Original Article

A Comparative Study of Recombinant Human Basic Fibroblast Growth Factor (bFGF) and Erythropoietin (EPO) in Prevention of Skin Flap Ischemic Necrosis in Rats

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Abstract

Background: Impaired wound healing in ischemic tissues such as skin flaps resulting from inefficient perfusion is one major cause of complications in plastic surgery. In present experimental study, we investigated the effects of fibroblast growth factor-2 (FGF-2 or bFGF) and erythropoietin (EPO) in prevention of skin flap necrosis in rats.

Methods: 30 adult albino rats were randomized into 3 groups: in control group, normal saline solution; in EPO group, erythropoietin (100U/kg/day); and in FGF-2 group, fibroblast growth factor-2 (2.5μg/day) were injected subcutaneously in 3 daily consecutive doses in the designated flap areas before creating 4:1 random pattern skin flaps on the dorsum of animals. Areas of ischemic (S_I) and necrotic (S_N) zones were measured and compared in all groups one week after the flap creations.

Results: The necrotic zone (S_N), as well as the ratio of the necrotic zone to the total discolored zone ($S_N/[S_I+S_N]$) were substantially larger in the control group (41%±7%, 90%±6%) compared to the EPO (20%±2%, 42%±4%) and the FGF-2 (8%±2%, 19%±3%) groups ($p<0.001$). The differences in these values were also meaningful between the EPO and FGF-2 groups ($p<0.001$).Vascular density in ischemic area of the control group was less than those in the EPO and the FGF-2 groups; however, the differences were not statistically significant between any of the groups ($p>0.05$).

Conclusion: Local administration of erythropoietin or fibroblast growth factor-2 in skin flaps could remarkably increase tissue viability and accelerate the wound healing process. However, the therapeutic effect of fibroblast growth factor-2 in preventing the necrotic event in ischemic zones of skin flaps is much more considerable than that of erythropoietin.

Keywords: Basic fibroblast growth factor; erythropoietin; rat; skin flap survival

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Introduction

Tissue ischemia and subsequent necrosis continues to be one of the major challenges in plastic surgery. Impaired perfusion in ischemic tissues may lead to delayed or imperfect healing in various wound models such as diabetic feet, bed sores, skin flaps and other pathologic conditions. Recent advances in vascular biology have suggested that angiogenesis is a primary target for therapeutic interventions to prevent necrosis in ischemic wounds.[1] Studies using various angiogenic factors such as fibroblast growth factor (FGF)[2–6] and erythropoietin (EPO)[7–11] have shown that these factors can improve tissue survival in skin flaps via new blood vessel formation; however, the results of these studies have been equivocal depending on the drug doses used or timing of their administration; therefore, there is no universal agreement on the optimal dose, interval, and number of drug administrations. On the other hand, there are no studies directly comparing the effects of angiogenic factors in preventing ischemic necrosis in skin flaps or other areas within the body. The current experiment compares the potency of basic fibroblast growth factor (bFGF), also known as FGF-2, and EPO in skin flap survival.

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Materials and Methods

The experiment was performed following Institutional Board approval and in accordance with the 'Guide for the Care and Use of Laboratory Animals' (NIH publication No. 85-23, revised 1996). Thirty Sprague-Dawley male rats (weighing 250-300 g) were included in the study. The animals were housed in separate clean cages at a constant temperature of 20-24°C under 12 h light/12 h dark cycles with chow pellets and tap water available ad libitum. The specimens were observed daily until the end of the study.

After anesthetizing the rats with intraperitoneal injections of ketamine hydrochloride (70 mg/kg) and xylazine (7 mg/kg), the skin was properly shaved by electric clippers. During surgery, animals were kept on warming blankets and strict aseptic measures observed. Full-thickness rectangular skin incisions (two, 8 cm parasagittal incisions and one, 2 cm cephalad horizontal incision) were made on the dorsum of each animal (Figure 1), then immediately sewed back with interrupted 4-0 nylon sutures (day 0). Three days later, the animals underwent a second surgery and the subdermal tissues of the designated flap areas were undermined, thus creating 4:1 caudally-based random pattern skin flaps that included the

entire thickness of the skin and skin muscle (panniculus carnosus). Dimensions of the flaps were 8 × 2 cm with their bases located at the level of the iliac crests.

