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ERYTHROPOIETIN ACCELERATES FRACTURE HEALING AN EXPERIMENTAL ANIMAL STUDY

Eritropoetin Kırık İyileşmesini Hızlandırıyor Deneysel Hayvan Çalışması

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ABSTRACT

Aim: The efficacy of erythropoietin (EPO) on wound healing has been shown before. There is limited data about the efficacy of EPO on fracture healing. In this study, the efficacy of EPO on closed forearm fracture healing is searched by an experimental model.

Material and method: Twenty eight rats were picked randomly and divided into EPO and Control groups. On the first day, fracture was performed on the right ulna and radius of all rats. 500 U/kg daily low dose EPO was administered for each rat in EPO group and the same volume of isotonic sodium chloride solution was given intraperitoneally in the control group for five days. Seven rats from each group were sacrificed at the end of the first week; the others were sacrificed at the end of the third week.

Results: At the end of the first week and third week, bone healing scores of EPO group is significantly higher than the control group according to the histological, clinic and radiologic analysis.

Conclusion: EPO could have positive effects on healing of forearm fracture on rats in acute and subacute period. Low dose EPO may be a new protective agent against delayed union or nonunion in the risk population to go or not to go to surgical treatment.

Key Words: Erythropoietin, fracture healing, rat.

ÖZET

Giriş: Eritropoetin'in yara iyileşmesi üzerine olan etkisi daha önce gösterilmiştir. Kırık iyileşmesi üzerine olan etkisi üzerine sınırlı sayıda bilgi mevcuttur. Bu çalışmada eritropoetinin kapalı kırık iyileşmesi üzerine olan etkileri deneysel bir model kullanılarak araştırılmıştır.

Yöntem: Yirmisekiz rat alınıp rastgele şçilerek Eritropoetin ve Kontrol grupları oluşturuldu. Çalışmanın ilk gününde tüm ratların sağ önkollarında kapalı ulna ve radius cisim kırıkları oluşturuldu. Beş gün boyunca Eritropoetin grubundaki ratlarda 500 U/kg düşük doz eritropoetin intraperitoneal olarak verilirken; Kontrol grubundaki ratlarda ise aynı hacimde izotonik intraperitoneal olarak verildi. Gruplardaki yedişer det rat ilk hafta sonunda, kalan yedişer adet ratda üçüncü haftanın sonunda sakrifiye edildi.

Bulgular: Birinci ve üçüncü hafta sonunda kırık iyileşme skorları histolojik, radyolojik ve klinik olarak değerlendirildiğinde Eritropoetin grubunda Kontrol grubuna gore belirgin olarak daha yüksek olduğu saptandı.

Sonuç: Ratların kapalı önkol kırıklarının iyileşmesi üzerine akut ve subakut peryotta eritropoetinin pozitif etkileri olabilir. Düşük doz eritropoetin kullanımı cerrahiye gidecek veya gitmeyecek olan riskli hasta gruplarında kaynama gecikmesi veya kaynamamaya karşı koruyucu ajan olarak verilebilir.

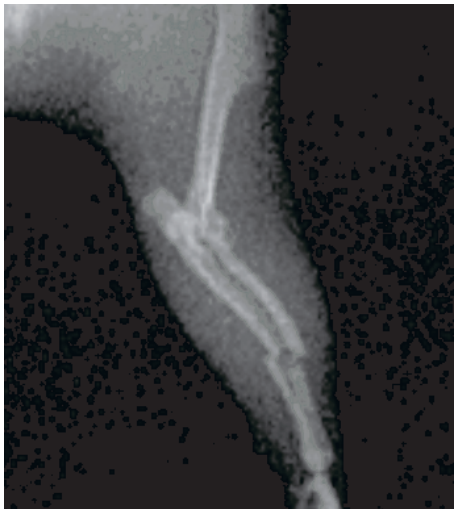
Anahtar kelimeler: Eritropoetin, kırık iyileşmesi, rat.

