Educated platelets promote wound healing via anti-inflammatory effect and down-regulated VEGF and MMP-9

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Abstract

Aim: This study aims to investigate the effect of educated platelets histological and immunohistochemical (MMP-9 and VEGF) in wound treatment.

Materials and Methods: 28 female Wistar albino 180-225 gr rats were divided into four groups randomly. The control group (C) is unburnt which blood samples are extracted or PRP with ordinary platelets. Burn group (B) is a burn group in which blood samples were extracted with the educated platelets for PRP. The Burn and uneducated platelet group (B+P) were the burn groups given PRP with uneducated platelets to their blood circulation. Burn and educated platelet group (B+EP) was the burn group given PRP with educated platelets to their blood circulation. The histological analysis was scored 14 days after injury, and MMP-9, VEGF were immunostained.

Results: The results clearly showed higher reepithelialization, lower inflammation, and granulation in the B+EP group. Immunohistochemical staining for MMP-9 and VEGF in the B+EP group were statistically lower than other groups. Educated platelet treatment significantly suppressed MMP-9 and VEGF.

Conclusion: Our findings suggest that educated-platelet treatment can be a promising strategy for promoting burn cutaneous wound healing. Further and more extensive clinical studies are needed to determine the effectiveness of this treatment method definitively.

Introduction

A wound is the disruption of the continuity of the anatomical structures and functions of the tissues as a result of physical, chemical, or trauma. Wound healing is the result of systematic, cellular, and biochemical events initiated by trauma with the formation of new tissue. The basic principle of wound healing is to minimize tissue damage, ensure adequate tissue perfusion and oxygen intake, and proper nutrition and moistening of the tissue [1]. Signal molecules, i.e., cytokines and growth factors secreted during wound healing, regulate this process [2]. A local microcirculation should provide oxygen and nutrients to meet the increased metabolic needs of regenerated tissues. For this purpose, under the influence of growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor-2 (FGF-2), endothelial cells migrate from the wound edges towards the wound center. This creates a microvascular network containing many new capillaries [3,4]. This structure, in which new capillary vessels are formed by capillary budding of existing vessels, is called angiogenesis. After angiogenesis, blood flow increases in the wound area. Thus, inflammatory cells can interact with each other and pass through the blood vessel endothelial basement membrane. Angiogenesis is required to synthesize and deposit the new extracellular matrix (ECM) [5,6]. VEGF plays an essential role in the formation of biomolecules during the wound healing process. It is believed that VEGF is the most effective in all growth factors due to trigger angiogenesis in the wound. VEGF also triggers collagen production and epithelization [7,8]. Matrix metalloproteinases (MMPs) regulate collagen degradation in the remodeling phase of wound healing [9]. MMPs largely regulate collagen degradation during the remodeling phase, where construction and degradation events are important. MMP activity is regulated by tissue inhibitor metalloproteinases (TIMP). TIMPs provide tight control of proteolytic activ-