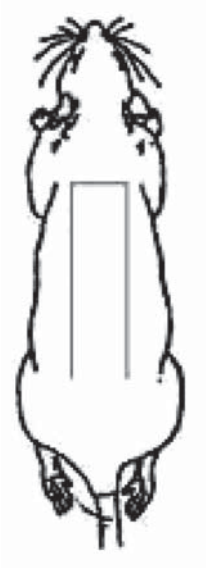

Figure 1. Schematic diagram of dorsal skin incisions designating outlines of the flap (8×2 cm). Drugs were injected subcutaneously into the cephalad half of the flap.

The animals were randomly assigned into three groups of ten samples as follows: group 1 (control) received 0.9% NaCl solution; group 2 (EPO) was administered 100 U/kg/day of recombinant human erythropoietin (Janssen-Cilag, Switzerland); and group 3 (FGF-2) received 2.5 µg/day of basic fibroblast growth factor (Peprotech, USA). All substances were administered by subcutaneous injections (1 ml) at the distal halves of the designated flap areas, once daily, for three consecutive days following the initial surgery.

On day 10, the animals were euthanized by lethal inhalation of chloroform. The skin at the site of the flaps was excised, and simultaneously, a photograph of each flap was taken. Flap necrosis was judged on the basis of skin blackening and induration at the cephalad area. The border between necrotic and discolored but still viable (ischemic) areas was determined by a low power microscope. The areas of tissue necrosis (S_N) and ischemia (S_I) were calculated by ImageJ version 1.34 software (NIH, USA) and expressed as relative percentages to the total flap area.

Two skin strips, 0.5 × 1.0 cm in size that contained the wound margins were sampled from each specimen at distances of 2 cm and 4 cm from the distal portion of the flap. The skin samples were fixed in 10% formalin, embedded in paraffin, and 5 µm sagittal sections were obtained for hematoxylin and eosin staining. All slides were histologically examined under high power (400x) light microscopy by two experts blinded to the study. The number of blood vessel sections was measured in 100 microscopic fields. Fibroblast and endothelial cell proliferation was also evaluated using a four-scale scoring system.

Data were analyzed by ANOVA one-way test using the SPSS ver. 14.0 statistical software (SPSS Inc., USA). Bonferroni post hoc test was performed for multiple comparisons when appropriate. Data have been presented as mean ± SD. *P* values less than 0.05 are statistically significant.

Results

After raising the flaps on day 3, the distal portion of the flaps invariably underwent cyanotic discoloration in all animals (Figure 2). The area of discoloration consisted of a zone of apparent clinical necrosis (S_N), as well as a zone of ischemic but still viable tissue (S_I), as judged by the skin color, skin stiffness and microscopic examination.

According to planimetric examination on day 10, the percents of ischemic plus necrotic areas ($S_N + S_I$) were approximately the same among the study groups, as follows: control (45.61% ± 7.47%), EPO (46.95 % ± 5.35%) and FGF-2 (40.03% ± 5.21%). There were no meaningful differences between the groups ($P = 0.06$); however, the amount of necrosis in the control group (41.07% ± 7.01%) was significantly higher than those in the EPO (19.79% ± 1.56%) and FGF-2 (7.67% ± 1.79%) groups ($P < 0.001$). The difference between the EPO and FGF-2 groups was also statistically significant ($P < 0.001$). The relative area of ischemic but apparently still viable zone in the control group (4.54 %± 3.08%) was significantly lower than those in the EPO (27.16% ± 4.60%) and FGF-2 (32.36% ± 4.34%) groups ($P < 0.001$). The difference between the EPO and FGF-2 groups was also meaningful ($P = 0.024$). The ratio of the necrotic zone to total discolored area was 90.20% ± 6.29% in the control group, 42.45% ± 3.90% in the EPO group, and 19.13% ± 3.42% in the FGF-2 group ($P < 0.001$ for comparison between all groups).

