New treatment strategies to reduce burn wound progression

Neue Behandlungsstrategien zur Verringerung der Brandwundenprogression

Abstract

**Background:** After a burn injury certain superficial partial-thickness burn wounds spontaneously progress into deep partial-thickness or full-thickness burn wounds. This poorly understood phenomenon is called burn wound progression. The aim of this study was to investigate whether treatment strategies using warm water (preservation of microcirculation) on the one side and erythropoietin (EPO) (molecule with anti-inflammatory, anti-apoptotic, vasodilatory and neoangiogenic properties) can prevent, delay and/or reduce secondary burn wound progression in a rat model.

**Methods:** We used a burn comb model in 63 rats, creating eight rectangular contact burns (2x1 cm each) intercalated by unburned zones (2x0.5 cm) prone to burn wound progression. In a first experimental set we treated burn wounds with locally applied warm (37 °C) or cold (17 °C) water for 20 minutes.

In a second experimental set, animals were treated systemically with EPO at two different dosages of 500 and 2,500 IU/kg bodyweight (bw) and initiated at 2 different time-points (45 minutes vs. 6 hours after burn injury).

Evaluation of microcirculatory perfusion, interspace necrosis and burn depth was performed using respectively laser Doppler flowmetry, planimetry and histology. For statistical analysis the two-way ANOVA-test followed by an adequate post-hoc test (Bonferroni) were used.

**Results:** In untreated control animals a conversion from superficial to full-thickness burns was observed within 24 hours. Warm and cold water treatment significantly delayed burn depth progression, nevertheless after 4 days, burn depth was similar in all three groups. Warm water significantly reduced interspace necrosis compared to untreated controls and cold water with a significantly improved perfusion in the warm water group. Surface extension and particularly burn depth progression were significantly decreased by EPO only if administered at a dosage of 500 IU/kg bw and initiated 45 minutes after burn injury. EPO administration was associated with an early hyperperfusion resulting from an increase in inducible nitric oxide synthase (iNOS) and a late angiogenic response indicated by increased microvascular density.

**Conclusion:** First aid treatment with warm water prevents extension of the burn on the surface and delays burn depth progression, thereby creating a “therapeutic window” of approximately 4–6 hours after burn injury. This window might be used for systemic use of EPO which prevents burn progression both at the surface and into the depth if first administered 45 minutes after burn induction at a dosage of 500 IU/kg bw.

**Keywords:** burn, burn wound progression, Epoetin, EPO, water treatment, zone of stasis, Penumbra, Falx lunatica
Zusammenfassung


Methode: Mittels Metallstempel („burn comb model“) wurden 63 Ratten je acht 2x1 cm große Kontaktverbrennungen zugefügt, getrennt durch unversehrte Zwischenräume von 2x0,5 cm. In einem ersten Versuch wurde direkt nach der Verbrennung für 20 Minuten lokal gekühlt (17 °C) oder mit 37 °C warmen Wasser behandelt, mit der Hypothese, dass Wärmeapplikation die Zirkulation verbessert und die Stasezone gerettet werden kann. In einer zweiten Versuchsreihe wurde systemisch mit EPO in zwei verschiedenen Dosierungen (500 vs. 2.500 IE/kg Körpergewicht (KG)) und Verabreichungszeitpunkten (45 Minuten vs. 6 Stunden nach Verbrennungsverletzung) behandelt. Die Messungen der Tiefe der Verbrennung, der Nekrose der Zwischenräume und der mikrozirkulären Perfusion wurden anhand von Histologie, Planimetrie respektive Laser-Doppler-Perfusionsmessung durchgeführt. Die statistische Analyse erfolgte mit dem 2-Weg ANOVA-Test gefolgt vom geeigneten Post-hoc-Test (Bonferroni).


