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Efficacy of In Vivo Electroporation-Mediated IL-10 Gene Delivery on Survival of Skin Flaps

S. Morteza Seyed Jafari[1,2,3] · Maziar Shafighi[2] · Helmut Beltraminelli[1] · Benedikt Weber[1] · Ralph. A. Schmid[4] · Thomas Geiser[2,5] · Amiq Gazdhar[2,5] · Robert E. Hunger[1,2]

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Abstract Despite advances in understanding the underlying mechanisms of flap necrosis and improvement in surgical techniques, skin flap necrosis after reconstructive surgery remains a crucial issue. We investigated the efficacy of electroporation-mediated IL-10 gene transfer to random skin flap with an aim to accelerate wound healing and improve skin flap survival. Nine male Wistar rats (300–330 g) were divided in two groups (a) control group ($n = 5$), only surgery no gene transfer, and (b) experimental group, received electroporation-mediated IL-10 gene transfer 24 h before the surgery as prophylaxis ($n = 4$). Random skin flap (McFarlane) was performed in both groups. Planimetry, Laser Doppler imaging, and immunohistochemistry were used to evaluate the effect of IL-10 gene transfer between study groups at day 7. Electroporation-mediated IL-10 gene transfer decreased percentage of flap necrosis (p value = 0.0159) and increased cutaneous perfusion compared to the control group (p value = 0.0159). In addition, Spearman's rank correlation showed a significant negative correlation between percentage of flap necrosis and Laser Index (p value = 0.0083, r −0.83, respectively). Furthermore, significantly higher mean $CD31^+$ vessel density was detected in the experimental group compared to the control group (p value = 0.0159). Additionally, semi-quantitative image analysis showed lower inflammatory cell count in experimental group compared to control group (p value = 0.0317). In vivo electroporation-mediated IL-10 gene transfer reduced necrosis, enhanced survival and vascularity in the ischemic skin flap.

Keywords IL-10 · In vivo electroporation · Skin flap necrosis · Non-viral gene therapy

Introduction

Although, significant improvements in surgical techniques and in basic understanding of mechanisms for flap survival have been made recently, surgeons and clinicians are faced with critical issues regarding skin flap necrosis, tissue ischemia (Basu et al. 2014; Kryger et al. 2000). This daunting challenge provides an opportunity to explore new strategies to improve flap survival (Basu et al. 2014). A number of cytokines and growth factors have been tested with an aim to promote angiogenesis and reduce necrosis/fibrosis with an aim to improve graft survival (Zhang et al. 2004).

IL-10 is a pleiotropic immunoregulatory homodimeric cytokine produced by $CD4^+$ and $CD8^+$ T cells, B cells, monocytes, macrophages, and keratinocytes (Moore et al. 1993), and regulates cell-mediated immunity (Fiorentino et al. 1989; Kieran et al. 2014). It also exerts important effect on neovascularization in the wounds by increasing the number of endothelial progenitor cells, in circulation and in the wound (Balaji et al. 2014). Moreover, IL-10

✉ Amiq Gazdhar
amiq.gazdhar@dkf.unibe.ch

1 Department of Dermatology, Inselspital, Bern University Hospital, Bern, Switzerland

2 Department of Clinical Research, University of Bern, Bern, Switzerland

3 Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland

4 Department of General Thoracic Surgery, University Hospital, Bern, Switzerland

5 Department of Pulmonary Medicine, University Hospital, Inselspital Bern, Bern, Switzerland

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strengthens the ''scavenger'' function and contributes to inhibit alloreactivity in vivo by induction of tolerance (Delvaux et al. 1994; Sabat et al. 2010; Wang et al. 1994). However, in order to achieve its therapeutic effect repeated or continuous administration is needed (Tavakoli et al. 2006). Gene therapy provides a reliable approach to achieve sustained therapeutic levels. Electroporation-mediated non-viral gene therapy offers a promising solution to improve treatment efficiency (Tavakoli et al. 2006; Zhang et al. 2004). Electroporation-mediated gene delivery is a safe method for prolonged transgene expression, and works on the principle of application of short electric pulses of high voltage to facilitate the DNA transfer (Basu et al. 2014; Gehl 2003). In the current study, we investigated the effects of electroporation-mediated IL-10 gene transfer to random skin flap in a rat model with an aim to accelerate wound healing, facilitate vascularization, and reduce skin flap necrosis.

Materials and Methods

Animals

Adult male Wistar rats (290–330 g) were used in this study protocol (Charles River Laboratories Sulzfeld, Germany). The rats were kept in individual cages in a temperature and light-controlled animal facility and standard food and water were provided ad libitum. The study was approved by the Bern cantonal animal experiment commission committee (protocol No. 17/14). All animals received humane care according to the guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85–23, revised 1996). Electroporation alone (without plasmid) has not shown any significant permanent effects on the outcomes of previous studies; (Fakin et al. 2011; Gehl 2003; Tavakoli et al. 2006) therefore, this group was not included in the current study to reduce the number of animals. Control group ($n = 5$) underwent surgery only and received no treatment (Basu et al. 2014). The experimental group received electroporation-mediated IL-10 gene transfer 24 h before the surgery ($n = 4$).

