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Long-term pre- and postconditioning with low doses of erythropoietin protects critically perfused musculocutaneous tissue from necrosis

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Summary It has been shown that pre- and postconditioning of ischemically challenged tissue with erythropoietin (EPO) is able to reduce necrosis in a dose-dependent manner. The aim of this study was to determine the tissue-protective effects of different EPO dosages and administration regimes.
Three groups of six C57Bl/6-mice each were analyzed: (1) pre- and postconditioning with initial high doses of EPO (starting at 2500 I.U./kg bw i.p.) followed by low doses of EPO (125 I.U./kg bw i.p.) (EPO-high-dose); (2) pre- and postconditioning with low doses of EPO (125 I.U./kg bw i.p.) (EPO-low-dose); and (3) untreated control group. Randomly perfused musculocutaneous flaps were mounted on dorsal skinfold chambers undergoing acute persistent ischemia and developing ~50% necrosis without treatment. Intravital epifluorescence microscopy was performed at days 1, 3, 5, 7, and 10 after surgery, assessing flap necrosis, microcirculation, and angiogenesis. The hematocrit was measured at days 0, 3, 7, and 10.

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Only the EPO-low-dose regimen was associated with a significant reduction of necrosis when compared to untreated controls. EPO-low-dose showed a higher increase in both arteriolar diameter and velocity, thereby resulting in a significantly increased arteriolar blood flow and a hence higher functional capillary density (FCD) of the critically perfused zone. EPO-induced angiogenesis was significantly increased in EPO-low-dose at days 7 and 10. Only EPO-high-dose reached a significant hematocrit increase by day 10.
Tissue pre- and postconditioning with low doses of EPO protects the critically perfused musculocutaneous tissue by maintaining capillary perfusion because of increased arteriolar blood flow mediated by nitric oxide (NO) expression.

Introduction

Tissue transfer in reconstructive surgery currently is based on a large variety of flaps, and continuous progress has been made during the last decades. However, surgeons performing flap surgery are still encountering ischemia-induced complications. These complications include wound breakdown, tissue necrosis, and even partial flap necrosis, which occur in 20–33% of pedicled flaps.[1,2]

The percentage of tissue necrosis is determined by the ability of the vascular pedicle to perfuse the distal zone of the flap (i.e., zone distant to the flap pedicle), which will result in failure of capillary perfusion, loss of adequate oxygen supply, and, eventually, irreversible ischemic tissue damage.[3,4]

Although there are several effective options to increase the tolerance of a tissue to ischemia, such as "surgical delay",[5] local heat,[6] or mechanical stress,[7] tissue preconditioning with pleiotropic substances such as erythropoietin (EPO) might represent a noninvasive and deviceless alternative to improve microcirculation and tolerance for ischemia of the critically perfused tissue.

EPO is a glycoprotein released from the kidney that was first known for its regulatory function in erythropoiesis.[8,9] It is released in response to hypoxia and transmits its effects through a conformational change in the EPO receptor (EPO-R).[10] Studies indicate an EPO-R-based elimination, with a decrease in clearance with increasing doses suggesting a saturable mechanism.[11]

Although its dose-dependent increase in hematocrit may be counterproductive for blood rheology and thus tissue perfusion due to blood thickening,[12,13] EPO has also been attributed to tissue-protective effects including an NO-mediated endothelium-dependent relaxation of arterial vessels,[14] induction of angiogenesis,[15] and tissue regeneration.[16] These effects can be seen in a variety of ischemically challenged tissues such as the heart,[17] kidney,[18] liver,[19] and brain.[20,21]

In previous studies, we have demonstrated that both pre- and postconditioning of the critically perfused musculocutaneous tissue with EPO using repetitive administration of equal EPO doses were able to protect the musculocutaneous tissue by reducing flap necrosis, if the hematocrit was not significantly altered.[22,23] Because hematocrit-based blood thickening should be avoided at any cost in the ischemically challenged flap tissue, we aimed to determine whether tissue pre- and postconditioning using a combination of initial high doses, subsequently followed by low doses of EPO, is superior to the repetitive administration of equal low doses of EPO to protect the ischemically challenged tissue.

Materials and methods

Animals

All experiments were performed in accordance with the German Animal Welfare Act for animal experimentation and were approved by the responsible Bavarian authorities (file number: 55.2-1-54-2531-89-12). The mice (C57BL/6; 12–24 weeks old; 26–30 g bw; Charles River Laboratories, Sulzfeld, Germany) were housed in individual cages at room temperature of 22–24 °C and at a relative humidity of 60–65% with a 12-h day–night cycle. Animals had free access to standard pellet chow (Altromin, Lage, Germany) and tap water.

Anesthesia

For all surgical procedures and microscopic analyses, animals were anesthetized using intraperitoneal injections of ketamine hydrochloride (90 mg/kg bw, Ketavet; Parke-Davis, Freiburg, Germany) and xylazine hydrochloride (25 mg/kg bw, Rompun; Bayer, Leverkusen, Germany).