Schlussfolgerung: Eine Notfallbehandlung mit warmem Wasser verhindert die oberflächliche Ausbreitung der Brandwunde und verzögert die Progression in die Tiefe. Dadurch wird ein „therapeutisches Fenster“ von ca. 4–6 Stunden eröffnet. Dieses kann für die systemische Administration von EPO genutzt werden, welches die Brandwundenprogression an der Oberfläche und in die Tiefe verhindert, sofern es nach 45 Minuten in einer Dosierung von 500 IE/kg KG verabreicht wird.

Schlüsselwörter: Verbrennung, Brandwundenprogression, Epoetin, EPO, Wasser-Behandlung, Stasezone, Penumbra, Falx lunatica

Introduction

Within the first 48 to 72 hours after thermal injury, burns may progress both into surface and depth [1]. This phenomenon is called burn wound progression and has been described by Jackson in 1953. He characterized this thermally injured area in three distinct zones [2]: A central core zone with irreversibly damaged tissue developing necrosis due to direct thermal injury, which is surrounded by a zone of stasis that is characterized by stagnant microcirculation and consequently hypoperfused tissue. The peripheral zone displays hyperperfusion with high metabolic activity and rapid regeneration. The intercalated zone of stasis is primarily alive, but might be recruited into the core zone and so become necrotic if kept untreated. A partial thickness lesion usually heals by conservative means with very few stigmata due to intact skin appendages in the deeper dermal layers that...
allow for spontaneous regeneration. Accordingly, one particularly fears burn progression of an initially partial thickness to full thickness, which almost always requires surgical debridement, wound conditioning and reconstructive surgery. The latter will result in high morbidity, pain, scar contracture, impaired joint mobility, recurrent and prolonged treatment and so, increased health care costs [3], [4].

Despite improved treatment strategies and outcome after burn injuries over the last decades [5], there is currently no therapeutic concept to prevent burn wound progression in superficial burn wounds. Nevertheless, before an injured patient reaches a hospital, a general practitioner or a pharmacist, where treatment could be applied, he requires first-aid treatment. To date, immediate application of cold water for ~20 minutes is still considered the gold standard, because it allows rapid heat dissipation and additionally has an analgetic effect [6]. Unfortunately, cooling is also known to induce vasoconstriction, potentially further impairing marginal perfusion conditions especially within the zone of stasis and so making the tissue even more susceptible to necrosis. After first aid treatment the focus should then be directed at strategies preventing burn wound progression, be it by applying agents topically or systemically. So far, different agents have been used targeting the main pathophysiological processes during burn wound progression, i.e. vasoconstriction, inflammation, hypercoagulability, oedema formation and thrombus formation [1], [2].

Erythropoietin (EPO) is a well-known protein increasing hematocrit in normal and anemic individuals by stimulating survival, proliferation, differentiation and migration of immature erythroid cells [7]. Besides its erythropoietic effect it has additional tissue protective effects, including mediation of vascular tone by over-expression of nitric oxide (NO) [8], prevention of ischaemia-induced apoptotic cell death and inflammation [9], induction of angiogenesis [9], [10], as well as tissue regeneration [11], [12], [13]. The study aim was therefore two-fold: (i) to evaluate warm water cooling alone for 20 min and three treatment groups with EPO and additionally an analgetic effect [6]. Unfortunately, cooling is also known to induce vasoconstriction, potentially further impairing marginal perfusion conditions especially within the zone of stasis and so making the tissue even more susceptible to necrosis. After first aid treatment the focus should then be directed at strategies preventing burn wound progression, be it by applying agents topically or systemically. So far, different agents have been used targeting the main pathophysiological processes during burn wound progression, i.e. vasoconstriction, inflammation, hypercoagulability, oedema formation and thrombus formation [1], [2].

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Material and methods

All experiments were carried out in accordance with the Swiss guidelines for animal experimentation and were approved by the Geneva Cantonal Veterinary authority. A total of 63 male Wistar rats weighing 461±8 g (Charles River Laboratories, L’Arbresle Cedex, France) were used in this study. Animals were housed in singles cages, at a room temperature of 22–24°C and at relative humidity of 60–65% with a 12 hour day-and-night cycle. Chow and water were available ad libitum.

