Secondary Burn Progression Decreased by Erythropoietin

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Objective: To investigate whether systemic erythropoietin administration can prevent secondary burn progression in an experimental model and to elucidate the underlying mechanisms.

Design: Prospective study.

Setting: University-based laboratory research.

Subjects: Twenty-one male Wistar rats.

Interventions: The burn comb model creates four rectangular burned surfaces that are intercalated by three unburned zones (interspaces) prone to secondary necrosis. Twenty-one animals were randomized to three experimental groups: 1) Local cooling with water for 20 min (control, 17°C); 2) and 3) local cooling with water and intraperitoneal erythropoietin once a day for five days starting 45 min after burn injury (600 IU/kg body weight: EPO500 or 2500 IU/kg body weight: EPO2500).

Measurements and Main Results: Secondary burn progression—both in depth (histology) and in surface (planimetry)—as well as inter space perfusion (laser Doppler flowmetry) and hematocrit were analyzed. Further, dilatary response (inducible nitric oxide synthase expression), inflammation (leukocyte count), and angiogenesis (CD31 expression) were assessed. Finally, wound healing time and contracture rate were reported. Burn progression resulted in complete dermal destruction as well as in important interspace necrosis in control animals, whereas burn progression was significantly reduced in a dose-dependent manner in animals treated with erythropoietin. Tissue protection was associated with an increased interspace perfusion with EPO500, but not with EPO2500, and was paralleled by a significant increase in inducible nitric oxide synthase expression and decreased inflammation, independent of the erythropoietin dosage. EPO2500 led to a significant increase of hematocrit at day 4. Finally, faster wound healing and less contracture were observed in animals treated with EPO500 only.

Conclusions: Erythropoietin represents an easy-to-use therapeutic approach to prevent secondary burn progression, i.e., to control damage after burn injury. It preserves microcirculatory perfusion within the endangered areas in a dose-dependent manner. (Crit Care Med 2013; 41:0:0)

Key Words: inflammation; ischemia; microcirculation; neoangiogenesis; resuscitation; thermal injury; vasodilation

Advances in intensive care medicine over the last two decades led to increased survival rate of patients suffering from large surface area burn injuries. Yet, morbidity, return to preburn activity level and quality of life mainly depend on the burn depth, which again determines the final quality of the skin, in both large and small surface area burn victims (1, 2). The local care of acute burn injuries can basically be divided into three critical phases: (I) first aid in the emergency setting, (II) prevention of burn progression, and (III) support in wound healing, either with conservative or surgical measures. Currently, clear recommendations exist for first aid. Multiple options and measures are available to support wound healing. However no strategy addresses the prevention of burn progression to control damage.

The secondary deepening of the burn wound after the initial insult has ceased can significantly alter the therapeutic strategy and influence the clinical outcome. A superficial second-degree burn injury healing spontaneously without major stigmata can convert to a deep lesion requiring surgical treatment with inferior outcome, risk for scar contracture and recurrent ulcerations, as well as additional donor site morbidity. This dynamic process of
burn progression therefore has a major impact on the final patient outcome in terms of morbidity and quality of life (1, 2).

Irreversible skin necrosis develops depending on the temperature and duration of the burn. However, the border between coagulated necrotic and surrounding healthy tissue is not clearly defined, and a transition zone, which may not survive if kept untreated, is intercalated. This so-called zone of stasis is characterized by ischemia, inflammation, and coagulation (3). Restoration of adequate perfusion and attenuation of inflammatory reactions may salvage this zone and decrease morbidity associated with burn injury.

Erythropoietin has been attributed nonhematopoietic effects that interfere with ischemia, including a nitric oxide-mediated relaxation of arterial vessels (4), induction of angiogenesis (5), prevention of apoptotic cell death and inflammation (6), as well as accelerated tissue regeneration (7). While the influence of erythropoietin on burn progression is not known, these effects were found protective against ischemia (8) and ischemia-reperfusion injury in a variety of organ systems (9–11), including the skin (11, 12). Thus, we hypothesized that erythropoietin, by virtue of its nonhematopoietic properties, prevents burn progression in the rat comb burn model.

MATERIAL AND METHODS

Animals
All experiments were carried out in accordance with Swiss guidelines for animal experimentation and following approval by the Geneva Cantonal Veterinary authority.