Flap preparation

The dorsal skinfold chamber model was used, as described and visualized in previous studies, for the experiments.[24-26] The chamber contains a random pattern musculocutaneous flap consisting of the skin and the underlying panniculus carnosus. Because of the width-to-length-ratio of the flap, it is subjected to acute persistent ischemia to develop approximately 50% necrosis if kept untreated.[26] The animals were depilated, and a flap of 11 × 15 mm was elevated perpendicular to the spine. The flap was then sutured back to the adjacent skin and fixed in between the two halves of the titanium chamber. The observation window of the chamber was sealed using a cover glass fixed with a snap ring for later microscopy. After surgery, the animals did not show any changes in sleeping or feeding habits and tolerated well the frame of total weight of ∼3 g.

Experimental protocol

A total of 18 animals were operated and assigned to three experimental groups of six animals each: (i) EPO-high-dose: animals were preconditioned (i.e., before induction of acute persistent ischemia; flap elevation) and postconditioned (i.e., after flap elevation) with initial high doses of EPO and subsequent low doses of EPO (2500 I.U./kg bw i.p. 30 min before flap elevation, 1250 I.U./kg bw i.p. 30 min after flap elevation, 500 I.U./kg bw i.p. 24 h after flap elevation, 125 I.U./kg bw i.p. every 12 h starting 48 h after flap elevation until day 10); (ii) EPO-low-dose: animals were pre- and postconditioned with low doses of EPO (125 I.U./kg bw i.p. administered at the same time points as those in group (i)); (iii) Control animals received injections of saline in equidoses. To keep conditions as steady and as comparable as possible, a dose of 125 I.U./kg bw i.p. was applied for the entire observation period.

The dosage was selected on the basis of that reported in previous studies, which demonstrated that administration of 5000 I.U./kg bw is hematocrit relevant, when started 24 h before the induction of ischemia and continued for 3 consecutive days. A dose of 500 I.U./kg bw marginally elevated the hematocrit when administered for 3 days but significantly elevated the hematocrit when administered for 10 consecutive days.[23,27] We therefore used 2500 I.U./kg bw as half of the previously used dose in combination with an instant decrease for our high-dose group. The maintenance dose, i.e., low dose of 125 I.U./kg bw every 12 h, was selected to again reach half of the previously used dose of 500 I.U./kg bw in 24 h.

Microscopic analyses were performed at days 1, 3, 5, 7, and 10 after surgery; blood samples were collected at days 0, 3, 7, and 10. At the end of the experiment, all animals were killed by an overdose of the anesthetic agent. Blood draws were not performed on days 1 and 5 due to the limited blood volume in the used animals.

Test substance

Epoetin alfa (recombinant human EPO, Erypo®; Janssen-Cilag, Neuss, Germany) was mixed with NaCl (0.9%) to achieve the needed concentrations for administering doses ranging from 125 I.U./kg bw to 2500 I.U./kg bw and were administered i.p. according to the study protocols. I.p. injections were preferred compared to i.v. injections for repeated administration of EPO because they are technically easier to perform and peak serum concentration takes half as long to be reached when compared to s.c. injections.[28]

The injected volume per administration was approximately 0.1 ml per animal. The solution was stored at a maximum temperature of 8 °C until usage.

Blood analysis

Blood samples of 150 μl were drawn from the tail vein. The first sample (day 0) was collected during the preparation of the flap elevation before any EPO or saline solution was administered, as well as days 3, 5, and 10 after flap elevation. The samples were immediately analyzed for hematocrit, hemoglobin concentration, and RBC count using an analyzer designed for animal blood (pocH-100iV Diff, Sysmex, Norderstedt, Germany).

Intravital epifluorescence microscopy

For in vivo microscopic analysis, the anesthetized animals were placed on a Plexiglas platform and were injected with 0.05 mL of 5% FITC-labeled dextran (molecular weight 150,000 Da; 50 mg/mL saline; Sigma-Aldrich, Taufkirchen, Germany) into the retrobulbar venous plexus for intravascular contrast enhancement. The animals were then placed under a Zeiss Axioscope microscope (Zeiss®, Oberkochen, Germany) equipped with a high-end LED-lamp (Zeiss Colibri; Zeiss®, Oberkochen, Germany) and a filter set for green light (530–560 nm excitation, >580 nm emission wavelength). Microscopic images were captured using a video camera (AxioCam HSm; Zeiss®, Oberkochen, Germany) and recorded on an external hard drive. The microscope was operated using ZEN software (Zeiss®, Oberkochen, Germany). All parameters were subsequently analyzed offline using image analysis software (Cap-Image; Zeintl® Software, Heidelberg, Germany).[29] The microscopic procedures were performed at constant room temperature of 22–24 °C.

