Histological Evaluation of Erythropoietin Application on Bone Healing in Rat Calvaria

Original Article

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ABSTRACT

Introduction: Erythropoietin (EPO) is a glycoprotein hormone whose primary physiological functions is regulation of erythropoiesis. During the last decade, its additional, so-called pleiotropic functions, have become also important. As skeletal regeneration is concerned, EPO successfully combines haemopoiesis with bone formation. Due to its osteogenic and angiogenic potential, EPO promotes endochondral ossification, formation of osteoblasts and blood vessels.

Aim of the Work: The purpose of the present study was to perform histological evaluation of effects from either local (independent and combined with bone substitute) or systemic administration of EPO using critical-size calvarial bone defect model in rats.

Materials and Methods: Thirty-six male Wistar albino rats, 6 months of age, weighing 250-300 g were used in the experiments. Experimental animals were randomly assigned to three groups. Two symmetrical defects were created in the calvaria of each of rats. Thirty and ninety days after the surgical procedure, rats from each group were euthanised to obtain material for histological examination after staining with haematoxylin-eosin and Schmorl's stain.

Results: The results showed that EPO was successful in coupling haemopoiesis and bone formation. Due to its osteogenic and angiogenic potential, EPO stimulated the formation of osteoblasts and blood vessels at the site of defect.

Conclusion: Local erythropoietin application resulted in bone formation and could be successfully used for bone regeneration. Combined with bone substitute, EPO potentiated its effect and improved bone healing. Contrary to expectations, the effect of EPO systemic application was found to be unsatisfactory.

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Key Words: Bone regeneration, calvarial model, critical defect, erythropoietin, histology.

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INTRODUCTION

Bone healing is an intricate process involving mainly bone and bone marrow stromal cells as well as inflammatory cells along with a number of growth factors, signalling molecules, vitamins and hormones.[1-4] Also, the formation of new blood vessels stimulated by production of proangiogenic factors, e.g. vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor beta (TGF-beta), insulin-like growth factor 1 (IGF-1) and erythropoietin (EPO), is extremely important for bone regeneration.[5-8]

Erythropoietin (EPO) promotes the growth, development and differentiation of red blood cell progenitor cells in the bone marrow.[9-10] In hypoxic conditions, its secretion in the blood circulation increased as response to released hypoxia-inducible factor 1 and 2 (HIF-1 and HIF-2).[11-14]

As skeletal regeneration is concerned, EPO successfully combines haemopoiesis with bone formation.[15] Due to its osteogenic and angiogenic potential, EPO promotes endochondral ossification, formation of osteoblasts and blood vessels.[16-17] According to other researchers, EPO directly promotes the precursors of osteoclasts and monocytes, thus inducing bone resorption.[18-20] This, however, does not lead to their activation, meaning that EPO increases the number of osteoclasts without influencing their function.[15]

Despite the stimulating effect of EPO on osteogenesis, the available information on its combined use with various bone substitutes and bone cements, as well as on its inclusion in biological carriers applied in bone implantology is still scarce.[21-22]

The purpose of the present study was to perform histological evaluation of effects from either local (independent and combined with bone substitute) or systemic administration of EPO using critical-size calvarial bone defect model in rats.

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MATERIAL AND METHODS

Ethical approval

This study was approved by the Ethics Committee to the Bulgarian Food and Safety Agency.

Animals

Thirty-six male Wistar albino rats, six months of age, weighing 250-300 g were used in the experiments. The rearing and housing of experimental animals was fully compliant with conditions stipulated by Ordinance 20 of 1.11.2012 on the minimum requirements for protection and welfare of experimental animals and site requirements for use, rearing and/or their delivery transposed from Directive 2010/63/EU.

Materials

Erythropoietin: Binocrit (Sandoz GmbH, Biochemiestr. 10, A-6250 Kundl, Austria) was used in this study. Binocrit 2000 IU is an injection solution, each mL containing 2,000 IU epoietin alpha equivalent to 16.8 μg/ml.