Animal preparation

The animals were anesthetized for preparation and all measurements using an inhalation of a 2% isoflurane-air mixture. Analgesia consisted of the administration of subcutaneous buprenorphine at a dose of 0.05 mg/kg bodyweight (bw) 15 minutes (min) before burn induction, and thereafter every 12 hours (hrs) for the first 3 days. The animals were prepared 24 hours prior to the experiments including hair removal of the dorsal skin (shaving and depilation with a depilatory cream (Veet® Reckitt Benckiser, Wallisellen, Switzerland), marking the areas to be burned with a marking pen and the corners with intradermal ink.

Burn induction

We used the comb burn model to create the burn wounds [17]. A chromium-nickel steel (V2A) template (Ornaplast Kunststofftechnik, Dagmersellen, Switzerland) weighting 136 g was immersed in boiling water for 15 minutes. Thereafter, with the animal in a prone position, the template was applied to the previously shaved and marked area on the back, parallel to the spine and perpendicular to the skin. No pressure was applied and the template was removed after 60 seconds. This procedure resulted in four burned areas (20x10 mm) separated by three unburned interspaces (20x5 mm) on each side of the back as defined by the notches of the template. These interspaces represent the zone of stasis. No dressing was applied.

Experimental groups and protocols

Animals were randomly assigned to the experimental groups. In the first section of the study each group consisted of 7 animals, i.e. a control group (CO) without treatment, a group treated with cold water at 17°C for 20 min (CW) and a group treated with warm water at 37°C for 20 min (WW). Water treatment was performed immediately after burn induction with soaked gauzes (10x10 cm), directly applied to the burn area and changed every minute.

In the second section of the study each group consisted of 7 animals with a control group (CO) treated with local cooling alone for 20 min and three treatment groups with EPO: Two groups received local cooling for 20 min followed by intraperitoneal (ip) administration of 500 IU EPO/kg bw initiated at 45 minutes (EPO500 45 min) or 6 hours (EPO500 6 hrs) after burn induction and further administered once a day for the following five days. In a third experimental group, the animals received 2,500 IU EPO/kg bw first administered 45 minutes after the thermal injury (EPO2,500 45 min).

24 hours before burn induction animal preparation and baseline measurements were performed. The parameters were further assessed at the following time-points: 1 h and 24 hours, as well as 4 days and 7 days after burn induction. Wound healing and contracture rate were as-
The wound was considered as completely healed if the entire burned zone was free of eschar and epithelialized.

**Erythropoietin**

EPO, a glycoprotein, acts as the main regulator of erythropoiesis and participates in the proliferation, differentiation and migration of erythroid progenitor cells and thus regulates the amount of the red blood cell mass [18]. Both, hypoxia and anaemia are main stimulators of EPO-production in the adult kidney, which transmits its effects through a conformational change in the EPO-receptor [19]. The main undesirable adverse effects due to increased haematocrit are thrombus formation and increased mortality [20]. Epoetin βeta (recombinant human erythropoietin, NeoRecormon; Roche® Pharma, Basel, Switzerland) was mixed with 1 mL of NaCl 0.9% in order to achieve final concentrations of 500 and 2,500 IU/kg bw. The solution was stored at a maximum temperature of 8°C until usage. We administered EPO using the i.p. route.

**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).

**Microcirculatory perfusion**

Skin perfusion within the interspaces was measured using a Laser Doppler device (PIM II Laser Doppler Perfusion Imager, LDPIwin 2.0.6 software, Liscab AB Berzelius Science Park, Linköping, Sweden). The surface probe measured microcirculatory blood flow to a depth of approximately 1 mm. The Laser Doppler unit was calibrated according to the guidelines of the manufacturer. The data are given in percentages of baseline due to high intersite variability [21], [22].

**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). In order to guarantee subsequent planimetric analysis, photographs were performed in a standardized manner with the camera fixed on a tripod. A computer-assisted image analysis system (Cap Image, Zeintl Software; Heidelberg, Germany) was used to determine the total amount of interspace necrosis (percent 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 percent.