A total of 21 male Wistar rats weighing 455 ± 23 g (Charles River Laboratories, L’Arbresle Cedex, France) were housed in single cages at a room temperature of 22–24°C and at a relative humidity of 60–65% with a 12-hrs day–night cycle. Chow and water were available ad libitum.

Anesthesia and Analgesia
The rats were anesthetized for preparation, and all measurements with an inhalational anesthesia with a 2% isoflurane–air mixture. Animals received subcutaneous buprenorphine at a dose of 0.05 mg/kg body weight (bw), 15 min before burn induction, and thereafter, every 12 hrs for the first three days.

Animal Preparation
The animals were prepared 24 hrs prior to the experiments, including hair removal of the dorsal skin (shaving and chemical depilation with Veet®, Reckitt Benckiser, Dansom Lane Hull, UK). The areas to be burned were outlined with a marking pen.

Burn Induction
The comb burn model was used to create the burn wounds (13). A chromium–nickel steel (V2A) template (Ornaplast Kunststofftechnik, Dagmersellen, Switzerland) weighing 136 g was immersed in boiling water for 15 min until equilibration of the temperature. With the animal in prone position, the template was then applied to the previously prepared area on the back, perpendicular to the skin surface and parallel to the spine at a distance of approximately 5 mm on both sides. No pressure was applied while inducing the burn, and the template was removed after 60 s. Application of the template resulted in four 20 × 10 mm burn areas separated by three 20 × 5 mm unburned interspaces as defined by the notches of the template. No dressing or topical treatment was applied except for local cooling.

Experimental Groups and Protocol
Animal preparation and baseline measurements were performed 24 hrs after burn induction. Animals were randomly assigned to three experimental groups of seven rats each: i) control group treated with local cooling alone for 20 min (control: LCW); ii) two treatment groups, receiving cooling for 20 min followed by intraperitoneal (i.p.) administration of erythropoietin 45 min after burn and once a day for five days at a dose of 500 IU/kg bw (EPO500) or 2500 IU/kg bw (EPO2500).

Cooling was performed immediately after burn induction with cold water (17°C) soaked gauzes (10 × 10 cm) applied directly to the burn area. They were changed every minute for 20 min.

After burn induction, the parameters were assessed after one hour, 24 hrs, four days, and seven days. At the end of every measurement except the baseline measurement, one animal was sacrificed in order to harvest skin for histological purposes, i.e., there were no repeat biopsies in the same animal. Analysis of necrosis (depth and surface) and perfusion are based on 4 and 5 timepoints, respectively (baseline, 1 hour, day 1, day 4 and 7). This results in four animals per group at the end of the experiments at day 7. Skin for baseline analyses was harvested on separate animals (histologic data not shown in the manuscript).

Time to complete wound healing and contracture rate were assessed once a week. The timepoint of complete wound healing was defined as the moment when there was no more crust and re-epithelialization was complete. Since hair regrowth can mechanically hinder the crust from dropping off, the duration of complete wound healing as indicated in the results might be longer than the actual interval of complete re-epithelialization, particularly in superficial or intermediate thickness burns with intact or partial hair growth and consequently adherence of the crust. Thereafter, the animals were sacrificed with an intraperitoneal overdose of pentobarbital under anesthesia.

Test Substance
Epoetin (recombinant human erythropoietin, Neo-Recormon®; Roche Pharma, Basel, Switzerland) was admixed with 1 mL of NaCl 0.9% to achieve final concentrations of 500 and 2500 IU/kg bw. The solution was stored at a maximum temperature of 8°C until usage. Erythropoietin has been administered using the i.p. route because i) subcutaneous (s.c.) administration has shown to last nearly twice as long to reach peak serum concentrations (14), ii) higher average doses of i.p. administration were not able to significantly alter hemoglobin level in comparison with s.c. administration (15), and iii) i.p. administration is technically easier to perform, especially if repeated applications are needed.

Histology and Immunohistochemistry
The entire area, including the directly burned tissue and the interspaces, was harvested en bloc at 1 hr as well as at one, four,
and seven days after burn on both sides. This tissue was immediately fixed in 10% formalin, stored in 70% alcohol for 24 hrs, and embedded in paraffin. Thereafter 4-μm sections were obtained for hematoxylin and eosin (H&E) and immunohistochemical staining.