On microscopic examination (Figure 3), the injured epidermis was visible in the control group specimens. Diffuse accumulation of inflammatory cells and widespread granulation tissue was also noted. There were variable degrees of epidermal healing in the EPO and FGF-2 groups. The amount of inflammatory cells and granulation tissue in these two groups was strikingly lower. There was organized fibrous tissue which occupied most of the dermis, especially in the FGF-2 group. The number of endothelial cells was also much higher in the latter two groups (data not shown). A slightly higher number of vessel sections were counted under high power microscope (400x) in the FGF-2 group specimens. However, the differences among the groups were not statistically significant (43.49 ± 15.22 for the control group, 45.25 ± 25.34 for the EPO group, and 51.74 ± 5.47 for the FGF-2 group; $P = 0.56$).

Discussion

After introduction of the animal model for random skin flaps by McFarlane in 1965,[12] many substances and growth factors with angiogenic potential have been examined with the intent to improve wound healing and prevent necrosis in the distal ischemic portion of the flap.[13–18] FGF-2 [2–6,19–22] and EPO[7–11,23] are among the drugs widely used in these studies and could be considered as possible candidates for clinical applications. FGF-2 is the most potent angiogenic factor in the FGF family; its effects are exerted in all steps of blood vessel formation and it plays an important role in wound healing by the induction of migration of macrophages, fibroblasts and endothelial cells into injured tissues, collagen, fibronectin, and proteoglycan synthesis, wound contraction, and in the formation of a new epidermis. It is also implied that FGFs are notable mediators in the growth and development of various tissues and hematopoiesis.[13–18,24] EPO is traditionally known for its effects on mitosis induction, differentiation and activation of a variety of cell lines

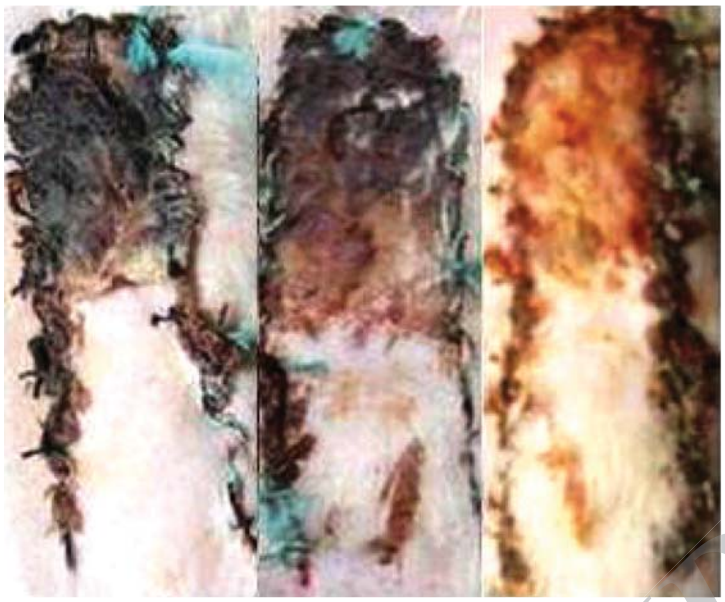

Figure 2. Photograph of necrotic (black) and ischemic (reddish brown) discoloration in the control (left), EPO (center), and FGF-2 (right) groups.

