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Erythropoietin Improves the Healing of Skin Necrosis Resulting From Doxorubicin Extravasation in a Rat Model

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ABSTRACT

BACKGROUND: Doxorubicin is an antineoplastic agent that causes skin necrosis when extravasated. Various agents have been tried to reduce tissue damage owing to extravasation. Erythropoietin (EPO) is an obligatory growth factor for red blood cells and has beneficial effects on wound healing.

OBJECTIVE: The aim of this study was to test the hypothesis that local EPO injection can prevent and improve healing of necrosis at the doxorubicin injection site in rats.

METHODS: We used 31 female Sprague-Dawley rats. The dorsal area of each rat was shaved, and 2 mg of doxorubicin in 0.5 mL saline was injected intradermally. The rats were then divided into 3 groups: control; control with intradermal injection of saline; and treatment, which received an intradermal injection of EPO. EPO in saline was injected into 4 quadrants of the same site where doxorubicin was injected 1 hour before. The rats were monitored and the area of each ulcer was measured. Skin biopsies were excised at the end of 4 weeks using anesthetic pentobarbital. Inflammation, edema, epithelization, neovascularization, necrosis, fibroblast proliferation, and collagen synthesis were evaluated and compared between groups.

RESULTS: The average areas of the lesions were significantly smaller in the EPO-injected rats ($P = 0.03$). The histopathologic evaluation revealed that the scores for epithelization, neovascularization, fibroblast proliferation, and collagen synthesis were higher ($P < 0.001$, $P < 0.001$, $P = 0.002$, and $P = 0.04$, respectively) and the score for necrosis was lower ($P < 0.001$) in the EPO-injected group than in both the saline-injected and control groups.

CONCLUSIONS: In this study using female Sprague-Dawley rats, EPO treatment improved the healing of skin necrosis caused by doxorubicin injection. This finding may lead to a new therapeutic approach for the management of skin necrosis caused by doxorubicin extravasation. (*Curr Ther Res Clin Exp.* 2011;72:141-149)

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INTRODUCTION

Doxorubicin is an anthracycline antibiotic widely used as an antineoplastic agent.[1] It is used in standard chemotherapeutic regimens for many hematopoietic malignancies and solid tumors. Leakage of intravenous infusions from the intravascular region into the interstitial space is called extravasation, and local skin necrosis after extravasation of doxorubicin is a common problem.[2] In large series, the incidence of necrosis owing to extravasation has been reported to occur in 6% of cancer patients treated with chemotherapy.[3] The lesion starts as a local toxicity with pain and erythema and may progress to tissue necrosis.[2] Doxorubicin causes cellular death with direct toxic effects to living cells and results in the release of a doxorubicin-DNA complex into the intercellular space.[2] This process prevents the release of the cytokines and growth factors that participate in wound healing.[4,5] Thus, this type of ulcer heals much more slowly than other types.[6]

Wound healing is a complex process that is initiated by tissue injury and involves inflammation, proliferation, migration, angiogenesis, matrix synthesis, collagen deposition, re-epithelization, neovascularization, and formation of granulation tissue.[7,8] The process uses an interplay of cells, mediators, growth factors, and cytokines.[9] It begins with clotting and the recruitment of inflammatory cells and proceeds to a highly proliferative state. During the proliferative phase angiogenesis occurs, and the formation of new blood vessels provides oxygen and nutrient delivery.[9] Healing is also concomitant with the release of angiogenic growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor, and platelet-derived growth factor.[10]

Erythropoietin (EPO) is a 34-kDa glycoprotein and an obligatory growth factor for red blood cell proliferation, maturation, and differentiation.[7,11] The biological effects of EPO are mediated by its specific cell-surface receptor, erythropoietin receptor (EPOR), which is a type of cytokine receptor that is present on erythroid progenitor cells as well as several nonhematopoietic cell types.[8,12,13] Many studies suggest that the functions of EPO and EPOR are not strictly limited to red blood cells.[14] EPOR expression has been shown in other tissues, such as the kidneys, muscle cells, and intestine, and is associated with cellular proliferation. Vascular endothelial cells also express EPOR, and studies have shown that EPO stimulates angiogenesis.[8] EPO can stimulate the first phase of angiogenesis, which includes increased cellular motility, breakdown of the cell matrix, and cellular proliferation.[12] In addition, EPO is known to induce a pro-angiogenic phenotype in cultured endothelial cells and to stimulate neovascularization in the chick chorioallantoic membrane.[15] Carlini et al[16] demonstrated that EPO may stimulate vessel growth of rat aortic rings embedded in culture. In a review by Burger et al,[17] the angiogenesis-promoting effect of EPO, which is independent of its erythropoietic effect, was emphasized, although all the molecular mechanisms could not be explained. EPO also improves wound repair by promoting revascularization and microvascular remodeling.[18] Moreover, the interaction between EPO and VEGF may be important in the complex phenomenon of wound healing.[9]