Microcirculatory and cellular analysis

At each observation time point, the chamber window was first scanned using a 5x magnification to determine the surface of the nonperfused nonviable zone of the flap. Using planimetrical evaluation, the percentage of the total nonviable nonperfused zone was calculated offline and is expressed in percentage of the total visible flap. This definition of the nonviable tissue is based on clinical techniques such as indocyanine green fluorescence angiography, where the intraoperative visualization of perfusion is used to predict necrosis.[30] Furthermore, microcirculatory parameters were measured in the perfused tissue. For these measurements, we selected easily identifiable branching patterns consisting of second-order (vessel branched out twice) or third-order arterioles (vessel branched out three times); their accompanying collecting venules; and capillary fields in the proximal, medial, and distal zones of the flap. First-order vessels were excluded, as they were usually too big to be adequately visualized and thus analyzed with a 20× magnification. The selected bundles including their surrounding regions were documented in a schematic sketch of the chamber to allow exact relocalization during repetitive measurement during the 10-day period. In addition, a representative section within the transition zone between the viable and nonviable flap zones was selected, documented, and observed with time for arteriogenesis and angiogenesis. The following parameters were investigated in arterioles (inflow), capillaries (nutrient vessels), and venules (out-flow) using a 20× magnification: (I) RBC velocity (mm/s) was analyzed using the line-shift-method, in which the shift (mm) of a grey-level pattern formed by the RBCs is measured with time (seconds). (II) Vessel diameter (μm) was measured perpendicular to the vessel path. (III)

Table 1 Hematocrit values showing a clear increase in EPO-treated animals. Although control animals show a decrease without complete compensation to the baseline value by day 10, EPO-low-dose and EPO-high-dose animals show continuous increase in hematocrit levels.

Parameter	Group	Day 0	Day 3	Day 7	Day 10
Hematocrit [%]	EPO-low	41.4 ± 3.8	42.0 ± 2.4	43.4 ± 2.4	47.4 ± 4.3
	EPO-high	41.4 ± 2.9	43.0 ± 2.3	47.9 ± 3.8	53.3 ± 2.9*
	Control	45.2 ± 0.7	37.2 ± 2.2	34.5 ± 2.8	39.7 ± 2.7

Values are mean ± SEM.
* $p < 0.05$ vs. control.

Volumetric blood flow (pl/s) was calculated from RBC velocity and the surface area of the vessel cross-section ($\pi^* r^2$) using the equation of Groos and Aroesty ($Q = V^* \pi^* r^2$).[31] (IV) Functional capillary density (FCD; cm/cm^2) was defined as the length of all RBC-perfused capillaries per observation field. (V) Tortuosity of capillary vessels was calculated by selecting two individual branching points and calculating the ratio of the actual path length and the straight line, i.e., the shortest distance between the two branching points. After selection of two individual branching points, tortuosity of capillaries was calculated from the ratio of the actual path length and the straight line, i.e., the shortest distance between the two branching points. (VI) Angiogenic response was measured as the length of all newly formed, capillary-like microvessels developing perpendicular to the existing capillary vessels.

Immunohistochemical analysis of vasoendothelial growth factor (VEGF) and endothelial NO-Synthase (eNOS)

The flap tissue was analyzed for VEGF to complement the data regarding the formation of new blood vessels, whereas eNOS provides further information regarding vessel dilation. After the mounting of the chamber, the tissue resected during the elevation of the flap was formalin-fixed. At day 10, the flap itself was formalin-fixed and the tissue samples were subsequently embedded in paraffin and cut into 6 μm sections. The endogenous peroxidase was blocked by incubating the sections for 5 min in 3% H_2O_2 in TBS solution. The sections were then incubated over night at 4 °C with a polyclonal rabbit antimouse antibody against VEGF (1:100 dilution; Abcam, Cambridge, UK) or eNOS (prediluted, Abcam, Cambridge, UK). A horseradish peroxidase-conjugated goat-antirabbit antibody was used as a secondary antibody (1:100 dilution, Abcam, Cambridge, UK) and 3,3-diaminobenzidine tetrahydrochloride was used as a chromogen. The slides were counterstained with Mayer Haemalaun (Carl Roth, Karlsruhe, Germany) and examined by light microscopy. Tissue sections from each sample incubated under the same conditions with the antibody incubation buffer alone instead of a primary antibody served as negative controls.

The tissue sections were analyzed using a semi-quantitative scoring system. For each stained tissue sample, five representative visual fields were rated with a score from 0 (= no staining) to 3 (= strongly positive), and their mean value was calculated.

Statistical analysis

All values are indicated as mean ± SEM. The experimental groups were compared using the ANOVA test, followed by Tukey's Honest Significant Difference method to adjust for multiple comparisons. Differences were considered significant at $p < 0.05$. For comparison between individual time points, a linear mixed model was applied including a fixed effect for treatment, point in time, and a random effect for the individual animal. *P* values are calculated according to the Kenward-Roger approximation for degrees of freedom.

Results

Hematocrit

An increase in hematocrit was observed in both EPO-treated groups when compared to untreated animals. However, the increase was significant only in the EPO-high-dose group at day 10 (day 10: EPO-high-dose: 53.3 ± 2.9%; EPO-low-dose: 47.4 ± 4.3%; control: 39.7 ± 2.7%; $p < 0.05$; Table 1).