**Inducible Nitric Oxide Synthase Immunostaining.** Antigen retrieval was performed for 30 s in citrate buffer at pH 6. Peroxidase blocking was carried out using Dako real peroxidase blocking solution (Dako Schweiz AG, Baar, Switzerland) for 10 min. This was followed by incubation for 60 min with primary antibody (1:800 rabbit polyclonal anti-rat/anti-mouse iNOS antibody, BD Biosciences, Heidelberg, Germany) and for 30 min with the secondary antibody (rabbit EnVision HRP antibody, Dako). The peroxidase activity was detected by incubation with AEC (Biogenex, Basel, Switzerland) for 10 min. Slides were counterstained with Mayer's hemalum and mounted in Aquatex (Merck, Geneva, Switzerland).

**CD31 Immunostaining.** Antigen retrieval was performed for 30 s in EDTA buffer at pH 7 within a pressurized heating chamber (Dako, Glostrup, Denmark). This was followed by incubation for 60 min with primary antibody (1:100 goat polyclonal antiserum CD31 PECA-1 antibody (M-20, Dako), and for 30 min with secondary antibody (1:150 rabbit antigoat biotin 1:150, Dako). CD31-stained sections were incubated for 5 min with Dako real peroxidase blocking solution and for 15 min with streptavidin peroxidase (Dako). The peroxidase activity was detected with diaminobenzidine for 10 min. Slides were counterstained with Mayer's hemalum and mounted in Ultrakitt (J.T. Baker, Deventer, The Netherlands).

All sections were recorded with a Zeiss® Axiophot microscope (Carl Zeiss®, Jena, Germany). To assess burn depth, a pathologist blinded to the treatment group observed H&E-stained sections (n = 6 per group) and rated burn depth based on a validated scale from 1 to 5, i.e., ranging from epidermis (1), superficial dermis (2), intermediate dermis (3), deep dermis (4), to muscle (5, 16). To assess inflammation, dilatatory response and angiogenesis, three visual fields per H&E, inducible nitric oxide synthase (iNOS), and CD31-stained sections were randomly selected, recorded with a charged coupled device camera (Axiocam, Carl Zeiss®, Jena, Germany) using Axiovision software (Carl Zeiss®, Jena, Germany). Thereafter, leukocytes as well as iNOS- and CD31-positive vessels were quantified and are given per mm².

**Observation and Planimetry**

Daily observations for the first week, followed by weekly observation until total healing, were recorded using a digital camera (Panasonic DMC-TZ1, John Lay Electronics AG, Lucerne, Switzerland). Photographs were performed in a standardized manner with the camera fixed on a tripod to guarantee subsequent planimetric analysis. A computer-assisted image analysis system (Cap Image®, Zeintl Software; Heidelberg, Germany) was used to determine the total amount of interspace necrosis (per cent of the interspace surface) and the contracture rate. The latter was determined as the decrease of the total surface area delimited by the corner points applied with ink before skin burn administration. Contracture rate was given in per cent.
Because of the high intersite variability, the data are given in percentage of baseline (17, 18).

**Blood Samples**
Samples of 250 µL of blood were withdrawn from the tail vein and analyzed for hematocrit using an analyzer designed for rat blood (OSM3; Radiometer, Copenhagen, Denmark).

**Statistical Analysis**
All values are expressed as mean ± standard error of the mean (±SEM). To control for inflation of type I error probability in the course of multiple data analysis, hierarchical modeling and hypothesis testing were performed. Linear mixed regression models comprising the main effect terms time and treatment group and the interaction term by treatment group were used in order to perform a two-way analysis of variance (ANOVA). If the global test of the main effect treatment group was significant at a level of 0.05, one-way ANOVA group comparison was done for each time point (followed by the appropriate post hoc test, which considered α-error correction according to the Bonferroni method). In case of right skewed data distribution, log transformation was applied to normalize data before applying the respective ANOVA model, and back transformed marginal means and confidence intervals were provided as effect measures. To adjust for baseline group differences in the statistical analysis, ANOVA models were extended with the respective baseline covariate.