Introduction

Fracture healing is a physiologic process assisted by many organised cell and cell products. Biologic, mechanic and environmental conditions play effective role on bone metabolism. The blood supply around fractured bone and better fixation methods are known as main reasons for better fracture healing (1). Exact reason for inadequate bone healing remained unclear. Nonsteroid anti-inflammatory drug uptake is a reason for inadequate fracture healing like smoking especially in long bones by inhibition of angiogenesis (2).

EPO is the most responsible hormone on erithropoesis during fetal, neonatal and adult life. Secretion of this hormone is tethered by tissue hypoxia. It is secreted against anemia and hypoxia (3). In clinical practice it is usually used in the treatment of anemia related with chronic kidney insufficiency and premature anemia. EPO has further been demonstrated to promote

Figure 1: Fracture was corrected radiologically.



angiogenesis and tethered to accelerate wound healing. It plays a role in wound healing by its' proangiogenic effects (4).

There is limited data regarding its efficacy in fracture healing. Therefore the aim of this study was to investigate the efficacy of erythropoietin on fracture healing by using a closed radius and ulna fracture model on rats. Histologic, radiologic and clinic examination scores are used in the study from the literature as objective examination models (5-7).

Material and methods

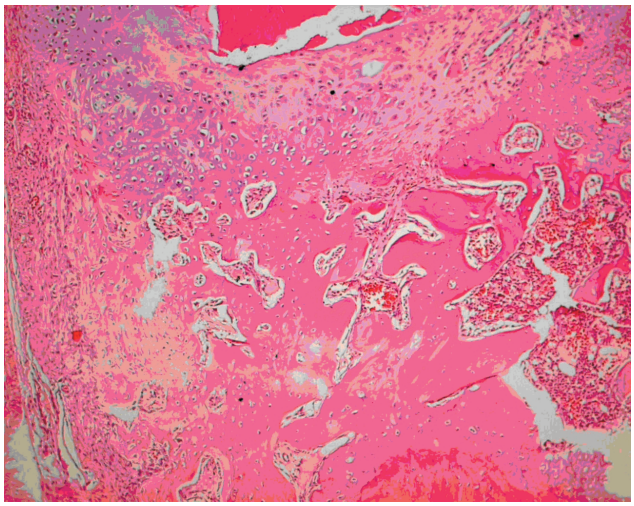
All experiments were performed under protocols approved by the local animal use and care committee and complied with the standards in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources and published by the National Academy Pres.

Twenty eight rats were randomly divided into two groups as the EPO group and the control (saline) group. Fourteen rats were selected randomly for each group. The animals were kept in temperature-controlled rooms (24°C) on a 12-hour light-dark cycle with access to rodent chow and bottled tap water ad libidum. Rats stayed healthy throughout the study period. Rats were anesthetized by an intraperitoneal injection of 15 mg/100 bodyweight (bw) ketamine (Ketalar, 50mg/ml, Eczacıbaşı). Fracture was performed on the right ulna and radius of all rats by manual three point bending technique like in femur fracture model in rats (8). Fracture was corrected by radiography (Figure 1). The rats in the EPO group were treated with 500 U/kg (bw) of intraperitoneal EPO (9) (Eprex, Santa-Farma). Injections were started on the day of fracture (two hours fracture formation) and

Table 1: The numeric system used for histologic assessment.

Histologic signs of fracture line	number
Fibrotic tissue	1
More fibrotic tissue and less cartilage tissue	2
Equal portions of fibrotic and cartilage tissues	3
More cartilage tissue and less fibrotic tissue	4
Cartilage tissue	5
More cartilage tissue and less immature bone tissue	6
Equal portions of cartilage and bone tissue	7
More immature bone tissue and less cartilage tissue	8
İmmature fracture healing	9
Mature fracture healing	10

Figure 2: Mostly immature osseos callus and chondral callus on the fracture line.



