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Research Article

Effects of Erythropoietin-Treatment on Wound Healing in Burn Patients. Investigated Using Immunohistochemical and Histological Methods, Analyzed Using AI. Post Hoc Analysis of the Randomized, Placebo-Controlled Clinical Trial "EPO in Burns"

Christina Irene Günter[1*], Silvia Egert-Schwender[2], Augustinus Bader[3], Hans-Günter Machens[4] and the "Epo in Burns" Study group

[1]Clinic for Plastic Surgery and Hand Surgery, Klinikum rechts der Isar, Technical University Munich, Munich, Germany

[2]Müncher Studienzentrum, Klinikum rechts der Isar, Technical University Munich, Munich, Germany

[3]Institute for Cell Techniques and Applied Stem Cell Biology, University of Leipzig, Leipzig, Germany

[4]Clinic for Plastic and Hand Surgery, Klinikum rechts der Isar, Technical University Munich, Munich, Germany

*Corresponding author: Christina Irene Günter, Clinic for Plastic Surgery and Hand Surgery, Klinikum rechts der Isar, Technical University Munich, Munich, Germany

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Abstract

Background: Scars often negatively impact individuals' self-esteem, making them self-conscious and lowering their self-confidence. To help patients avoid scar formation after burns or accidents, researchers are trying to better understand the biological mechanisms leading to scar formation and develop effective clinical treatments. Burns are leading causes of mortality and morbidity, including prolonged hospitalization, disfigurement, and disability. Erythropoietin (EPO) is a well-known hormone causing erythropoiesis. However, EPO may play a role in healing acute and chronic wounds due to its anti-inflammatory and pro-regenerative effects. Losing the protective barrier of their skin puts burn patients at high risk. Typical manifestations of a severe burn injury are immunosuppression, hyper-metabolism, and complications such as wound infection and sepsis, with subsequent multi-organ failure and death. Therefore, burns lead to the risk of delayed and poor recovery. For this reason, early wound closure is essential for the prognosis and rehabilitation of burn patients.The large, prospective, placebo-controlled, randomized, double-blind, multi-center clinical trial "EPO in Burns" was initiated to investigate the effects of EPO versus placebo treatment in severely burned patients. The primary endpoint of „EPO in Burns" was defined as the time elapsed until complete re-epithelialization of a designated split skin graft donor site.

Methods: Previous pre-clinical studies have shown that low-dose Erythropoietin (EPO) has regenerative and cytoprotective effects, for example, in animal models and cell cultures. However, the molecular mechanisms by which EPO influences scar formation or wound healing in human skin tissue are still unclear. This study evaluates the effect of EPO on skin graft donor site wound healing in human patients using immuno-histochemical methods. The verum (n 45) and control (n 39) groups were compared with regard to the time it took for study wounds (a predefined split skin graft donor site) to reach the three stages of wound healing (re-epithelialization

levels). The effect of systemic application of EPO on immunohistochemical indicators of wound healing is tested using skin samples from a clinical trial that compared EPO-treated to placebo-treated groups.

Results: The immuno-histochemical analyses failed to uncover any strong patterns differentiating treatment and control groups, except Von Willebrand Factor (VWF), which showed signs of decreased expression in the EPO-treated group.

Conclusion: To test whether EPO is one of the key pro-regenerative agents in skin regeneration, as suggested in previous studies [1], a clinical trial was conducted to assess how wound healing is affected by systemic treatment with EPO. IHC analyses failed to uncover any strong effects differentiating treatment and control groups, except VWF, which showed decreased expression in the EPO treatment group. More research is needed to determine if different dosages, application schemes, or topical applications of EPO may improve wound healing at the donor site of skin grafts and if the effect of EPO on wound healing is dose-dependent.

Keywords: Erythropoietin; EPO; Skin regeneration; Wound healing

Introduction

Cutaneous wound healing and scar tissue formation involve complex biological and physiological processes comprising different cellular and extracellular mechanisms. These include multiple cell populations, epidermal and dermal cell interactions, chemotaxis, mitosis, angiogenesis, ECM synthesis, and soluble mediators such as growth factors and cytokines [2,3]. In previous publications, Günter et al. [1] pointed out that several cytokines and growth factors play a role in stem cell activation in wound sites, suggesting that EPO is one of the vital active agents in skin regeneration, increases the proliferation rate of MSCs when added under hypoxic conditions, and stimulates angiogenesis, mitosis, vascularization, and cell-cycle activation [1]. EPO seems to be crucial in skin regeneration, especially in treating acute and chronic tissue injuries [4,5]. It is expressed in hematopoietic and several non-hematopoietic tissues, improving cell survival and delaying inflammation processes caused by hypoxia, toxicity, or injury [1]. There is also a possibility that EPO restores impaired wound healing and stimulates angiogenesis and cell cycle in burn-damaged tissues [6]. Understanding the molecular and cellular mechanisms responsible for wound healing may provide clues that enable the development of faster – and possibly scar-less – wound healing treatments. It is the objective of medical research to develop such alternative, rapid, efficient, and cost-saving therapies to treat wounds. This study reviews the molecular and cellular basis of regeneration and skin tissue repair, with particular attention to dermal Mesenchymal Stem Cells (MSC). Differences in the speed of healing in various regenerative models can provide critical insights for developing therapeutic strategies that may alleviate some of the morbidity associated with scarring after traumatic injuries.