**Histology**

The entire burn area consisting of 8 burns and 6 interspaces per animal was harvested en bloc at each time point in all groups. The tissue was fixed in 10% formalin, stored in 70% alcohol for 24 hours and embedded in paraffin. Thereafter, 4-μm sections were obtained for hematoxylin and eosin (H&E) and immune-histochemical staining.

**Inducible nitric oxide synthase immunostaining**

Nitric oxide (NO), known as a mediator of numerous processes such as vasomotor tone, metabolism, signal transduction, cell-to-cell interaction and immune modulation, is generated by N0 synthases (NOS), which catalyze the conversion of Larginine to L-citrulline. Inducible (iNOS) and endothelial (eNOS) are the two most commonly analyzed NOS, and are generally expressed after cellular stimulation via cytokines and other stimuli respectively angiogenic and vascular remodelling processes and serve as a response to injury [23]. iNOS immunostaining was done in the EPO-groups. Antigen retrieval was performed for 30 seconds (s) in citrate buffer at pH6. Peroxidase blocking was carried out using Dako real peroxidase blocking solution (Dako Schweiz, Baar, Switzerland) for 10 min. This was followed by incubation for 60 min with the primary antibody (1:800 rabbit polyclonal antirat/antimouse iNOS antibody; BD Biosciences, Heidelberg, Germany) and for 30 min with the secondary antibody (rabbitEnVision™ HRP antibody; Dako). The peroxidase activity was detected by incubation with 3-amino-9-ethyl carbazole (BioGenex, Basle, Switzerland) for 10 min. Slides were counterstained with Mayer’s haemalum and mounted in Aquatex® (Merck, Geneva, Switzerland).

**CD31 immunostaining**

Cluster of differentiation 31 (CD 31), which is also known as platelet endothelial cell adhesion molecule (PECAM-1), is normally expressed at the surface of endothelial cells, platelets, monocytes and neutrophils, accounting for a large amount of endothelial cell intercellular junctions. Main characteristics of this protein, belonging to the immunoglobulin superfamily, are the involvement in angiogenesis, migration of leukocytes and the activation of integrins [24]. CD31 immunostaining was done in the EPO groups. 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 the primary antibody (1:100 goat polyclonal antirat/antimouse CD31 platelet endothelial cell adhesion molecule 1 antibody (M-20); Dako) and for 30 min with the secondary antibody (1:150 rabbit antigoat biotin; 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 haemalum and mounted in Ultrakitt™ (J. T. Baker, Deventer, the Netherlands).

All sections were recorded with a Zeiss® Axiophoto microscope (Carl Zeiss, Jena, Germany). To assess burn depth, a pathologist blinded to the treatment groups examined H&E-stained sections (6 per group), and rated burn depth based on a validated scale from 1 to 5: 1. epidermis; 2. superficial dermis; 3. intermediate dermis; 4. deep dermis; and 5. muscle [25]. To assess inflammation, dilatory response and angiogenesis, three visual fields per H&E-, iNOS- and CD31-stained section were selected randomly, and recorded with a charged coupled-device camera (Zeiss® Axiocam) using Axiovision software. Thereafter, leukocytes as well as iNOS- and CD31-positive vessels were quantified.

Statistics

All values are expressed as mean and standard error of the mean (s.e.m.). To control for inflation of type I error probability in the course of multiple data analysis, hierarchical modelling and hypothesis testing was performed. Linear mixed regression models comprising the main-effect terms time and treatment group and the interaction term time by treatment group were used in order to perform a two-way ANOVA. If the global test of the main-effect treatment group was significant at a level of 0.05, one-way ANOVA group comparison for each time point was carried out (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 co-variable. Group differences in ordinal data at a single time point were assessed by means of the non-parametric Kruskal-Wallis test followed by Bonferroni-adjusted pairwise comparisons using the Mann-Whitney U test. Statistical testing was done with SPSS® (IBM, Armonk, New York, USA).