Collagen sponge: Collacone (Botiss biomaterials GmbH, Germany) is a collagen cone on the basis of porcine collagen. It served to elaborate cylindrical pads with diameter 5 mm and thickness 1 mm. Immediately before their placement in the defect site, they were soaked with either erythropoietin or physiological saline.

Bone substitute: Bio-Gen (BiOTECK, Italy) are cancellous bone granules of equine origin. The remodelling time is from 4 to 6 months.

Experimental design

Thirty six Wistar albino rats were divided randomly into 3 groups. Each group contained 12 rats. Two symmetrical defects were created in the calvaria of each of rats.

Group I (local EPO): In rats from this group a membrane soaked in physiological saline was placed in the right defect site, whereas a membrane soaked with erythropoietin – in the left defect site.

Group II (bone substitute with or without local EPO): In rats from this group a combination of bone substitute and physiological saline was placed in the right defect site, whereas bone substitute plus erythropoietin – in the left defect site.

Group III (systemic EPO): In animals from this group the right defect was left empty, and a collagen cone was placed in the left one. Erythropoietin was applied at a dose of 4,900 IU/kg via single intraperitoneal injection.

Experimental procedure

Rats were anaesthesised with 80 mg/100 g ketamine hydrochloride 10% (Anaket®, Richter Pharma AG, Austria) and 10 mg/100 g xylazine hydrochloride 2% (Xylazin®, Bioveta, Czech Republic) applied intramuscularly. After aseptic preparation, standardised 5-mm critical-size calvarial bone defects were created. The different stages of the procedure are illustrated on (Figure 1).

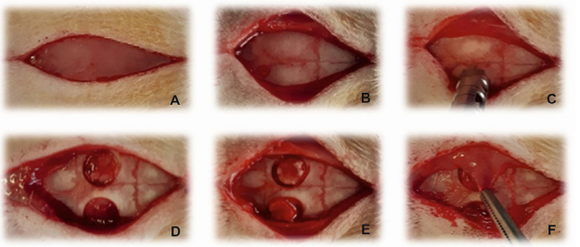

Fig. 1: Stages of creation of standardized calvarial bone defects. A) creation of skin incision from from the nasal bone to just caudal to the middle sagittal crest or bregma. B) dissection of the periosteum; C) creation of bone defects with a trephine; D) creation of the second calvarial defect; E) placement of biomaterials into the defect sites; F) retraction of periosteum and closure of skin incision.

Samples collection

Rats from each experimental group were euthanised on post operative 30th and 90th day. Calvarial specimens were harvested and fixed in 10% formalin and processed for paraffin blocks formation, sectioned and subjected to the following staining techniques:

I. Hematoxylin and Eosin staining (H&E)

II. Schmorl's staining[23]

Histological preparations were observed on binocular light digital microscope Leica DM1000 (Leica Microsystems, UK) at magnifications ×40, ×100 and ×200 and LEICA DFC 290 (Leica Microsystems, UK) camera for detection of newly formed bone and bone substitute remnants. Healing was evaluated using the histological criteria of Emery[24]: empty (score 0), fibrous tissue only (score 1), more fibrous tissue than fibrocartilage (score 2), more fibrocartilage than fibrous tissue (score 3), fibrocartilage only (score 4), more fibrocartilage than bone (score 5), more bone than fibrocartilage (score 6) and bone only (score 7).

Statistical analysis

The collected data were analyzed using statistical software MedCalc v.15.8 (Belgium). *P value* was considered highly significant if $P < 0.05$. All data were expressed as median (min-max). Statistical analysis of within- and between-group differences of histological scores was done by the Wilcoxon test for paired samples.

RESULTS

The scores obtained in the different treatment groups by the 30th and by the 90th day are presented in (Table 1).

By the 30th day

In animals from the first experimental group (Figure 2), resorption of implanted cone was complete. Right defects were filled with fibrous tissue (score 1). Mild infiltration of fibroblasts and fibrocytes in the centre with single osteoblasts on the periphery could be seen. The central part of left defects was occupied by fibrous tissue. Bone healing began from the periphery, close to bone defect margins, where osteocytes were detected (score 3).