The nonparametric Kruskal–Wallis test followed by Bonferroni-adjusted pairwise comparisons via the Mann–Whitney U test assessed group differences in regard to ordinal data at a single time-point. Statistical testing has been performed with SPSS (Statistical Package for the Social Sciences: IBM Corporation, Armonk, NY).

**RESULTS**

**Burn Depth**
Histologic evaluation of burn depth 1 hr after burn induction showed consistent and uniform damage to the epidermis and superficial dermis in all animals (2.0 ± 0.0) (Fig. 1). Burn progression resulted in complete dermal destruction in control animals and was significantly reduced with EPO500 to an intermediate level at which substantial regeneration with minimal morbidity can be expected (5 ± 0 for control vs. 3.3 ± 0.6 for EPO500 (p = 0.039) and 4.2 ± 0.3 for EPO2500 (p = 0.0203).

**Interspace Necrosis**
Interspace necrosis mainly occurred during the first four days in all animals (Fig. 2). Compared to controls, EPO500 and EPO2500 decreased the surface extension of necrosis. During the whole observation period, EPO500 resulted in significantly less necrosis than EPO2500 (38% ± 7% vs. 68% ± 4%, p = 0.029) and controls (81% ± 4%, p = 0.002).

**Interspace Perfusion**
EPO500 improved perfusion already after one day (86% ± 4% for EPO500 vs. 67% ± 3% for control and 73% ± 4% for EPO2500;
TABLE 1. Quantitative Analysis of Hematocrit (%) Before Burn Induction (Baseline) and Over the 2-week Observation Period in Control Animals, As Well As in Animals Receiving EPO 2500 and EPO 500 (n = 6 per Group)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>d1</th>
<th>d4</th>
<th>d7</th>
<th>w2</th>
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<tr>
<td>Control</td>
<td>38 ± 1</td>
<td>38 ± 1</td>
<td>42 ± 2</td>
<td>40 ± 0</td>
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<tr>
<td>EPO 2500</td>
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<td>39 ± 1</td>
<td>50 ± 1*#</td>
<td>53 ± 1*#</td>
<td>50 ± 1*#</td>
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<td>EPO 500</td>
<td>37 ± 1</td>
<td>37 ± 2</td>
<td>45 ± 2#</td>
<td>49 ± 2#</td>
<td>47 ± 3#</td>
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Values are mean ± SEM.
* p < 0.05 vs. control.
*p < 0.05 vs. baseline.

F3

p < 0.05) (Fig. 3). The observed group heterogeneity was mostly due to a significant difference between EPO500 and controls at day 1 (p = 0.011).

iNOS expression

One day after burn injury, erythropoietin showed significantly increased iNOS expression when compared with controls (23% ± 8% for control vs. 73% ± 14% for EPO500 (p = 0.006) and 66% ± 8% for EPO2500 (p = 0.066)) (Fig. 4).

Hematocrit

Erythropoietin increased hematocrit from day 4 when compared with baseline, in a dose-dependent manner. However, compared with control animals, similar values were observed at day 1 (Table 1). Significant differences between EPO500 and controls were observed for days 7 and 14 (p < 0.001 and p = 0.005), whereas comparison between EPO2500 and controls revealed statistical significance at days 4, 7, and 14 (p = 0.014, p < 0.001 and p < 0.001).

Leukocyte Count

The inflammatory response represented by leukocyte extravasation into the interstitial space of the critically perfused transition zone was reduced following administration of erythropoietin (613 ± 82 cells/mm² for control vs. 400 ± 32 cells/mm² for EPO500 (p = 0.090) and 366 ± 50 cells/mm² for EPO2500 (p = 0.022) (Fig. 5). The maximal leukocyte count in erythropoietin-treated animals was reduced by approximately 30–40% during the initial four days when compared with control animals.

Angiogenesis

CD31 protein expression remained stable in control animals, whereas administration of erythropoietin induced an angiogenic response in a dose-dependent manner with a certain delay in the EPO500 group when compared with the EPO2500 group (50 ± 10 cells/mm² for control vs. 92 ± 10 cells/mm² for EPO500 and 106 ± 9 cells/mm² for EPO2500). The differences between the erythropoietin groups and controls were statistically significant at day 7 (global test for group heterogeneity: p = 0.002) (Fig. 6).