EPO is reported to bind to the homodimeric EPO receptor (EPOR) and the Tissue Protective Receptor (TPR). By binding to EPOR, EPO promotes erythropoiesis; by binding to TPR, however, it can act differently, for example, by suppressing pro-inflammatory cytokines, promoting wound healing, protecting cells from apoptosis, and modulating the activation, differentiation, and function of immune cells [7]. The healing, immuno-regulatory, and tissue-protective effects at injury sites depend on high EPO concentrations and elevated expression of the TPR receptor so that EPO can bind effectively to TPR. It activates signaling pathways in different cells that suppress pro-inflammatory cytokines, alter the inflammatory response, and regulate the immune system. Consequently, the immuno-regulatory effects of EPO can down-regulate apoptosis, promote tissue survival, and accelerate wound healing [7]. Within the framework of the "EPO in Burns" project [1,8] that investigated the effect of EPO on vascularization, re-epithelialization, and dermal stem cell activation at skin-graft donor sites, the following study focuses on the role of Recombinant Human Erythropoietin (rHuEPO) plays in wound healing and gene expression.

Methods

In our study, the effects of systemic EPO application on the healing process of defined skin graft donor sites were investigated using new histological and immuno-histochemical analyses applied to skin samples collected by the published "EPO in Burns" project [9], with a particular focus on potential acceleration of skin regeneration. We have also investigated if systemic EPO application can lead to faster stem cell activation, faster re-epithelialization, and better vascularization in split skin donor areas, as measured by cell marker activation. Skin samples from the donor sites of skin grafts were used to test if systemic application of EPO can accelerate wound healing. In both treatment and placebo groups, the expression of cell surface markers over 16 days was quantified using histology, immunohistochemistry, and image analysis. The study data was analyzed using the R software package [10]. The effect of EPO on re-epithelialization at the skin graft donor sites was measured using CK10 and CK14 expression levels since these

markers are especially suited to measure epidermal development. CK10 is a type-I cytokeratin and creates intermediate filament structures. It can be used to visualize neo-epidermis formation and epithelialization [11]. CK14 is a type-1 keratin and an intermediate filament protein. It forms the structure of the cytoskeleton of epithelial cells and plays a critical role in maintaining the cell nuclei in epidermal cells. This study aims to identify if there is a cytoprotective or regenerative effect of systemically administered EPO if healing outcomes are improved at skin graft donor sites of patients suffering severe burn injuries after EPO administration and to understand its cellular effects, if any. Specifically, the study will evaluate if EPO activates dermal stem cell markers (e.g., CD90, CD105, and CD271) at split skin graft donor sites if EPO promotes vascularization and up-regulates the expression of CD31 and VWF at split skin graft donor sites.

Finally, it will evaluate if the expression of biomarkers linked to re-epithelialization and granulation tissue formation is more significant in the treatment group than in the control group. The data used in this study were collected as part of a large, prospective, randomized, double-blind, multi-center study funded by the German Federal Ministry of Education and Research and fully approved by the designated ethics committee. Tissue samples from adult patients with deep second- or third-degree burn injuries were obtained from the "EPO in Burns" project [9]. Patients were randomly allocated to study medication (EPO) or a matched placebo. The samples were processed and stained to assay the expression of several key cell markers. The stained tissue was photographed, and next-generation image processing software was used to quantify expression levels. The EPO-treated group received 150 IU of EPO per kg body weight subcutaneously at the abdomen, starting on day 2 of the study and continuing every other day for 21 days. The EPO treatment was prepared by dissolving multi-dosage vials (Neo Recormon 50,000 IU EPO; Roche Diagnostics GmbH, Germany) into a placebo solution. The placebo group received only the placebo solution itself, without EPO. The tissue samples from the skin graft donor sites were collected on days 2, 10, 12, and 16 [9]. Split skin grafts from the lateral thigh were harvested at dimensions of 8 cm x 8 cm and a thickness of 0.3 mm using a dermatome. Samples were obtained using punch biopsies (5 mm) and prepared for histological and immuno-histological analyses. Pieces of skin were placed in liquid nitrogen immediately after sampling and shipped to the Biotechnologisch-Biomedizinische Zentrum at the University of Leipzig. At the BBZ, the samples were kept in liquid nitrogen until further analysis. Tissue-Tek cryomolds were prepared and marked with the orientation of the tissue.