Results

Warm water

1. Interspace necrosis and burn depth

The interspaces of all groups were intact after 1 hour with equal progression in all animals within the first 24 hours. Between day 1 and 4, interspace necrosis occurred in all groups with a significantly reduced extension of tissue damage at the skin’s surface in the WW group compared to untreated controls and the CW-group after 4 days respectively 7 days (CO: 94±2% vs. CW: 81±4% and WW: 65±4%; p<0.05) (see Figure 1, A and C–F). Histologic analysis of burn depth 1 hour after burn induction showed equal damage to the epidermis and superficial dermis in all groups (score 2±0). Within 24 hours, burn injury progressed to the deep dermal layers in untreated control animals while no further progression was observed in animals treated with cold or warm water (CO: 4.3±0.2 vs. CW and WW: 2±0; p<0.05). However, over the next 72 hours the tissue damage in the two treatment groups progressed to the deep dermis (CO: 5±0 vs. CW: 4.3±0.2 and WW: 4.8±0.1; n. s.), to progress to full thickness lesion in all groups after 7 days (see Figure 1, B).

2. Interspace perfusion

Interspace perfusion 1 hour after burn induction showed decreased perfusion conditions in all groups with already significantly improved perfusion in the WW group (CO: 63±1% vs. CW: 62±2% and WW: 81±2%; p<0.05). Treatment with WW continued to improve the local perfusion over the next days compared to the untreated controls and the CW-group with complete recovery to baseline values at day 4 and even induction of hyperperfusion at day 7. Neither untreated controls nor cold water treatment were able to re-establish baseline values (CO: 80±2% vs. CW: 91±3% and WW: 103±4% at day 4; p<0.05) (see Figure 2).

3. Total healing time and contracture rate

There was no difference in contracture rate (25–29%) and healing time (median 11 weeks) between the three groups (see Table 1).

Erythropoietin

1. Interspace necrosis and burn depth

Necrosis of the healthy interspaces mainly occurred during the first 4 days after burn induction (see Figure 3).
Figure 1: Time course of burn surface progression given in percent of the total interspace area in untreated animals (controls: white bars), as well as in animals treated with cold water (blue bars) respectively warm water (red bars) (A). Time course of burn depth progression in untreated animals (white bars), as well as in animals treated with cold water (blue bars) respectively warm water (red bars) (B). Mean ± SEM; *p<0.05 vs. controls; †p<0.05 vs. cold water treatment; n=7/group. Local treatment of either temperature delays secondary burn depth progression without preventing final burn depth at day 7. Burn depth progression score: 1. epidermis; 2. superficial dermis; 3. intermediate dermis; 4. deep dermis; 5. muscle. n=7/group. Tissue morphology 1 h (C) and 7 days after burn in an animal without treatment (D), as well as after cold water (E) or warm water-treatment (F). Note the decreased width of interspace necrosis after cold water and particularly after warm water-treatment.

Figure 2: Time course of interspace perfusion in untreated animals (controls; white bars), as well as in animals treated with cold water (blue bars) respectively warm water (red bars) (A). Mean ± SEM; *p<0.05 vs. controls; †p<0.05 vs. cold water; n=7/group. Flow pattern before (B) and 1 h after burn in an untreated animal (C), as well as after cold water (D) and warm water-treatment (E). Note the maintained interspace perfusion after warm water application (E) indicated by red and yellow pixels.
Figure 3: Time course of interspace perfusion in cold water-treated animals (controls; blue bars), as well as in animals first treated with EPO at a dosage of 500 IU/kg bw after 45 minutes (light grey, EPO500 45 min) or after 6 hours (intermediate grey, EPO500 6hrs) or at a dosage of 2500 IU/kg bw after 45 minutes (dark grey, EPO2,500 45 min) (A). Mean ± SEM; *p<0.05 vs. controls; **p<0.05 vs. EPO500 6 hrs and EPO2,500 45 min; n=7/group. Tissue morphology 7 days after burn in a control animal (B) as well as in animals treated with EPO500 45 min (C), EPO500 6 hrs (D) or EPO2,500 45 min (E). Note the decreased width of interspace and overall necrosis after EPO treatment, especially after administration of EPO500 45 min.

