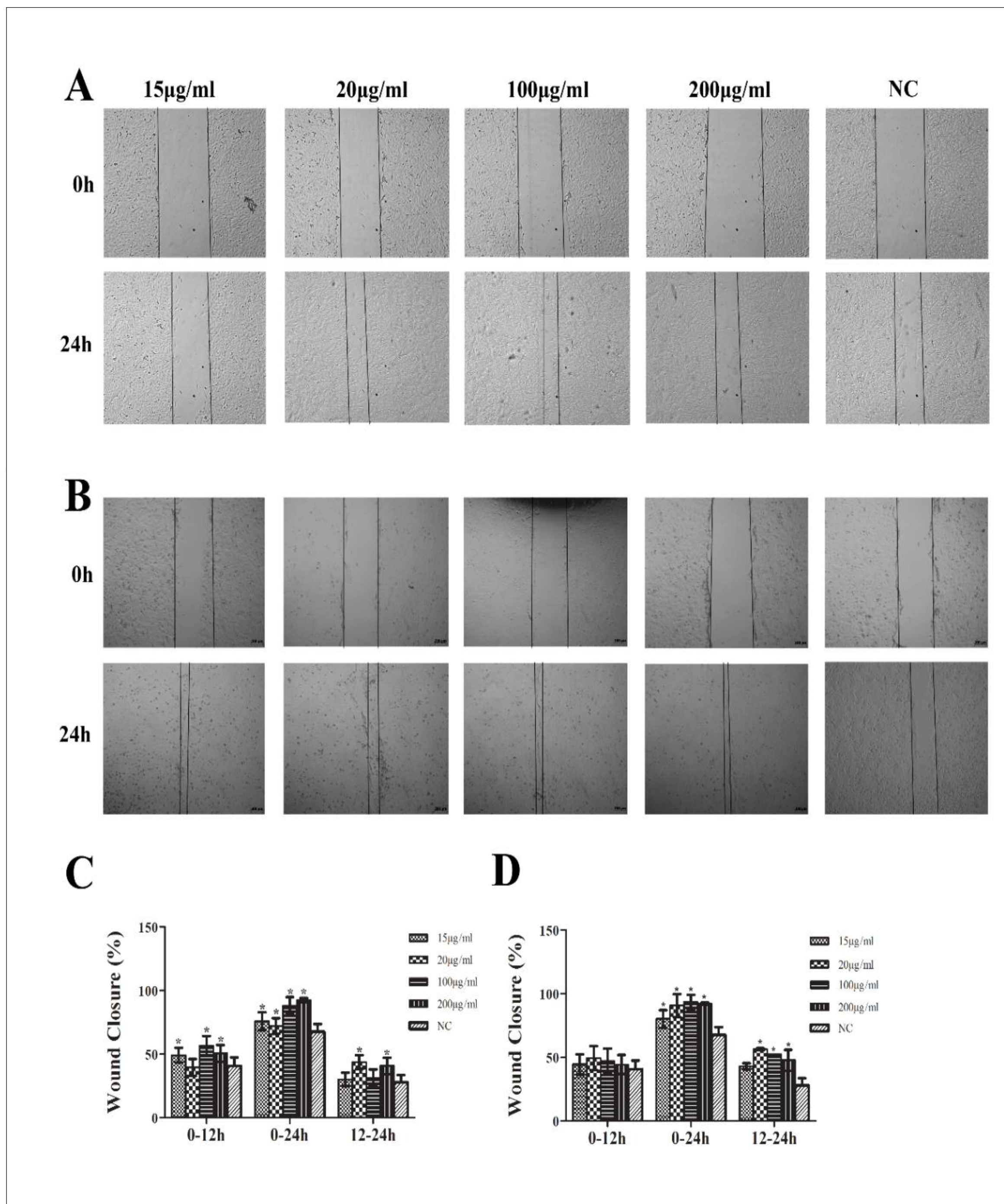


Figure 3.7. Scratch assay analysis of HaCaT cells treated with various concentrations of boric acid (BA) and sodium pentaborate pentahydrate (NaB). (A) Representative images of BA treated HaCaT scratches taken using inverted light microscope. (B) Representative images of NaB treated HaCaT scratches taken using inverted light microscope. (C) Wound closure rates of HaCaT cells after BA application. (D) Wound closure rates of HaCaT cells after NaB application. NC: Growth medium treated cells, *$P<0.05$, magnification: 40x.

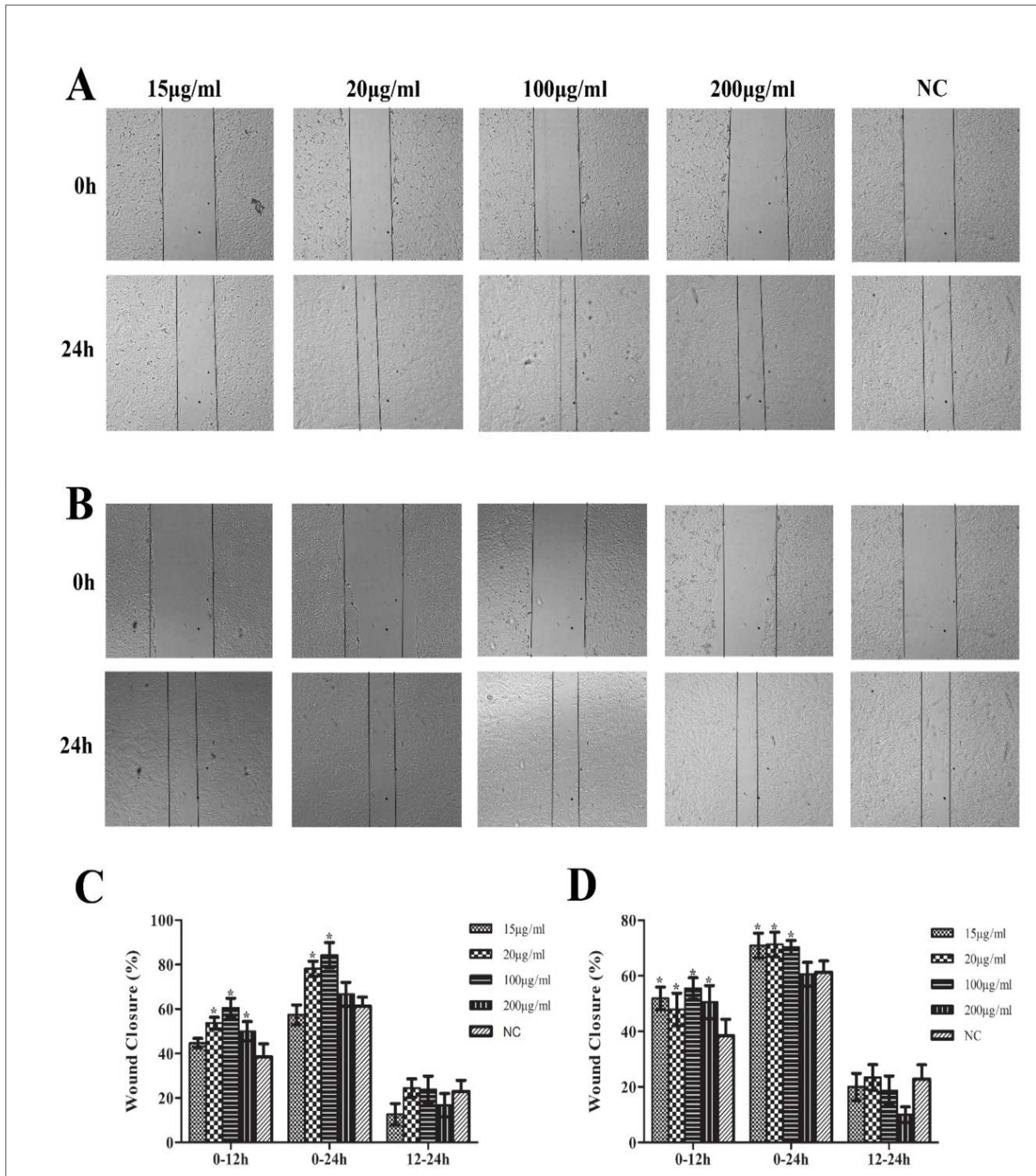


Figure 3.8. Scratch assay analysis of L-929 cells treated with various concentrations of boric acid (BA) and sodium pentaborate pentahydrate (NaB). (A) Representative images of BA treated L-929 scratches taken using inverted light microscope. (B) Representative images of NaB treated L-929 scratches taken using inverted light microscope. (C) Wound closure rates of L-929 cells after BA application. (D) Wound closure rates of L-929 cells after NaB application. NC: Growth medium treated cells, *P<0.05, magnification: 40x

Mouse fibroblast cells gave slightly different response to BA treatment compared to HF cells. While BA application did not change AKT, laminin and fibronectin expression levels in HF cells, it enhanced the mRNA levels in L-929 cells (Figure 3.11), indicating possible species-specific roles of various boron compounds. In consistent with RT-PCR assays conducted with HF cells, NaB treatment resulted in an approximately 150-fold increase in AKT, laminin and fibronectin mRNA levels in L-929 cells (Figure 3.12). Moreover, collagen type I, MMP2 and MMP9 mRNA levels were found to be upregulated by both BA and NaB treatments.

Keratinocyte cells (HaCaT) treated with specified concentrations of BA and NaB for 24h found to express remarkable levels of fibronectin and laminin mRNAs compared to growth medium treated control cells (Figure 3.13 and 3.14). AKT mRNA expression was diminished in BA treated cells. NaB treatment, however, moderately augmented AKT mRNAs at 20 and 200µg/ml concentrations and it did not result in any significant change at 15 and 100µg/ml concentrations. MMP2 and MMP9 levels were significantly upregulated by both boron administrations for all concentrations.

3.4. GROWTH FACTOR ARRAY ANALYSIS

HF and HaCaT cells were treated with 100µg/ml of BA or NaB for 24h and 41 human growth factors of boron treated fibroblast and keratinocyte cells were evaluated by array-based assay (Figure 3.15 and 3.16). The results were normalized to control samples and fold changes of 0.77< and >1.3 were designated as the definition of down-regulation and upregulation, respectively. Figure 3.15 depicts that BA treatment resulted in overexpression of beta-NGF, GM-CSF, insulin-like growth factor-binding protein (IGFBP)-1 and VEGFR2 in HF cells, whereas NaB treated HF cells expressed high levels of bNGF, granulocyte colony stimulating factor (G-CSF), GM-CSF, IGFBP-1 and VEGFR2. Growth factor prolife of keratinocyte cells were much more affected by boron treatment compared to fibroblast cells. For HaCaT cells treated with 100µg/ml of BA, FGF-7, G-CSF, GM-CSF and TGF-β1, and low levels of FGF-6 and glial cell line-derived neurotrophic factor (GDNF) were noted. NaB treated keratinocyte cells overexpressed more growth factors including bFGF, EGF, FGF-7, G-CSF, GM-CSF, IGFBP-6, TGF-β1, TGF-β3 and VEGFR3 and down-regulated GDNF.

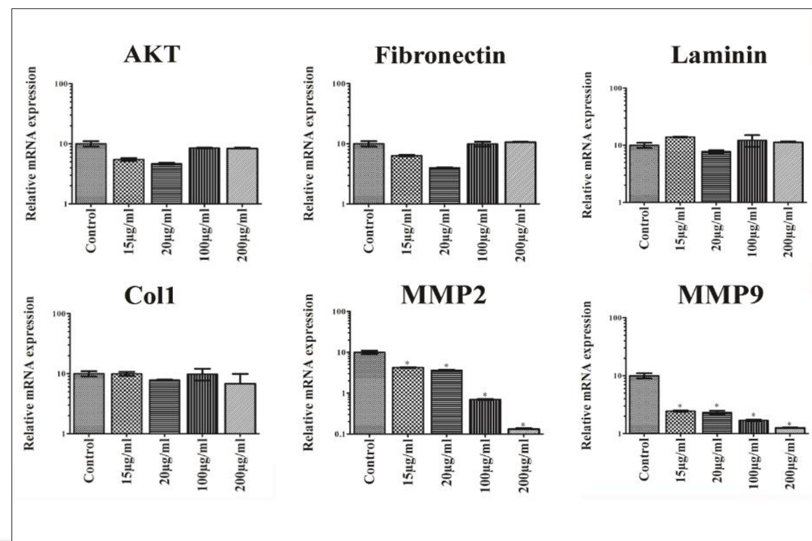


Figure 3.9. . Effect of boric acid on mRNA expression levels of wound healing associated genes in human fibroblast (HF) cells. Abbreviations: AKT: protein kinase B, Col1: Collagen type I, MMP2 and MMP9: Matrix metalloproteinase-2 and 9, Control: Growth medium treated HF cells. Notes: Results were analyzed by one-way ANOVA and Tukey’s post hoc test. *P<0.05.