The cryomolds were filled with chilled Tissue-Tek O.C.T. solution (Leica, Germany), and the frozen tissue was immediately placed in the cryomold and covered with Tissue-Tek O.C.T. solution. Subsequently, the cryomold was put into a liquid nitrogen bath until the Tissue-Tek solution turned white and was completely frozen. The embedded tissue was then stored in liquid nitrogen. To ensure good performance, a microtome was used to section the skin tissue cooled to -20°C. Two drops of Tissue-Tek O.C.T. solution were added to the cold specimen stage, and frozen tissue samples were then inserted. The metal holder and sectioning blade were adjusted, and the tissue was cut to 10 µm thick. The tissue was then placed on Visium Spatial Slides for the next step.Cryosections were obtained from the skin samples using standard methods and then fixed in acetone (NeoLab, Germany) for 10 minutes. They were air-dried and placed on slides, and a Dako pen (Sigma, USA) was used to draw a border around each sample. The slides were then put in a ready-made hematoxylin solution (Hämalaun nach Mayer; Carl Roth, Germany) for another 10 minutes. After staining, the slides were rinsed with tap water for several minutes and kept in tap water for 15 minutes. The slides were washed with deionized water and air-dried. Eosin solution (Carl Roth, Germany) was added to the samples for 1 minute, and the samples were washed with deionized water. In the next step, the samples were put into different concentrations of alcohol (70%, 80%, and 96%, respectively) for 10-20 seconds each and then in 100% alcohol (Carl Roth, Germany) for 3 minutes. The slides were air-dried, covered with a mounting medium, and closed with slide covers. The samples were then checked under a microscope. After staining, the cell plasma appears pink, and the cell nuclei blue or purple.

For immunohistochemistry procedures, cryosections were prepared as before. The samples were then blocked by incubating in goat serum (in a 1:10 dilution with PBS) for approx. 20 minutes. The sections were then incubated with primary antibodies for one hour at room temperature (25°C). Afterward, the sections were washed twice with PBS for 5 minutes, placed in a humidified chamber, and incubated with a secondary antibody, peroxidase-conjugated goat-anti-mouse IgG (Jackson ImmunoResearch, UK; diluted 1:100 in PBS), for 45 minutes. The samples were then rinsed with PBS two times for 5 minutes, and 3-amino-9-ethyl-carbazol substrate (AEC; Sigma, USA) was added to the specimens. They were then incubated in a humidified chamber at room temperature for 10 minutes. The sections were washed two more times with PBS. Double-Distilled Water (ddH2O) was added to the slides to reveal the color of the antibody staining. Once the desired color intensity was reached, cell nuclei were counter-stained with Mayer's Haematoxylin (Lillie's Modification; Dako Cyomation, Germany) for one or two minutes. The slides were rinsed with tap water twice more for 15 minutes and then dehydrated and mounted with glass coverslips. As a result, antibodies were stained dark red, the cell plasma pink, and the cell nuclei blue. Positive, negative,

and isotype controls were used to validate all test results. To quantify the relative area of stain types, the Orbit image analysis software [12] version 3.64 was used. Each image was opened individually in the software; three cell-type classes were set up by manually selecting example regions of each cell type, including the background (purple), the stained cells (blue), and the unstained cells (green). These target areas were selected using a polygon pen. After each model fit, the image was visually checked to ensure that the background was accurately colored purple and the stained and unstained cells were accurately classified as blue and green, respectively. A "Region Of Interest" (ROI) was defined using a pen to highlight a constant-width band of tissue with the boundary as one side to ensure a standardized interpretation of expression between images. After determining the ROI for each image, the relative density of cell types in the ROI was calculated. The data were then saved in an Excel table and statistically analyzed using the R software package.