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ity within the scar by creating a natural counterbalance against MMPs. When this balance is disturbed in any way, it results in excessive or insufficient matrix formation or open wound [10]. MMPs play a role in completing wound healing, and high levels of MMP-9 have been shown in many chronic wound types [11] and increased in acute burn wounds [12]. Platelets are involved in many biological functions such as immunity, angiogenesis, arterial thrombosis, haemostasis, inflammation, vessel remodeling, etc. Moreover, platelets secrete lysophosphatidic acid (LPA), one of the main lipids involved in signaling these growth factors. Also, LPA has been found to increase MMP2, MMP9, and MMP7 activity in tumour cells. MMPs are important endopeptidases that play a role in the entry and metastasis of the circulatory system by moving from the source of tumour cells. In order to do this, they degrade and remodel the ECM. Platelets contain antigenic proteins known to inhibit proliferation and vascularization in endothelial cells. These inhibitors are Platelet factor-4, endostatin, thrombospondin-1, and pro-angiogenic factors (VEGF). Although platelets secrete both factor groups, the result is usually angiogenesis [13]. Platelets have the capability to transfer their mRNA to monocyte and change their genetic material. Certain growth factors secreted by tumours interact with platelets, causing changes in the mRNAs of platelets. In other words, growth factors such as PGDF (platelet-derived growth factor), VEGF, and PF4 (angiostatic platelet factor-4) cause proliferation of tumour cells by changing the genetics of platelets. Therefore, mRNA in platelets potentially differs in healthy and cancer patients. Biomarkers to be created from these different platelet mRNAs can be used to detect the tumor early or indicate metastasis. These platelets genetically modified by the tumour are called tumour-educated platelets. Therefore, these platelets have the potential to be biomarkers in tumour detection. Cancer does not only cause mRNA changes in platelets. In addition, characteristic features such as thrombocyte count, protein content, and volume vary in the platelets affected by the tumour. Therefore, using these features separately or in combination can be used in cancer diagnosis to improve the prognosis of cancer and the efficiency of existing treatments [13]. Educated-plateles are genetically modified and/or changed version of thrombocyte as a result of encountering disease/pathological conditions. And they adapted to the new situation. This description has been made with detail in cancer cases. Nowadays, scientists research that educated-platelets (genetically modified platelets) may be used in diagnosis. Wound healing after burns can affect long-term complications such as survival, loss of function, and cosmetic problems. Quick wound healing reduces the risk of infection and dehydration, and also it has been shown to reduce mortality, length of stay in the hospital, and subsequent hypertrophic scarring. In other words, the applied treatment is the main determinant of the wound healing process. There are many potential therapies now to limit inflammation and oxidative stress and improve dermal circulation. Thus, they aimed to reduce the progression and transformation of burn injury [14]. Because the pathophysiology of wound healing is multifactorial and may vary from patient to patient, more than one agent or therapeutic intervention may be required tailored to burns. Therefore, the need for alternative treatment methods still continues today. In our previous study, we macroscopically evaluated the effects of educated-platelet in wound treatment [15]. This study evaluated the effects of educated-platelet at microscopic level on wound healing by histological and immunohistochemical methods.

Material and Methods

Animals

Experimental procedures were approved by the Dokuz Eylul University, Faculty of Medicine, Local Ethical Committee of Animal Experiments (Protocol no. 18/2021). This study was done at Dokuz Eylul University, Experimental Animal Laboratory. In this study, 28 Wistar female rats were used that were obtained from the Research Unit of Dokuz Eylul University Faculty of Medicine. All rats were housed in the standard animal room and cages at 20–22C room temperature with 50-60% relative humidity in 12/12 hours of dark/bright periods, and fed with rested tap water and standard pellet feed in Experimental Animal Laboratories until the end of the experiment (ad libitum). Our study design is a prospective experimental study. Twenty-eight female rats were randomly divided into four groups. Each group consisted of 7 rats. Group C rats (control group, unburnt; n:7) were exposed to platelet-rich plasma (PRP) with the uneducated-platelet application; group B rats (burn group; n:7) were exposed to the educated-platelet application after a burn injury; group B+EP rats (burn and treated educated-platelet group; n:7) were given PRP with educated platelets to their blood circulation; and group B+P rats (burn and treated uneducated-platelet group; n:7) were given PRP with uneducated platelets to their blood circulation.

Experimental design

A burn wound model was created in rats as described in the literature. Rats were anesthetized with 50mg/kg and 10mg/kg xylazine hydrochloride IP (intraperitoneal injection). The 5x2x2 cm3 sized metal plate was heated in boiling water for 5 minutes and touched the back of the shaved rat with its weight for 10 seconds (16). For post-operative analgesia, rats were given fentanyl citrate (0.002 µg/kg) twice a day. They were sacrificed following the administration of anesthesia (60mg/kg IP ketamine) on the fourteenth day of the burn injury; the burn sites were excised for histological analysis. Group C, group B rats were sacrificed to take enough blood samples for PRP preparation. Non-educated platelets were derived from group C, and educated platelets were derived from group B. After 1 hour, the prepared PRP was injected into the B+P and B+EP groups.