Surgical Model (McFarlane Flap)

A modified McFarlane skin flap, was used as the surgical model in this study. The animals received Buprenorphine (0.1 mg/kg) subcutaneous (s.c.) 30 min before surgery. Animals were anesthetized by allowing them to inhale 5% isoflurane in a glass chamber. Anesthesia was maintained during surgery by 2–3% isoflurane via nasal inhalation. The skin of the dorsum was shaved with electric clippers. Before raising the flap, a caudally pedicled flap measuring 9 × 3 cm was marked on the dorsum of the animals symmetric to the dorsal midline. The flap was raised in the plane deep to the panniculus carnosus and after careful hemostasis, re-sutured back in its own place using 4-0 Monocryl sutures. Animals were observed for 6 h before being returned to their cages. Further doses of Buprenorphine (0.1 mg/kg) were administered s.c. twice daily during the 72 h follow-up.

Plasmid

Plasmid pCik IL-10 was constructed by inserting hIL-10 cDNA (537 bp) into a unique backbone of pCIk driven by human CMV early promoter enhancer at Not 1 and Nhe 1 site (Pierog et al. 2005). The plasmid was purified and produced in the quantity required at (PlasmidFactory Gmbh & Co, Bielefeld Germany).

Electroporation-Mediated Gene Transfer to the Flap

For gene delivery, the plasmid was suspended in endotoxin-free water at a concentration of 1 μg/μl. Total of 100 μg of plasmid (100 μL solution) was injected intradermally (one midline longitudinal injection 1.5 cm away from the edge of the flap) (as described in Fig. 1) using a 25-gauge needle, at this place usually the necrosis and rejection occur predominantly (Basu et al. 2014; Seyed Jafari et al. 2017). After injection of plasmid, in vivo electroporation-mediated gene transfer was performed at the site of plasmid injection (midline, 1.5 cm from the edge of the flap) with a series of eight pulses of 200 V/cm, for 10 ms, using a stainless tweezertrode Electrode, 10 mm diameter. (Pulse generator: BTX Gemini X2 electroporation system, Harvard Apparatus, USA). The distance between the electrodes was 5 mm; however, the flap was held in between the tweezer electrode and gentle constant pressure was applied during the electroporation procedure to hold the flap.

Assessment

Laser Doppler Perfusion Imaging of Skin Flap

The animals were anesthetized as described above by inhalation of 5% isoflurane. The Laser Doppler imaging of the McFarlane flap was performed using commercially available microcirculation-imaging camera (EasyLDI; Aimago SA, Lausanne, Switzerland) on the seventh post-operative day, before the animals were sacrificed. The analysis was subsequently performed using EasyLDI Studio software (Aimago SA, Lausanne, Switzerland) by

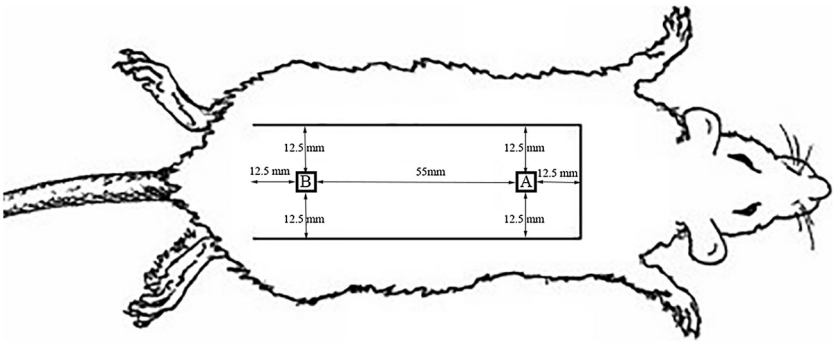

Fig. 1 Tissue collection protocol. After resection of the skin flap with the underlying tissue, transverse segments (5 × 5 mm) were taken 12.5–17.5 mm (proximal part) and 72.5–77.5 mm (distal part) from the cranial margin of the flap. Furthermore a third segment was taken from the margin of necrotic and survived regions (intermediate part)

recording the blood flow from the predefined point on the tip of the flap (midline 1.5 cm to the distal end of the flap), as discussed before (Tschumi et al. 2015). The values were then expressed as percentage of blood flow related to the measurement at the base of the flap (Laser Index).

$$\text{Laser Index } (\%) = \text{Easy LDI value on the tip of the flap (APU)} / \text{reference Easy LDI value (APU)} \times 100$$