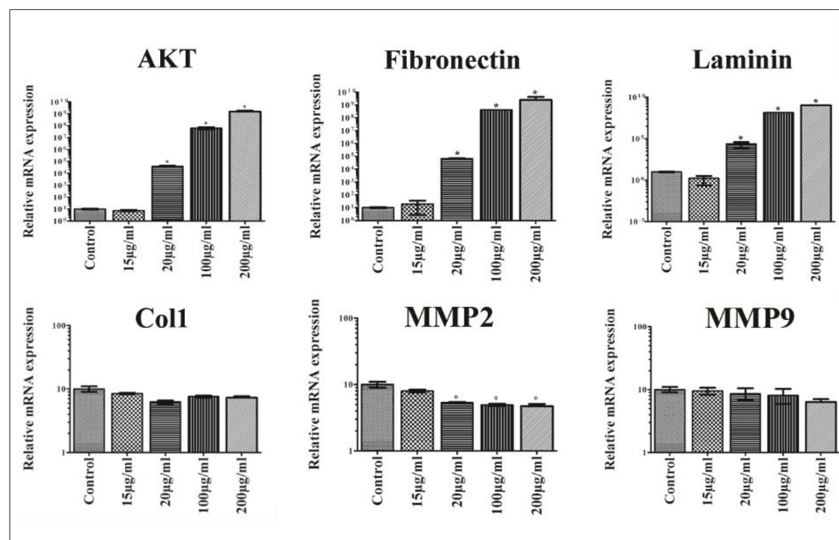


Figure 3.10. Effect of sodium pentaborate pentahydrate on mRNA expression levels of wound healing associated genes in human fibroblast (HF) cells. Abbreviations: AKT: protein kinase B, Col1: Collagen type I, MMP2 and MMP9: Matrix metalloproteinase-2 and 9, Control: Growth medium treated HF cells. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

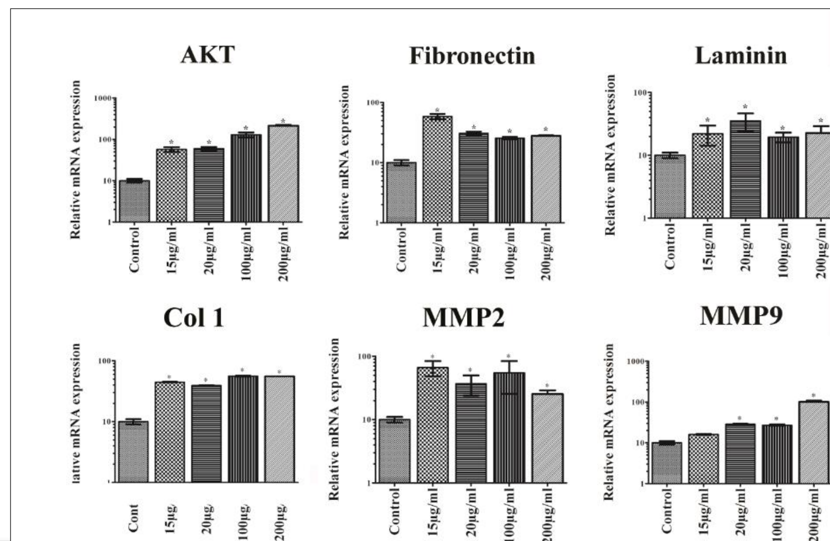


Figure 3.11. Effect of boric acid on mRNA expression levels of wound healing associated genes in L-929 cells. Abbreviations: AKT: protein kinase B, Col 1: Collagen type I, MMP2 and MMP9: Matrix metalloproteinase-2 and 9, Control: Growth medium treated cells. Results were analyzed by one-way ANOVA and Tukey’s post hoc test. *P<0.05.

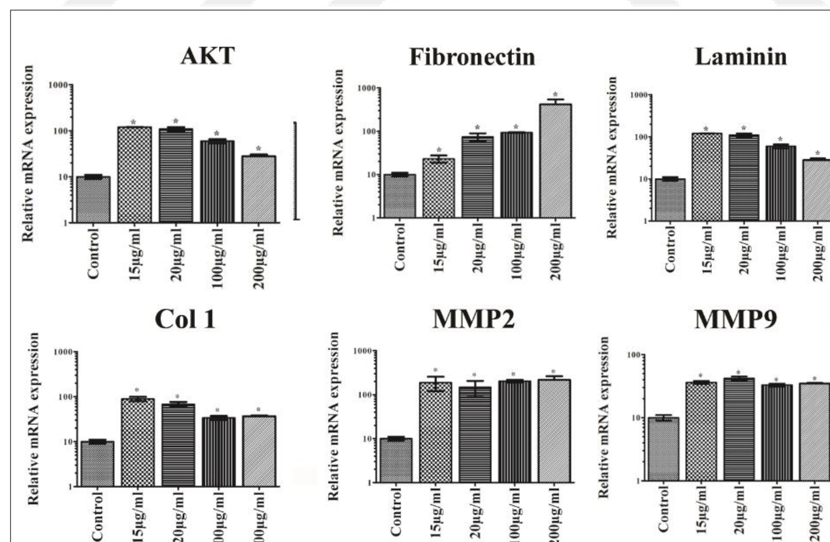


Figure 3.12. Effect of sodium pentaborate pentahydrate on mRNA expression levels of wound healing associated genes in L-929 cells. Abbreviations: AKT: protein kinase B, Col 1: Collagen type I, MMP2 and MMP9: Matrix metalloproteinase-2 and 9, Control: Growth medium treated L-929 cells. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

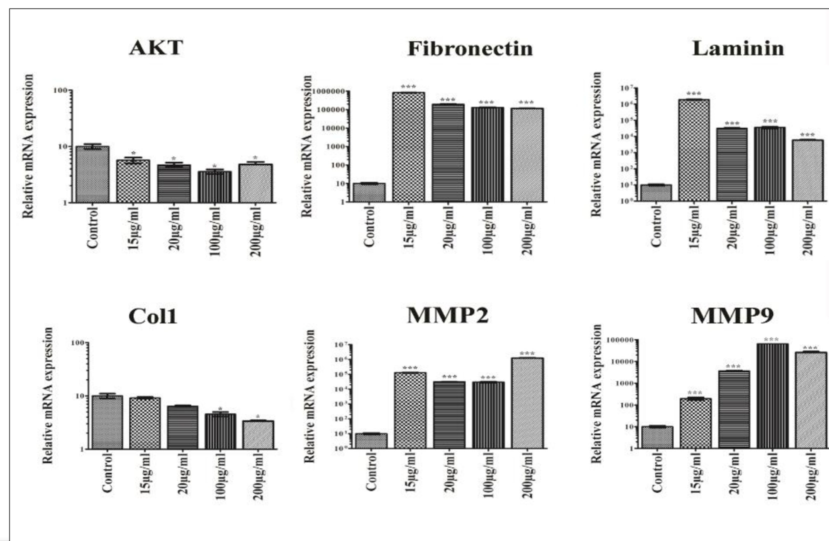


Figure 3.13. Effect of boric acid on mRNA expression levels of wound healing associated genes in HaCaT cells. Abbreviations: AKT: protein kinase B, Col1: Collagen type I, MMP2 and MMP9: Matrix metalloproteinase-2 and 9, Control: Growth medium treated HaCaT cells. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

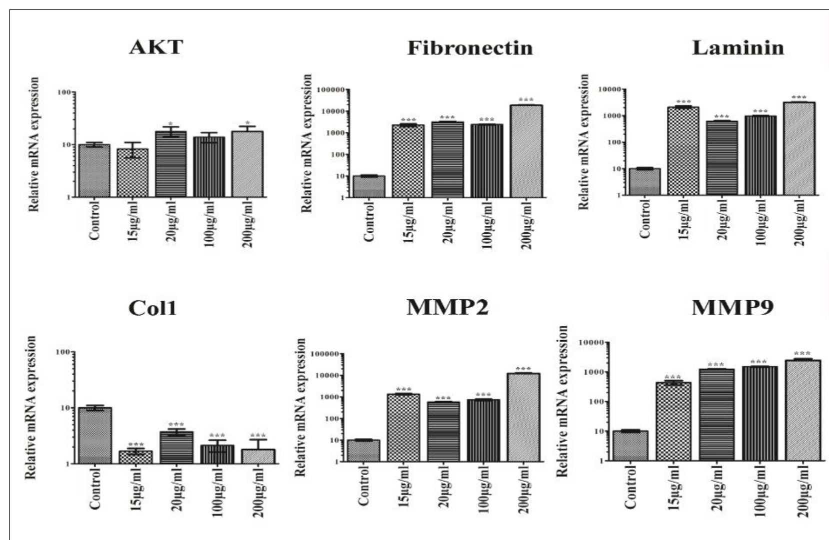


Figure 3.14. Effect of sodium pentaborate pentahydrate on mRNA expression levels of wound healing associated genes in HaCaT cells. Abbreviations: AKT: protein kinase B, Col1: Collagen type I, MMP2 and MMP9: Matrix metalloproteinase-2 and 9, Control: Growth medium treated HaCaT cells. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

3.5. ANGIOGENESIS ASSAY

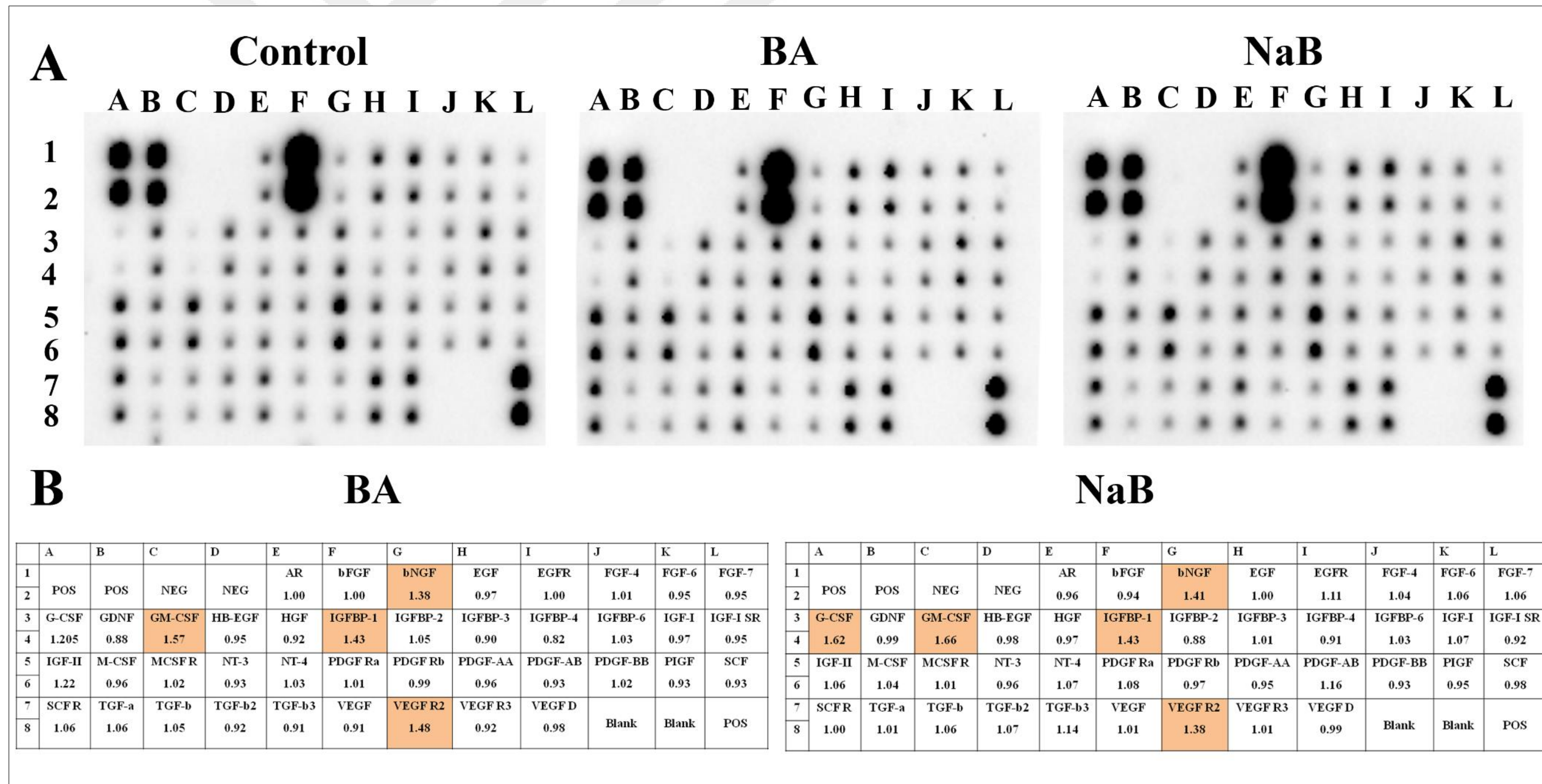
In order to investigate potential effects of boron derivatives on vascularization, a vital process for a healthy wound healing, HUVEC cells were treated with indicated concentrations of boron derivatives. Two different experimental models were used for the evaluation of angiogenesis: tube formation assay and rat aortic ring assay.

