THE EFFECT OF ERYTHROPOIETIN ON BONE REGENERATION "AN EXPERIMENTAL STUDY"

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ABSTRACT

Background: Erythropoietin (EPO) is a hematopoietic growth factor stimulating the formation of red blood cells. EPO is notoriously known as a doping substance in high-performance sports, and in cycling in particular. In recent years, the non-hematopoietic functions of EPO, also known as pleiotropic functions which include osteogenic and angiogenic potencies have been intensively investigated.

Aim of study: The objectives of the present study were to address and investigate the efficacy of EPO in regenerating bone and facilitating bone healing by using a single local dose of 1ml EPO 4000 IU per animal to augments bony ingrowth in rabbit calvarial bony defect.

Material and methods: 12 New Zealand White rabbits received a calvarial defect. Absorbable oxidized cellulose was soaked with 1ml EPO 4000 IU (local treatment group) were implanted into the gap, the other side used as (control group). Histological analysis was performed 7, 14 and 30 days postoperatively. Vascularization was evaluated histologically.

Results: Histopathological findings of bone gap after 30 days of surgery, untreated animals showed granulation tissue which invaded numerous number of inflammatory cells mainly macrophages. Bony gap showed more fibrous tissue than fibrocartilage. In treated animals group, the periosteal cells proximal to the fracture gap developed into osteoblasts. The animals group, fracture induced gap was filled with well-organized bone trabeculae. The healed bone gap revealed numerous number of proliferative osteocytes .

KEY WORDS: Erythropoietin, angiogenesis, osteogenesis, neovascularization

INTRODUCTION

Erythropoietin (EPO) is a well-known hormone that regulates red blood cell generation. Previous studies have found that EPO additionally plays roles in bone homeostasis. EPO can enhance bone formation by increasing the expression of vascular endothelial growth factor and bone morphogenetic protein. In addition, other studies reported beneficial effects of EPO on the non-hematopoietic system and on the bone fracture repair process, including stimulation of angiogenesis as well as regulates bone formation through mTOR signaling. [1-4]

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Delayed or insufficient bone healing with development of non-union is a major clinical problem. Several groups have described increased bone healing by vascular-endothelial growth factor- (VEGF-) induced effects on angiogenesis and endochondral bone formation. [5] Structural and functional conformities between VEGF and therefore glycoprotein erythropoietin (EPO) [6] have caused growing interest in EPO to improve bone healing.

Based on bone-related pleiotropic effects, EPO seems to be attractive for enhancement of bone healing. Despite increased osteoclast stimulation [7], other several studies have been done to evaluate the effect of EPO induced mechanism on invivo bone healing in animal models. [2,7-14]

Characterization, production, function and degradation of EPO; after a century of extensive research in erythropoietin (EPO), its structure, production and hematopoietic way of action have been described in detail, while the clearance and degradation of EPO is not yet entirely understood .[15,16]

EPO consists of 60% protein and 40% carbohydrates. A chain of 165 amino acids constitutes the protein core. The peptide terminals of the core mediate functionality via binding to the receptor, while the four carbohydrate side chains protect EPO from degradation in the blood. [15]

In 1985, the discovery of the nucleotide sequence of EPO made it possible to produce recombinant human EPO (rhEPO) for clinical use. [17] Endogenous EPO and rhEPO are identical apart from minor differences in glycosylation. [18]

In 1989, the U.S. Food and Drug Administration (FDA) approved the use of rhEPO for the treatment of anemia caused by insufficient endogenous EPO production due to chronic renal failure. Structural and functional conformities between VEGF and the glycoprotein erythropoietin (EPO) have caused growing interest in EPO to improve bone healing.

EPO is routinely used to treat anemia, especially in patients with chronic renal failure.[15]

The approval has since been extended to a wide range of indications, e.g. anemia induced by chemotherapy or HIV, and to decrease the need for transfusion in patients scheduled for certain types of surgery . [19]

It is also used in patients who refuse blood transfusions, e.g. Jehovah's Witnesses. The kidney is the primary production site of EPO. Upon hypoxia, renal peritubular fibroblasts increase EPO gene expression via hypoxia-inducible transcription factor and subsequently release EPO into the circulation. Before birth, the liver is the primary production site and it still accounts for about 10% of production in adult life .[16] In the past decades, it has become well established that EPO is also expressed locally in many tissues and that it acts in a paracrine fashion figure (1).