Skin Flap Survival Assessment (Planimetry)

Flap survival was evaluated by standardized digital photographs of the dorsum of each rat on postoperative day 7. Distal flap necrosis was quantified by measuring the percentage flap survival. Regions of pink and pliable soft skin with evidence of new hair growth were considered healthy, while regions of thickened, hard, contracted, and dark-colored tissue without new hair growth were considered necrotic for planimetric analyses (Richter et al. 2006). ImageJ Software (NIH, Bethesda, MD, USA) was administered to calculate the percentage flap necrosis after assessing the digital images of the skin flaps (Basu et al. 2014; Shafighi et al. 2012). The results of this analysis were reported on an Excel sheet to calculate the percentage of necrotic area observed on each flap:

$$\text{Percentage of necrosis } (\%) = \text{Area of necrosis} / \text{Area of the whole flap} \times 100$$

Tissue Collection and Histology Assessment

Seven days after surgery, the animals were sacrificed by intraperitoneal injection of 50 mg/kg of pentobarbital. The skin sections were obtained from both necrotic and survived regions (Fig. 1).

Histologic examination was performed on tissue fixed in 10% buffered formalin, routinely processed and subsequently embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E). After general evaluation of H&E stained sections, presence of inflammatory cells in the biopsies was evaluated semi-quantitatively by a trained staff (Score 1–4: slight, mild, moderate, severe) (Myers et al 1961). For all microscopy examinations Nikon Eclipse E600 microscope (Nikon Cooperation, Japan) was used.

Immunohistochemistry and Quantitative Image Analysis

In order to assess immunohistochemical IL-10 protein expression, formalin-fixed tissue sections were deparaffinized in a xylene series and rehydrated through a decreasing ethanol series. Sections were incubated with the anti-IL-10 antibody (Biorbyt Ltd., UK) at 1:400 dilution followed by the second antibody horseradish peroxidase (HRP) conjugate and staining with 3,3-diaminobenzidine (DAB). For IL-10 protein expression comparison between the group's quantitative analysis was performed using IHC Profiler *and* Image J Software (NIH, Bethesda, MD, USA) and DAB optical density (DAB OD Score: 1–4) which is proportional to the amount of IL-10 protein, and was calculated for each slide as reported previously (Seyed Jafari and Hunger 2017).

Furthermore, to evaluate angiogenesis, similar immunohistochemical protocol was used for staining CD31 (1:100; Biorbyt Ltd., UK) as a marker for neovascularization on the endothelial surface of skin vasculature as reported previously (Khan et al. 2004). In each section, a total of 10 different fields in one flap section at 400× magnification were randomly selected, and the vessel number was counted. The vessel density was calculated as number of vessels per square millimeter field (0.55 mm^2

each field), and the mean was reported for each flap (Khan et al. 2004).

Immunofluorescence Staining

In order to assess co-localization, immunofluorescence, staining was done using anti-IL10, anti-VEGF, anti-CD200 (as the specific marker for hair follicle stem cells) antibodies. The slides were pre-treated by microwave in citrate buffer (100 mM, pH 6.0) for 8 min and then washed with TBS before incubation overnight at 4 °C with primary antibodies in different combinations, anti-IL-10 antibody and anti-VEGF164 antibody (R&D Systems, Abingdon, UK); and anti-CD200 antibody (Biorbyt Ltd., UK). The sections were washed and incubated with the corresponding secondary antibody for 2 h at room temperature. The images were taken with Nikon Eclipse 80i microscope (Nikon Cooperation, Japan).

Statistical Analysis

All analyses were conducted using the GraphPad Prism version 6.01 (GraphPad Software, Inc. USA). Descriptive statistics were presented as mean ± SD. All p values relate to two-sided tests with an alpha level of 0.05. In order to assess non-parametric measurement of statistical relation between Laser Index and Flap necrosis percentage, Spearman's rank correlation coefficient was computed across groups. Additionally, Mann–Whitney test was applied to detect differences between groups.

Result

Effect of In Vivo Electroporation-Mediated IL-10 Gene Transfer on Flap Necrosis

Seven days after surgical procedure, flaps were evaluated clinically. Skin ischemic necrosis was observed only in the distal portion of the skin flaps. Prophylactic electroporation-mediated IL10 gene delivery significantly reduced flap necrosis percentage compared to the control group (Flap necrosis percentage: 18.74 ± 4.70 vs. 35.23 ± 3.90; respectively) (p value = 0.0159). (Figs. 2, 3).

Effect of In Vivo Electroporation-Mediated IL-10 Gene Transfer on Skin Flap Perfusion

Spearman's rank correlation showed a strong significant negative correlation between Laser Index and Flap necrosis percentage (p value = 0.0083, r −0.83, respectively). IL10 gene transfer led to a significant increase in skin perfusion compared to the control group (Laser index: 61.72 ± 13.63 vs. 33.96 ± 10.92, respectively); (p value = 0.0159). (Figs. 3, 4).