Flap necrosis

Acute persistent ischemia over time resulted in irreversible morphological changes in every single flap, displaying a proximal viable zone at the base of the musculocutaneous flap, a critically perfused medial zone of transition and a distal zone of nonperfused necrotic tissue. The transition zone could further be divided into a proximal hyperemic red fringe, indicating vasodilation and microvascular remodeling, and a distal white falx lunatica, already lacking nutritive capillary perfusion but not yet showing the desiccation and thus transparency of the necrotic zone (Figure. 1(A)-(C)).

The lack of treatment in control animals resulted in a total of 58 ± 6% necrosis of the flap by day 10 after flap elevation. The EPO-high-dose regimen did not result in a decreased rate of tissue necrosis (day 10: 60 ± 5%), whereas the EPO-low-dose regimen was able to significantly reduce the extent of tissue undergoing necrosis to 37 ± 7%. Of interest is that, at day 1 after flap elevation, these animals revealed a 70% perfusion of the total flap surface, whereas untreated animals and animals receiving EPO at a high dosage showed ca. 55% and 45% of flap perfusion (Figure. 2).

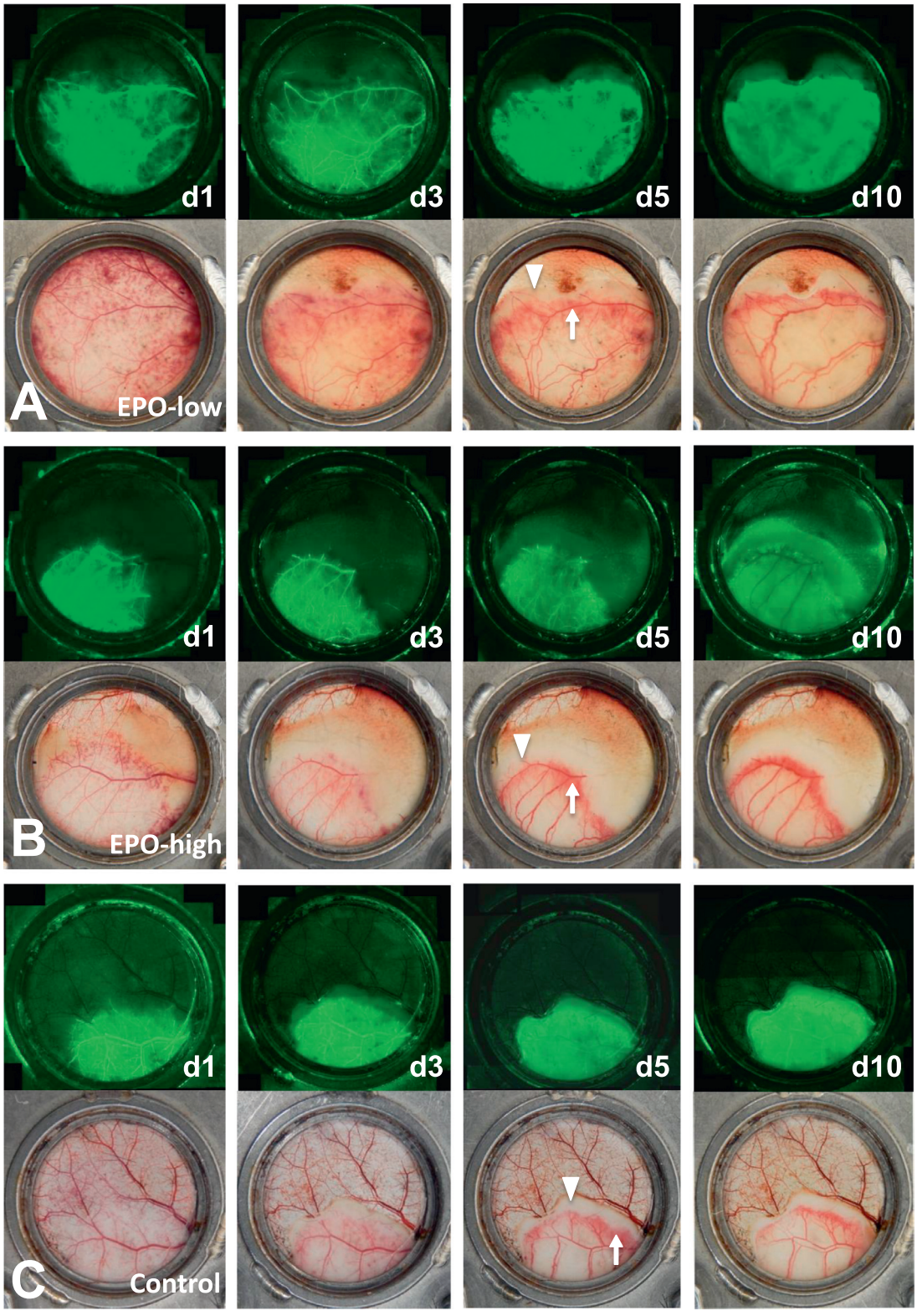

Figure 1 Intravital microscopy images and corresponding photographs of observation chambers of the EPO-low-dose (A), EPO-high-dose (B), and control (C) groups during the observation period. All experimental groups develop a proximal zone of the viable tissue at the flap base, which is well demarcated from the distal necrotic flap zone, intercalated by a zone of transition, consisting of a hyperemic red fringe proximally (arrow) and a white falx distally (arrowhead), here marked in day 5 images. Macroscopically, EPO-low-dose results in a significantly lower rate of tissue necrosis at day 10 than both EPO-high-dose-administered animals and untreated control animals.