Many treatment options, such as local and systemic pharmacological agents and surgery, have been tried for skin necrosis caused by doxorubicin.[2] In the present study, we investigated the potential beneficial effects of local EPO injection on preventing and healing tissue damage in a rat model of doxorubicin extravasation.

MATERIALS AND METHODS

Study Design

The experiment was performed in 31 female Sprague-Dawley rats, weighing 200 to 250 g, supplied by Eskisehir Osmangazi University Medical Faculty, Medical and Surgical Research Center (Eskisehir, Turkey). Laboratory food and water were provided ad libitum. Following ether anesthesia, 2 mg of doxorubicin in 0.5 mL saline was injected intradermally into the dorsal skin of all animals. The animals were then divided into 3 groups: control, control with saline, and treatment. In the control group (n = 11), no additional injection was given. In the control group with saline (n = 10), 0.1 mL saline was administered intradermally into 4 quadrants of the doxorubicin injection site 1 hour after the doxorubicin injection. In the treatment group (n = 10), EPO (400 IU/kg epoetin beta [recombinant human erythropoietin]) in saline was administered intradermally into 4 quadrants of the doxorubicin injection site 1 hour after the doxorubicin injection. The total volume of EPO and saline was equal to the volume of saline injected into the control with saline group.

The development and progression of skin necrosis was monitored at a regular interval. The area of necrosis was measured every 7 days by an investigator who was blind to group allocation. Ulcers developed in all doxorubicin-injected animals. At the end of 1 month, we calculated the area of each ulcer by measuring the 2 greatest perpendicular diameters of the lesion and using those measurements in the formula reported in the study by Vargel et al[19]: $(a \times b)/2$.

At conclusion of the experiment, 1 month after doxorubicin injection, the entire ulcerated skin and underlying tissues from all animals were excised under pentobarbital anesthesia (80 mg/kg injected intraperitoneally) and the remaining dorsal areas were sutured. The tissues underwent routine tissue processing procedures for paraffin embedding following fixation in 10% formalin and were stained with hematoxylin and eosin.

The study design was approved by the local ethics committee and is in accordance with the Declaration of Helsinki.

Histopathologic Evaluation

The pathologist who examined the slides was blind to group allocation. Under a light microscope, inflammation, edema, epithelization, neovascularization, necrosis, fibroblast proliferation, and collagen synthesis were evaluated and compared separately for each group. The following scoring system was used in the evaluations: 0 = absent; 1 = minimal; 2 = mild; 3 = severe.[20]

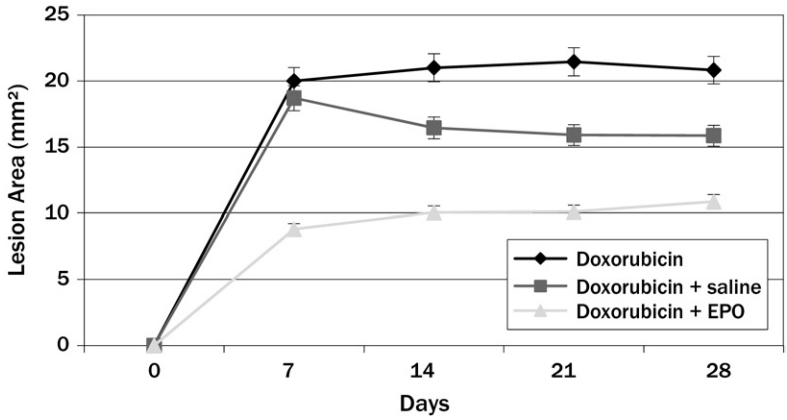

Figure. Weekly comparison of lesion areas after administering doxorubicin, doxorubicin plus saline, or doxorubicin plus erythropoietin (EPO) in this study of the effects of EPO on skin necrosis from doxorubicin extravasation in rats.