Histological Evaluation of Skin Sections

Proximal sections revealed a regularly stratified epithelium with ordinary developed hair follicles. Distal sections were similar in the control and experimental groups and showed an inflammation with monocytes and neutrophils. However, the semi-quantitative assessment of the stained tissues revealed a significantly higher quantity of inflammatory cells in the control group compared to experimental group (Inflammatory cells score: 1.80 ± 0.18 vs. 1.25 ± 0.32; p value = 0.0317, respectively). Additionally, a significantly higher mean CD 31^+ vessel density was found in the experimental group than in the control group. (Mean CD 31^+ vessel density (/mm^2): 3.43 ± 0.41 vs. 1.73 ± 0.62; p value = 0.0159, respectively). (Figs. 3, 5).

Figure 6 shows the expression of IL-10 in experimental and control group. In addition, IL-10/VEGF double immunofluorescence staining showed co-localization of IL-10 and VEGF protein expression (Fig. 7). In IL-10/CD200 double immunofluorescence staining showed some IL-10^+/CD200 $^+$ cells around hair follicles. (Fig. 8).

Discussion

In the current study we show in vivo electroporation-mediated IL10 gene transfer as a novel approach for management of ischemic skin flap. Improved neovascularization and reduced necrosis and inflammation were observed when IL-10 gene transfer was performed to the flap bed of recipient 24 h before raising the flap.

Even in very small amount the growth factors and cytokines can affect wound healing (Waller et al. 2004). IL-10, is a potent anti-inflammatory cytokine produced by multiple immune cells early in wound healing (Balaji et al. 2015; Moore et al. 1993). IL-10 affects both inflammatory and proliferative phases of wound healing, like regulatory effects in inflammatory response, extracellular matrix formation, and fibroblast function (Balaji et al. 2015; Kieran et al. 2014; Krishnamurthy et al. 2011). However, in order to achieve its effects repeated or continuous administration is warranted (Tavakoli et al. 2006). To overcome this limitation, various gene delivery strategies have been applied using either viral or chemical vectors, each method however has shortcoming and adverse effects, ranging from inflammatory response, hepatotoxicity, insertional mutagenesis, to host immune system activation (Basu et al. 2014; Byrnes et al. 2001; Gazdhar et al. 2007; von Degenfeld et al. 2006). To avoid