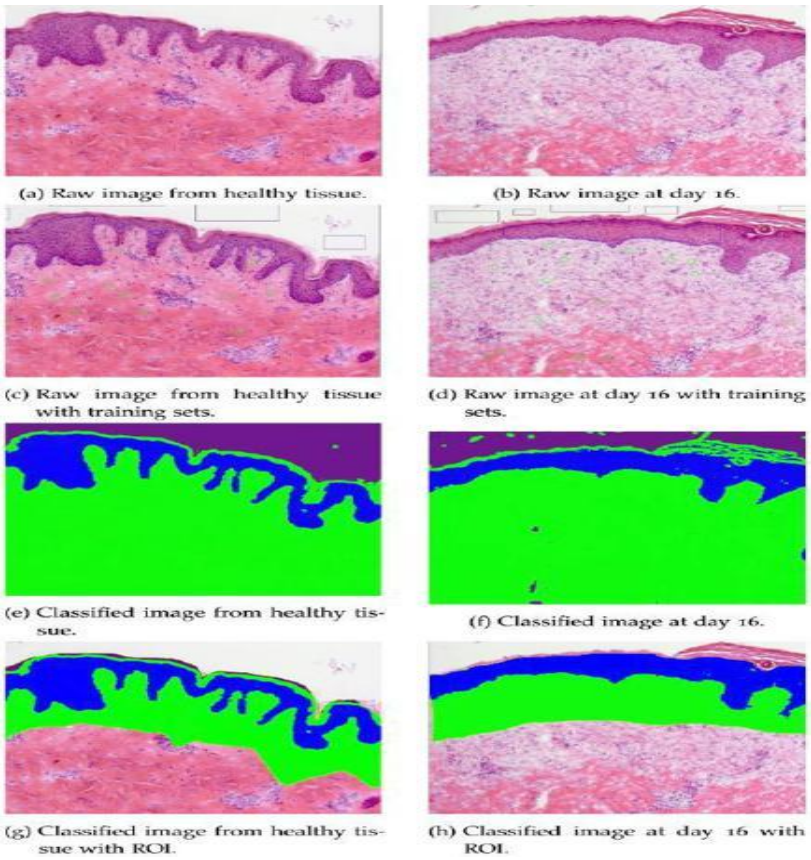

Figure 1: The steps involved in image analysis with Orbit software.

Results

This study used the expression of various cell markers to evaluate dermal stem cell proliferation, vascularization, re-epithelialization, and wound closure. The relative levels of areas of Immunohistochemical staining (IHC) can be used to measure the rate and activation of stem cells in the wound healing process. The results of IHC staining are presented in (Figures 2-4), respectively. It is suggested that CD90 or Thymocyte differentiation antigen 1 (Thy1) participates in the self-renewal and differentiation of MSCs [13] and is involved in producing pro-inflammatory cytokines. Figure 2 shows the log-odds of CD_{90} marker expression in tissue samples as a function of day and treatment group.

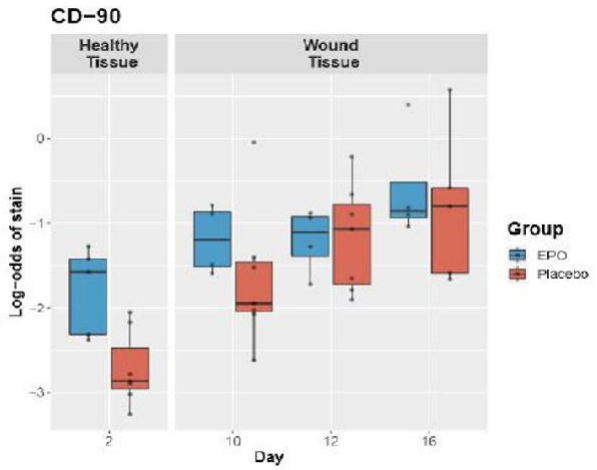

Figure 2: Results of staining for CD90 markers. Data are presented using boxplots. Each color represents a group, and each set of boxes represents a day post-treatment. There is no apparent difference in the log odds of CD90 staining as a function of the treatment group.

As wound healing progressed from days 10 to 16, more extensive areas were stained in both treatment and placebo groups. Averaging across days, the effect of EPO treatment on the expression of CD_{90} stain is b= 0.26 (p = 0.33), indicating that in these study patients, EPO has little to no effect on the rate of activation of CD90+ cells during wound healing. Endoglin, or CD_{105}, is a cell surface marker and type 1 membrane glycoprotein. To investigate whether CD_{105} marker expression is affected by EPO, the log odds of CD_{105} marker expression in tissue samples as a function of day and treatment group were plotted, as shown in Figure 3.