EPO500 45 min and EPO2,500 45 min showed significant reduction of interspace necrosis after 4 days with EPO500 45 min being superior to EPO2,500 after 7 days. EPO500 6 hrs had no significant effect to prevent interspace necrosis compared to control animals (see Figure 3, B–E). All groups suffered uniform damage of the epidermal and superficial dermal layers of the skin. During the first 7 days, control animals – rats that received local cooling for emergency treatment – and animals treated with EPO500 initiated 6 hours after burn suffered secondary depth progression through the entire dermis. In contrast EPO500-treatment started 45 minutes after burn significantly reduced dermal destruction at a level of intermediate dermis compared to local cooling alone (control) and the EPO500 6 hrs group (see Figure 4). This level of burn progression allows spontaneous healing and regeneration due to intact skin appendages within the deeper dermal layers. Treatment with EPO2,500 45 min showed a reduction of burn wound progression compared to controls, yet the progression reached the deep dermis.

2. Interspace perfusion

Within the first 24 hours after burn injury the perfusion decreased substantially in all animals with however significantly better perfusion conditions in the EPO500 45 min group compared to control animals and the other EPO groups. EPO500 45 min treated animals reached baseline values within 4 days, to even develop hyperperfusion of 114% of baseline. EPO500 6 hrs group reached baseline values only after 7 days. After local cooling (and EPO2,500 45 min), the perfusion was not able to re-establish to baseline values (see Figure 3, A).

3. Hematocrit

Administration of EPO was associated with a significant increase of the hematocrit in a dose dependent manner that was observed from day 4 on (see Table 2).

4. Inducible nitric oxide synthase

EPO administration significantly increased iNOS expression one day after burn injury (see Figure 5), when compared to controls.

5. Angiogenesis

A significant increase in CD31 protein expression that correlated with an angiogenic response was observed from day 7 in EPO-treated animals, irrespective of dosage or administration time (see Figure 6).

6. Total healing time and contracture rate

Only animals treated with EPO500 45 min showed a significantly reduced healing time compared to control animals. Similarly, EPO500 45 min was the only treatment regimen that was able to significantly reduce contracture rate compared to control animals (see Table 1).
Figure 4: Time course of burn depth progression in cold water-treated animals (controls; blue bars), as well as in animals treated with EPO500 45 min (light grey), EPO500 6 hrs (intermediate grey) or EPO2500 45 min (dark grey) (A). Burn depth progression score: 1. epidermis; 2. superficial dermis; 3. intermediate dermis; 4. deep dermis; 5. muscle. Mean ± SEM; *p<0.05 vs. controls; n=7/group. Histology of dermis (B, D, F, H) and muscle (panniculus carnosus: C, E, G, I) at day 7 after burn with complete architectural destruction of dermis, skin appendages and muscle in cold water-treated animals (B, C). Note the partial preservation of both, skin appendages (D) and muscle (E), in animals treated with EPO500 45 min, indicating that burn progression is limited to the intermediate dermis. The other EPO-regimens also reduced depth progression, affecting skin appendages (F) and preserving muscle (EPO2500 45 min; G) or affecting both (EPO500 6 hrs; H, I). Scale bar 100 µm.

Table 2: Quantitative analysis of haematocrit (%) before burn induction (baseline) and over the 2-week observation period in control animals and in animals that were administered 500 IU EPO/kg bw at 45 minutes (EPO500 45 min) and 6 hours (EPO500 6 hrs) or 2,500 IU EPO/kg bw at 45 minutes (EPO2500 45 min; n=7 per group). Mean ± SEM, *p<0.05 vs. baseline, +p<0.05 vs. control.