3.5.1. Tube Formation Assay

HUVEC cells cultured on matrigel were exposed to aforementioned concentrations of BA and NaB, and tube-like structures were examined. In contrast to proliferative and migrative activities of boron compounds, number of branches in both BA and NaB treated groups decreased in a dose-dependent manner (Figure 3.17). An average decrease of 33%, 37%, 36% and 58% in tube-like structures was observed for 15, 20, 100 and 200µg/ml of BA applications, respectively, with respect to control group. As in BA treatment, significant reduction in tube formation was obtained for 15, 20, 100 and 200µg/ml NaB groups in percentages of 49%, 44%, 39% and 40% respectively, showing boron-specific antiangiogenic effect.

3.5.2. Rat Aortic Ring Assay

Rat aortic ring assay was performed to evaluate whether boron derivatives inhibit the microvessel sprouting from an existing vessel. In consistent with tube-formation assay results, increasing concentration of both boron derivatives were found to decrease microvessel growth from aortas (Figure 3.18). Although there was not any significantly difference between low boron concentrations and negative control (growth medium treated aortic rings), higher concentrations apparently reduced cell spreading, indicating potential anti-angiogenic activity of boron compounds.



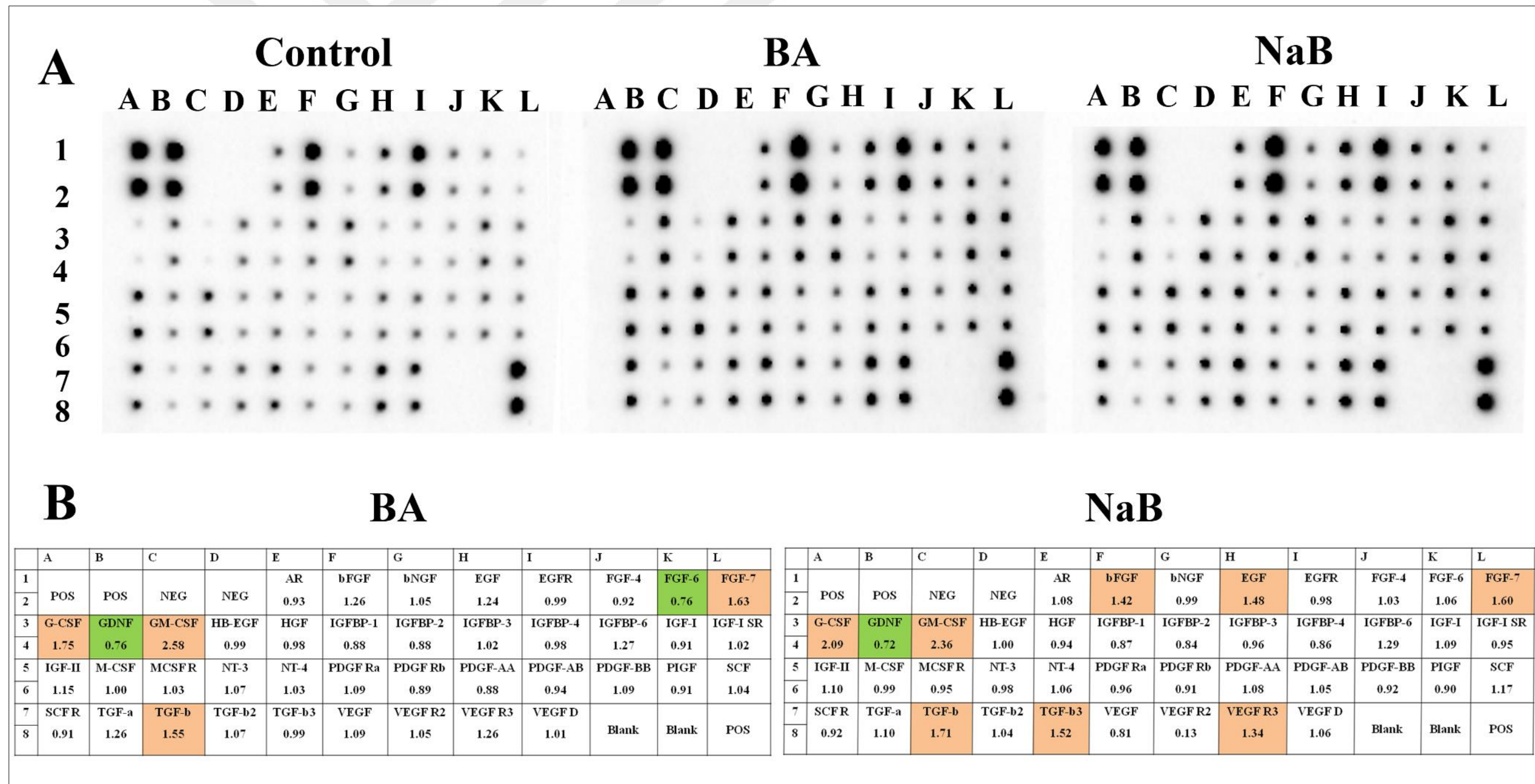
BA

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	AR	bFGF	bNGF	EGF	EGFR	FGF-4	FGF-6	FGF-7
2					1.00	1.00	1.38	0.97	1.00	1.01	0.95	0.95
3	G-CSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
4	1.205	0.88	1.57	0.95	0.92	1.43	1.05	0.90	0.82	1.03	0.97	0.95
5	IGF-II	M-CSF	MCSF R	NT-3	NT-4	PDGF Ra	PDGF Rb	PDGF-AA	PDGF-AB	PDGF-BB	PIGF	SCF
6	1.22	0.96	1.02	0.93	1.03	1.01	0.99	0.96	0.93	1.02	0.93	0.93
7	SCF R	TGF-a	TGF-b	TGF-b2	TGF-b3	VEGF	VEGF R2	VEGF R3	VEGF D	Blank	Blank	POS
8	1.06	1.06	1.05	0.92	0.91	0.91	1.48	0.92	0.98			

NaB

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	AR	bFGF	bNGF	EGF	EGFR	FGF-4	FGF-6	FGF-7
2					0.96	0.94	1.41	1.00	1.11	1.04	1.06	1.06
3	G-CSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
4	1.62	0.99	1.66	0.98	0.97	1.43	0.88	1.01	0.91	1.03	1.07	0.92
5	IGF-II	M-CSF	MCSF R	NT-3	NT-4	PDGF Ra	PDGF Rb	PDGF-AA	PDGF-AB	PDGF-BB	PIGF	SCF
6	1.06	1.04	1.01	0.96	1.07	1.08	0.97	0.95	1.16	0.93	0.95	0.98
7	SCF R	TGF-a	TGF-b	TGF-b2	TGF-b3	VEGF	VEGF R2	VEGF R3	VEGF D	Blank	Blank	POS
8	1.00	1.01	1.06	1.07	1.14	1.01	1.38	1.01	0.99			

Figure 3.15. Growth factor analysis of human fibroblasts treated with 100µg/ml of boric acid (BA) or sodium pentaborate pentahydrate (NaB). (A) Photographic representation of antibody printed membranes. (B) Upregulated (Pink boxes) growth factors in boron treated human fibroblast cells. Representative band intensities of each growth factor was normalized with control.



BA

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	AR	bFGF	bNGF	EGF	EGFR	FGF-4	FGF-6	FGF-7
2					0.93	1.26	1.05	1.24	0.99	0.92	0.76	1.63
3	G-CSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
4	1.75	0.76	2.58	0.99	0.98	0.88	0.88	1.02	0.98	1.27	0.91	1.02
5	IGF-II	M-CSF	MCSF R	NT-3	NT-4	PDGF Ra	PDGF Rb	PDGF-AA	PDGF-AB	PDGF-BB	PIGF	SCF
6	1.15	1.00	1.03	1.07	1.03	1.09	0.89	0.88	0.94	1.09	0.91	1.04
7	SCF R	TGF-a	TGF-b	TGF-b2	TGF-b3	VEGF	VEGF R2	VEGF R3	VEGF D	Blank	Blank	POS
8	0.91	1.26	1.55	1.07	0.99	1.09	1.05	1.26	1.01			

NaB

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	AR	bFGF	bNGF	EGF	EGFR	FGF-4	FGF-6	FGF-7
2					1.08	1.42	0.99	1.48	0.98	1.03	1.06	1.60
3	G-CSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
4	2.09	0.72	2.36	1.00	0.94	0.87	0.84	0.96	0.86	1.29	1.09	0.95
5	IGF-II	M-CSF	MCSF R	NT-3	NT-4	PDGF Ra	PDGF Rb	PDGF-AA	PDGF-AB	PDGF-BB	PIGF	SCF
6	1.10	0.99	0.95	0.98	1.06	0.96	0.91	1.08	1.05	0.92	0.90	1.17
7	SCF R	TGF-a	TGF-b	TGF-b2	TGF-b3	VEGF	VEGF R2	VEGF R3	VEGF D	Blank	Blank	POS
8	0.92	1.10	1.71	1.04	1.52	0.81	0.13	1.34	1.06			

Figure 3.16. Growth factor analysis of HaCaT cells treated with 100µg/ml of boric acid (BA) or sodium pentaborate pentahydrate (NaB). (A) Photographic representation of antibody printed membranes. (B) Upregulated (Pink boxes) and down-regulated (Green boxes) growth factors in boron treated HaCaT cells. Representative band intensities of each growth factor was normalized with control.

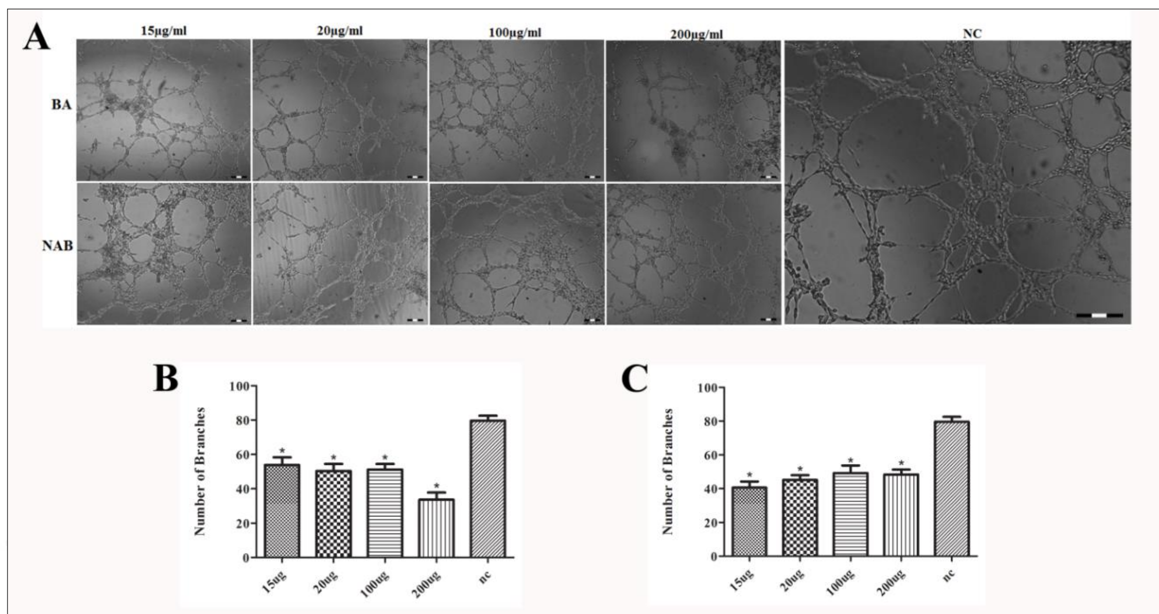


Figure 3.17. Effect of boric acid (BA) and sodium pentaborate pentahydrate (NaB) on angiogenic properties of HUVEC cells. (A) Tube-like structure formation in the presence of boron derivatives, (B) Number of branches after BA treatment, (C) Number of branches after NaB treatment. *$P<0.05$. Notes: Results were analyzed by one-way ANOVA and Tukey's post-test.