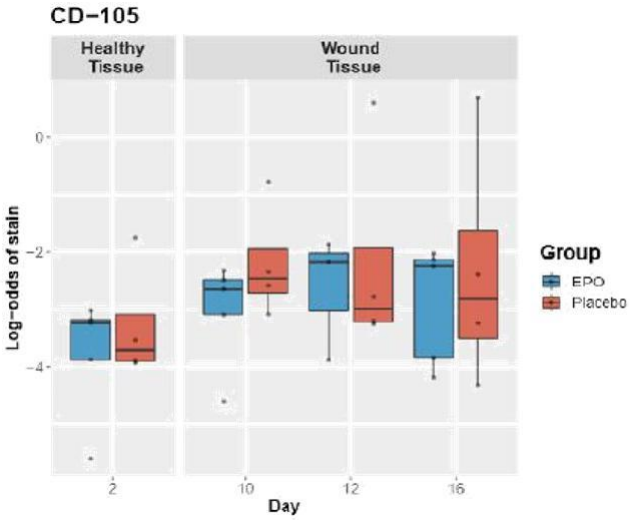

Figure 3: Results of staining for CD_{105} markers using boxplots. As before, each color represents a group, and each set of boxes represents a day post-treatment. There is no apparent difference in the log odds of CD_{105} staining as a function of the treatment group.

As before, no detectable differences in IHC staining between treatment and placebo groups were found. Averaging across days, the effect of EPO treatment on the expression of CD_{105} stain is b = -0.65 (p = 0.21). This indicates that, in these study patients, EPO has little to no effect on the activation rate of CD105+ cells during wound healing. CD_{271+} dermal cells produce various cytokines, growth factors, and neurotrophins essential in wound healing. To test if CD_{271} marker expression is affected by EPO, the log odds of CD_{271} marker expression were plotted in tissue samples as a function of day and treatment group.

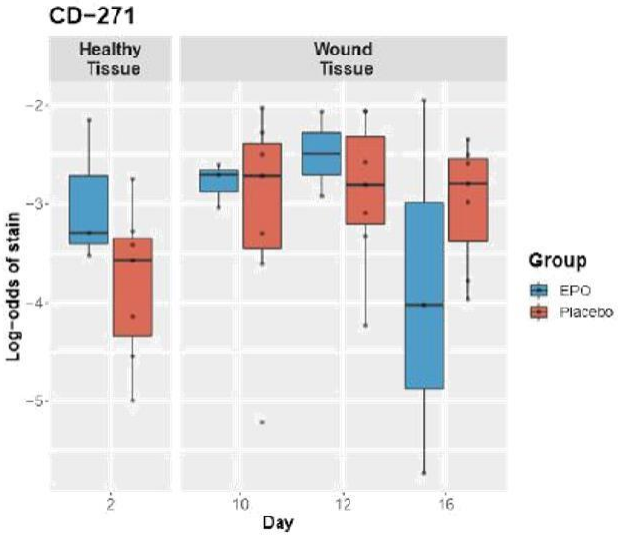

Figure 4: CD271 marker expression. Data are presented using boxplots, each color presenting a group, and each box set representing a day post-treatment. There is no apparent difference in the log odds of CD271 staining as a function of the treatment group.

On day 16, the placebo group appears to have slightly higher CD_{271} expression, but expression levels vary highly across patients. There is thus no apparent effect of EPO on outcomes. Averaging across days, the effect of EPO treatment on the expression of CD_{271} stain is b = -0.14 (p=0.72). This indicates that EPO has little to no impact on the activation rate of CD_{271+} cells during wound healing in these patient groups. In addition, we used CD_{31} and VWF expression as endothelial cell markers to evaluate vascularization.

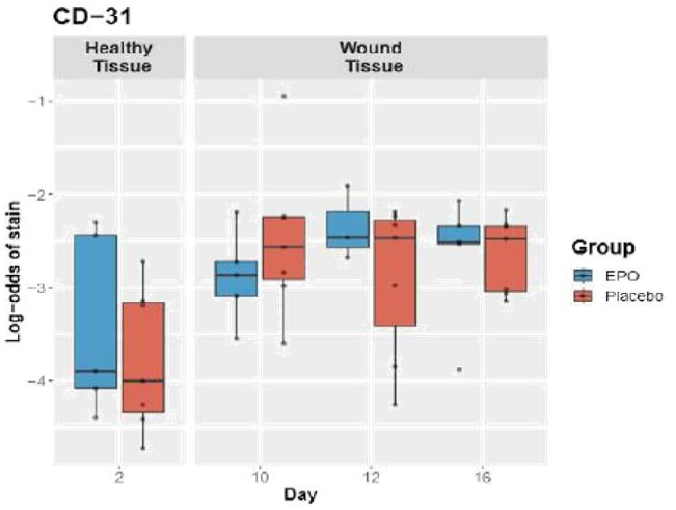

Figure 5: Results of staining for CD_{31} markers. Data are presented using boxplots, with each color representing a group and each box set representing 1 day post-treatment. There is no apparent difference in the log odds of CD_{31} staining as a function of the treatment group.