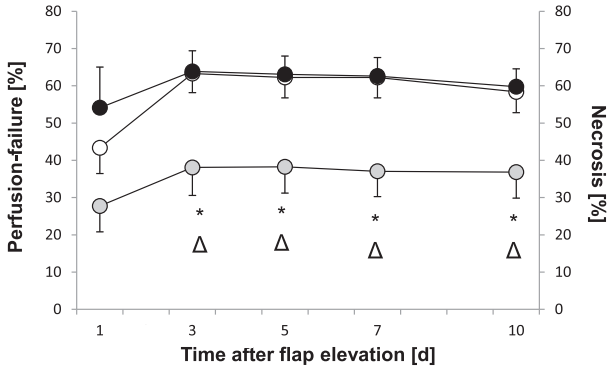

Figure 2 After an initial perfusion failure of ~45% in untreated animals (white circles) and ~55% in EPO-high-dose-treated animals (black circles), EPO-low-dose-treated animals (~30% perfusion failure; grey circles) develop significantly less necrosis at day 10 (B). Note that necrosis takes ~3 days to fully develop after initial perfusion failure. d = day; * = $p < 0.05$ vs. control and Δ = $p < 0.05$ vs. EPO-high-dose.

Arteriolar diameter and blood flow

Animals of all groups showed an increase in arteriolar blood flow over time after flap elevation, although only the EPO-low-dose regimen was associated with a significant increase in arterial blood flow within the proximal (base) zone and the critically perfused central zone (transition zone) of the flap. This elevated blood flow in both zones could already be seen on day 1 after flap elevation, and the maximal blood flow was observed 5–7 days after induction of ischemia (Figure. 3(A) and (B)).

The increase in arteriolar blood flow is correlated with an overexpression of NO and increased arteriolar dilation, as supported by the fact that the immunohistological analysis for eNOS showed a more intense staining in all samples of EPO-treated animals (Figure 3(C)-(E)). On day 10, the EPO-treated groups had a semiquantitative score of 1.70 ± 0.18 (EPO-low-dose) and 1.63 ± 0.34 (EPO-high-dose) compared to 1.40 ± 0.26 (Control).

Capillary perfusion

In line with the gradual increase, respectively the maintenance of high values of arteriolar blood flow, both in the proximal and in the central zone of the flap, only low dose of EPO was associated with a maintained density of perfused capillaries (FCD) in the critically perfused central zone of the flap (FCD of ~250 cm/cm^2; $p < 0.05$). Neither EPO-high-dose nor untreated controls were able to maintain FCD at this level (Figure 4(A) and (B)). Furthermore, capillaries of the EPO-low-dose animals showed a significantly higher index of tortuosity in both the proximal and central zones of the flap, thus indicating a larger surface for oxygen delivery to the critically perfused tissue (Figure 4(C) and (D)).