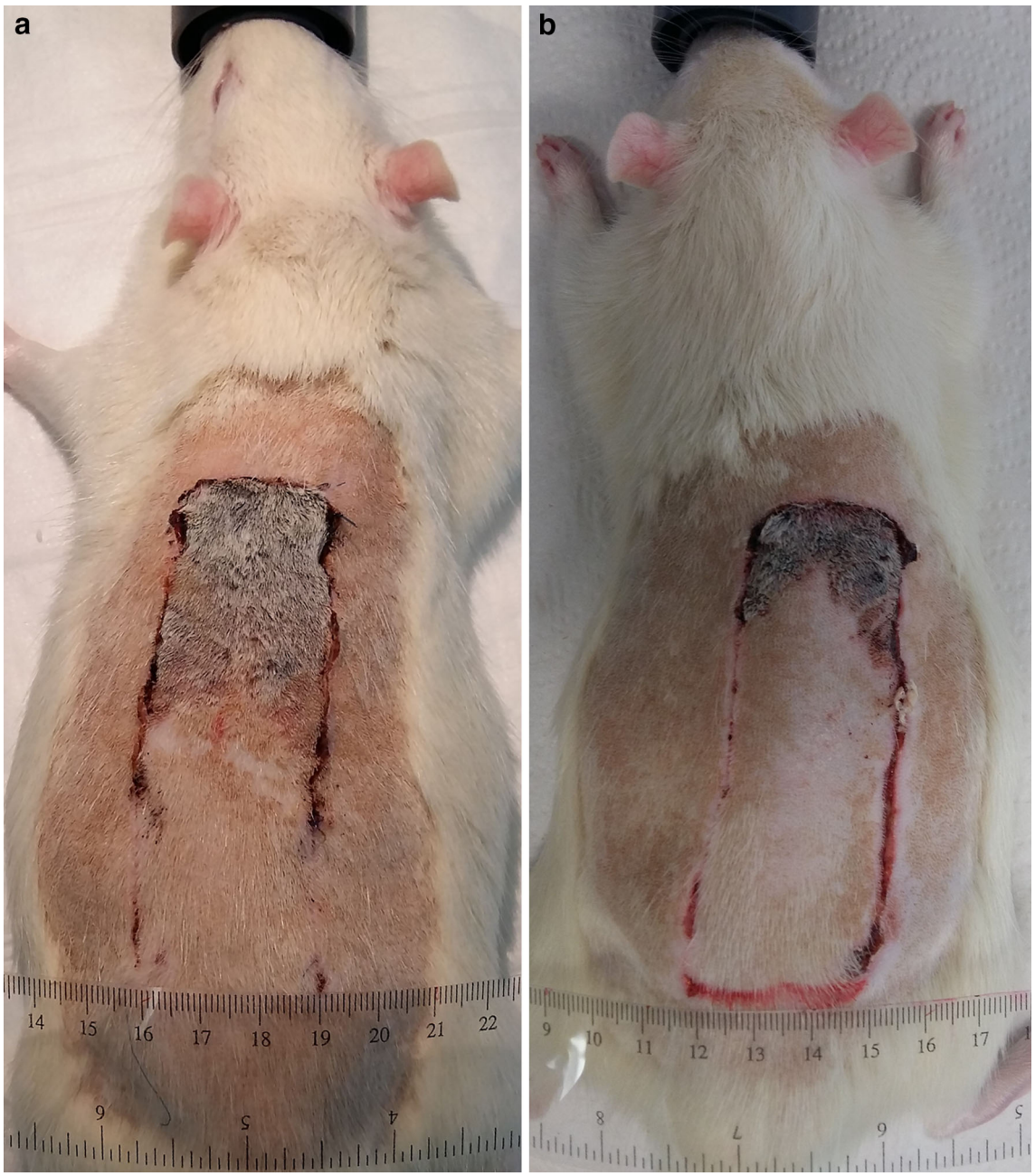

Fig. 2 Comparison of tissue necrosis between control group (**a**), and experimental group (**b**)

these drawbacks and to enhance the efficiency of IL-10 gene transfer we applied the physical method of in vivo electroporation.

In vivo electroporation-mediated gene transfer is a reproducible and efficient gene delivery method and has been successfully applied in various organs in experimental settings (Basu et al. 2014; Gazdhar et al. 2007; Gehl 2003; Murakami and Sunada 2011). In the current experiment not only significantly lower inflammation and better vascularization were observed in IL-10 gene transfer group but also necrosis was decreased. These findings are related to the fact that overexpression of IL-10 recapitulates fetal-like regenerative and scarless wound healing phenotype in postnatal tissue (King et al. 2014). Various effects of IL-10 like attenuation of the inflammatory response, regulation of the extracellular matrix, induction of postnatal hyaluronan production, optimization of fibroblast function and differentiation, increase in endothelial progenitor cells (EPCs), (Balaji et al. 2014; King et al. 2014; King et al. 2013; Krishnamurthy et al. 2011; Peranteau et al. 2008) further justify the anti-necrotic and anti-fibrotic properties of IL-10 (Fiorentino et al. 1989; Kieran et al. 2014).