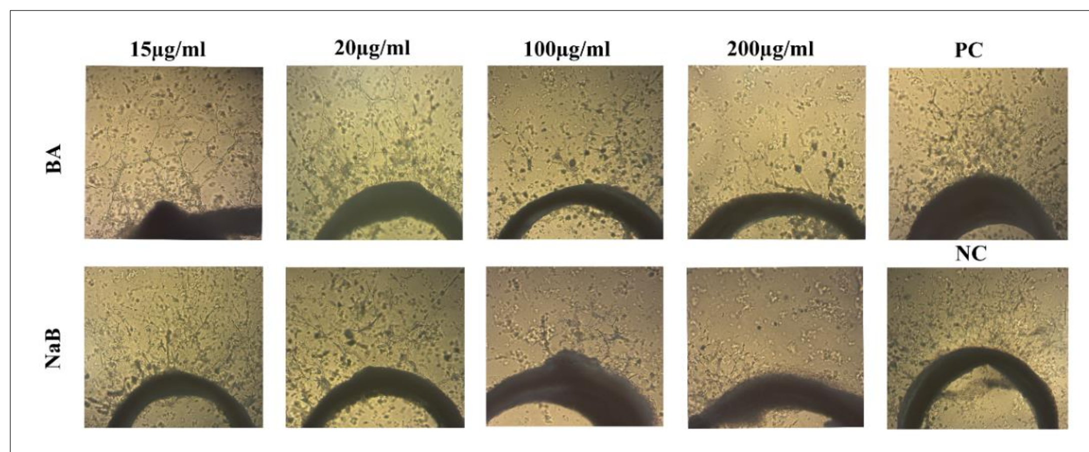


Figure 3.18. Representative images of microvessel sprouting from matrigel coated rat aortic rings exposed to various concentrations of boric acid (BA) and sodium pentaborate pentahydrate (NaB) for 7 day. Abbreviations: NC: Endothelial basal medium-2 (EBM-2) containing 2% FBS and 1% PSA, PC: EBM-2 containing 2% FBS, 1% PSA and 15ng/ml Vascular endothelial growth factor (VEGF). Magnification: 40x

3.6. MIC/MBC-MFC ASSAYS

MBC/MFC values were determined as the lowest concentration of boron derivatives at which no microbial growth were observed. The results revealed that both boron compounds displayed antimicrobial activity against microbial species tested (bacteria, yeast and fungus). They exhibited moderate levels of antibacterial activity while their anticandidal and antifungal activity were found to be remarkable (Table 3.1). MIC values of NaB were in the range of 1.875-7.5mg/ml for bacteria and it was 0.469mg/ml for both *C. albicans* and *Aspergillus niger*. Similarly, MIC level of BA was 7.5mg/ml for all bacteria tested, but it was 0.937mg/ml and 1.875mg/ml for *C. albicans* and *A. niger*, respectively. Considering from a wide perspective, NaB was found to exhibit greater antimicrobial activity against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *C. albicans* and *A. niger* than BA.

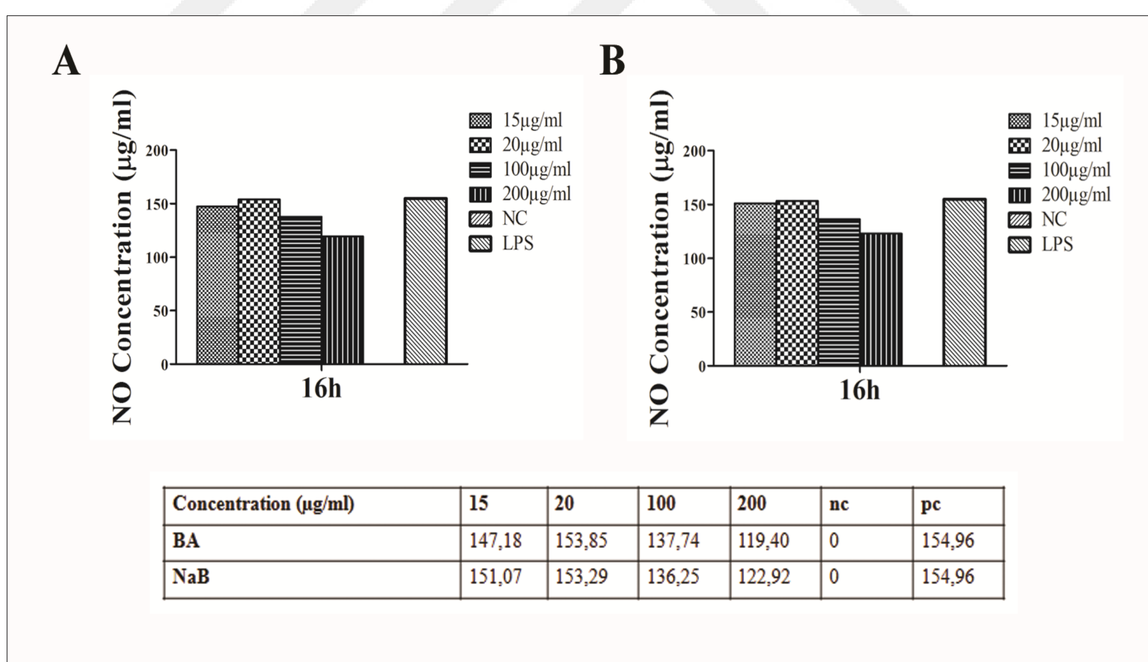
Table 3.1. MIC and MBC/MFC values of boron compounds determined by micro-well dilution assay

	NaB		**BA**	
Microbial Species	**MIC**	**MBC/MFC**	**MIC**	**MBC/MFC**
Escherichia coli ATCC 10536	7.5mg/ml	>15µg/ml	7.5mg/ml	7.5mg/ml
Staphylococcus aureus ATCC 6538	1.875mg/ml	3.75mg/ml	7.5mg/ml	7.5mg/ml
Pseudomonas aeruginosa ATCC 15442	7.5mg/ml	7.5mg/ml	7.5mg/ml	7.5mg/ml
Klebsiella pneumoniae ATCC 13883	3.75mg/ml	3.75mg/ml	7.5mg/ml	7.5mg/ml
Aspergillus niger ATCC 16404	0.469mg/ml	3.75mg/ml	1.875mg/ml	7.5mg/ml
Candida albicans ATCC 10231	0.469mg/ml	>15µg/ml	0.937mg/ml	>15µg/ml

NaB: Sodium pentaborate pentahydrate, BA: Boric acid, MIC: Minimum inhibition concentration, MBC: Minimum bactericidal concentration, MFC: Minimum fungicidal concentration

3.7. INFLAMMATION ASSAY

The effect of boron derivatives on inflammation process was evaluated by determining expression levels of inflammation-related genes and total nitric oxide (NO) levels in cell culture supernatant of LPS treated macrophage cells as an indicator of inflammatory response. Results showed that NaB and BA displayed almost the same patterns. While no significant difference in NO levels was detected at low doses (15 and 20µg/ml), 100 and 200µg/ml of NaB and BA inhibited approximately 20% of total NO levels compared to control group (Figure 3.19). Moreover, important inflammation-related gene levels (iNOS and COX-2) in LPS-induced RAW264.7 macrophage cells were determined using RT-PCR. The results proved that all concentrations of BA and NaB tested resulted in a significant decrease in iNOS and COX-2 mRNA levels. In comparison with the control cells, approximately 50% and 60% decrease were observed in BA and NaB treated groups for COX-2 and iNOS mRNA expression levels, respectively (Figure 3.20).



Concentration (µg/ml)	15	20	100	200	nc	pc
BA	147,18	153,85	137,74	119,40	0	154,96
NaB	151,07	153,29	136,25	122,92	0	154,96

Figure 3.19. Anti-inflammatory effects of (A) boric acid (BA) and (B) sodium pentaborate pentahydrate (NaB) on lipopolysaccharide (LPS) induced mouse macrophage RAW 264.7 cells. Abbreviations: NO: Nitric oxide, NC: Negative control (growth medium).

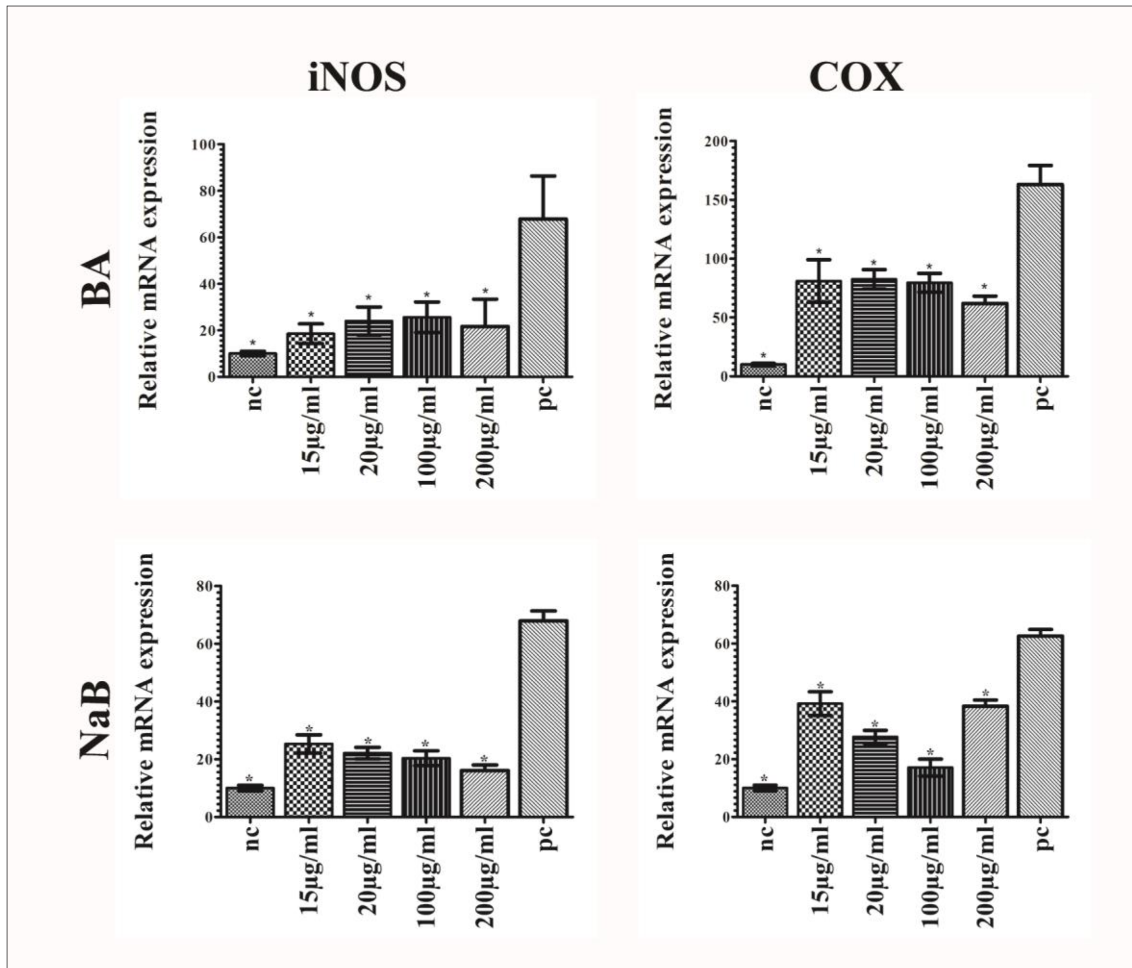


Figure 3.20. Effect of boric acid (BA) and sodium pentaborate pentahydrate (NaB) on mRNA expression levels of iNOS and COX-2 genes in lipopolysaccharide treated RAW 264.7 cells. *P<0.05. Notes: Results were analyzed by one-way ANOVA and Tukey's post-test.

3.8. *IN VIVO* DIABETIC WOUND HEALING

3.8.1. Fasting Blood Glucose Levels and Body Weights

After administration of STZ (65mg/kg), animals were kept for 7 days to stabilize their blood glucose levels. Blood glucose levels of animals fasted for 16h were measured using Accu-check glucometer to define the diabetic status. According to the results, while fasting blood glucose levels for non-diabetic animals were 86.12±3.04mg/dl, glucose levels for diabetic groups were 372.83±32.41mg/dl on average (Figure 3.21), indicating STZ injection

significantly increased fasting blood glucose levels (*P*<0.001). After determining diabetic status of animals, they were kept in normal conditions for further 14 days to stabilize their diabetic symptoms. Animals were weighed every week and changes in initial body weights were recorded throughout the experiment (Figure 3.22). There was an increase in weights of non-diabetic animals, whereas a significant reduction in average diabetic body weights was noted. Mean body weights for diabetic and non-diabetic animals were 270.15±30.21g and 395.75±36.18g, respectively.