continued for five days. In control group, the same volume of isotonic sodium chloride solution was given intraperitoneally for five days. On the seventh day of experiment, radiographies of all the fractured extremities were recorded. Seven rats from each group were selected randomly and sacrificed. After clinical examinations of fractured bones and fracture lines, specimens were kept in a 10% phosphate buffered formalin solution for 24 hours. On the second day specimens were taken in formic aside solution for decalcification. At the end of the second day all specimens were washed by water and cleaned from formic aside. Two coronal plane sections for each fracture specimen were taken. One of them was embedded into a paraffin block and the other was kept in formaldehyde till the end of the study. For histologic studies 4- 5 micron thicknesses of longitudinal sections in paraffin blocks including the site of callus formation between fractured bone sides were stained by hemotocsilen eosin. The same method was applied for the retained 14 animals at the end of the third week.

Histological examination was performed under light microscope by a pathologist. Callus tissue was assessed by the method of Hou et all (6) (Table 1). The radiologic examination and clinic assessment of the fracture callus was done by three orthopedists blinded to the treatment modality, according to the methods of Lane and Sandhu (7) (Table 2), and Dimar and friends (5) (Table 3), respectively.

Statistical Analyses

All values are given as mean ± standard deviation. Statistical Package for Social Sciences software (SPSS 15, Chicago, IL, USA) was used for analysis. Unpaired Student t test was used for group comparisons. Categorical data were compared with the chi-square test. Abnormally distributed data were evaluated using Mann Whitney U test. P value of <0.05 was considered significant.

Table 2: The numeric system for radiologic assessment.

Radiologic signs	number
No healing	0
Callus formation	1
Starting fractured bone healing	2
Diseppearing of fracture line	3
Finishing fractured bone healing	4

Table 3: The numeric system for clinic assessment.

Clinic signs at the fracture line	number
No movement on both of the two plane (union)	2
Movement on one plane (modarete union)	1
Movement on both of the two plane (nonunion)	0

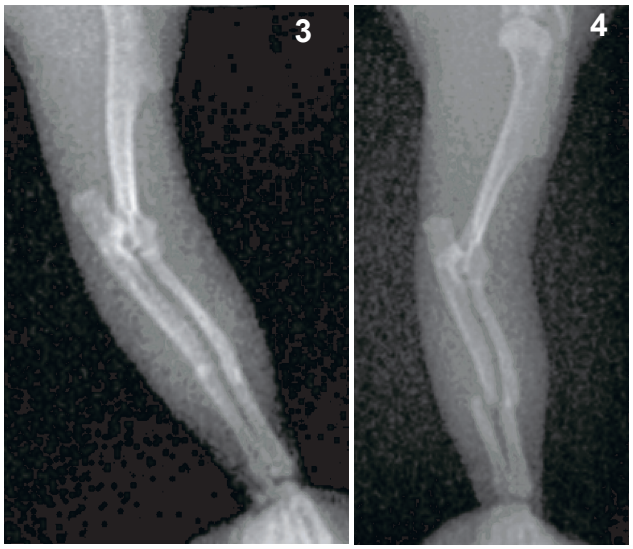
Results

Mean body weight of the rats in the EPO group was 220 g and 210 g in the control group at baseline. At the end of the study mean weight of rats in EPO group was increased 25 g whereas there was only 10 g of mean body weight increase in the control group. The fibrotic callus formation between the fractured bone sides in EPO group is larger than the Control group at the end of the first week. Because of the short period for fracture healing, the clinic assessment of fracture healing is not studied for the first week. According to the clinic assessment method of Dimar and friends, the mean numeric score was 1.14 ± 0.69 in control group and 1.86 ± 0.38 in EPO group at the end of the third week which was statistically significant ($p<0.05$)

Radiologic assessment is measured at the first week and at the end of the study according to the Lane and Sandhu method (Figure 2-6). The mean numeric score of radiologic fracture healing was 0.93± 0.73 in the control group and 1.50 ± 0.65 in the EPO group at first week which was statistically insignificant ($p> 0.05$). However the mean score of radiologic fracture healing was 1.14 ± 0.69 in the control group and 2.29 ±0.49 in the EPO group at the third week which reached statistically significance ($p< 0.05$).