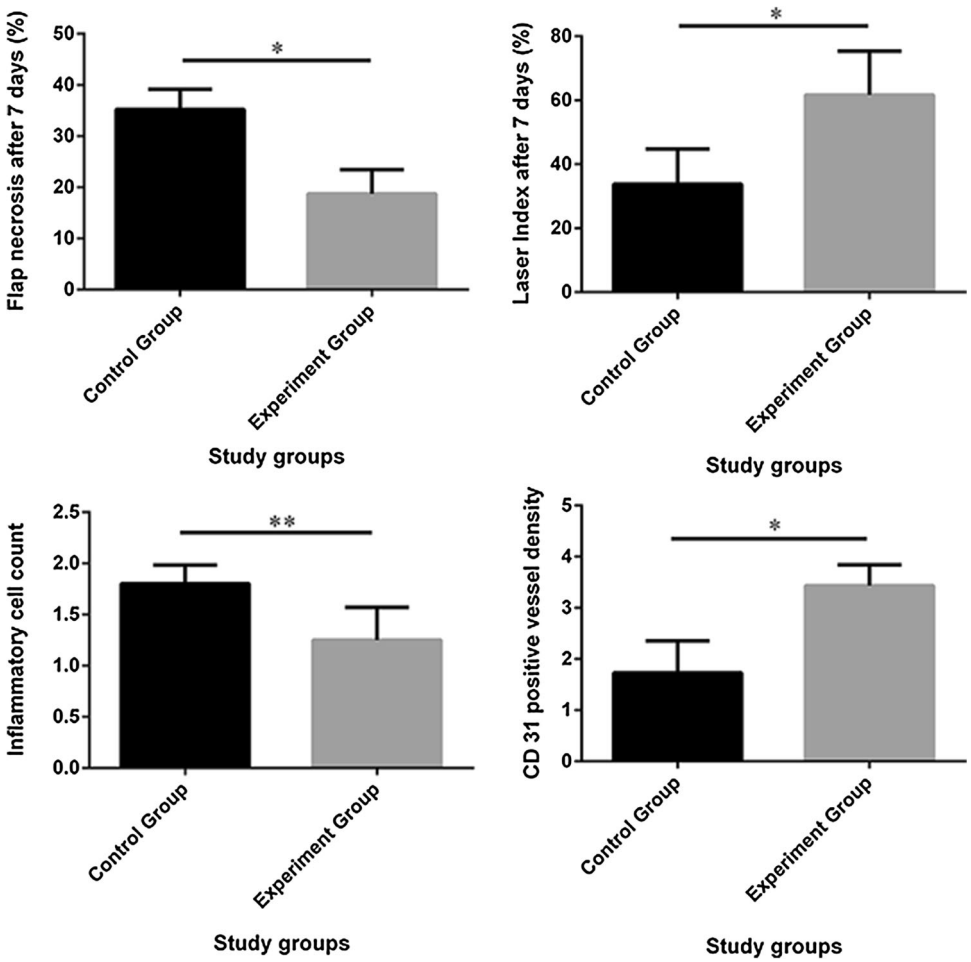

Fig. 3 Comparison of tissue necrosis between control and experimental group showed a significant decrease in flap necrosis percentage. Additionally, skin flap perfusion evaluation between control and experimental group showed a significant increase in Laser Index. In semi-quantitative image analysis statistically significant lower inflammatory cell count was detected in experimental group than that of control group. Furthermore, a significantly higher mean CD 31^{+} vessel density was detected in the experimental group compared to the control group (*p value = 0.0159, **p value = 0.0317)

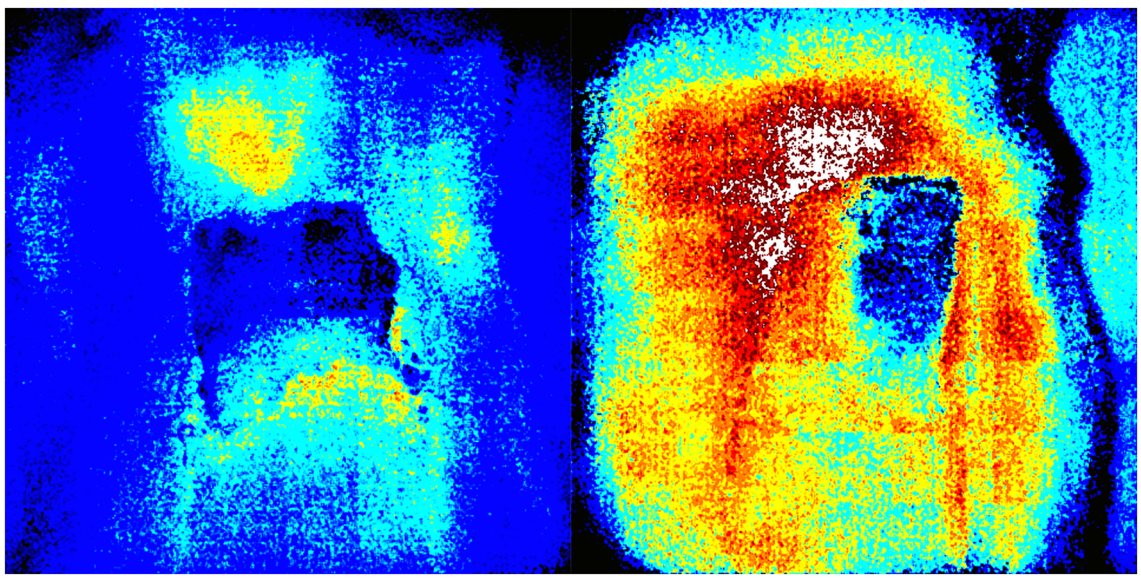

Fig. 4 Comparison of skin flap perfusion between control group (*left*) and experimental group (*right*). The *blue area* in the skin flap shows the area with critical skin perfusion. However, it should be noted that LDI assessment is a relative measurement and flap perfusion in each animal should be compared with the healthy skin perfusion in the same animal (Color figure online)