Erythropoietin (EPO) is most recognized for its function as a hematopoietic hormone secreted from
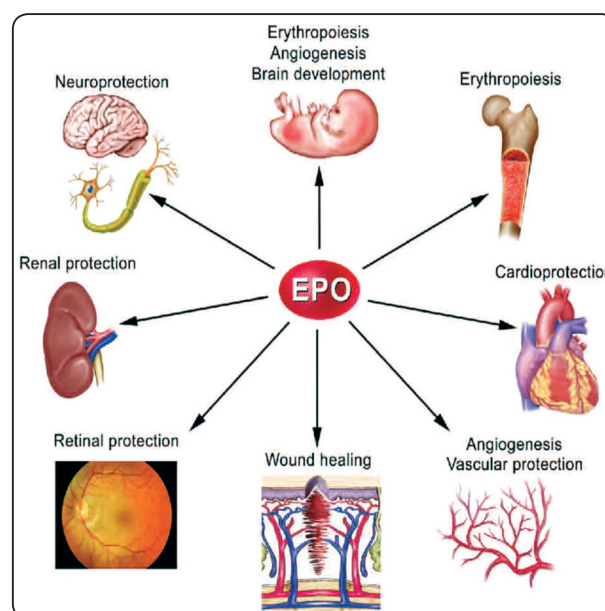

Fig. (1): Non-erythroid effects of erythropoietin. Copyright: Arcasoy MO, Haematologica 2010; 95: 1803-1805. Obtained from Haematologica/the Hematology Journal website http:// www.haematologica.org

the kidneys and liver in adult mammals in response to hypoxia by activating HIF-1a. [20,21]

In reaction to low oxygen levels, EPO is released into the peripheral circulation. EPO binds to a preformed homodimer transmembrane receptor (EPO-R) resulting in downstream phosphorylation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signal transduction cascades to induce proliferation and differentiation of hematopoietic precursors, thus expanding the production of erythrocytes.[21,22]

EPO has also been demonstrated to have a significant promise for tissue protection in the cardiovascular system. Multiple studies have demonstrated the potential that EPO has as a treatment for myocardial infarction [23-27] figure (1). After ischemia–reperfusion injury, Epo has been shown to aid in the survival of cardiomyocytes as well as the remodeling of the left ventricle.[28]

Influence of Epo on bone

The finding that Epo has pleiotropic roles in nonhematopoietic tissues has prompted a search for a role of Epo in bone formation and homeostasis. The rationale for such a search is that crosstalk between hematopoietic and osteogenic cells is known to affect each other's function.[29-31]

Therefore, the coupling of hematopoiesis with skeletal homeostasis through Epo signaling makes considerable sense. In fact, it has been shown that osteogenic activities in vivo are enhanced following direct marrow ablation or trauma.[32-36]

Locally delivered EPO to the extraction defect area. It has been reported in the in vivo study that EPO inhibits pro-inflammatory pathways and apoptosis, promotes endochondral ossification, enhances osteoblastogenesis, improves vascularization, and promotes new bone formation. [2,10,37]

Studies have repeatedly shown that Epo improves bone healing; however, the mechanisms regulating the process is not clear. One proposal is that Epo is able to play a key role in regeneration of newly resorbed bone by stimulating JAK-STAT signaling pathways in HSCs through Epo-R. This triggers BMPs production. The resulting production of BMPs is able to induce osteogenic progenitor cells to differentiate into osteoblasts and stimulate cartilage formation through the cell surface interactions with BMP-Rs.[3]

Aim of study

The objectives of the present study were to address and investigate the efficacy of EPO in regenerating bone and facilitating bone healing by using a single local dose of 1ml EPO 4000 IU per animal to increase bony ingrowth in rabbit calvarial bony defect .

MATERIAL AND METHOD

Animals and surgical procedure

The present study was conducted on 12 skeletally mature New Zealand rabbits; mean weight was 4.2 (3.6–4.8) kg.

Linear incision was made, after stripping off periosteum; 1×1 cm bony defects were created on both, right and left side in the calvarial bone, as shown in figure (2).

Absorbable oxidized cellulose was soaked with single local single dose of 1 ml EPO 4000 IU* on the right side, while the left side used as control group, figure (3).

In vivo histological analysis was performed at 7, 14 and 30 days post-operatively, vascularization was evaluated histologically.

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* 1 ml EPO 4000 IU SEDICO Pharmaceutical Co. - Egypt.

Fig. (2): Linear incision was made, after stripping off periosteum; 1×1 cm bony defects were created on both, right and left side in the calvarial bone.