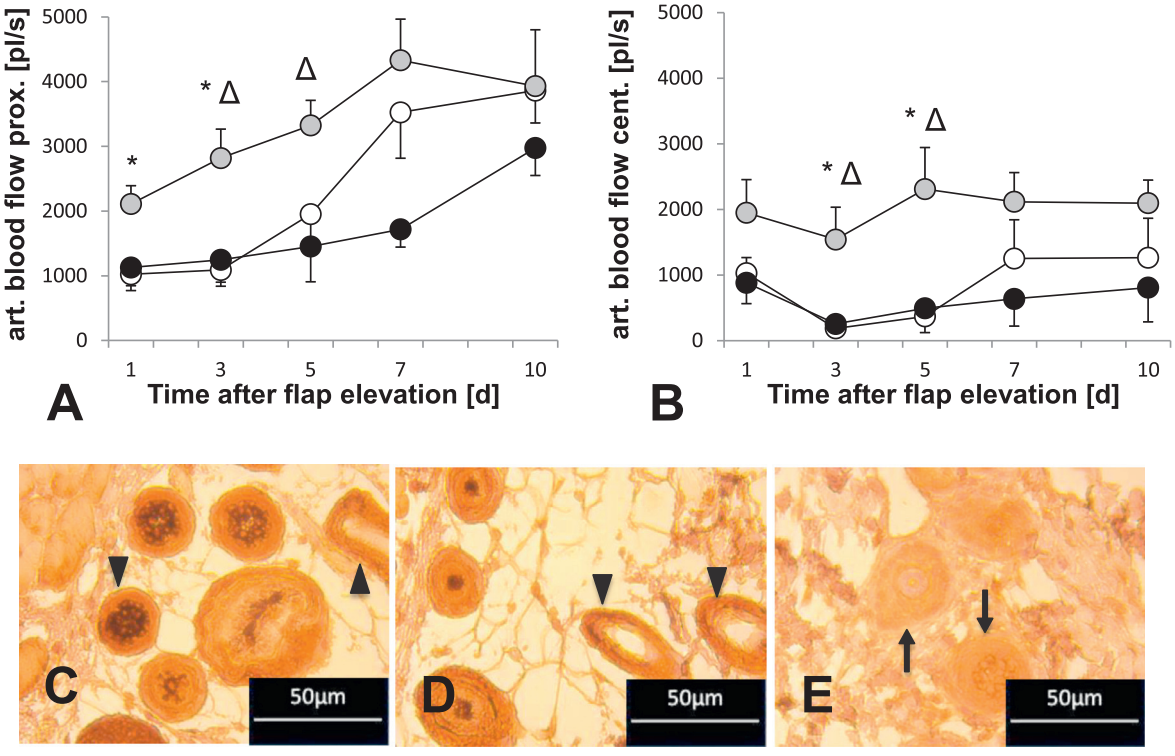

Figure 3 EPO-low-dose-treated animals (grey circles) show higher arteriolar blood flow within both the proximal (A) and the central (B) zone of the flap from day 1 after flap elevation. Controls: white circles; EPO-high-dose: black circles. d = day; * = $p < 0.05$ vs. control; Δ = $p < 0.05$ vs. EPO-high-dose.
Results of immunohistochemical staining for endothelial NO-synthase reveal more intense staining in EPO-treated animals (arrowhead; EPO-high-dose: C; EPO-low-dose: D) than untreated controls (arrow; E).

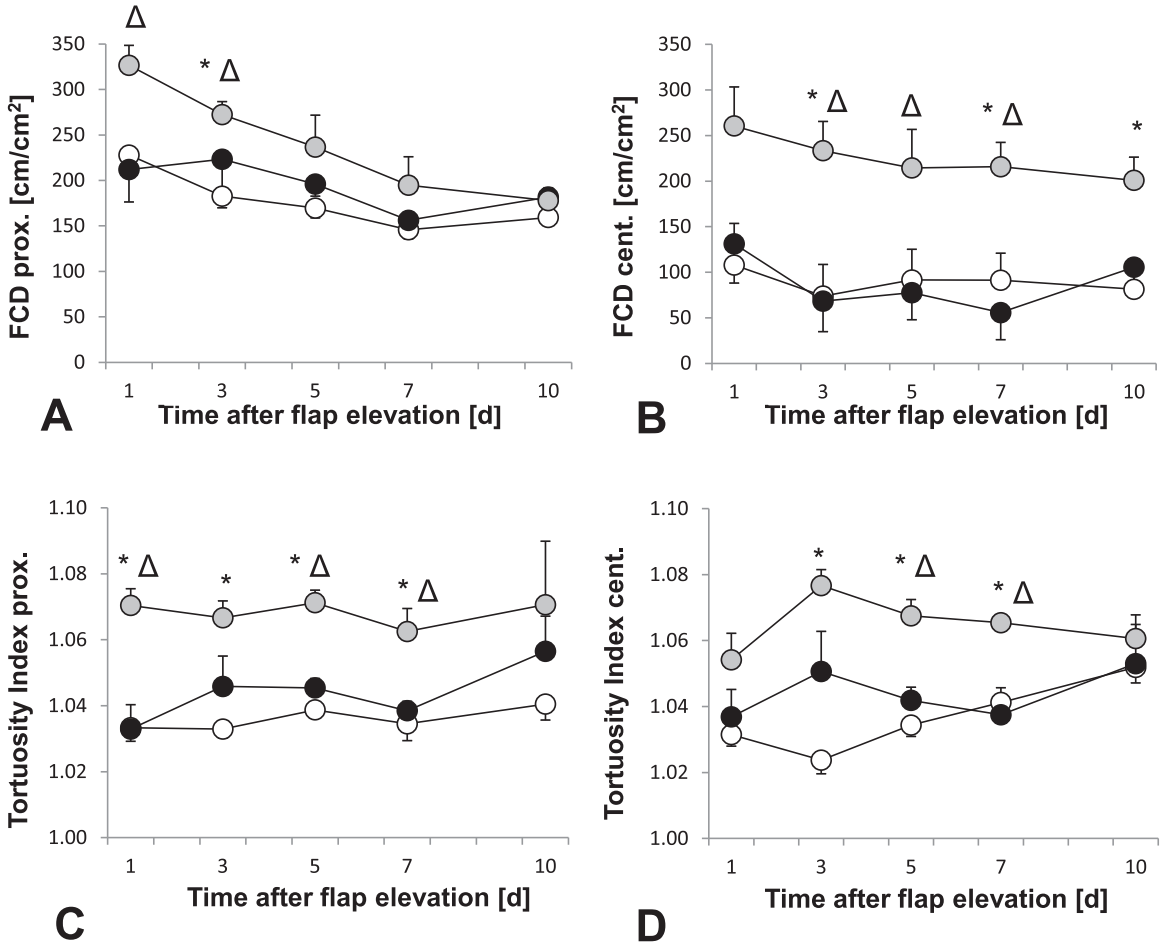

Figure 4 Functional capillary density (FCD) in the proximal zone of the flap (A) shows adequate nutritive perfusion, which is significantly higher in EPO-low-dose-treated animals (grey circles) than in control animals (white circles) and EPO-high-dose-treated animals (black circles). This difference becomes much more significant in the critically perfused central zone of the flap (B), with only EPO-low-dose being able to maintain adequate FCD.
In line with the FCD, EPO-low-dose animals also show a significantly higher index of tortuosity in both the proximal (C) and the central (D) zone of the flap, which indicates dilated and elongated microvessels. d = day; * = $p < 0.05$ vs. control; Δ = $p < 0.05$ vs. EPO-high-dose.