The numeric scores for histological assessment of fracture healing were studied according to Huo and friends at the end of first and third weeks. Mean score of histological fracture healing was 1.86 ± 0.69 in the control group and 2.86 ± 0.90 in the EPO group at the end of the first week. The difference was statistically significant ($p<0.05$). The mean score of histological fracture healing was 3.00 ± 0.82 in the control group and 4.86 ± 1.46 in the EPO group at the end of the third week which was again statistically significant ($p<0.05$).

Figure 3: EPO group 1.week radiography.
Figure 4: Control group 1. week radiography.



Discussion

Fracture healing is a basic model of wound healing. Both of them are inflammation process. This study demonstrates the positive effect of EPO on closed forearm fracture healing on rats. We decided to take forearm fracture model instead of any other bones. As there is much more study about wound healing, this complex process has not known the real entities that affect fracture healing (10) . We wanted to determine if EPO has positive effect on fracture healing. Histological, radiologic and clinical examination methods are used to evaluate the efficacy of EPO.

The exact mechanism of hypoxia-induced erythropoietin production has not been resolved yet. The oxygen receptors in or around the cells were supposed to be the main responsible mechanism. EPO receptors were found in brain, overs, tubas and testicular tissues (11). Erythropoietin production is possibly related not only with the hormonal system, but also involves paracrine and otocrine systems. EPO was successfully used in experimental myocardial infarction (12), necrotisane enterocolitis

Figure 5: EPO 3.week radiography.

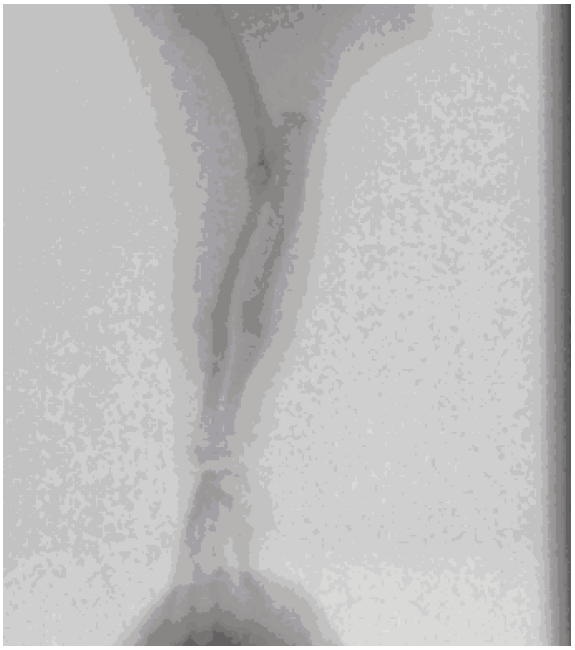
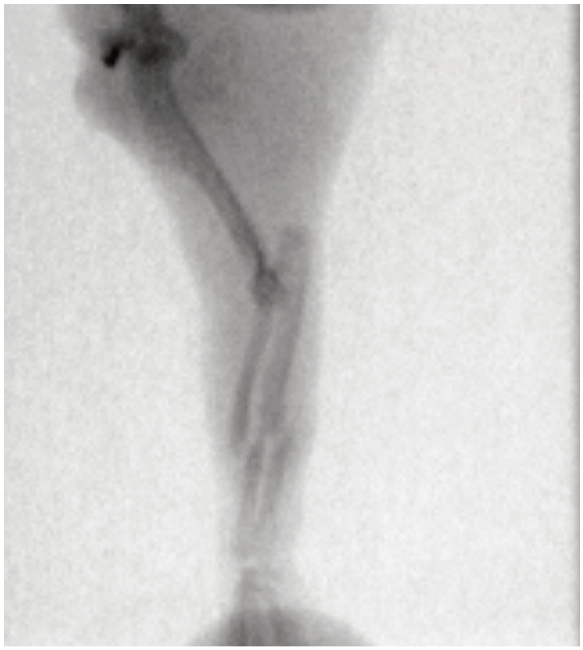


Figure 6: Control 3.week radiography.