PRP (Platelet- Rich Plasma) preparation

Blood samples were taken from the inferior vena cava and buffered with sodium citrate at a ratio of 1:10 in tubes. After the first centrifugation at 1700 rpm for 7 minutes, plasma and buffy coats were collected, and the second centrifugation was performed at 3200 rpm for 5 minutes. Then the lower half of the plasma, called platelet-rich

plasma (PRP), was obtained. The platelet count in PRP was measured by an automated cell counter (CDA-1000; Sysmex) [17].

Histological examination

Tissue samples were collected 14 days after injury and fixated in 10% formaldehyde for 48-hours. Then by applying a routine tissue processing procedure, the tissues were embedded in paraffin blocks. Tissue blocks sections of 5 µm were cut and stained with H&E (05-06002/L, Bio-Optica, Milano, Italy), Masson Trichrome Staining (04-010802 Bio-Optica, Milano, Italy). We also examined histological features between groups B, B+P, and B+EP. Stained sections were analyzed by a pathologist, blinded to treatment, using light microscopy. The maturity of wound healing was scored according to the parameters given in the previous article [18].

Immunohistochemistry

MMP-9 (Bioss USA, bs-4593R) and VEGF (Bioss USA, bs-16165R) antibodies were used for immunohistochemistry. After deparaffinization and rehydration, the slides were incubated for 10 and 5 minutes with 10 mM citrate buffer (Cat No. AP-9003-125 Labvision) and 3% H2O2 solution respectively. Then, the slides were washed with PBS. They were incubated for eighteen hours at $+4\ ^{\circ}$C with anti- MMP-9 (diluted 1:100) and anti-VEGF (diluted 1:100) antibody, followed by a secondary antibody (Invitrogen-Plus Broad Spectrum 85-9043). Finally, they have been stained with DAB and Mayer hematoxylin, respectively. We used a light microscope for analysis. We evaluated: MMP-9 immunostaining in the epidermis and dermis, VEGF immunostaining in the dermis using a semi-quantitative scale. MMP-9 and VEGF immunoreactivity has been scored, by a pathologist, for staining intensity. Immunostaining intensity has been given scores 0 (negative), 1(weak), 2 (moderate), and 3 (strong), respectively [19].

Statistical analysis

Statistical analyses were performed using the SPSS (Statistical Package for Social Sciences) software version 26. Before starting to study, we performed power analysis. The power analysis indicated that 7 rats per group would be required to have 80% power with 5% type 1 error level to detect a statistical significant at $p < 0.05$ level. We evaluated the score of the wound healing process and the immunohistochemical staining. The difference between the groups was analyzed with Kruskal Wallis. The Mann-Whitney U test was performed to test the significance of pairwise differences using Bonferroni correction to adjust for multiple comparisons [20].

Results

Histologically, normal epidermis and dermis were evaluated in the group control (Figure 1C). Re-epithelialisation was significantly more intense in group B+EP ($p < 0.05$) (Figure 1 and 2). Granulation and inflammation reaction was significantly lower in group B+EP ($p < 0.05$) (Figure 1 and 2). There was no difference between the B and B+P
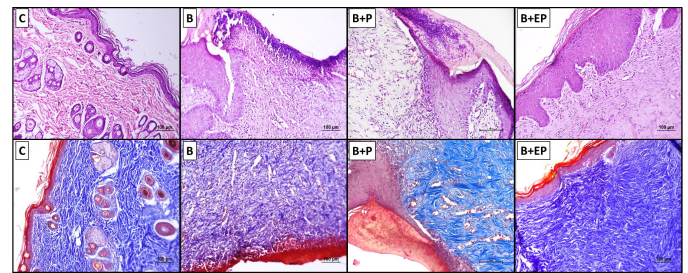