Cluster Of Differentiation 31 (CD_{31}) is a cell surface marker associated with vascularization. As such, it may be used to measure the effectiveness of various treatments by increasing the number of endothelial and blood vessels. To test if CD_{31} marker expression is affected by EPO, the log odds of CD_{31} marker expression in tissue samples were plotted as a function of day and treatment group. No detectable differences in IHC staining were found between the treatment and placebo groups. Averaging across days, the effect of EPO treatment on the expression of CD_{31} stain is b = 0.001 (p = 0.9). Von Willebrand Factor (VWF) is an endothelial cell-specific marker promoting angiogenesis, smooth muscle cell proliferation, and tissue regeneration, thus accelerating tissue repair and maintaining normal hemostasis [14,15]. VWF promotes wound healing, and a lack of VWF may decrease growth factors at the wound site, delaying wound healing [14]. Figure 6 plots the log odds of VWF marker expression in tissue samples as a function of day and treatment group:

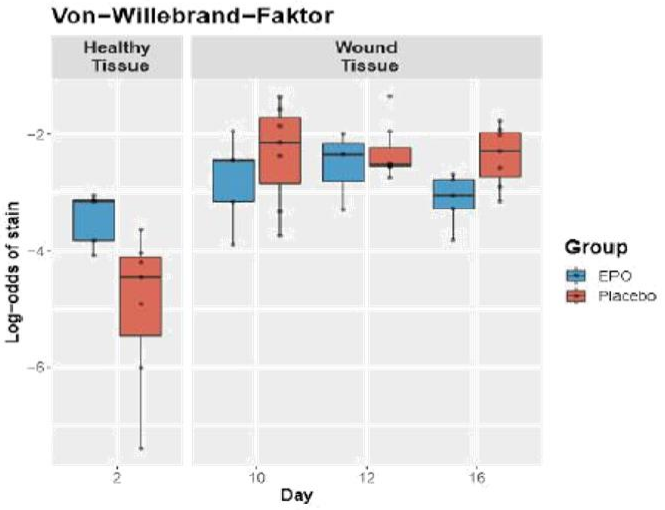

Figure 6: Results of staining for VWF markers, with data presented as box plots. Each color represents a group, and each box set a day post-treatment. There is a significant difference in the log odds of VWF staining as a function of the treatment group, with expression higher in the placebo group.

In both treatment and placebo groups, there is a higher expression of VWF markers at later days in wound tissue relative to healthy tissue at day 2. Averaging across days, the effect of EPO treatment on the expression of VWF stain is b = -0.51 (p = 0.02). This indicates that EPO has a significant adverse impact on the intensity of VWF expression during wound healing in these patients. Finally, we investigated whether EPO increases the rate of re-epithelialization and tested if the application of EPO on skin graft sites improves overall wound healing. The results of histological staining using Hematoxylin and Eosin (H&E) are shown in Figure 7.

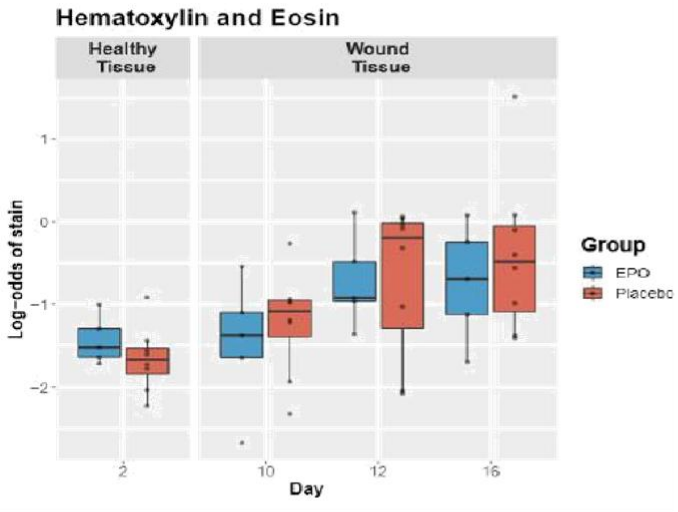

Figure 7: Results of H&E staining. Data are presented using boxplots, with each color representing a group and each box set representing a day post-treatment. There is no apparent difference in the log odds of H&E staining as a function of the treatment group.

Since H&E staining measures re-epithelialization, the relative levels of stained areas on histological slides may be used to measure the rate and completeness of wound healing. As wound healing progressed from day 10 to day 16, we found larger areas stained in both treatment and placebo groups. However, we did not find detectable differences in H&E staining between treatment and placebo groups. Averaging across days, the effect of EPO treatment on the expression of H&E stain is b = -0.2 (p = 0.95). This indicates that EPO has little to no impact on the rate or completeness of wound healing in these study patients. Cytokeratin 10 (CK_{10}) creates intermediate filament structures and can be used as an early marker of keratinocyte differentiation and to show neo-epidermis formation and epithelialization. Cytokeratin 14 (CK_{14}) is a keratinocyte proliferation marker in the basal layer of the epidermis and may also be used to detect neo-epidermal structures and epithelialization [11,16,17]. In Figure 8, the CK10 marker expression is plotted to test if CK10 marker expression is affected by EPO.