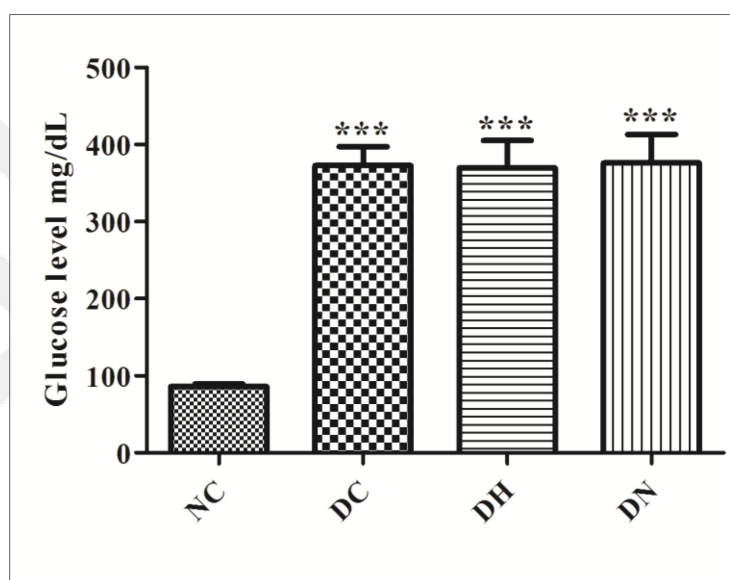


Figure 3.21. Mean fasting blood glucose levels (mg/dl) of each experimental group. Abbreviations: NC: non-diabetic control, DC: Diabetic untreated control, DH: Diabetic rats treated with hydrogel, DN: Diabetic rats treated with NaB-Gel. Results were analyzed by one-way ANOVA and Tukey's post-test, ***P<0.001.

3.8.2. Daily Water and Food Consumption

Food and water intake for non-diabetic and diabetic animals were recorded daily. Table 3.2 depicts that STZ-induction significantly increased water and food consumptions (*P*<0.05). Mean food intake per rat for non-diabetic, diabetic control, diabetic animals treated with hydrogel or NaB-gel were 81.125±6.79, 103.68±20.31, 109.18±17.95 and 97.56±16.29g/day, while mean water consumption per rat in each group were 37.62±5.62, 135±15.35, 140.62±19.89 and 143.37±8.26, respectively.

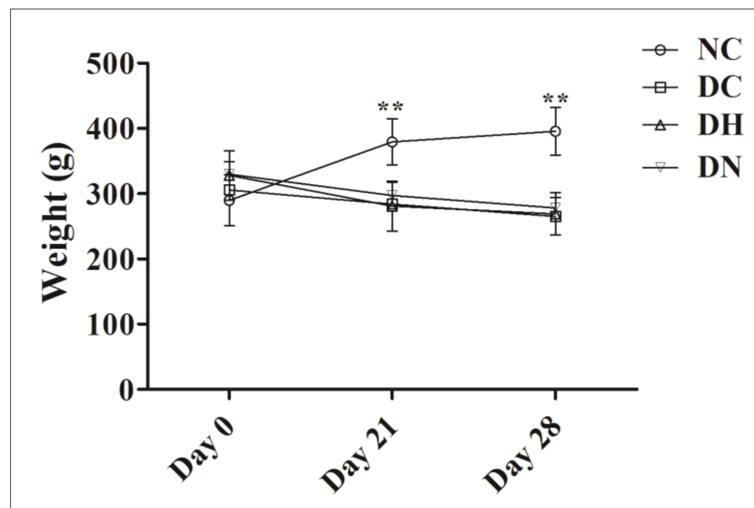


Figure 3.22. Changes in mean body weights of non-diabetic and diabetic animals. Abbreviations: Day 0: Time of Streptozotocin injection, Day 21: Time of full-thickness wounding, Day 28: End of experiment, NC: non-diabetic control, DC: Diabetic untreated control, DH: Diabetic rats treated with vehicle hydrogel, DN: Diabetic rats treated with NaB-Gel. Results were analyzed by one-way ANOVA and Tukey's post-test, ***P*<0.01.

Table 3.2. Mean daily food and water consumption per rat of each experimental group

Groups	Food consumption (g/day)	Water consumption (ml/day)
NC	81.125±6.79	37.62±5.62
DC	103.68±20.31	135±15.35
DH	109.18±17.95	140.62±19.89
DN	97.56±16.29	143.37±8.26

Abbreviations: NC: non-diabetic control, DC: Diabetic untreated control, DH: Diabetic rats treated with hydrogel, DN: Diabetic rats treated with NaB-Gel. Results were analyzed by one-way ANOVA and Tukey's post-test, ****P*<0.001.

3.8.3. Wound Contraction

Animals with diabetic status were wounded at day 21 of STZ injection and treated with hydrogel (vehicle), 3% (w/v) NaB containing hydrogel (NaB-Gel) once a day or left untreated for 7 days. Photographs of wounds were taken every other day and wound gap was represented as percentage of original wound area. The results revealed that while hydrogel partially ameliorated wound healing in STZ-induced diabetic rats compared to untreated control wounds, NaB-Gel significantly increased wound contraction and re-epithelization ($P<0.01$) (Figure 3.23). NaB-Gel treatment significantly augmented wound healing rate of diabetic animals starting from day 2 of operation. However, there was not any significant difference between hydrogel group and untreated control animals until the last 6th day of experiment. At day 8 post-wounding, wound areas for control, hydrogel and NaB-Gel groups were 53.43±9.92%, 32.15±4.05% and 13.02±4%, respectively, indicating potential wound healing activities of NaB in healing impaired rats (Figure-3.24).

3.8.4. Histopathological Examinations

Skin tissue samples were excised from each animal and fixed in formaldehyde to be evaluated histopathologically. Histopathological outcomes of multiple serial cross sections from each experimental group (Control, Hydrogel and NaB-Gel) were determined using routine H&E and Masson trichrome stains (Figure 3.25). The sections were examined in points of morphology, fibroblast density, granulation tissue formation and inflammatory cell infiltration. In addition, wound healing was assessed in terms of collagen deposition and fiber organization. In general, there were dense inflammatory cell infiltration and fewer fibroblast cells in control cross-sections with respect to other treatment groups. Moreover, there was a wide-spread granulation tissue formation and severe lost in epithelial integrity in control samples while regular epithelial organization was observed in hydrogel and NaB-Gel treated animals. There was a remarkable decrease in leukocyte cell number, and increase in collagen bundle formation and fibroblast cells in hydrogel treated animals compared to untreated control wounds.

Although collagen deposition is evident in all experimental groups, obvious difference was noted in NaB-Gel treated animals. There were abundance and well-organized collagen

deposition in NaB-Gel group, whereas sparse collagen organization was detected in hydrogel (vehicle) and control groups. Lower inflammatory cell infiltration, higher number of fibroblast cells, reduced granulation area and relatively thicker dermal formation were observed in NaB-Gel treated wounds at day 8 post-wounding with respect to untreated or hydrogel treated wounds, indicating NaB is a potent wound healing agent for STZ-induced diabetic animals.

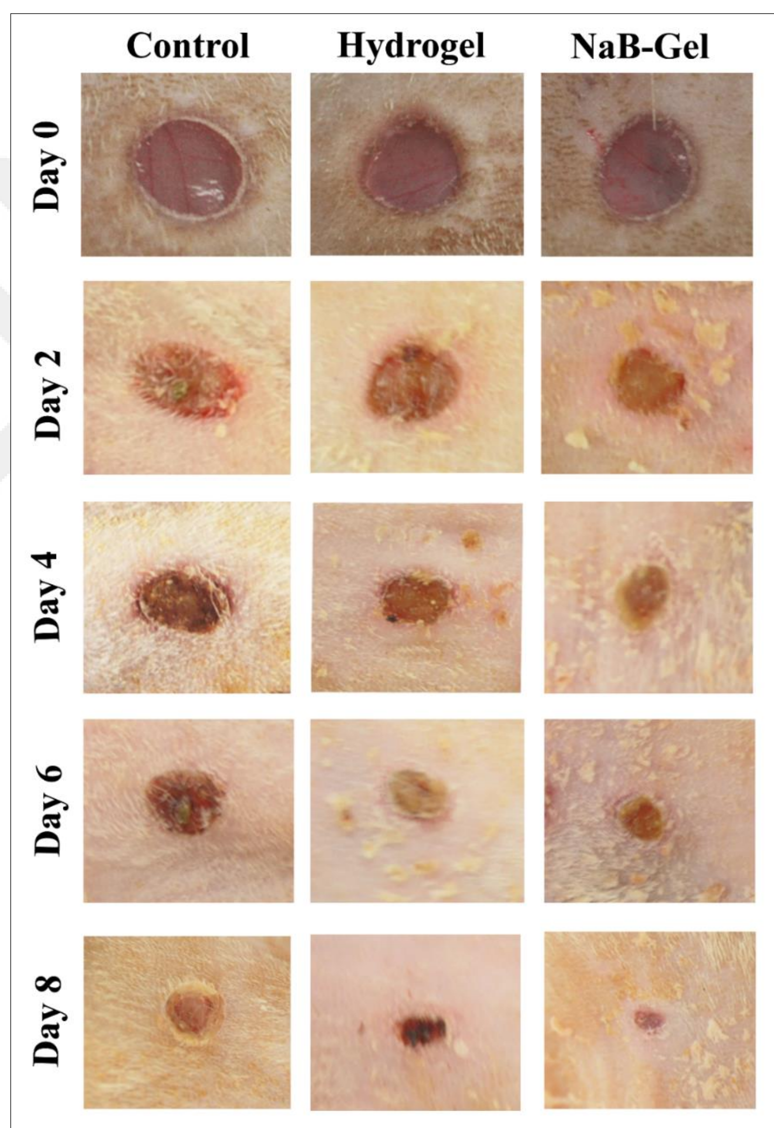


Figure 3.23. Macroscopic evaluation of wound contraction in all experimental groups for 8 days. Control: Untreated diabetic animals, Hydrogel: Animals treated with vehicle (1% (w/v) Carbopol based hydrogel), NaB-Gel: 3% (w/v) Sodium pentaborate pentahydrate (NaB) containing carbopol based hydrogel.