Fig. (3): Absorbable oxidized cellulose was soaked with single local single dose of 1 ml EPO 4000 IU on the right side, while the left side used as control group

Histopathological examination:

The animal groups were sacrificed via pentobarbital overdose (100mg/kg) intervals on the 7, 14 and 30 days after the surgery. Then, the calvarial bone was removed and fixed in 10% neutral buffered formalin for 48 h, then decalcified in 10% ethylene di-amine tetra-acetic acid (EDTA).

Tissue processing, including dehydration, clearing, impregnation, and embedding, was done through graded ethanol, xylol, and paraffin.

Histologic sections with a thickness of 6 μm

were prepared from each defect containing an intact border of the bone, and then the samples were routinely stained with hematoxylin and eosin [47]. For histological evaluation of healing process, Emery's histopathological healing criteria were used [48], which are explained in Table 1.

TABLE (1) Emery's histopathological healing criteria:

Score (point)	Tissue present
0	Empty cavity
1	Fibrous tissue only
2	More fibrous tissue than fibrocartilage
3	More fibrocartilage than fibrous tissue
4	Fibrocartilage only
5	More fibrocartilage than bone
6	More bone than fibrocartilage
7	Bone only

RESULTS

Histopathological findings regarding to bone specimens collected after 7 days of surgery in untreated group, revealed empty space contained bone fragments (score 0) fig.4a-b Treated animal group showed extravascular blood cells form a blood clot, known as a hematoma. All the cells within the blood clot degenerated and died. Activation of mesenchymal cells in the soft tissues and bone surrounding the fracture gap differentiate into fibroblast and angioblast with newly formed capillaries which appeared engorged with blood known as granulation tissue. More fibrous tissue was seen proximal to the fracture gap were seen (score 1) fig. 4c-d.
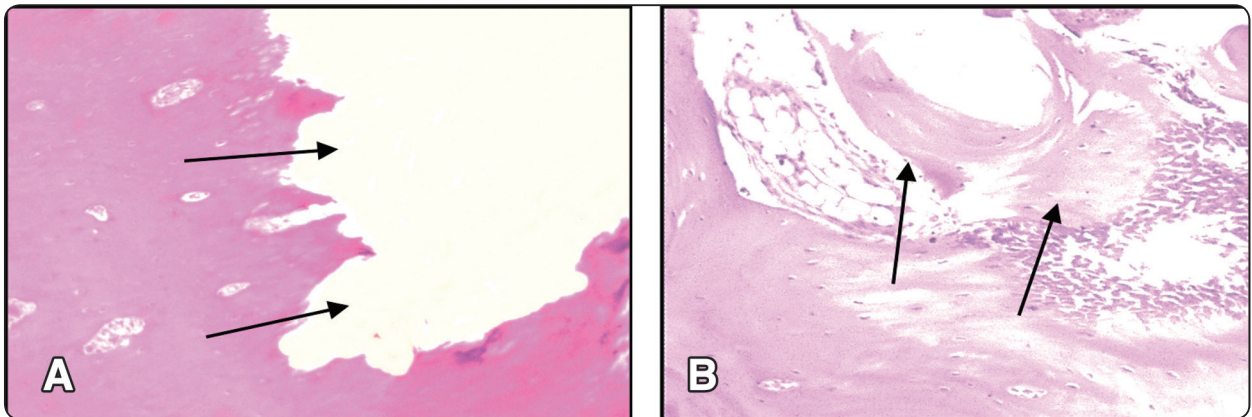
7 Days

Untreated (L)