In rats from the second experimental group, both defects were filled with bone tissue (Figure 3), yet more blood vessels were identified in the left defect (score 6).

In rats from the third experimental group (Figure 4), right defects were left empty (score 0). In left ones, collagen cone was resorbed, with fibrous tissue in the centre with fibrocytes, chondrocytes and single osteoblasts on the periphery (score 1).

By the 90th day

Right defects in animals from first experimental group demonstrated scant formation of new bone tissue with predominance of fibrous tissue (score 2). Left defects were fully regenerated (Figure 5). Lamellar bone was observed on the periphery. There were no inflammatory cells, yet more and more osteocytes and blood vessels (score 5).

In the second group (Figure 6), both defects were completely regenerated with filled with new bone. The number of blood vessels in left defects was higher (score 7). In rats from the third group, right defects were empty (score 0) while scant formation of new trabeculae in left defects was detected only in some of animals (Figure 7).

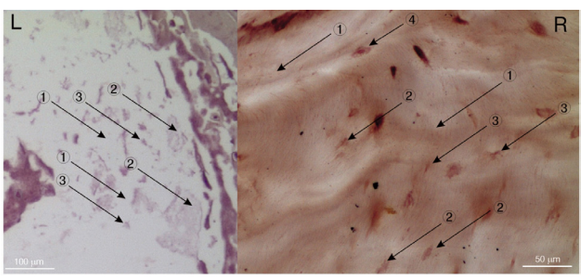

Fig. 2: Calvarial bone from group I on day 30 shows fibrous tissue at the centre (1) and bone healing at the periphery (2) of the left defect (L). Osteocytes (3) are also present (H&E, x 200). The right (R) bone defect shows mild infiltration of fibroblasts and fibrocytes (1, 2, 3) in the centre with single osteoblasts (4) at the periphery (Schmorl, x 400).

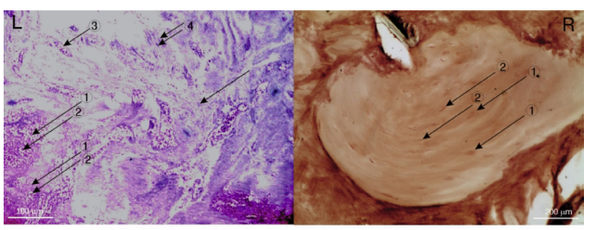

Fig. 3: Calvarial bone from group II on day 30 shows fibrous tissue (1), chondroblasts (2), chondrocytes (3) and osteoblasts (4) in the left defect (L) (H&E, x 200). The right (R) bone defect shows osteoblasts (1) and osteocytes (2) (Schmorl, x 100).

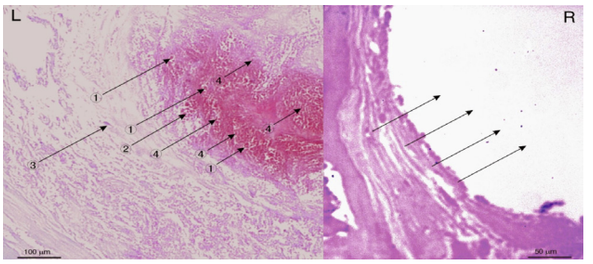

Fig. 4: Calvarial bone from group III on day 30 shows fibrous tissue formation with chondrocytes(1), blood vessels (2), osteoblast (3) and fibrocytes (4) in the left (L) defect (H&E, x 200), whereas the right (R) one is empty (black arrow) (H&E, x 100).

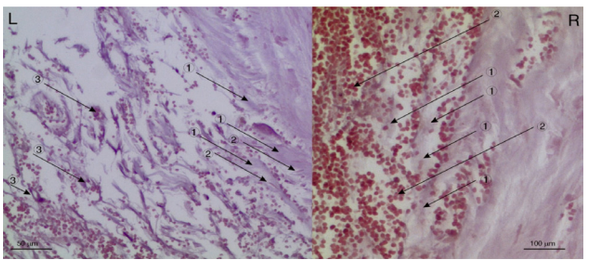

Fig. 5: Calvarial bone of group I on day 90 shows newly formed bone tissue (1), osteocytes proliferation (2) and newly formed blood vessels (3) on the left defect (L) (H&E, x 100). The right (R) defect shows newly formed bone tissue (1) with predomination of fibrous tissue (2). (H&E, x 200).