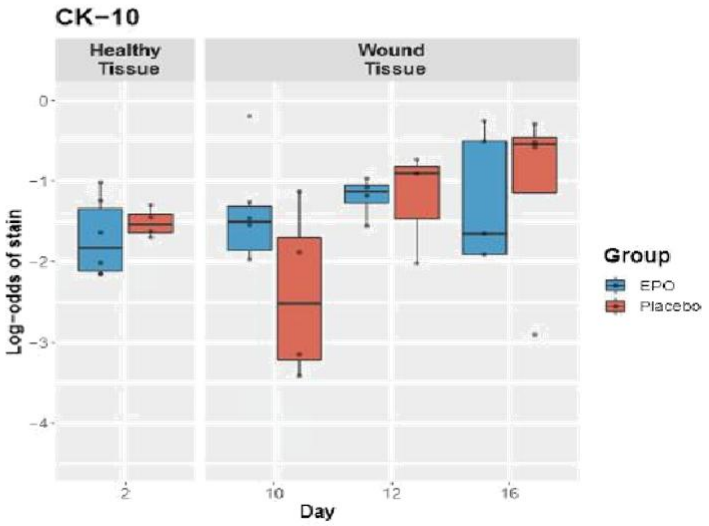

Figure 8: Results of staining with CK_{10}. Data are presented using boxplots. Each color represents a group, and each box set represents a day post-treatment. There is no apparent difference in the log odds of CK_{10} staining as a function of the treatment group.

In both treatment and placebo groups, there is slightly higher expression of CK_{10} markers at later days, but treatment with EPO does not appear to lead to higher CK10 expression. We did not find detectable differences in IHC staining between the treatment and placebo groups. Averaging across days, the effect of EPO treatment on the expression of CK_{10} stain is b = -0.09 (p = 0.9). To test if CK_{14} marker expression is affected by EPO, Figure 9 plots the log odds of CK_{14} marker expression in tissue samples as a function of day and treatment group.

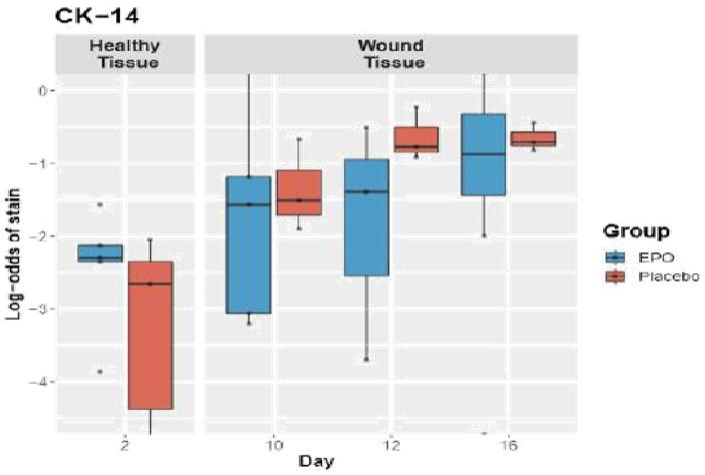

Figure 9: Results of staining with CK_{14}. Data are presented using box plots. Each color represents a group, and each box set represents a day post-treatment. There is no apparent difference in the log odds of CK_{14} staining as a function of the treatment group.

In both treatment and placebo groups, there is a slightly higher expression of CK_{14} markers at later days, but treatment with EPO does not appear to lead to higher CK_{14} expression. We did not find detectable differences in IHC staining between the treatment and placebo groups. Averaging across days, the effect of EPO treatment on CK_{14} stain expression is b= -07.23 (p = 0.15). This indicates that EPO has little to no effect on the expression of CK_{14} during wound healing in these patients. Alpha Smooth Muscle Actin (a-SMA) is a marker of activated fibroblasts or differentiated myofibroblasts and shows granulation tissue formation, which accelerates wound closure [15]. We tested if a-SMA marker expression is affected by EPO and plotted the log odds of a-SMA marker expression in tissue samples as a function of day and treatment group.