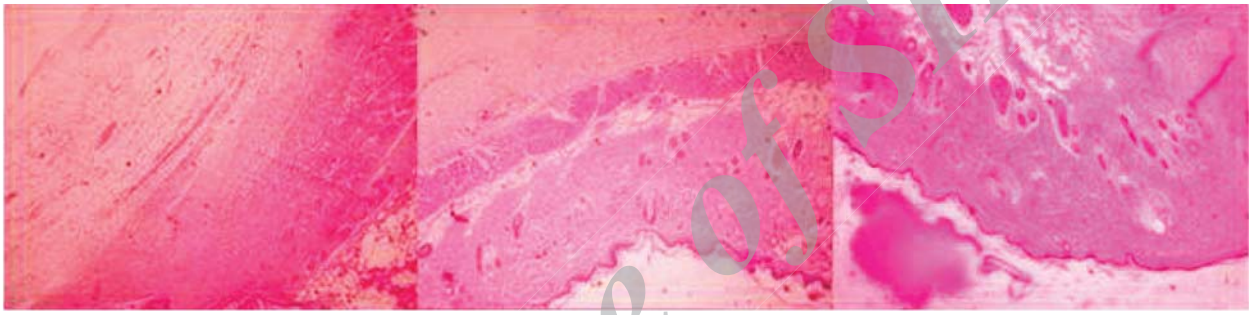

Figure 3. Microscopic evaluation of the specimens at the end of the study shows extensive granulation tissue interspersed by fibroblasts in the control group (left), while in the EPO (center) and FGF-2 (right) groups, there are moderate to high grades of fibroblast proliferation, respectively, and an accelerated process of wound healing.

that include mesangial cells, cardiac and smooth muscles, and vascular endothelium. After discovery of EPO receptors in endothelial cells, this substance has been considered as a growth factor and it has been proposed that EPO can directly or indirectly act in certain steps of angiogenesis.[25,26]

Studies administering FGF-2 at a dose of 2-2.7 μg in single or multiple injections have all demonstrated promising results in terms of increasing flap survival.[5,6,20,22] Results of the experiments using EPO in preventing skin flap necrosis have also been favorable;[9–11] however, the drug dose in these studies has varied significantly, mostly from 50 to 400 U/kg. Saray et al.[11] have reported that high (150 U/kg) or chronic (beyond one week) use of EPO detrimentally effects flap viability with results that are not different from the control group. They have concluded that the best results could be achieved by short-term use of low or therapeutic (50-100 U/kg) doses of EPO. In a pilot study previously performed (data not shown), we have also observed that increasing the dose of EPO from 100 U/kg to 300 U/kg increases flap necrosis. Results of the current study were comparable to studies which have used similar doses of drugs and similar methods of administration;[5,6,9–11] however, most of these studies administered drug injections after the flap was created. We administered the substances prior to creating the flaps in order to provide sufficient time for the drugs to exert their effects. The designated flap length to width ratio in our study was 4:1 in order to invariably cause significant necrosis in the distal portion of the flap in the control group. We have noted that the discolored area in the flaps could be further divided into two zones; a distal blackened and indurated portion with clear clinical necrosis and a more proximal ischemic part with apparently viable tissue. We have measured these two zones separately and concluded that although the area of total discoloration might not vary significantly among the study groups, there would be an obvious reduction in the area of the apparent necrotic zone in the FGF-2 and EPO groups. Particularly, most of the discolored area in the FGF-2 group consisted of ischemic but viable tissue. However in the control group, the necrotic zone nearly covered the entire discolored area. In the EPO group, there was a well-defined border between the two zones.

Based on the finding that the numbers of visible blood vessels in high power (400x) light microscopy did not meaningfully increase in the EPO and FGF-2 groups, it could be implied that the increased flap viability which followed administration of these substances could be due to improvement of tissue perfusion in microcirculation and very small forming vessels which were not countable by our assessment tools. Hence the use of further assessment methods (such as immunohistochemistry and laser Doppler) is recommended in future similar studies for a more quantitative evaluation of angiogenesis and/or other possible underlying mechanisms.

In conclusion, FGF-2 and EPO have been both shown to, with variable degrees, prevent the flap from undergoing necrosis in an

area threatened by tissue ischemia. The therapeutic effects of FGF-2 are far more advantageous in terms of preventing flap necrosis and increasing tissue viability during the 10 day study period; however, EPO still remains as a beneficial choice in improving flap survival.

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