Healing Time and Wound Contracture

Burn wounds of animals treated with EPO500 were completely healed after a mean time of 7 ± 1 weeks, which was significantly faster than the healing time observed in control animals (p = 0.027 vs. control). Animals receiving EPO2500 took 10 ± 1 weeks to heal. Burn wounds treated with EPO500 but not with EPO2500 healed with significantly less contracture than controls (34% ± 2% for control vs. 23% ± 4%, p = 0.031 for EPO500 and 30% ± 3% for EPO2500 p = 0.800) (Table 2).

DISCUSSION

This study shows that erythropoietin treatment significantly decreases—in a dose-dependent manner—secondary burn progression, which halts at an intermediate level with preservation of skin appendages necessary for spontaneous healing in contrast to control animals that develop complete destruction of the dermis and part of the panniculus carnosus. This was primarily the result of a significant improvement of the microcirculation, which was most likely independent of an erythropoietin-induced anti-inflammatory and angiogenic effect.

EPO500 prevented the decrease of interspace perfusion of about 40% observed in untreated control animals and promoted rapid re-establishment of perfusion after burn injury. In contrast, EPO2500 did not significantly improve perfusion when compared with control animals. It has been shown that a single dose of 400 IU erythropoietin per kilogram of body weight (bw) rapidly normalized perfusion in skeletal muscle of septic mice in contrast to higher dose erythropoietin administration (19). Harder et al demonstrated that erythropoietin reduced necrosis

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in musculocutaneous tissue exposed to acute persistent ischemia by maintaining perfusion within the critical transition zone in a dose-dependent manner (20). The inferior outcome of the high-dose regimen was partly the result of a significant reduction of red blood cell velocity and an increased hematocrit resulting in altered blood rheology. The dose-dependent outcome in the present study is most probably not due to rheologic alterations secondary to the hematopoietic effect of erythropoietin, because the differences in blood flow were already present within the first hours after burn, i.e., before any increase in hematocrit.

Erythropoietin up-regulated the expression of iNOS in both groups to a similar extent. The beneficial, vasodilatory effect of erythropoietin by an increase in nitric oxide bioavailability, especially on the cerebral circulation has previously been reported.
(21). While nitric oxide is well known for its vasodilatory effect (22), high levels can also inversely induce vascular collapse and injury (23).

The ischemia-induced inflammatory response has been reduced by erythropoietin, independently of the administered dose. Contaldo et al demonstrated that a single dose of 5000 IU erythropoietin per kilogram of body weight effectively attenuated ischemia-reperfusion injury by maintaining capillary perfusion via a reduced leukocyte-endothelium interaction (11). Also, improved tissue survival by preventing neutrophil infiltration and plugging of the microvasculature of reperfused flaps was observed (24). In the present study, the reduction of the inflammatory response to thermal and ischemic injury does not seem to play a major role, since the significant reduction in leukocyte extravasation within the critically perfused tissue did not result in substantial decrease in burn progression in the higher dose erythropoietin group, and the differences observed do not clarify why interspace perfusion was significantly improved in the lower dose group. Likewise, we have demonstrated that an erythropoietin-driven anti-inflammatory and antiapoptotic effect was only effective, if the administration happened before the induction of ischemia (12).

Signs of neoangiogenesis were observed in a dose-dependent manner from day 4, a timepoint at which necrosis was already demarcated. Consequently, erythropoietin-induced neoangiogenesis does not appear to contribute to the prevention of secondary burn progression in this model. This is in line with a previous study where the first new functional vessels were detected five days after induction of ischemia in a musculocutaneous flap pretreated with erythropoietin (25). Of interest, co-administration of a monoclonal anti-VEGF antibody and erythropoietin completely blocked the erythropoietin-induced angiogenic response without affecting the pro-survival effect of erythropoietin on ischemic tissue. This is in contrast to studies that have shown that neoangiogenesis with high-dose and/or prolonged systemic administration of erythropoietin during at least seven days after onset of the ischemic injury was associated with improvement in ischemia-reperfusion injury (11), wound healing (26), and flap survival (27).