Angiogenesis

The formation of new functional capillaries at the zone of transition was observed from day 3 onward. EPO-treated animals developed a higher density of new capillaries more rapidly, which resulted in a significant difference only in EPO-low-dose-treated animals (Figure 5(A)).

The increased formation of new microvessels after EPO treatment was also associated with an increased expression of VEGF (Figure 5(B)-(D)). By day 10, the EPO-treated groups have reached a semiquantitative score of 1.80 ± 0.21 (EPO-low-dose) and 1.82 ± 0.22 (EPO-high-dose) compared to 1.33 ± 0.12 (Control).

Discussion

This study demonstrates that low doses of 125 I.U./kg bw EPO, administered every 12 h before and after the induction of acute persistent ischemia, are able to improve the survival of the critically perfused musculocutaneous tissue. This appears to relate to an increased arterial blood flow and maintained FCD, which improves nutritive perfusion. These findings are in line with those reported in previous works, which were used to determine our dosage regimen, showing that 500 I.U./kg bw is an effective dosage for tissue protection but with altered blood rheology, a finding in contrast to that reported in our current work using 125 I.U./kg bw.[22]

This effect seems to be mediated by an overexpression of NO resulting in sufficient perfusion of the ischemically challenged tissue and eventually reducing flap necrosis. Furthermore, repeated EPO administration was associated with an angiogenic response represented by the new formation of functional microvessels and an increased VEGF expression in treated tissues.

Flap elevation consistently causes acute persistent ischemia with impairment of perfusion of approximately 50-60% of the flap's surface, which results in tissue necrosis over the following 10-day observation period. Doses of EPO