Fig. (4) Histopathological section of induced bone gap: (a) untreated group showing empty gap arrow (b) gap contained bone fragments arrow (H&E X200)
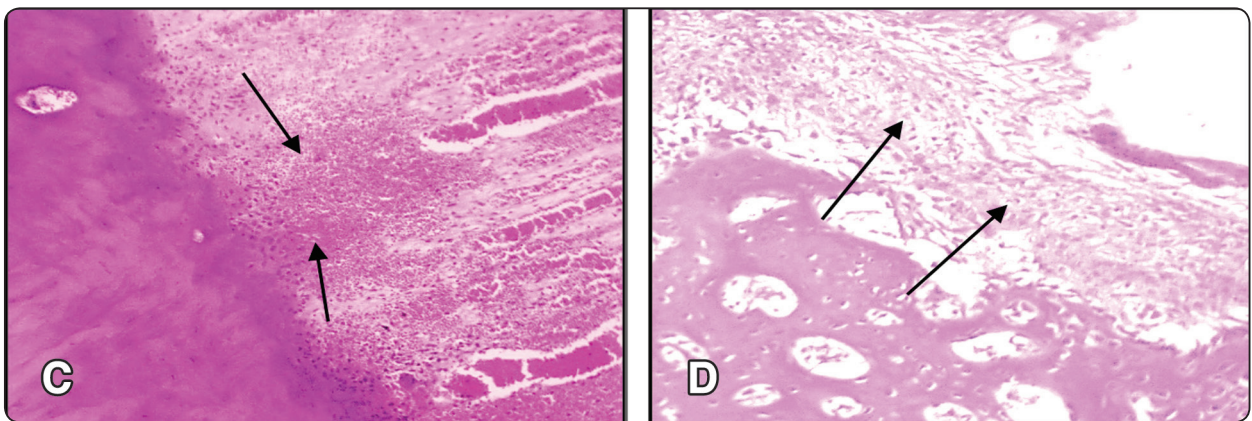
Treated (R)

Fig. (4) Histopathological section of induced bone gap: (c) treated group showing extravasation of blood cells arrow (d) fibrous tissue proliferation proximal to the fracture gap (H&E X200)

Histopathological findings of bone gap after 14 days of surgery, untreated animals showed granulation tissue which invaded numerous number of blood capillaries. Fibrous tissue only was noticed in fracture gap (score 1) fig.5a-b. In treated animals group, the periosteal cells proximal to the fracture gap developed into chondroblasts, which formed hyaline cartilage. On the other hand, the periosteal cells distal to the fracture gap develop into osteoblasts. In this animals group, the gap was filled with trabecular bone characterized by acellular osteocyte lacunae and fibrocartilage tissues. The healed bone gap inhabited osteocyte lacunae and delimited by osteoblasts that forming a network of bone connected to the reactive trabeculae deposited elsewhere in the medullary cavity and beneath the periosteum (score 3) fig. 5c-d
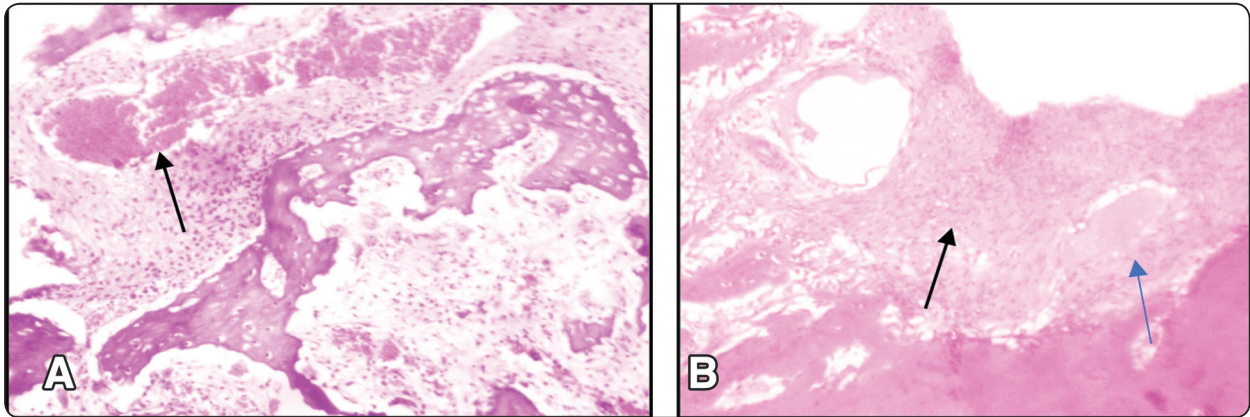
14 Days

Untreated (L)