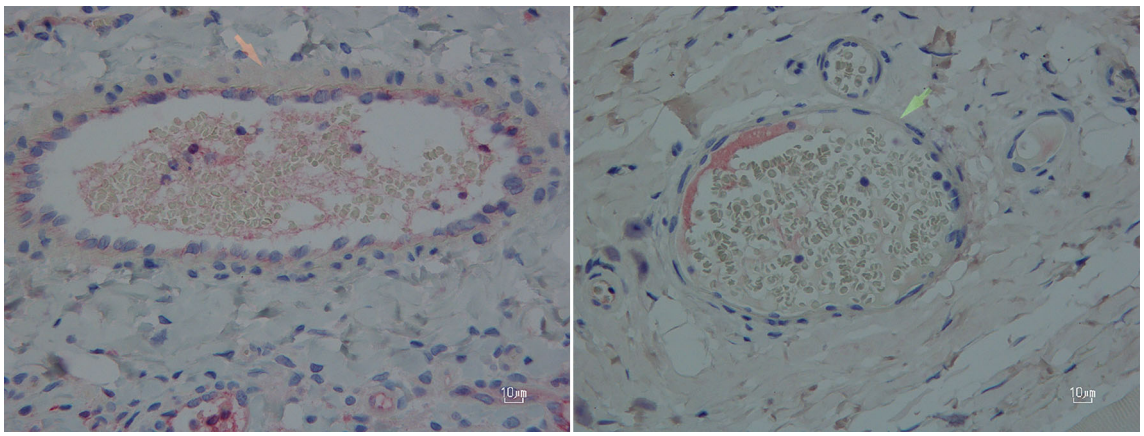

Fig. 5 CD 31^{+} vessels in the experimental group Streptavidin/alkaline phosphatase staining; 400× magnification. The *arrow* in the *left image* points to a vessel stained for endothelial cells with anti-CD31 (PECAM-1) antibody. The *arrow* in the *right image* points to a non-stained vessel (control)

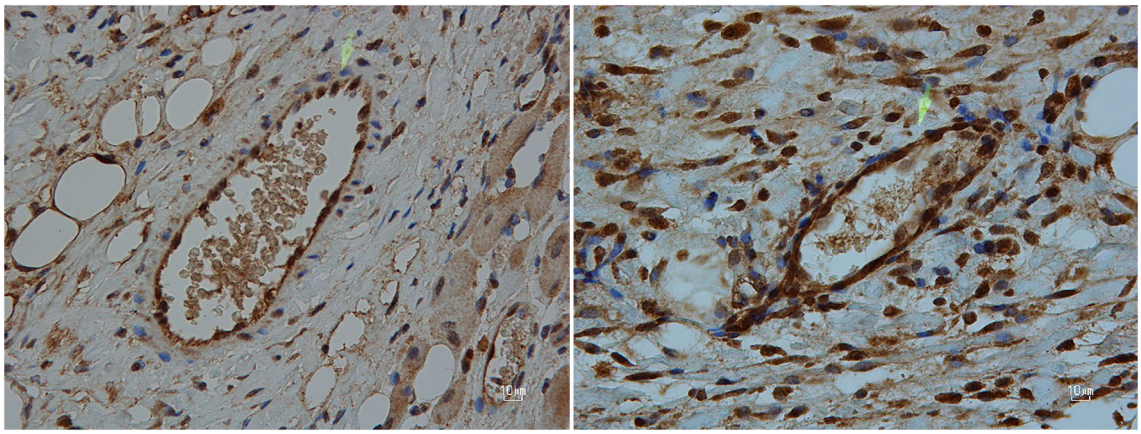

Fig. 6 Image analysis and IL-10 expression comparison by Image J, plus IHC Profiler plugin, 400× magnification. DAB optical density score was calculated for the *left* (control group) and the *right* (experimental group) images as 1.78 and 2.21, respectively, based on IHC Profiler percentage contributions. The *yellow arrows* point to the vessels in the images (Color figure online)

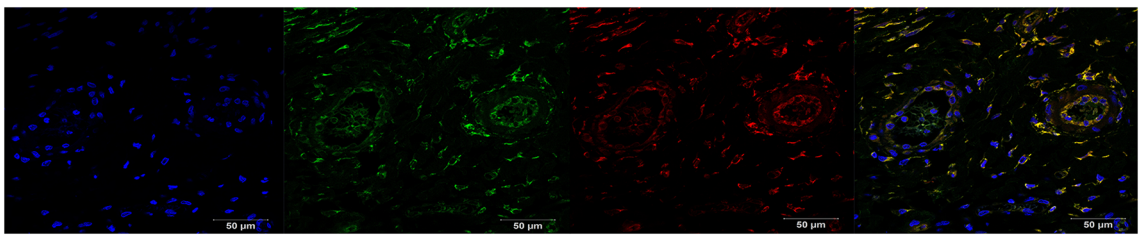

Fig. 7 IL-10/VEGF double staining anti-IL-10 antibody (*red*) and anti-VEGF164 antibody (*green*); DAPI (*blue*); 400× magnification. Primary Ab: anti-IL10 antibody and anti-VEGF164 antibody; Secondary Ab: Alexa anti-Gout 546, Alexa anti-Mouse 488 (Color figure online)