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Discussion

This study shows that first aid treatment with warm water prevents extension of the burn on the surface and delays burn depth progression, thereby opening a “therapeutic window” for further therapeutic intervention. This window might be used for systemical administration of EPO which prevents burn progression on the surface and into the depth if administered daily for 5 days starting 45 minutes after burn induction with a dosage of 500 IU/kg bw. Emergency treatment as soon as possible after local thermal injury attempts to interrupt the vicious circle of burn wound progression by dissipating heat from the affected tissues. Local application of tap water for ~20 minutes is still regarded as the “gold standard” [6]. Experimental data currently showed that application of warm water of 37°C shortly after burn injury was more effective than local application of cold water of 17°C. Both water regimens delayed burn depth progression without reducing tissue injury in the depth compared to the untreated control group. However, only warm water was able to significantly reduce secondary extension of tissue necrosis at the skin’s surface. Vasodilatation and capillary recruitment are known effects of the application of warmth leading to an increased blood flow [26]. Treatment with warm water was associated with less perfusion breakdown after burn within the critically intercalated zone of stasis and normalization of perfusion conditions compared to baseline within 4 days and further
Figure 5: Inducible nitric oxide synthase (iNOS)-positive cells in cold water-treated animals (controls; blue bars), as well as in animals treated with EPO500 45 min (light grey), EPO500 6 hrs (intermediate grey) or EPO2,500 45 min (dark grey) (A). Mean ± SEM; *p < 0.05 vs. controls; †p < 0.05 vs. EPO500 6 hrs; n=7/group. Staining for iNOS at day 1 in a controls animal (B), as well as in an animal receiving EPO500 45 min (C), EPO500 6 hrs (D) and EPO2,500 45 min (E), respectively. Positive cells are represented by red staining, which is only seen in EPO-treated animals (arrow heads). Scale bar 15 µm.

Figure 6: Time course of CD-31 positive vessels in cold water-treated animals (controls; blue bars), as well as in animals treated with EPO500 45 min (light grey), EPO500 6 hrs (intermediate grey) or EPO2,500 45 min (dark grey) (A). Mean ± SEM; *p<0.05 vs. controls; n=7/group. Staining for CD31 at day 7 after burn in a control animal (B), as well as in an animal receiving EPO500 45 min (C), EPO500 6 hrs (D), and EPO2,500 45 min (E) respectively. Positive vessels indicating vessel density are represented by brown staining, which is almost only seen in EPO-treated animals (arrow heads). Scale bar 30 µm.
induction of hyperperfusion. Neither cold water-treated animals nor untreated animals were able to restore perfusion of the interspace after burn. Most people instinctively apply cold tap water on burn injuries. Cooling is widely known to dissipate heat and to reduce pain. It is further associated with anti-inflammatory properties and so counteracts oedema-formation and improves wound healing capacity [27], [28], [29], [30] and wound healing capacity [31], [32], [33], [34].

Prolonged application of cold water to the skin results in vasoconstriction and eventually in impaired perfusion as currently demonstrated. Delay in burn depth progression after local cooling - despite significantly impaired perfusion within the critically intercalated zone of stasis over approximately 7 days – may result from decreased inflammation (data not shown), reduced oedema formation and increased ischemic tolerance of the critically perfused tissue [22], [35].

Short term cooling after local burns has undeniably shown to have beneficial effects. Nevertheless, if local cooling is applied too long, tissue survival might be jeopardized due to impaired perfusion [36], [37]. Currently, no exact data is available with regard to the optimal temperature and duration of cooling to rapidly dissipate heat without irreversibly impairing microcirculation of the zone of stasis. In order to use the advantages of both cold water and warm water for emergency treatment of local burns, an alternated application seems to be obvious. This approach is simple and available almost anywhere. It could significantly reduce burn wound progression at the skin’s surface and delay burn depth progression to some days in order to open a “therapeutic window” that allows induction of treatment aiming at preventing burn wound progression to the depth.