Figure 1. Histomorphological evaluation of wound healing in different groups. (C); Normal epidermis and dermis in the group control, (B); burn group, (B+P); burn+ uneducated platelet group, (B+EP); burn+ educated platelet group. The first line is Hematoxylin – Eosin staining, and the second line is Masson Trichrome staining, X 20, scale bar:100µm.
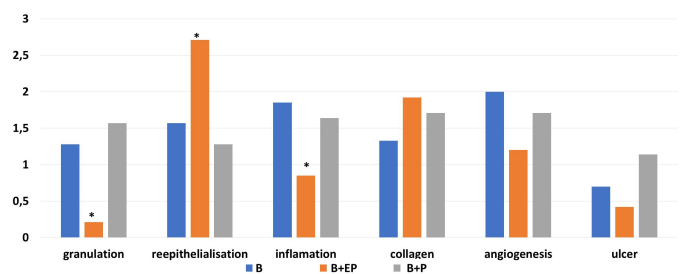

Figure 2. Semiquantitative scores of histological analysis in wound healing. (* $p<0,05$, respectively). (B); burn group, (B+P); burn+ uneducated platelet group, (B+EP); burn+ educated platelet group.
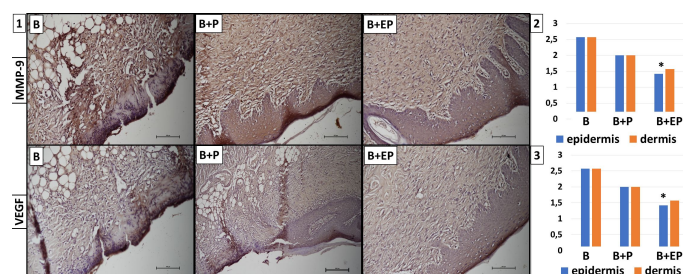

Figure 3. 3.1. Immunohistochemical expression of MMP-9 and VEGF in all groups (X20, scale bar:100µm.). 3.2. Semiquantitative scores of MMP-9. 3.2. Semiquantitative scores of VEGF . (* $p<0,05$). (B); burn group, (B+P); burn+ uneducated platelet group, (B+EP); burn+ educated platelet group.

groups. Histological analysis of wound healing showed that epithelialization, granulation tissue formation, collagen deposition was successful. Immunohistochemical staining for MMP-9 and VEGF in the B+EP group were statistically lower than other groups (p < 0.05). Educated platelet treatment significantly suppressed MMP-9 and VEGF on the 14th day (Figure 3).

Discussion

Many variables such as type of Burn, duration of contact, width, depth, burning body area, and cause of Burn affect the type and duration of burn treatment. The correct analysis of burn wound healing processes helps to choose an appropriate treatment plan to decrease the risk of complications [21]. Our study examined the effects of educated-platelet treatment, which we think can be an alternative method. Our result showed that treatment by educated platelet resulted in a significant reduction in inflammation, granulation, and increased reepithelialization during the healing period compared to the other groups. Prolongation of inflammation in burn patients causes complications. It may also cause systemic inflammatory response syndrome due to intense stimulation with mediators released from burn injuries [22]. Avsar et al. examined the effect of argan oil on the second-degree burn models in their created study. The study compared data for the 3rd, 7th, and 14th days. While re-epithelization was successful, no difference was noted in the inflammatory reaction [23]. On the other hand, we included the anti-inflammatory effect in our study. Studies showing that the anti-inflammatory effect accelerates the wound healing process also support our treatment method. Ozcelik et al. examined the topical effect of PRP on burn wound healing in their study and performed histopathological analysis on the tissues obtained by scarification on the 7th day of wound healing [24]. However, any marker that plays a role in this process has not been included in the study. Our study analyzed wound healing on the 14th day, and similarly, histomorphological features were evaluated. Also, systemically applied PRP has been shown to be therapeutic. Wound healing requires various developmental processes such as cell migration, extracellular matrix degradation, and tissue reorganization. The keratinocytes at the edge of the wound have to migrate to re-epithelize the wound surface, and then the fibrin-rich temporary matrix that is exposed after the wound where MMP is required must be removed. It has been shown that MMP-9 expression increased in the previous inflammatory phase and decreased in the later phases of wound healing [25]. The gradual increase in MMP-9 was noted in examinations on different days of wound healing. In mRNA analysis, it was shown to decrease in the following days after peaking on the 7th day, and MMP-9 deficiency was recorded in groups with insufficient wound healing [25]. In addition, MMP-9 deficient mice have been reported to cause delayed reepithelialization and irregular matrix remodeling in later stages of wound healing, in unsuccessful wound healing and keratinocyte migration and collagen fibrillogenesis. In addition, irregular re-epithelization and delayed matrix remodeling, which may be due to decreased MMP-9 expression, were observed [25]. MMP-9 was up-regulated levels in var-