Studies using viral-mediated IL-10 overexpression showed that sustained release of IL-10 in the dermis would create an environment conducive for regenerative wound healing by decreasing the inflammatory response to injury (Gordon et al. 2008; Peranteau et al. 2008). Likewise, the semi-quantitative evaluation of the skin flaps in our study demonstrated significantly low inflammatory cells in the IL-10 group compared to the control group. Similarly, two preclinical and phase II randomized control studies (Kieran et al. 2013) report significant reduction in inflammatory cells after repeated injection of rhIL-10. This immunomodulatory and anti-inflammatory properties of IL-10 have been demonstrated to induce tolerance and prolong the graft survival significantly (Moore et al. 2001; Pierog et al. 2005; Sembeil et al. 2004). Similar beneficial effect has been recently reported to improve survival of

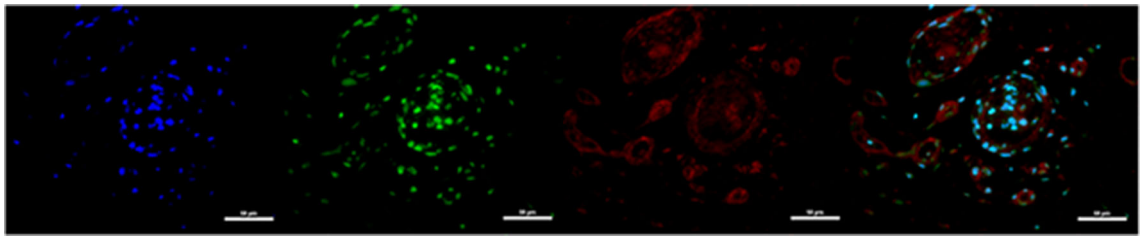

Fig. 8 IL-10/CD200 double staining anti-IL-10 antibody (*green*) and anti-CD 200 antibody (*red*); DAPI (*Blue*); 400× magnification. Primary Ab: anti-IL10 antibody and anti-CD 200 antibody; Secondary Ab: Alexa anti-Gout 488, Alexa anti-Rabbit 594 (Color figure online)

lung and heart allografts using electroporation-mediated hIL-10 overexpression (Pierog et al. 2005; Tavakoli et al. 2006).

Furthermore, IL-10 enhances VEGF expression and improves neovascularization of the wounds. Co-localization of IL-10 and VEGF protein expression as observed by fluorescence microscopy supports the proposed effect. Moreover, Balaji S. et al. (2014) demonstrated significantly increased VEGF expression mediated by IL-10, which leads to an increase in vessel density as observed, moreover they also demonstrated that IL- 10 also significantly increased VEGF expression by dermal fibroblasts. However, previous study by Krishnamurthy et al. (2011) showed overexpression of IL-10 results in increased EPCs in circulation and in the wound after injury, leading to increased VEGF expression by EPCs. Similarly, the significant increase in $CD31^+$ vessels in our study also shows the direct effect of electroporation-mediated IL-10 gene transfer on endothelial cells to induce angiogenesis.

In histological sections of IL-10-treated group, new vessels were detected to be centered around hair shafts. Additionally, we also detected some unique $IL\text{-}10^+/CD200^+$ double positive cells around hair follicles; this is an important finding, since the bulge of the follicle has been shown to contain pluripotent progenitor cells of different developmental origin with a fundamental role in the acute wound healing response (Ito et al. 2005; Lako et al. 2002; Tiede et al. 2007). It has also been stated that in response to skin damage and wounding, bulge cells mobilize, leave their stem cell niche, and contribute to repopulation of the epidermis (Ito et al. 2005; Lako et al. 2002; Tiede et al. 2007). These findings could also be due to modulatory effect of IL-10 overexpression on stem cell function (King et al. 2014). We therefore, speculate that the progenitor cells are transduced with IL-10 after in vivo electroporation-mediated gene transfer; thus enhancing their efficiency.

The limitation of the current study is that we have not evaluated the long-term effect of IL-10 gene transfer. However, the information obtained from this study provides insight into the electroporation-mediated IL-10 gene therapy as a simple, safe, and economical local gene delivery strategy to counter flap necrosis. It is a promising anti-inflammatory, anti-fibrotic, and anti-necrotic therapeutic alternative (Kieran et al. 2014) to improve skin flap survival. The beneficial effects are governed by enhancement of angiogenesis, improved wound healing, and induction of tolerance. The approach to have a constitutively stable cytokines at therapeutic levels offers a promising alternative for reconstructive surgeons and dermatologists to overcome various complications; (Vogel 2000; Waller et al. 2004) the epidermis is an important target organ as it is accessible and can be easily evaluated for the expression of an induced gene (Hengge et al. 1995; Vogel 2000). However, future studies are required to assess this method on larger animal model to test long-term safety before clinical translation of this promising system.

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Compliance with Ethical Standards

Conflict of interests The authors declare that they do not have conflict of interests.

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