Statistical Analysis

Statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois), and the results were analyzed using the Kruskal-Wallis test and the Tukey post-hoc (nonparametric) test.

RESULTS

Each week, the largest lesions observed were in the control group ($P = 0.03$) (Figure). The largest mean ulcer area measured at the end of week 3 was in the control group. Lesion areas were significantly smaller in the treatment group, in which EPO was administered ($P = 0.01$) (Figure). Final measurements were taken on the 28th day of the experiment. At that time, the largest ulcer area measured was in the control group, whereas the smallest area measured was in the treatment group (Table I).

The histopathologic evaluation revealed that the scores of epithelization and neovascularization were significantly higher in the treatment group than in the control and control with saline groups (both, $P < 0.001$), whereas the scores for fibroblast and collagen proliferation for the treatment group were only significantly higher than the scores for the control group ($P = 0.002$ and $P = 0.04$, respectively). However, the scores for inflammation ($P = 0.243$) and edema ($P = 0.130$) were not significantly different between any of the groups ($P > 0.05$). Finally, the scores for necrosis were significantly lower in the EPO-injected group than in the control groups ($P < 0.001$) (Table II).

DISCUSSION

Doxorubicin is a widely used antineoplastic agent, and local skin necrosis after the extravasation of doxorubicin is a common and serious problem in cancer patients.[2] In

Table I. Comparison of the lesion areas measured each week after administering doxorubicin, doxorubicin plus saline, or doxorubicin plus erythropoietin (EPO) in this study of the effects of EPO on skin necrosis from doxorubicin extravasation in rats.

	Lesion Areas (mean ranks)			
Group	Week 1	Week 2	Week 3	Week 4
1: Doxorubicin	20.09	21.00	21.45	20.82
2: Doxorubicin + saline	18.70	16.45	15.90	15.85
3: Doxorubicin + EPO	8.80*	10.05†	10.10†	10.85†
P	0.007	0.021	0.013	0.03

*$P < 0.05$: Group 3 is significantly different from groups 1 and 2.
†$P < 0.05$: Group 3 is significantly different from group 1.

the present study, EPO appeared to accelerate the healing of the wound caused by doxorubicin extravasation.

Various agents have been tested for their ability to reduce the tissue damage that results from doxorubicin extravasation. Melatonin is an effective alternative treatment because of its potent antioxidant properties, such as oxygen free radical scavenging, upregulation of antioxidants, and downregulation of pro-oxidant enzymes.[21] Dapsone is a leukocyte inhibitor and effectively reduces inflammation, but it plays only a small role in the treatment of doxorubicin extravasation injuries.[22] Heparin fractions have been tested and have been reported to have beneficial effects on ulcer size and rates by increasing new vessel formation and inhibiting coagulation.[23] Dexrazoxane, a topoisomerase II catalytic inhibitor that antagonizes doxorubicin, has been approved by the US Food and Drug Administration and protects against the free radical toxicity induced by anthracyclines.[11] The study by Vargel et al[19] monitored the effects of granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) on the healing of skin necrosis caused by doxorubicin extravasation. Both G-CSF and GM-CSF induce endothelial cell proliferation and differentiation, resulting in angiogenesis.[19]

EPO is known to have an angiogenic effect, which is an important step in wound healing.[20] It has proven beneficial effects on wound healing in genetically diabetic mice[9] and ischemic skin wounds.[5] We used the same study design used by Vargel et al,[19] and we chose EPO because of its possible beneficial effects on wound healing based on previously reported data.[5,6,9] In the present study we used the same dose of EPO that Galeano et al[9] used in the experimental study, in which EPO was reported to stimulate wound healing in genetically diabetic mice. We chose to use a single dose of EPO because it has been reported that a single dose accelerates wound epithelization, reduces wound cellularity, and induces the maturation of new microvascular formation, whereas using repetitive doses of EPO impairs the healing

Table II. Mean values of histopathologic scores and comparisons between the doxorubicin, doxorubicin plus saline, and doxorubicin plus erythropoietin (EPO) groups in this study of the effects of EPO on skin necrosis from doxorubicin extravasation in rats.