(13), ischemic skine flabs (14) and short intestine syndrome models (15). Its'antiinflamatory effect has been shown in autoimmune encephalitis (16). It is also responsible for new blood vessels formation; angiogenesis (17). EPO is mainly used in the treatment of anemia due to chronic kidney insufficiency. It was used between 300-5000 U/kg intraperitoneally in experimental studies (12,13,18). In this study EPO showed its positive effect at a fairly low dose (500 U/kg intraperitoneally). Several cilinical studies regarding EPO use in fracture healing have been published. Hasegawa and friends showed that EPO could be used on the patients who might not be an autologe blood donor before going under hip surgery (19). Schmidt and friends suggested that ferrum and EPO could be used in the treatment of the traumatic patients with pelvic and hip fractures who had massive blood lost (20). It is known that anemia has negative effect on fracture healing. Giglio and friends composed an experimental anemic rat model and showed anemia has negative effects on fracture healing according to the control group (21).

In traumatic patients hypovolemia and anemia are expected. Such patients with anemia, chronic kidney insufficiency, patient with risks of delayed union and nonunion could be target indication of EPO use as well. Patient with NSAID use, smoking, pulmonary oxygenation problems, diabetic disorders, and osteomyelitis or with open fractures and closed fractures that could not perfectly stabilized is potential risks for delayed union or nonunion.

Beyond anemia EPO has direct effects on fracture healing. Fracture-healing response consists of two major components: angiogenesis mediated by endothelial cells and pericytes; and callus formation orchestrated by fibroblasts and osteogenic cells with macrophages, the major cell type in callus tissue, playing a critical role in both regulation of angiogenesis and callus formation. The positive effect of EPO in degenerative vascular disease by inducing angiogenesis has been shown before . EPO increases the blood flow around the fractured bone and plays a positive role on fracture healing (22). EPO also increases the number of micro vessels and amount of collagen around the traumatic skin tissue (23) . All recent studies were thought EPO could have positive effects on fracture healing. Based these results it is predictable use of EPO as a drug on patients for earlier fracture healing.

It is the starting point for us to correlate the efficacy of EPO in this experimental animal study. Fracture healing in rat model is significantly increased histologically according to the control group at the end of the first week and also significantly increased histologically, radiologically and clinically with EPO use at the end of the third week. One more evaluation criteria might be biomechanical examination. Because of the short fracture healing period in rats it is excluded.

We hope as the efficacy of EPO on fracture healing is more clear, clinic studies about EPO will be started in the future. EPO is known as large safety profile and widely used drug. Low dose EPO may be a new protective agent against delayed union or nonunion in the risk population not to go to surgical treatment. However, the results of this study should be confirmed on human subjects with placebo controlled studies.