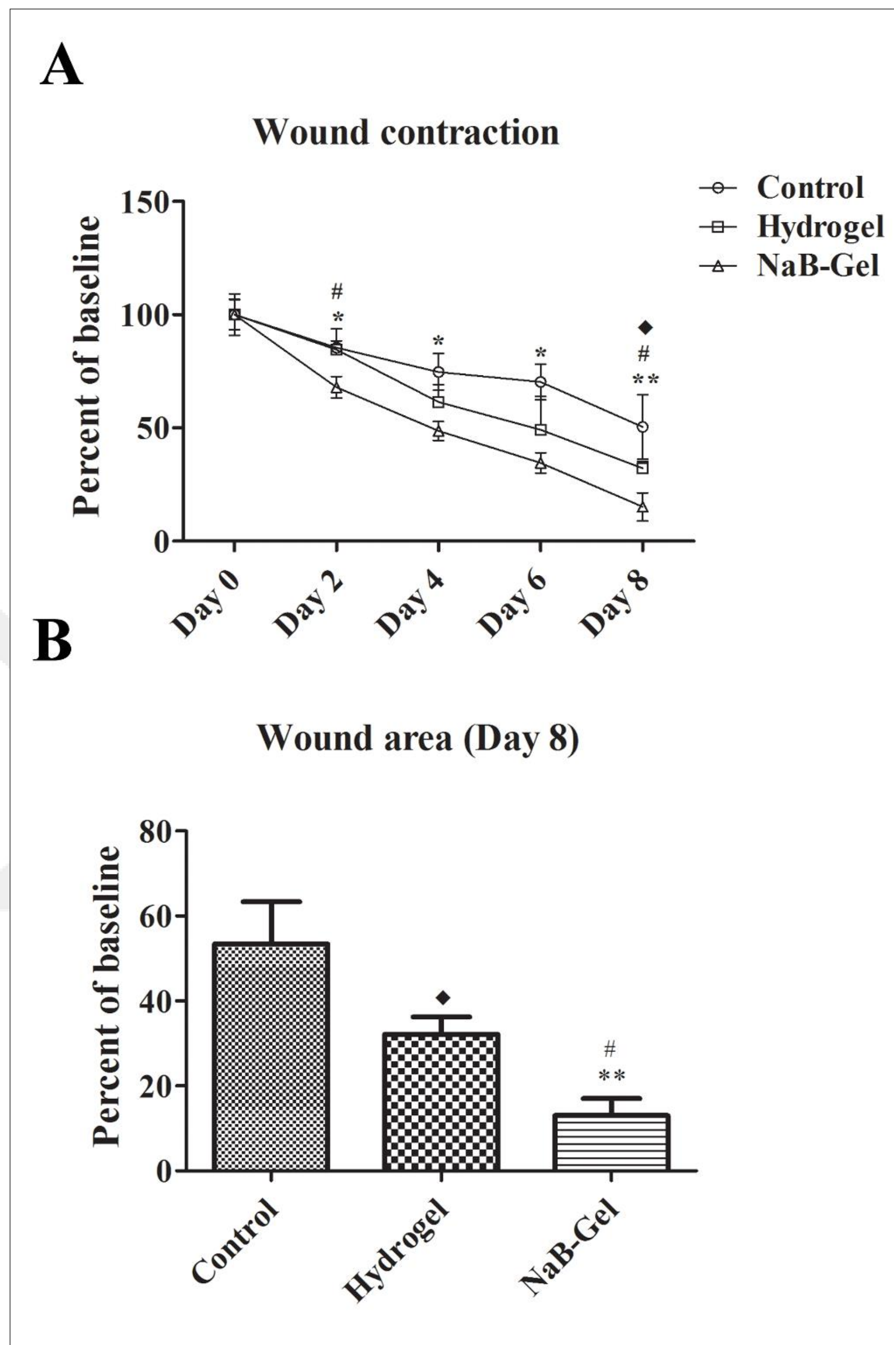


Figure 3.24. (A) Wound contraction rates of STZ-induced diabetic animals for each experimental groups for 8 days. (B) Percentage of wound area at day 8 post-wounding for each experimental group. Control: Untreated diabetic animals, Hydrogel: Animals treated with vehicle (1% (w/v) Carbopol based hydrogel), NaB-Gel: 3% (w/v) Sodium pentaborate pentahydrate (NaB) containing carbopol based hydrogel. Results were analyzed by one-way ANOVA and Tukey's post-test, * *P*<0.05, ***P*<0.01: Control vs NaB-Gel, $^{\#}$ *P*<0.05: Hydrogel vs NaB-Gel, $^{\blacklozenge}$ *P*<0.05: Control vs Hydrogel.

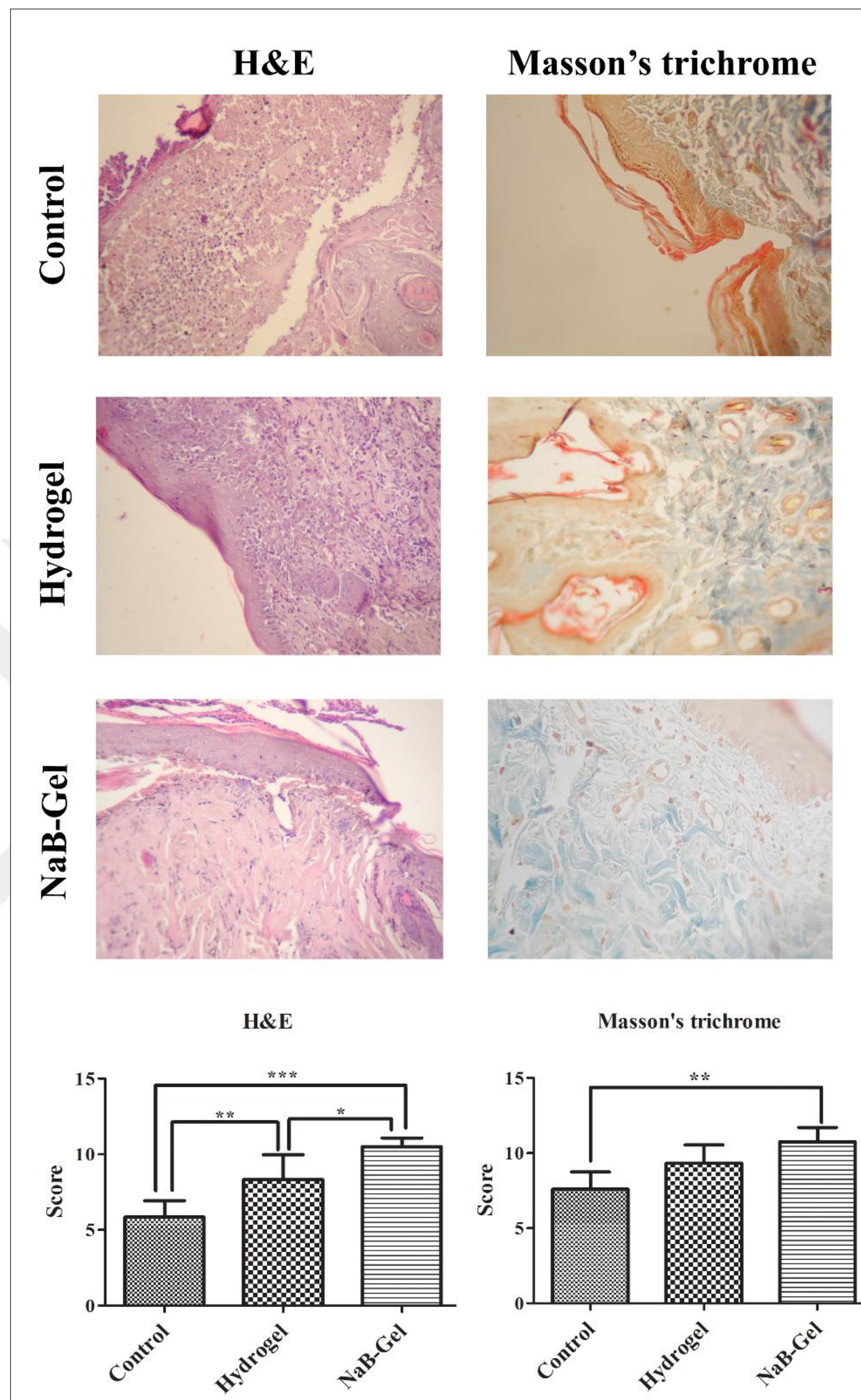


Figure 3.25. Histopathological examinations of wound tissue sections performed by H&E (Magnification=20×) and Masson's trichrome (Magnification=40×) stainings. Control: Untreated diabetic animals, Hydrogel: Animals treated with vehicle (1% (w/v) Carbopol based hydrogel), NaB-Gel: 3% (w/v) Sodium pentaborate pentahydrate containing carbopol based hydrogel.

4. DISCUSSION

Acute and chronic wounds are being faced by millions of people around the world and result in severe complications (even death), decreased life quality and enormous wound care costs. Recent studies in clinical dermatology have led to a better understanding of normal and delayed wound healing processes which allows scientists to develop new therapeutic modalities. Although many practitioners generally use “tried and true” techniques, a vast amount of products have still being introduced to the market, claiming to provide a better optimization of local and systemic conditions of patients and an ideal wound healing environment. Most of those products are improved versions of old ones and some of them are novel, holding several unanswered questions to be solved by long-term clinical studies and use. Although there are encouraging and promising approaches in the wound care field, they remain inadequate due to being expensive and not easily accessible, requiring wound care facilities, having patient-specific response, low efficiency and severe side-effects; hence, life-threatening infections, extremity amputations or severely reduced health conditions are still the major problems of dermatology [287-289]. Therefore, developing new, cheap, broad-range antimicrobial, self-applicable and safe wound healing approaches has always been the pivotal subject of international research.

The skin protects the body integrity from external factors including mechanical, chemical or biological influences [290]. The body becomes unsecure and susceptible to microbial infections immediately after the disruption of skin structure. Although all wounds are contaminated with bacteria coming from environment or surrounding tissues, transition from contamination to infection only occurs when the immune system of the patient is weaker than microbial proliferation [291]. Wound infection can be diagnosed in almost any kind of wounds, but most likely in surgical wounds, full-thickness burn wounds, and chronic ulcers including diabetic foot ulcers, pressure ulcers, venous leg ulcers and arterial leg ulcers [292]. Second- and third-degree burn wounds are highly sensitive to microbial infections and up to 75% of infected, high-degree and large surface burns are end up with death [293]. Apart from burn wounds, chronic wounds are also generally accompanied with infections that further improves chronicity. Infection at the wound site results in excessive neutrophil infiltration and maintenance, high expression levels of pro-inflammatory cytokines such as

TNF-α, IL-1α, IL-1β, and production of elevated levels of MMPs [294,295]. These cytokines and proteases alter inflammation response of the host and further enhances tissue degradation at the wound site, resulting in delayed healing. In addition, if the microbial proliferation is not controlled in the initial contamination step, these microorganisms such as *P. aeruginosa* and methicillin-resistant *S. aureus* would form biofilm which is approximately 500 times more resistant to conventional antibiotics and regimens than planktonic (unattached, freely living) cells [291]. Thus, designing wound care products with antimicrobial properties is an important criteria to cope with infection related wound healing complications. In other words, wound healing agents and approaches having antimicrobial properties are considerably advantageous compered to their counterparts. One possible solution to prevent infection is to design wound care products containing disinfectants, antiseptics or antibiotics. However, disinfectants and antiseptics containing alcohols, anilides, biguanides, halogen compounds, sodium hypochlorite, heavy metals, peroxygens and quaternary ammonium compounds are not selective to microorganisms and toxic to the host tissue in a dose-dependent manner [296,297]. As for antibiotics, they can be used only after the infection and diagnosis of the microbial flora, and they are useless against poly-microbial infections due to being effective against a particular group of microorganisms. Most importantly, overuse and misuse of antibiotics might result in resistant colony selection which often results in sepsis, amputation and death [298]. Besides, as these products generally neither improve nor delay wound healing process, they should be used with additional wound healing promoting agents or products, which increases the possibility of cross-reactions between drugs used and toxicity arisen from synergistic activity. To this end, non-toxic wound healing agents comprising broad-range antimicrobial activity is always superior compared to conventional therapies.

In the current study, boron derivatives (BA and NaB) were examined for their wound healing potentials using *in vitro* and *in vivo* approaches. Apart from their proliferative, migrative activities on dermal cells (HF, L-929, HUVEC, and HaCaT cells), their effects on angiogenesis, inflammation, gene expression and growth factor production profiles were investigated in *in vitro* conditions. Due to the main purpose of the wound healing process is to protect body from environmental threats such as infection, as mentioned above, antimicrobial properties of boron derivatives were also tested. Although both boron compounds displayed wide-range antimicrobial (antibacterial, anticandidal and antifungal)

activity against bacterial, candidal and fungal species tested, antimicrobial activity of NaB was found to be greater than BA against *S. aureus*, *K. pneumoniae, C. albicans* and *A. niger*. In consistent with the current study, BA and sodium tetraborate have been proven to be effective against *S. aureus*, *E. coli*, *P. aeruginosa*, *C. albicans* and *A. niger* in the literature [299,300]. Yılmaz and co-workers have shown that MIC levels for sodium tetraborate and boric acid have been different [300] as it was found in this study, indicating different boron compounds might reveal diverse outcomes. Although boron is essential for plant metabolism and micronutrient for many organisms, the distance between its necessity and toxicity is not too far [301]. Boric acids and its salts have been used as bactericide and fungicide since 1860s while the exact molecular mechanism of antimicrobial effect remains unclear [302]. However, it is a well-known issue that boron affects both enzymatic and non-enzymatic processes such as having high affinity to ribose molecule, a component of ATP, NADH, NADPH and RNA, destructing protein synthesis, mitochondrial function, cell division, development, cell membrane integrity, conformation and transport [303-306]. Antifungal properties of both boron derivatives were found to be higher than their antibacterial activities, indicating potential difference in antimicrobial targets for different microbial groups. While the known effects of boron on living cells are not enough to explain the difference between antifungal and antibacterial activities due to shared potential targets such as cell membrane, metabolic pathway, and gene expression systems, two independent reports have proposed prospective mechanisms for boron's antifungal activity [307,308]. Disrupted primary septum and morphogenesis during cytokinesis, incorrect cytoskeleton assembly, and most importantly disorganized cell wall have been observed in yeast cells treated with boric acids. The components of fungal cell wall, such as ergosterol, alter and the activity of cell wall repair enzymes such as chitin synthase 3 reduce, leading to irregular cell wall organization and integrity. Further studies are highly warranted to elucidate different responses of boron derivatives on various organisms and exact molecular details of antimicrobial activity to anticipate potential use and limitation of boron in clinics and industry.