ious types of slow-healing or non-healing wounds in different investigations [26]. The therapeutic effect of Curcumin was investigated in the wound model created in mice. In the experiment, it has been shown that MMP-9 is significantly suppressed and stimulates alpha-smooth muscle (SMA) levels in fibroblasts treated with tumour necrosis factor-alpha (TNF) via nuclear factor kappa B signaling. Thus, they have shown that topical Curcumin accelerates wound healing in mice by regulating the levels of various cytokines [26]. In the histological analysis of our study, we recorded low MMP-9 expression in the group (B + EP) where epithelization, granulation tissue formation, collagen accumulation were successful and interpreted this as an advanced stage in which wound healing was completed. We have described many studies showings that MMP deficiency affects wound healing negatively, but in our study, we recorded MMP low in the group where wound healing was successful, and we interpreted this finding as to the successful completion of wound healing and MMP reduction. In wound healing success, angiogenesis has a high effect, and angiogenesis proceeds quite regularly under the control of many growth factors. It is known that angiogenic and vascular growth factors are tightly regulated in a complex interaction. VEGF is one of the major growth factors of angiogenesis and plays a role in the early stages of angiogenesis. VEGF has a transformative role in wound healing through the extracellular matrix [25]. In one study, a decrease to basal levels in VEGF mRNA was observed in control mice during the period of granulation tissue formation on the 14th day of wound healing, while the maximum VEGF mRNA expression was also observed on 3th and seventh days. In the injury group where wound healing was unsuccessful, VEGF levels were significantly lower on day one and significantly higher on day 14 than in the control group. This was interpreted as indicating a delay in early angiogenesis and defective matrix formation [25]. VEGF is involved in the mechanism of wound healing, and an insufficient VEGF level can cause abnormal wound closure, while up-regulated VEGF exacerbates and induces skin scar formation. In another study, the group treated with 20% callicarpa nudiflora water extract (CNE) only on day 7 exhibited higher VEGF levels than the control and Vaseline groups, which differed significantly. VEGF levels of all groups did not differ on the 14th and 21st days. These results suggest that the CNE may accelerate wound healing by regulating VEGF early in the healing process [27]. Okur et al. investigated the effects of Capparis ovata on wound healing in mice and evaluated wound healing with TGF, VEGF, COL1A1(collagen type1 alpha1), and angiogenesis, granulation, regeneration levels on day 14. They noted an increase in VEGF in successful wound healing [28]. Since the pathophysiology of wound healing is multifactorial and can vary from patient to patient, there is no fixed and definitive treatment. Therefore, multimodal approaches using multiple agents or therapeutic interventions, particularly tailored to individual patients and burns, may be more effective than currently available single-mode approaches. However, a better understanding of the underlying pathophysiology and the contributions of individual interactions between the burned patient's genetic makeup and environmental factors may open new

avenues for research and development. Therefore, alternative treatment options are needed [14]. In summary, these results suggest educated-platelet is effective in healing experimentally created burns in rats. Our study showed that educated platelets are therapeutic by examining their effects on the stages of wound healing, including neutrophil migration, inflammation, reepithelialization, fibroblast increase, granulation, angiogenesis, and collagen deposition. We think that it accelerates this effect by regulating some cytokine levels. Also, educated-platelet treatment accelerated wound healing in rats by regulating the levels of various cytokines. As a result, more studies are needed to include trained platelets in treatment protocols. Further and larger clinical studies are needed to determine the effectiveness of this treatment method definitively.

Conflict of interest and financial support

Ethics approval

Experimental procedures were approved by the Dokuz Eylul University, Faculty of Medicine, Local Ethical Committee of Animal Experiments (Protocol no. 18/2021).

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