The improved microcirculatory perfusion in the critical zone of ischemia—that is at least partly independent from iNOS expression and anti-inflammatory properties of erythropoietin—did prevent burn progression both in surface and depth. This tissue protection resulted in accelerated wound healing and reduced wound contracture in the lower-dose erythropoietin group. Previously, Galeano et al have demonstrated that erythropoietin increased epithelial proliferation as well as maturation of the extracellular matrix and angiogenesis of experimental burn injuries (28). Presently, the angiogenic response that occurs in a dose-dependent manner from day 4 on—as represented by the deferred increase of CD31 expression in EPO500 when compared with EPO2500—does not seem to be directly responsible for accelerated wound healing. In fact, we postulate that improved wound healing and decreased contracture rate after erythropoietin treatment directly correlates with the total extension of the burn damage, both in depth and surface.

The rat comb burn model consistently created superficial second-degree burn lesions evolving to full-thickness burns and fusion of the healthy interspaces, a process prevented by erythropoietin. However, the anatomy of rodent skin differs fundamentally from human skin and also, it cannot be excluded that different thermal agents may result in different patterns of burn progression. Whereas the ischemic zone surrounding the thermally injured tissue on the surface represents the zone of interest prone to progression (interspaces), the measurement of burn depth gives additional information. Burn deepening is more rapid since the burn injury is relatively severe, and the rat skin in contrast to human skin is relatively thin, nonadherent and contains a panniculus carnosus.

In general, the exact time course of burn progression, an ischemic phenomenon different from the actual burn injury, is unknown and probably depends on the ischemic tolerance of the tissue and particularly on the amount of blood perfusion remaining adjacent to the burned tissue. Also, the interval that permits salvage of this zone of stasis is probably variable. In a previous study using the rat comb burn model, application of EPO500 at 6

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**TABLE 2.** Final healing time (weeks) and contracture rate (%) in control animals as well as in animals receiving EPO 500 and EPO 2500 (n = 3 per group)

<table>
<thead>
<tr>
<th></th>
<th>Final Healing Time</th>
<th>Contracture Rate</th>
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<tbody>
<tr>
<td>Control</td>
<td>10 ± 0</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>EPO 2500</td>
<td>10 ± 1</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>EPO 500</td>
<td>7 ± 1*</td>
<td>23 ± 4*</td>
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</table>

Values are mean ± SEM.

* p < 0.05 vs. control.
In conclusion, an impressive reduction of burn depth extension with preservation of skin appendages that are essential for dermal and epidermal regeneration and limitation of interspace necrosis was obtained with erythropoietin in a dose-dependent manner. This protection seems foremost to be a direct consequence of maintained microcirculatory perfusion. The angiogenic or anti-inflammatory properties of erythropoietin do not appear to play a significant role in the prevention of burn progression by erythropoietin. Accordingly, the results suggest that erythropoietin might constitute an effective therapeutic

hrs after burn induction did not result in prevention of burn progression as did early administration after 45 min, indicating that rapid action is necessary to prevent burn progression (29).

Potential side effects of erythropoietin in large surface area burn patients might preclude its systemic use due to its hematopoietic properties that may increase the risk of thromboembolic events. This may also be valid in patients with comorbidities and risk factors that predispose to such event. These limitations may be shortcircuit in the future by topical drug application or by non-erythropoietic erythropoietin-derived substances.

Figure 6. Scatter plot that displays the distribution of CD31 positive vessels in the treatment groups at day 1, 4, and 7. Significant effects of group (p = 0.001), day (p = 0.002) and significant different group effects within the days (p = 0.029) could be detected (A). At day 7, Bonferroni post hoc comparisons revealed significant differences between EPO2500 and controls (p = 0.002) as well as between EPO600 and controls (p = 0.023). Staining for CD31 at day 7 after burn in a control animal (B), as well as in an animal receiving EPO600 (C) and EPO2500 (D), respectively. Note the increased number of vessels that are represented by brown staining after EPO treatment (arrows).
approach for the future to improve clinical outcomes after thermal injury.

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AUTHOR QUERIES

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AQ1—Please provide the highest academic qualification of the first author.
AQ2—Please expand AEC.
AQ3—Please provide the missing information (specific day) here. Also please note that "A" has been deleted from the legend (as it does not seem to have significance here). Please check.