Exploring antimicrobial activity of boron compounds in safe concentrations is a good but not a sufficient reason to be used in advanced wound care products. Bactericidal and fungicidal activity of boron might shorten the inflammatory step resulting in increased healing rate in acute wounds. However, it would not be sufficient to overcome persistent

inflammatory response in chronic wounds. In acute wounds, the healing process is an orderly event that starts with hemostasis and inflammatory step lasting for a couple of days and switches to tissue regeneration (epithelialization, granulation and angiogenesis) and remodeling [309], whereas inflammatory response resists for a long time in chronic wounds resulting in severe tissue damage and ulceration [310]. Elevated expression levels of proinflammatory cytokines further activate inflammatory cells such as neutrophils and vice versa [311]. Thus, a wound care product to be used in the chronic wound management should weaken the inflammatory response to provide a healthy healing environment. Boron derivatives, in the current work, were examined for their anti-inflammatory effect using model macrophage cells *in vitro*. Both BA and NaB significantly decreased NO production levels in a dose-pendent manner and suppress important inflammatory mediator genes, iNOS and COX-2. NO is normally produced by macrophages and neutrophils via upregulating iNOS and COX-2 expressions due to various stimuli such as lipopolysaccharides [312,313]. Prolonged expression of NO, as a nonspecific host defense intermediate, is highly toxic to the surrounding tissue and cells, resulting in chronic inflammation and subsequently inducing healing impairments. It should also be noted that decreasing inflammatory response is not always a good solution for wound treatments because inflammatory cells protect the wound from potential microbial invaders. However, having antimicrobial characteristics along with anti-inflammatory activity take boron one step forward closer to practical applications. Therefore, inhibition of NO production by down-regulating iNOS and COX-2 gene levels may hold a therapeutic value in clinical dermatology (especially for chronic wounds). Dietary boron intake has important regulatory roles in normal inflammatory response in bones and joints, and its deprivation causes significant increase in inflammation related cytokine and growth factor levels [272,314]. Moreover, in line with the findings of the study, Scorei and his co-workers have published that calcium fructoborate treatment has decreased IL-1β and IL-6 protein expressions, and NO levels in the culture supernatant [273]. These studies along with the current study might direct future works to develop boron-based anti-inflammatory products and modalities for acute and/or chronic inflammatory diseases.

Main focus of the total healing process is to cover the denuded area for the protection of body from environmental danger. The tissue regeneration phase consisting re-epithelization, granulation tissue and vascular network formation is, therefore, relatively the most important

section for a healthy healing. Most of the wound care products aim to increase one or more of proliferative, migrative, angiogenic or extracellular matrix production inductive properties of dermal cells. The first move, after inflammation is toned down, is the proliferation and migration of keratinocytes and fibroblasts towards injured dermis [75]. Migration of the dermal cells is triggered by interaction of cell surface proteins with extracellular matrix proteins including provisional wound matrix, collagen, fibronectin, laminin and elastin [315]. These proteins are vital key players for a healthy wound healing and either directly or indirectly change the cell behavior such as adhesion, migration, proliferation and survival by regulating protease expression and stability or adjusting growth factor activity [316]. Matrix proteins (e.g. fibronectin) have been reported to be degraded in venous leg and diabetic foot ulcers, and present in low amounts resulting in a defected cell migration and proliferation [317]. Moreover, turnover of degradation and production of laminin and collagen fibers are important stimuli for keratinocyte and fibroblast migration and proliferation [10]. In this sense, upregulation of collagen, laminin and fibronectin gene expressions in HF, HaCaT and L-929 cells by boron treatments (especially NaB treatment) might be the primary reason for cell proliferative and migrative activity. An additional reason for cell proliferation and migration would be the upregulation of Akt/protein kinase B in NaB treated HF cells and both NaB and BA treated L-929 cells. Akt, core mediator of phosphoinositide 3-kinase (PI3K) signaling pathway, has been proven to induce cell proliferation, migration and invasion by regulation of matrix metalloproteinase production [318]. Metalloproteinases including MMP-1, MMP-2, MMP-8, MMP-9 and MMP10 degrade provisional wound matrix and extracellular matrix proteins to provide a suitable environment for cell migration [319]. Increase in MMP-2 and MMP-9 in mouse fibroblast cells and HaCaT keratinocytes treated with boron derivatives might have triggered the migratory activity. In consistent with the present work, cell migration and MMP expressions in HaCaT cells have been induced by boron application [268,267]. Interestingly, boron treatment was found to decrease the protease levels in human fibroblasts, indicating species-specific response of boron. This might not be a disadvantage for the management of ulcers because chronic wounds are mainly accompanied with high levels of MMPs and degraded matrix proteins [319]. Boron administration resulted in an unquestionable effect in gene expressions of extracellular matrix proteins, Akt and MMPs, but more works conducted with several cell lines and animal models are needed to evaluate their protein levels and activities. Performing further *in vitro* experiments with various cell types isolated from different

wounds would also allow to clarify the exact action of mechanisms and develop possible wound-specific healing products.

During the early phase of wound healing, dermal cells start to migrate to the injured area using newly formed extracellular matrix proteins. Therefore, transportation of waste materials, sufficient oxygen and nutrients supply should be provided for the immigrant cells. Endothelial cells in the blood vessels adjacent to the wound are activated by mainly resident inflammatory, fibroblast and keratinocyte cells, and move towards wound bed to form an immature vasculature, referred to as angiogenesis or neo-vascularization [19]. Chronic wounds are generally experienced by patients with vascular problems such as venous insufficiency and diabetic patients [320,119]. Healing in these chronic wounds is further delayed due to deficient or non-functional angiogenic activity. Enhancing angiogenesis is a standard way to increase wound healing rate in both acute and chronic wounds [321,322]. In contrary to the goal of the current study, boron derivatives inhibited tube-like structure formation and microvessel sprouting of endothelial cells in a dose-dependent manner. As the endothelial cells are players not regulators in angiogenesis process, boron's activity on inflammatory cells, fibroblasts and keratinocytes (main directors of angiogenesis) have the possibility to suppress boron's negative effects on endothelial cells. Although anti-angiogenic activity of boron derivatives cannot be proven by only two *in vitro* experiments and more works with animal models and clinical studies are highly required, dose management of boron containing wound healing products seems to be a vital subject in clinical studies. Even with the assumption of certain antiangiogenic activity of boron, wound healing was not repressed even augmented by boron containing gel formulation treatment in the diabetic full-thickness wound model. Several researches have proposed an unanticipated hypothesis that angiogenesis in normal wound healing is excessive and reducing (not inhibiting) vascularization for the treatment of angiogenesis-related disease (e.g. cancer) does not impair cutaneous wound healing [323,324]. Although the idea of vasculature formation repression does not delay tissue regeneration is satisfying alone, some reports have also suggested remarkable effects of lesser angiogenic response on healing process [325-327]. Apart from having no depressing effect on wound healing, partly inhibition of robust vascularity has been suggested to decrease scar formation [328,327]. The literature is contradictory in degree of angiogenesis required for a health wound healing but it is obvious that there are some applications like boron derivatives in the current study, which increase

the healing process along with decreasing angiogenesis. As a different point of view, elucidating the detailed molecular mechanisms of angiogenesis suppression would inspire scientists to develop new approaches for the treatment of diseases with hyper-angiogenesis conditions such as cancer, hemangioma, psoriasis, allergic dermatitis, diabetic retinopathy and arthritis [329].

Proliferation and migration of dermal cells, cellular network formation, production and degradation of extracellular matrix proteins, and interactions of cells with each other are regulated by a series of cytokines and growth factors secreted from various cell types. Insufficient production or elevated destruction of a certain or group of growth factors lead to chronic wound development. Although exogenous administration of growth factor for the treatment of ulcers is a potential option, rapid degradation due to high proteolytic activity in chronic wounds is the major problem [330]. Thus, administration of stable agents with growth factor production inductive properties remains as a more appropriate choice for the treatment of non-healing wounds. Determining growth factor production ability in *in vitro* monoculture conditions would not be a definitive explanation for wound healing effect of a compound because cell-cell interaction changes the cell behavior and there is an oscillating tendency of growth factor production, not a linear increase or decrease during the wound healing process [7]. However, evaluation of growth factor profile of boron treated fibroblasts and keratinocytes may allow to have an idea about potential pathways and mechanisms that boron is involved. Human fibroblast cells treated with BA and NaB gave quite similar responses with the exception of G-CSF upregulation in NaB treated HF cells. bNGF, GM-CSF, IGFBP-1 and VEGFR2 are other proteins enhanced in HF cells by boron application. In addition to G-CSF and GM-CSF, boron administration augmented FGF-7 and TGFβ-1 in keratinocyte cells. NaB treatment enhanced the expressions of more growth factors in number including bFGF (FGF-2), EGF, TGFβ-1 and VEGFR3 compared to BA treatment. All these growth factors have been shown to take either direct or indirect roles in wound healing process and affect one or more of proliferation, migration, extracellular matrix or growth factor production abilities of dermal cells. NGF, a member of neurotrophin family, is overexpressed in healthy wound healing process and exogenous application of NGF has accelerated the healing process in both acute and chronic wounds [126]. NGF mainly maintains proliferation, development and functional integrity of certain sympathetic and sensory neurons in the peripheral and central nervous systems [331]. Upregulation of NGF

by boron treatment might be of particular importance in diabetic patients with peripheral neuropathy which intensifies chronicity of diabetic foot ulcers [332]. The idea can be supported by reports mentioning protective effects of NGF administration on diabetic sensory neuropathy [333] and diminished levels of NGF in keratinocyte cells isolated from diabetic patients [334].

IGF-I and IGF-II are important proliferative growth factors for several cell types and their importance for a healthy wound healing is well documented [7]. They activate cells by interacting with their respective receptors with the help of six different binding proteins. Therefore, IGFBP-1 might have triggered IGF-I induced cell proliferation of dermal cells. Besides, IGFBPs have been shown to promote cell growth in a IGF-independent pathway [335]. Apart from proliferation, some of these upregulated growth factors direct cell motility. EGF pathway activation through EGF receptor triggers consecutive signaling pathways leading to cell proliferation and migration [32]. EGF is a vital stimulator for keratinocyte motility, hence, its importance for re-epithelization is undoubted [336]. Other important mediators affecting keratinocyte migration are bFGF and FGF-7. Their importance for a normal healing process and lack of these growth factors in non-healing wounds are presented in the literature [337]. They enhance migration and proliferation of fibroblast and keratinocyte cells via both autocrine and paracrine manners, augment extracellular matrix protein formation and neo-angiogenesis [286,338,339]. Positive contributions of FGF family proteins to angiogenesis might have reversed the negative effects of boron on endothelial cells. In addition, increase in VEGFR-2 and VEGFR-3 levels as in HF and HaCaT cells, respectively, treated with boron derivatives could be another positive parameter for the organization of newly formed vascular network in diabetic wound.

TGF family proteins secreted from several dermal cells have been shown to take place in almost every phase of wound healing process with variable levels [340]. Although, the exact role of TGF proteins in wound healing is not fully understood and there are some controversial report in the literature, TGF-β1 and TGF-β3 have been claimed to increase migration and proliferation of fibroblast and keratinocyte cells, granulation tissue formation, extracellular matrix production, growth factor secretion production, angiogenesis and tissue remodeling [337,341,340,342,343]. Moreover, administration of these growth factors have partially improved diabetic and pressure ulcers [344,345]. In consistent with the current

study, TGF-β1 production has been induced by boric acid treatment (10mM-618μg/ml) in placenta nuclei, whereas FGF-1 and TNF-α levels have remained constant [266].

The most prominent and certain effect of both boron derivatives on growth factor profiles of human fibroblast and keratinocytes seemed to be upregulation of G-CSF and GM-CSF. G-CSF has been suggested to increase angiogenesis and promote wound healing in ischemic diseases such as diabetes [346]. GM-CSF, on the other hand, is a well-studied growth factor and shown to be effective in re-epithelization, granulation tissue formation and inflammation steps [347]. Furthermore, GM-CSF is suggested to increase angiogenic factor production, endothelial cell proliferation and migration, indicating potential roles in angiogenesis [348]. Overall data of growth factor analysis suggest that boron treated fibroblast and keratinocyte cells enhance vital growth factors production which could affect one or more of inflammation, re-epithelization, granulation tissue formation, angiogenesis and remodeling phases.