	Light Microscopic Examination Scores (mean ranks)						
Group	Inflammation	Edema	Epithelization	Neovascularization	Necrosis	Fibroblast Proliferation	Collagen Formation
1: Doxorubicin	18.45	15.50	10.73	9.05	21.82	10.59	13.50
2: Doxorubicin + saline	16.75	20.05	13.00	13.75	18.15	14.80	15.05
3: Doxorubicin + EPO	12.55	12.50	25.90*	25.90*	7.45*	23.15†	19.70‡
P	0.243	0.130	<0.001	<0.001	<0.001	0.002	0.04

*$P < 0.001$: Group 3 is significantly different from groups 1 and 2.

†$P < 0.005$: Group 3 is significantly different from group 1.

‡$P < 0.05$: Group 3 is significantly different from group 1.

process, as indicated by delayed epithelization, high wound cellularity, and lack of maturation of the microvascular networks.[24]

We observed that doxorubicin caused a smaller ulcer in the group in which saline was administered than in the group in which only doxorubicin was injected. This result is in agreement with results of the study by Vargel et al.[19] However, Vargel et al[19] also observed that CSFs, like EPO, were statistically more effective, and the authors hypothesized that growth factors regulate and coordinate the important steps of wound healing by increasing angiogenesis. The sizes of the lesions in each group did not change after the first week. The EPO-injected group had the smallest lesion areas from the begining of the study. Once doxorubicin is extravasated, it can persist in tissue for weeks[2]; therefore, the stability of the necrosis area supports the hypothesis that EPO prevents the enlargement of tissue damage.

At the end of the experiment, ulcerated skin and the underlying tissues were excised to histopathologically evaluate inflammation, edema, epithelization, neovascularization, necrosis, fibroblast proliferation, and collagen formation. These parameters are the most important steps in skin lesion formation and wound healing. In previously published studies, investigators have stated that doxorubicin causes direct toxic effects in tissues without causing inflammation. In support of the literature, this study did not find significant differences in inflammation between the tested groups.[25]

Neovascularization and epithelization as important steps of wound healing were significantly better in the EPO-injected group. Jaquet et al[26] found that EPO stimulates capillary proliferation 220% more than physiologic conditions. Buemi et al[5] found that recombinant human erythropoietin (rHuEPO) induced the healing of ischemic skin necrosis by increasing neovascularization and dermal regeneration by promoting the proangiogenic phenotype and stimulating early and late angiogenic features. In an experimental study on diabetic rats, Galeano et al[9] observed that rHuEPO improves impaired wound healing both in diabetic and nondiabetic rats. These data support the hypothesis that EPO may have a physiologic role in angiogenesis, which is the most important step of wound healing.

In a separate study, Galeano et al[6] reported that rHuEPO enhanced burn wound healing by reducing inflammatory cell infiltration and edema and that it also stimulated dermal and epidermal regeneration, fibroblast proliferation, and new capillary formation. Sayan et al[20] investigated the effects of systemic EPO administration on wound healing. In the EPO-treated group, increased re-epithelization, collagen synthesis, and VEGF synthesis were reported. The histopathologic evaluation of the tissue samples revealed that rHuEPO stimulates dermal organization and angiogenesis as a result of induced VEGF synthesis, as stated in previously published studies.[20] We had similar results in our study, such as increased fibroblast proliferation, collagen synthesis, and epithelization in the EPO-treated group.

The limitations of this study are the short time period between doxorubicin extravasation and EPO injection and doxorubicin injection and the administration of EPO before the onset of skin necrosis. The small number of rats used in this study may be another limiting factor.

CONCLUSIONS

To our knowledge, this is the first study in which EPO has been used as an alternative treatment for doxorubicin-induced tissue necrosis. In the 31 female Sprague-Dawley rats tested, we observed that EPO prevents the augmentation of the necrotic area and improves the healing of skin necrosis caused by doxorubicin injection. Through clinical trials, EPO injection may be proven to be an alternative treatment for preventing skin necrosis resulting from doxorubicin extravasation.

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Dr. Yaşar Bilge designed and mainly performed the study, searched the literature, created the figures and tables, wrote the main document, and revised the manuscript. Dr. Dündar excised the biopcies, made pathological evaluation, helped to write material and methods, and helped to revise the manuscript. Mr. Şahin Mutlu performed the statistical analysis, helped to write material and methods, and helped to revise the manuscript. Dr. Gülbaş designed the study, helped to write the main document, helped to create the figures and tables, and helped to revise the manuscript.

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