To date, several approaches and agents have been used in experimental settings. All aim at saving the critically perfused zone of stasis to be recruited into the core zone of the burn lesion [38], [39], [40], [41]. We demonstrated that EPO could effectively reduce a secondary burn wound progression in a time- and dose-dependent manner. Administration of 500 IU EPO/kg bw initiated 6 hours after burn induction was too late to prevent wound progression to a deep dermal level, whereas the equal administration of EPO already 45 minutes after burn injury was capable of significantly decreasing surface necrosis and particularly depth progression to the level of intermediate dermis. A five-fold increase of the dosage to 2,500 IU EPO/kg bw first administered at 45 minutes after burn could not prevent burn wound progression to the deep dermal layers. Burn wound progression only to the intermediate dermal layers (EPO500 45 min) is synonymous with the preservation of skin appendages that are necessary for regeneration and spontaneous healing. Less tissue damage was primarily associated with significantly improved perfusion conditions within the zone of stasis. EPO2,500 45 min demonstrated a rapid and significant increase of the haematocrit already by day 4, potentially impairing perfusion of the interspaces. Harder et al. have shown that rapid increase of haematocrit was able to significantly impair blood rheology and so decrease tissue survival despite increased anti-inflammatory, vasodilatory and angiogenic response [9], [42]. We believe that the rapid haematocrit-increase over 4 days after first EPO-administration (EPO2,500 45 min) and the late normalization of perfusion conditions by day 7 (EPO500 6 hrs and EPO2,500 45 min) results in the worse outcome of these two groups compared to EPO500 45 min. Impaired rheology or reduced perfusion conditions over 7 days are sufficient to aggravate secondary burn wound progression.

EPO was associated with an early over-expression of iNOS that was dose-, but not time-dependent. The NO-mediated vasodilatory response seems to be partly responsible for the increased perfusion in EPO-treated animals.

EPO was further associated with an angiogenic response that was time- and dose-dependent and characterized by CD31-positive cells. These newly formed vessels were first seen at day 4 and particularly at day 7 after burn induction, a time-period when necrosis is already almost completely demarcated. This observation is in line with a previous study of our group that has first shown newly developed and functional microvessels five days after induction of acute persistent ischemia in a musculocutaneous flap pre-treated with EPO. It has also been shown that this rather delayed onset of blood-cell carrying microvessels was not able to prevent necrosis from ischemia-induced microvascular breakdown [43]. In line with this observation, we believe that the EPO-mediated angiogenic response does not play any role in the prevention of burn wound progression as does the iNOS-mediated dilatory response. However, EPO seems to support tissue regeneration that is partly mediated by neovascularization, which is in line with Galeano et al., who could first show the positive regenerative effect of EPO in treatment of burns [44]. After administration of 400 IU EPO/kg bw for 14 days starting 3 hours after burn, they observed increased epithelial proliferation and a quicker wound healing in treatment rats, represented by increased microvascular density secondary to a vascular endothelial growth factor-mediated angiogenic effect.

Only treatment with 500 IU EPO/kg bw initiated 45 minutes after injury was associated with a significantly reduced final healing time and contracture rate compared to control animals, warm water-treatment or the other EPO-regimes. We believe that improved wound healing and decreased contracture rate are directly correlated to the extension of the burn injury in surface and particularly in depth.

Noteworthy, Machens et al. currently conduct a multicenter randomized controlled trial on the regenerative effects of EPO in scalding injuries in humans [45].

Conclusions
This study shows that emergency treatment of burn injuries with warm water is superior to cold water by means of preventing surface extension of burn wound progres-
sion. Both treatment regimens equally delay burn depth progression without limiting it in depth, thereby creating a “therapeutic window” for further therapeutic intervention. Systemic administration of EPO has shown to limit burn-induced necrosis in surface and particularly in depth to an intermediate dermal level, if administered at a certain dose (500 IU/kg bw) and within a certain time-frame (<6 hours). Decreased burn wound progression results from maintained perfusion conditions, as well as from anti-inflammatory and from angiogenic effects mediated by EPO. Further studies are necessary to elaborate a sound and potent emergency protocol alternating cold and warm water followed by the administration of tissue protective agents. EPO-derivatives with increased half-lives (e.g. CERA) or without erythropoietic properties (e.g. ARA290, CEPO) administered systemically or topically might allow increasing the doses without increasing the rate of side-effects.

Notes

Competing interests

The authors declare that they have no competing interests.

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