REFERENCES

1. Brinker RM, Miller MD. Basic sciences, Review of Orthopaedics. 2nd ed. WB Saunders Co, Philadelphia, 1996: 1-26.
2. Murnaghan M, Li G, Marsh DR. Nonsteroidal anti-inflammatory drug-induced fracture nonunion: an inhibition of angiogenesis?. J Bone Joint Surg Am. 2006 Nov;88 Suppl 3:140-7.
3. Kertesz N, Wu J, Chen THP, Sucov HM, Wu H. The role of erythropoietin in regulating angiogenesis. Developmental Biology. 2004; 276: 101-110.
4. Buemi M, Galeano M, Sturiale A. Recombinant human erythropoietin stimulates angiogenesis and healing ischemic skin wounds. Shock, 2004; 22: 169-173.
5. Dimar JR, Ante WA, Zhang YP. The effect of nonsteroidal antiinflammatory drugs on posterior spinal fusions in the rats. Spine. 1996; 21: 1870-1876.
6. Huo MH, Troino NW, Pekler RR, Gundberg CM, Friedlaender GE. The influence of ibuprofen on fracture repair: biomechanical, histological, and histomorphometric parameters in rats. J Ortoph. Res. 1991; 9: 383-390.
7. Lane JM, Sandhu HS. Current approaches to experimental bone grafting. Orthop Clin North Am. 1987; 18: 213-225.
8. Altman RD, Latta LL, Keer R, Renfree K, Hornicek FJ, Banovac K. Effect of nonsteroidal antii-nflammatory drugs on fracture healing: a laboratory study in rats. J Orthop Trauma. 1995;9(5):392-400.
9. Erbayraktar Z, Erbayraktar S, Yilmaz O, Cerami A, Coleman T, Brines M. Nonerythropoietic tissue protective compounds are highly effective facilitators of wound healing. Mol Med. 2009 Jul-Aug;15(7-8):235-41.
10. Kabak Ş, Balkar F, Duygulu F. Effect of fracture hematoma on fracture healing. Acta Orthop Traum Turc. 2001; 35: 252-259.
11. Lappin T. The cellular biology of erythropoietin receptors. The Oncologist. 2003; 8: 15-18.
12. Moon C, Krawczyk M, Ahn D, Ahmet I, Paik D, Lakta EG; Talan MI. Erythropoietin reduces myocardial infarction and left ventricular functional decline after coronary artery ligation in rats. Proc Natl Acad Sci. 2003; 100: 11612-11617.
13. Kumral A, Baskin H, Duman N, Yilmaz O, Tatli M, Ozer E, Gokmen N, Genc S, Ozkan H. Erythropoietin protects against necrotizing enterocolitis of newborn rats by the inhibiting nitric oxide formation. Biol Neonate. 2003; 84: 325-329.
14. Buemi M, Vacorro M, Sturiale A, Galeano MR, Sansotta C, Cavallari V, Floccari F, D'Amico D, Torre V, Calapai G, Frisina N, Guarneri F, Vermiglio G. Recombinant human erythropoietin influences revascularization and healing in a rat model of random ischemic flaps. Acta DermVenoreol. 2002; 82: 411-417.
15. Noyan T, Onem O, Ramazan Sekeroglu M, Koseoglu B, Dulger H, Bayram I, Yalcinkaya AS, Bakan V. Effects of erythropoietin and pentoxifylline on the oxidant and antioxidant systems in the experimental short bowel syndrome. Cell Biochem Funct. 2003; 21: 49-54.
16. Agnello D, Bigini P, Villa P, Mennini T, Cerami A, Brines ML, Ghezzi P. Erythropoietin exerts an anti-inflammatory effect on the CNS in a model of experimental autoimmune encephalomyelitis. Brain. Res. 2002; 952:128-34.
17. Ribatti D, Vacca A, Roccaro AM, Crivelatto E, Presta M. Erythropoietin as an angiogenic factor. European Journal of Clinical İnvestigation. 2003; 33: 891-896.
18. Junk AK, Mammis A, Savitz Sİ, Singh M, Roth S, Malhotra S, Rosenbaum PS, Cerami A, Brines M, Rosenbaum DM. Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury. Proc Natl Acad Sci. 2002; 99:10659-10664.
19. Hasegawa Y, Takamatsu J, Iwase T, Iwasada S, Kitamura S, Iwata H. Effects of recombinant human erythropoietin on thrombosis and fibrinolysis in autologous transfusion for hip surgery. Arch Orthop Trauma Surg. 1999; 119: 384-387.
20. Schmidt HA, Templeman CD, Kyle RF. Blood conservation in hip trauma. Clin. Orthop An Related Research. 1998; 357: 68-73.
21. Giglio MJ, Gorustovich A, Guglielmotti MB. Bone healing under experimental anemia in rats. Acta Odontol Latinoam. 2000; 13: 63-72.
22. Chong ZZ, Kang JQ, Maiese K. Angiogenesis and plasticity: role of erythropoietin in vascular systems. J Hematother Stem Cell Res. 2002; 11: 863-871.
23. Frisina N. Wound care; recombinant human erythropoietin heals ischemic skin wounds. Drug week. 2004; 570.