Fig. (5) Histopathological section of induced bone gap: (a) untreated group showing granulation tissue which invaded by blood capillaries arrow (b) well developed organized fibrous tissue arrow (H&E X200)
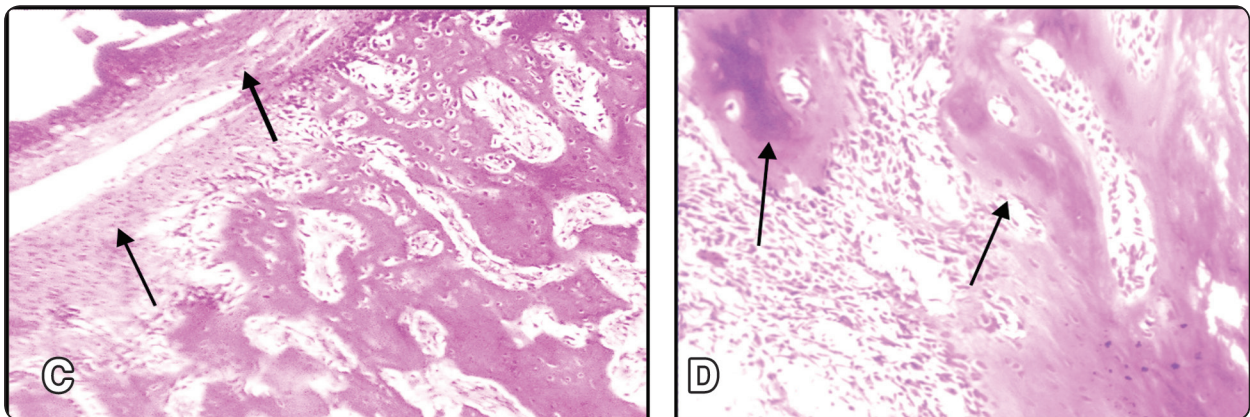
Treated (R)

Fig. (5) Histopathological section of induced bone gap: (c) treated group showing hyaline cartilage proximal to fracture gap arrow (d) osteoblasts that forming a network of bone connected to the reactive trabeculae deposited in the medullary cavity, arrow (H&E X200)

Histopathological findings of bone gap after 30 days of surgery, untreated animals showed granulation tissue which invaded numerous number of inflammatory cells mainly macrophages. Fracture gap showed more fibrous tissue than fibrocartilage (score 2) fig.6a-b. In treated animals group, the periosteal cells proximal to the fracture gap developed into osteoblasts. The animals group, fracture induced gap was filled with well-organized bone trabeculae. The healed bone gap revealed numerous number of proliferative osteocytes (score 6) fig.6c-d

TABLE (2) Emery's histopathological healing criteria evaluation of fracture bone gape

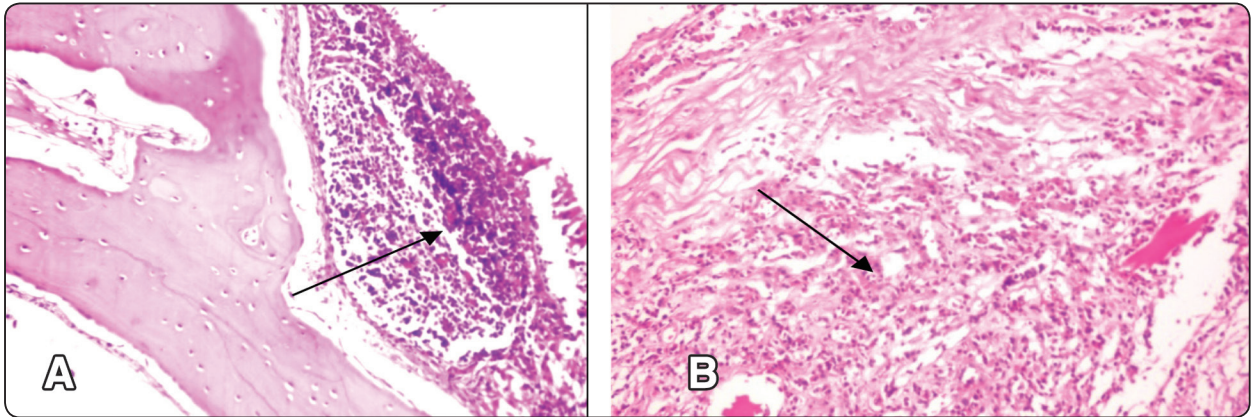| Day/score	Untreated Group	Treated Group
7	0	1
14	1	3
30	2	6

30 Days

Untreated (L)