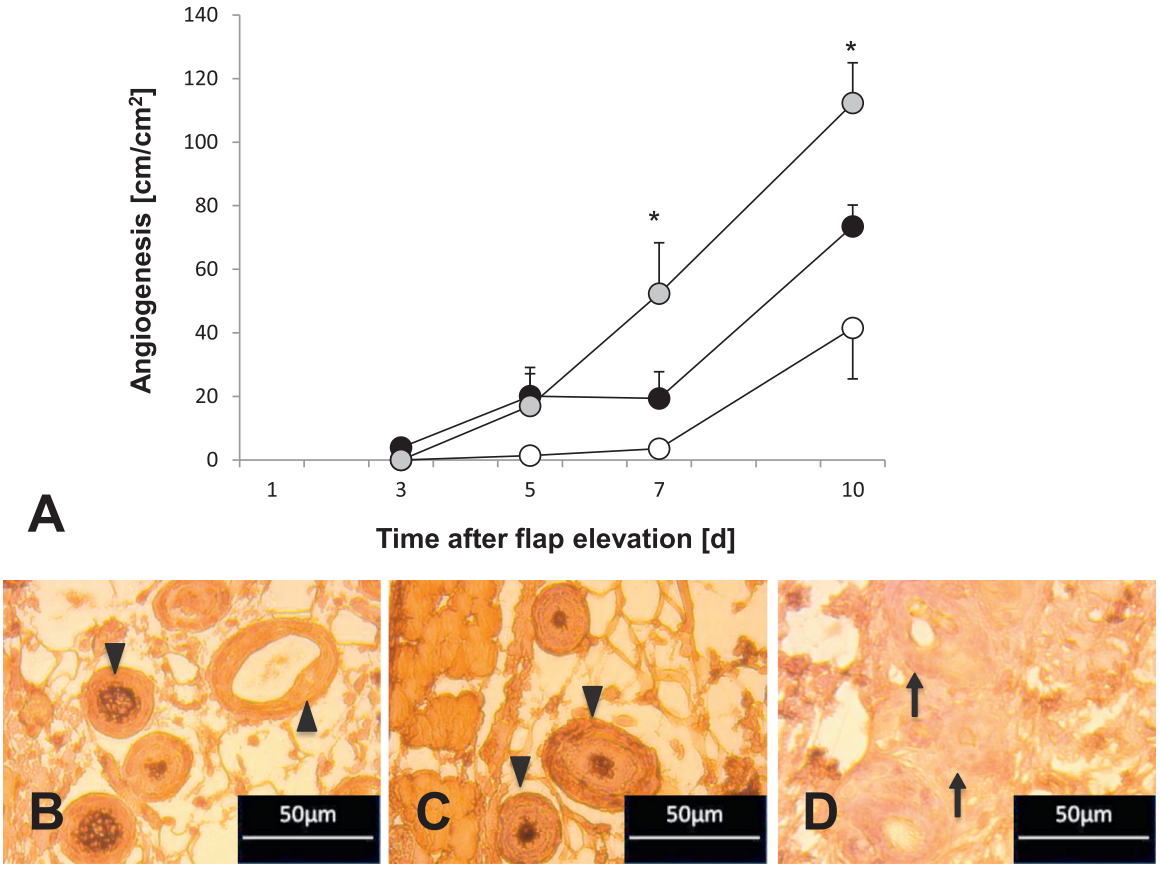

Figure 5 The formation of new functional capillaries is first observed between days 3 and 5. Of interest, control animals (white circles) and animals that were administered EPO at a high dose (black circles) show slower and less dense new microvascular formation than EPO-low-dose-treated animals (grey circles; A). d = day; * = $p < 0.05$ vs. control.
IHC staining for VEGF shows stronger staining in EPO-treated animals (arrowhead; B = EPO-high-dose, C = EPO-low-dose) than in untreated controls (arrow; D).

as low as 125 I.U./kg bw are sufficiently effective in preventing tissue necrosis without any associated increase in the hematocrit level. Thus far, EPO doses as low as 400–500 I.U./kg bw administered daily for 10–14 days have shown to be effective in increasing tissue survival, as observed after experimental burn wounds[32] and experimental flap elevation.[27] However, in both experimental settings, a significant increase in hematocrit was observed. This may counteract tissue survival because of hyperviscosity, increased peripheral vascular resistance, and microvascular thrombosis. This is in line with Saray et al., who demonstrated that daily administration of EPO starting 3 weeks before flap elevation, results in elevated hematocrit levels during flap elevation, and eventually impaired tissue survival.[33] Harder et al. were also able to demonstrate that hematocrit elevation due to repeated administration of 5000 I.U.EPO/kg bw negatively influenced tissue survival.[23] An EPO-mediated early peak in hematocrit may be one of the reasons for the lack of any protective effect in EPO administered at high doses when compared to the untreated control group.

This assumption might be strengthened by the fact that both EPO-treated groups were associated with an increase in eNOS-expression, while only animals that were administered EPO at a low dose showed an increase in arterial blood flow. A rapid increase in hematocrit and hence an altered rheology impairs the EPO-mediated and hematocrit-independent tissue-protective effects, if EPO is administered at high doses. The initial days after flap elevation are crucial for tissue survival, and the potential impact of EPO appears to mitigate against the reduced arterial blood flow after flap elevation at three to five days.

The early NO-mediated dilation of feeding arterioles and subsequently improved capillary perfusion is in line with the finding reported in the previous work of Rezaeian and coworkers, who showed a dilatory response in arterioles after preoperative administration of repetitive doses of 500 I.U. EPO/kg.[27,34] Similarly, Santhanam et al. and Wang et al. were able to demonstrate NO-mediated tissue-protective effects of EPO on cerebral circulation in rabbits[35] and aortic dilation in diabetic rats.[36]

The NO-mediated increase in arteriolar diameter leads to a higher arteriolar blood flow and increased FCD, subsequently resulting in improved perfusion of the tissue.

EPO at a low dosage showed a higher rate of FCD immediately after flap elevation than in the other experimental groups, which may indicate that EPO at this dosage is able to recruit pre-existing, nonperfused capillaries. Furthermore, an increase in capillary diameter and tortuosity indicates that the total microvascular surface for oxygen exchange between vessels and tissue is augmented to enhance perfusion of the ischemic tissues.

EPO-induced angiogenic response, as seen by newly formed microvessels in our results, may, however, play a more important role in subsequent tissue regeneration as described previously by Rezaiean et al. in the ischemic musculocutaneous tissue[27] and Rotter and coworkers in traumatized tissue.[37]

Limitations

The limitations of this study include the rather small experimental groups, which were based and experimentally powered on our previous studies, examining the first endpoint "tissue necrosis". It would have also been beneficial to measure hematocrit and red blood cell count at each time point for analysis, but we were limited by the total blood volume that could be extracted from repeat sampling of experimental animals. Because of the staining specificity, the immunohistochemistry was evaluated by grading and better stains could be used in the future to obtain measures more quantifiable.

Conclusion

In conclusion, the present study demonstrates that pharmacological manipulation of the ischemic musculocutaneous tissue with low doses of EPO of 125 I.U./kg bw repeatedly administered twice daily before and after flap elevation significantly reduces tissue necrosis without increasing hematocrit levels. In contrast, administration of potentially tissue-protective, high doses of EPO before and after induction of acute persistent ischemia compromises tissue survival by worsening blood rheology and impairing capillary perfusion of critically ischemic tissues.

The results of the present study show that tissue pre- and postconditioning with low doses of EPO that do not change hematocrit levels might represent a future approach to reduce ischemia-induced complications in flap surgery, including wound breakdown and tissue necrosis.

Alternatively, EPO derivatives without hematopoietic activity, yet including all tissue-protective properties of EPO, might even be preferable.[38,39]

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DS and AW contributed equally to this work.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.bjps.2019.01.003.

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