In vitro experiments of the current study clearly showed that boron derivatives (BA and NaB) affected microorganisms, fibroblast and keratinocyte cells in a different manner. While cell proliferation and migration properties of dermal cells treated with BA and NaB were quite similar, antimicrobial properties, gene and growth factor expression inductive properties of boron derivatives were different from each other, in favor of NaB. There is not enough convincing reports about different responses obtained from different boron compounds but the possible explanation might be positive ions released from boric acid salts such as Na^+. Positive ions including Na^+ and K^+ are required for activation of voltage-gated channels affecting cell growth, cell cycle and migration [349]. An additional reason for this dissimilar behavior would be different efficiency of boron uptake into the cell. Boron uptake was thought to be only passive diffusion through the cell membrane until the discovery of Na^+-coupled borate transporter (NaBC1) [249]. This co-transporter works in the presence of Na^+ and borate, selectively regulate the transportation of borate. NaBC1 expression patterns of various cells would be the main explanation for why some cells are more susceptible to boron derivatives and others not. Whereas additional studies using broad-range of cell types should strictly be provided to confirm this hypothesis, there is an incontestable evidence that NaB displayed greater antimicrobial activity, gene and protein expression inductive properties compared to BA. In addition, sodium borate compounds have been reported to be

less toxic compared to BA [350]. Therefore, NaB was selected for *in vivo* diabetic wound healing experiments.

To develop an *in vivo* chronic wound model to study wound healing potential of NaB, STZ-induced diabetic rats were used. STZ, a glucose analogue, selectively destructs insulin producing β-cells through the low affinity GLUT2 glucose transporters, and results in insulin-dependent diabetes mellitus [351]. Blood glucose level of animals increases rapidly, peaks 1-3 days after a single high dose STZ injection and remains constant thereafter [352]. High blood glucose is associated with impaired wound healing in diabetic patients [353]. STZ-induced diabetes reflects clinical symptoms of diabetes including hyperglycemia, polyuria, polyphagia and weight loss [354]. In line with these reports, severe weight loss and polyuria was observed in STZ-injected rats along with significant increase in daily food and water uptake. Animals were kept 21 days in their diabetic status to provide stable blood glucose levels and diabetic symptoms. The rats were wounded and applied NaB containing hydrogel formulation for seven days. Macroscopic evaluations revealed that NaB containing gel formulation increased closure rate of full-thickness wounds as expected. Histopathological examinations confirmed the presence of lower inflammatory cells, relatively thicker dermal tissue formation, higher numbers of fibroblast cells, well-organized and abundance collagen deposition in NaB-Gel treated groups compared to control rats, indicating wound healing promoting properties of boron containing gel formulation in STZ-induced diabetic wounds. These observations are strictly in correlation with *in vitro* results, confirming certain boron's anti-inflammatory action as well as promoting effects on fibroblast and keratinocyte proliferation, migration and extracellular matrix production.

Although there is not suitable animal model reflecting clinical signs of diabetic status literally, STZ induction is one of the best way to perform chronic wound healing experiments. However, as the STZ accumulates in GLUT2 transporter expressing organs, liver and kidney which also express significant levels of GLUT2 are highly affected [351]. Change in organ metabolisms might have affected wound healing process severely. Further limitation of the study is using type I diabetes mellitus model which is responsible for only 10% of total diabetic patients [355]. Genetically diabetic animals could be used as a model of type II diabetes (non-insulin-dependent diabetes mellitus) instead of chemically induced diabetic rats but this should be the next step after obtaining encouraging results due to being

highly expensive. Before starting new experimental designs, optimum dose, application volume and frequency should be determined to maximize wound healing rate. In addition, as diabetic wound is only a member of several chronic wounds, other chronic wounds including pressure and venous leg ulcers should be tested to extend and compare potential use area of boron in clinical dermatology.

Chronic wounds are generally accompanied with hard to heal infections as described above in detail. While broad-range of antimicrobial activities of boron derivatives against bacteria, yeast and fungus were shown *in vitro*, *in vivo* infection models developed using biofilm producing and antibiotic resistant microorganisms should be performed to determine degree of antimicrobial characteristics. These experiments would help to decide whether additional antibiotic therapy should be applied together with boron containing wound care products or not. Furthermore, dose arranging should be considered not only for healing promoting activity but also antimicrobial properties. As a different point of view, synergistic activity of boron with not only antimicrobial agents but also wound healing promoting agents should also be performed to maximize activity and use. Formulation base could be changed from hydrogel to cream or additional micelle forming agents such as pluronic block co-polymers could be included in the formulation in order to increase absorption rate of boron through cell membrane. Apart from daily applied products, biodegradable wound dressings releasing boron constantly for a long time could be a future solution to decrease psychological stress and promote healing. After determining optimum concentration, formulation, application volume and frequency, the next milestone of this project would be clinical trials performed with various acute and chronic wounds to confirm the findings of the study.

Major limitation of the study is lack of exact molecular mechanism evaluation of boron's activity on each wound healing phase in *in vivo* conditions. Designing time-point *in vivo* experiment with several intervals such as 3, 7, 10 and 14 days would be more informative and give insights into effects of boron containing formulations on inflammation, re-epithelization, granulation tissue formation, angiogenesis and extracellular matrix production. While this approach will increase the number of animals to be used, it will also raise the power and credibility of the study. Evaluation of tissue extract for protease and growth factor viability in each time-point would provide strong confirmation data for *in vitro* findings. Secondly, as cell-cell interaction is important factor during wound healing, using

monoculture experiments does not literally stimulate healing process in *in vitro* conditions. Different co-culture and 3-D model systems should be employed to mimic living body circumstances. One another limitation of the current work is uncertainty of boron containing gel formulation toxicity and irritation on skin cells for large wounds requiring prolonged treatment. Therefore, skin sensitization and irritation test must be completed before passing to clinical trials.

5. CONCLUSION

In today's science, there is not a satisfying therapy for chronic wounds suffered by millions of people around the world. New modalities with one or more of antimicrobial, proliferative, migrative, ECM or growth factor production inductive properties are strictly demanded in clinical dermatology. The data gathered from this study obviously demonstrates that boron derivatives exhibited proliferative and migrative effects on fibroblast and keratinocyte cells as well as having remarkable antimicrobial activities against bacteria, yeast and fungi tested. Extracellular matrix protein and matrix metalloproteinase gene expressions were induced by boron treatment. In addition, vital growth factors secreted from human fibroblast and keratinocyte cells were upregulated and wound healing rate in STZ-induced diabetic rats were augmented by boron application. Overall data suggest that boron (NaB) potentially provide a new treatment option for the treatment of diabetic chronic wounds. Provided that preclinical studies to be completed, clinical trials with various chronic wounds including DFU are on the way. However, further studies are required to determine signaling pathways and target metabolisms which boron is involved to elucidate detailed molecular of mechanisms and to extend its use and limitations in clinics.

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7. APPENDIX A: CHEMICAL PROPERTIES OF SOME BORON COMPOUNDS

Important boron compounds, their formula and chemical properties were illustrated in Table A.1 to Table A.6.

Table A.1. Important boron derivatives

Chemical Name	Cas No	Molecular Formula
Ammonium pentaborate tetrahydrate	12229-12-8	$NH_4B_5O_8.4H_2O$
Boric acid	10043-35-3	H_3BO_3
Boron dioxide	13840-88-5	BO_2
Boron oxide (6:1)	11056-99-8	B_6O
Diboron trioxide	1303-86-2	B_2O_3
Dicalcium hexaborate pentahydrate	12291-65-5	$2CaO.3B_2O_3.5H_2O$
Dipotassium tetraborate tetrahydrate	12045-78-2	$K_2O.2B_2O_3.4H_2O$
Disodium octaborate tetrahydrate	12280-03-4	$Na_2O.4B_2O_3.4H_2O$
Disodium tetraborate decahydrate	1303-96-4	$Na_2O.2B_2O_3.10H_2O$
Sodium calcium pentaborate octahydrate	1319-33-1	$NaCaB_5O_9.8H_2O$
Sodium metaborate tetrahydrate	10555-76-7	$NaBO_2.4H_2O$
Sodium perborate trihydrate	28962-65-4	$NaBO_3.3H_2O$
Sodium pentaborate pentahydrate	12046-75-2	$Na_2O.5B_2O_3.10H_2O$
Zinc borate	12447-61-9	$2ZnO.3B_2O_3.3.5H_2O$

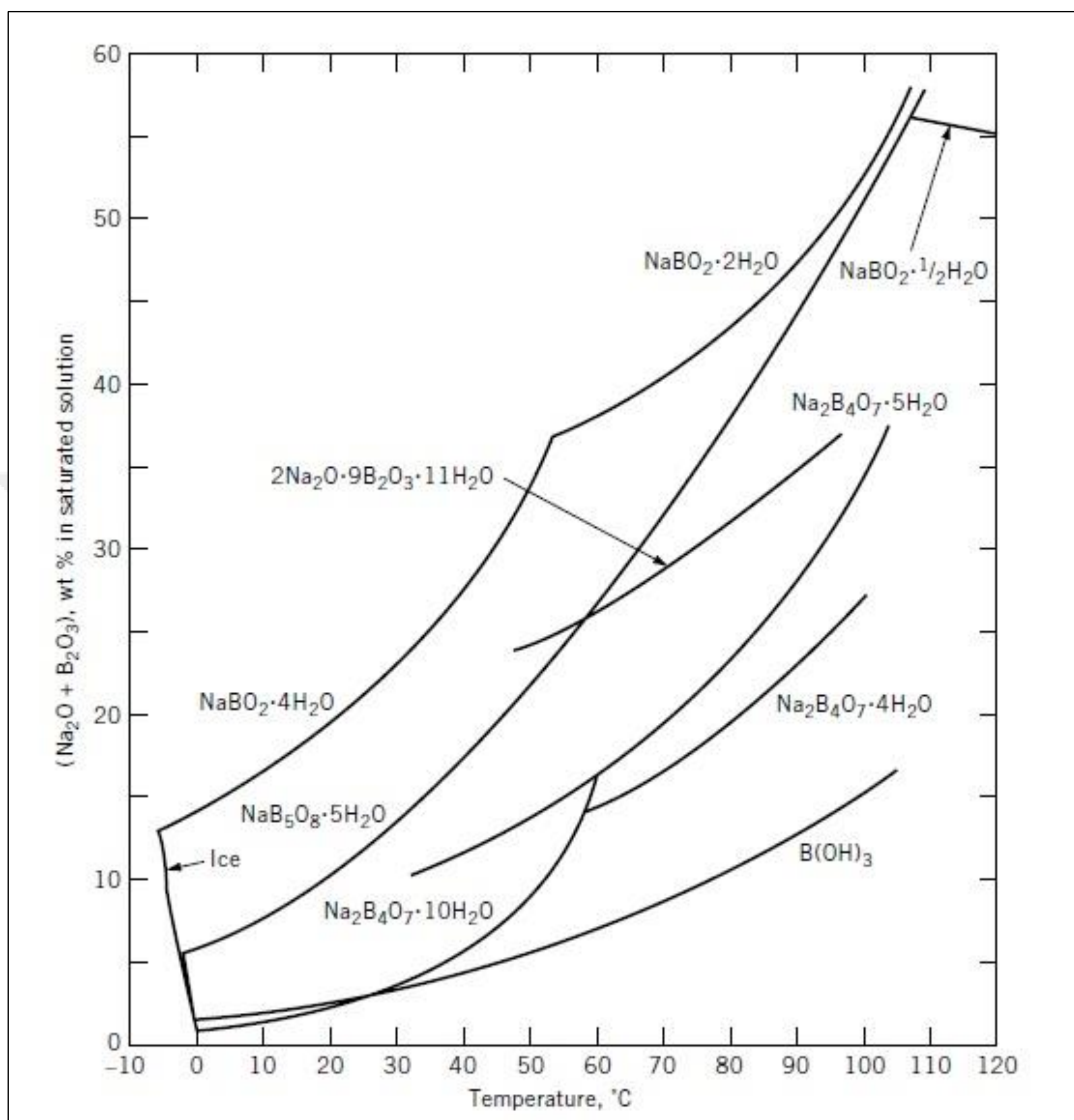

Figure 1A.1. Temperature dependent solubility curves of boric acid, borax, sodium pentaborate, and sodium metaborate [356].

Table A.2. pH values of boron solutions [357, 358].

Chemical Formula	Concentrations, wt %					
	0.1	1	2	4	4.5	10
$NaB_5O_8.5H_2O$ (Sodium pentaborate pentahydrate)		8.5	8.4	8.1		7.6
H_3BO_3 (Boric acid)	6.1	5.1	4.5		3.7	