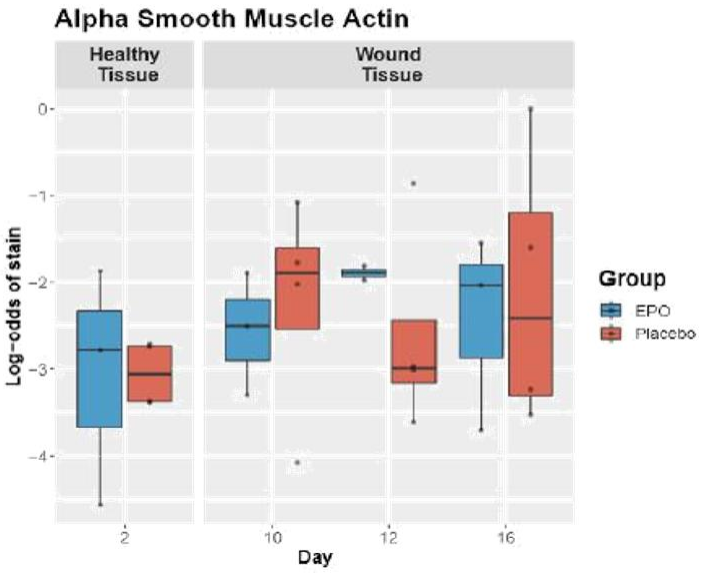

Figure 10: Results of staining with a-SMA. Data are presented using box blots. Each color represents a group, and each box set represents a day post-treatment. There is no apparent difference in the log odds of a-SMA staining as a function of the treatment group.

In both treatment and placebo groups, treatment with EPO does not appear to lead to higher a-SMA expression. We did not find detectable differences in IHC staining between the treatment and placebo groups. Averaging across days, the effect of EPO treatment on a-SMA stain expression is b = -0.03 (p = 0.94), indicating that EPO has little to no effect on a-SMA expression during wound healing in these patients.

Discussion

In summary, the results of this study provide a starting point for further preclinical and clinical investigations to gain a deeper understanding of the underlying mechanisms of the revealed effects. In contrast to many previous studies on the effects of EPO on wound healing, both in vitro and in animal models, the current study did not find any clear evidence for an effect of EPO on the rate of wound healing as measured by CD_{31}, CD_{90}, CD_{105}, CD_{271}, CK_{10}, CK_{14}, and a-SMA. However, there was some evidence that treatment with EPO led to decreased expression of VWF, which may have relevance to angiogenesis during wound healing. This study explored the effects of experimental treatment with EPO on markers linked to wound healing in a clinical trial setting. The first research question was to investigate if treatment with EPO activates dermal stem cell markers (e.g., CD_{90}, CD_{105}, and CD_{271}). The data, however, show no detectable differences in IHC staining for these markers between treatment and placebo groups, indicating that EPO has little to no effect on the rate of CD_{90}, CD_{105}, and CD_{271} expression during wound healing in these study patients. However, in both treatment and placebo groups, there was increased expression of CD_{90}, CD_{105}, and CD_{271} markers at SGDSs compared to healthy skin. This shows that dermal stem cells may proliferate and migrate to wound areas in both treatment and placebo groups. However, the placebo and treatment groups do not show significant differences in marker expression.

The effect of EPO on the rate of re-epithelialization at the donor site of skin grafts was investigated using H&E and the three cell-surface markers discussed above. The data showed no significant differences between treatment and placebo groups in H&E and IHC staining. This may indicate that injection of EPO has little to no effect on the CK_{10}, CK_{14}, and a-SMA expression rate during wound healing in these study patients. In both treatment and placebo groups, the epithelial layer was initially formed by day 10 and completed by day 16. Previous studies have argued that topical and/or systemic application of EPO has anti-inflammatory and anti-apoptotic effects, accelerates epithelialization and vascularization, and generally improves wound healing [8, 18-24]. However, the histological and immunohistochemical analyses presented here fail to replicate such findings, at least with the specific dosage levels investigated in the "EPO in Burns" protocol [20]. With regard to the Von Willebrand factor, an endothelial cell-specific marker, the current study has shown that EPO administration leads to statistically significant decreases in VWF expression in the treatment group, suggesting lower neovascularization at the wound sites.

Conclusion

The results of this study suggest that the effects of in vivo treatment with EPO in humans may lead to different outcomes than previous studies using animal and in vitro models suggested. Low doses, high doses, single doses, or repetitive doses of EPO may have other effects or even side effects on the body. The method of administration, i.e., topical vs. systemic application – may affect the results of EPO treatment in clinical settings [22]. Other factors that impact the scope of effects for EPO may be the kind of injury, the size of the graft, the depth of the donor site, and the individual characteristics of a patient [23]. The inferences that can be drawn from the current study are, therefore, limited, as only a single dosage and timing regime was tested using a single standardized graft profile [24-26].

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