Fig. (6) Histopathological section of induced bone gap: (a) untreated group showing granulation tissue which invaded by numerous numbers of inflammatory cells arrow (b) more fibrous tissue than fibrocartilage arrow (H&E X200)
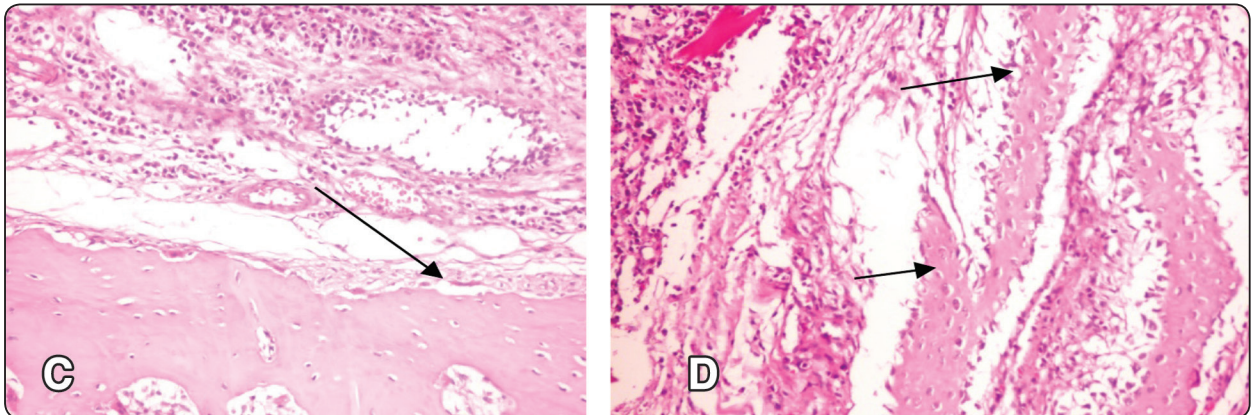
Treated (R)

Fig. (6) Histopathological section of induced bone gap: (c) treated group showing well organized bone trabeculae arrow (d) numerous number of proliferative osteocytes,arrow (H&E X200)

DISCUSSION

Our study clearly demonstrates the potency of EPO as an additional treatment option to increase bone healing by using local single dose of 1ml EPO 4000 IU.

Several studies demonstrated that hematopoietic stimulation improves bone formation both in the context of earlier ossification and fraction healing, and improvements in mechanical strength. [10,38-40]

In 2017, Zhang et al, [41] conducted a comprehensive assessment of EPO overexpression MSC on SC, combining with phytochemical and physiological studies. Conclusively, the overexpression of EPO promoted the therapeutic effects of MSC on diabetic neuropathy via inhibiting SC apoptosis. EPO is able to enhance bone formation and to increase neovascularization, which is likely to contribute to osteogenesis. Moreover, a direct way of action via stimulation of hMSCs

was documented. Besides these advantages, the limitation of repetitive systemic EPO administration was highlighted. Although no thromboembolisms or adverse effects were observed in the wake of EPO treatment, the hematocrit rose to a critically high level, which makes the clinical implementation of this treatment regime impossible. For the first time, the lowest effective dose was established in vitro. This is a prerequisite for commencing clinical trials as adverse effects including thromboembolism were described after high-dose EPO treatment.[42]

Our study was in agreement with in vivo study that has been reported that locally delivered EPO to extraction defect area [43] improves vascularization, promotes endochondral ossification, and enhances osteoblastogenesis and promotes new bone formation. [2,10-13,37]

Another study demonstrated a bone catabolic effect of rHuEPO using several adult mouse models, namely EPO overexpressing mice (Tg6), intermittent injections of low and high EPO dosage, and continuous administration of low doses. In Tg6 mice, the increase in EPO levels is similar to the stimulation of EPO at high altitude. [44]

However, our study was disagree with other study which indicated that EPO induces bone resorption and attenuates bone formation, as well as stimulates monocyte-derived cells in the bone marrow. Stimulation of osteoclastogenesis and bone resorption is likely attributable to EPO-R activation of the Jak2 and PI3K pathways in osteoclast precursors and therefore seems to occur independently of erythropoietic effects in the bone marrow. However, the attenuated bone formation in EPO overexpressing and injected animals does not support this conclusion. in vivo data indicate a reduced bone formation, whereas in vitro experiments failed to reveal any direct effect of EPO on osteoblast proliferation, differentiation, and activity at a dose of 10 U/ml.[45]

In 2016, Omlor et al, [46] conducted a study on 19 New Zealand White rabbits received a 15-

mm defect in the radius diaphysis. Thus, single application of EPO at an early time point appears to be most effective and from a clinical point of view, the intraoperative single local dose administration appears to be most attractive to improve bone healing. Apart from effects on ossification, systemic and local EPO treatment leads to increased callus vascularization. and that we concluded in our study, that the local EPO administration lead to callus vascularization and angiogenesis stimulation.

Further studies need to improve whether single local dose application to defects would be beneficial in comparison to single systemic dose treatment.

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