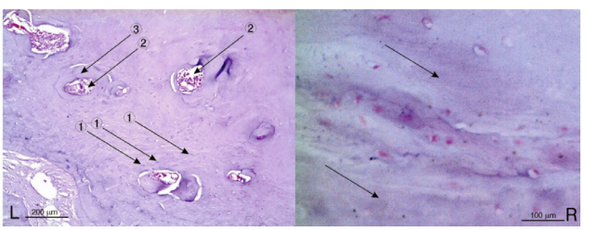

Fig. 6: Calvarial bone of group II on day 90 shows osteocytes (1) proliferation, blood vessels (2) and bone tissue (3) formation on the left defect (L). The right (R) defect shows newly formed bone tissue (black arrows) with less newly formed blood vessels. (H&E x 200).

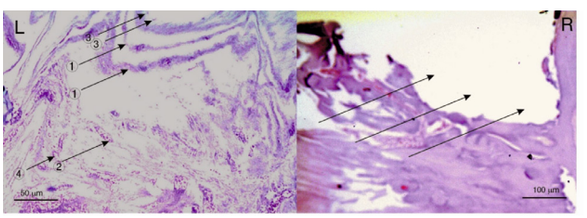

Fig. 7: Calvarial bone of group III on day 90 shows sparsely represented trabeculae (1), chondrocytes (2), osteoblasts (3) and blood vessel (4) formation on the left defect (L) (H&E, x 100), whereas the right (R) defect remains empty (black arrows) (H&E, x 200).

Table 1: Histological Emery's scores in the different treatment groups by the 30th and the 90th day. Data are presented as median (range), n=6

Treatments	Day 30	Day 90	P*
Cone + physiological saline	1 (1-1)^{AA}	2 (2-2)^{AA}	0.03
Cone + EPO	3 (3-4)^{BD}	5 (5-5)^{BD}	0.03
Bone substitute + physiological saline	6 (6-6)^{BF}	7 (7-7)^{BF}	0.03
Bone substitute + EPO	6 (6-6)^{BF}	7 (7-7)^{BF}	0.03
Empty defect + 4 900 EPO IU/kg i.p.	0^{BE}	0^{BE}	-
Cone + 4 900 IU/kg EPO i.p.	1 (1-2)^{AC}	2 (2-3)^{AA}	0.13

**P-value* between day 30 and day 90 within a group.
Different superscripts within a column indicate statistically significant differences (P<0.05)

DISCUSSION

So far, three mechanisms for stimulation of bone regeneration are acknowledged – osteogenesis, osteoinduction and osteoconduction[25]. Only fresh autogenous bone grafts and bone marrow cells perform osteogenesis[26]. Osteoconduction is biomaterials' property to serve as scaffold for bone healing, whereas osteoinduction – the ability of grafts to induce differentiation of stromal cells into mature bone cells[27]. In the present experiments, we tested a combination of two of these mechanisms, as collagen cone and bone substitute possessed osteoconductive properties, whereas erythropoietin – osteoinductive ability.

The exact mechanism of action of EPO is still unclear, but it is supposed to act through binding to EPO-receptors on the surface of haemopoietic stem cells (HSCs), which results in synthesis of bone morphogenic proteins (BMPs), in particular BMP2 and BMP6[28]. Their production leads to differentiation of osteogenic progenitor cells into osteoblasts and stimulates callus formation[6]. Another mechanism of EPO on bone healing is direct action by induction of differentiation of bone marrow stem cells (BMSCs) into osteoblasts[29].

The information available so far allows suggesting that erythropoietin combines successfully erythropoiesis and bone formation[30]. In order to investigate its effects in detail, symmetrical critical-size calvarial defects were created in the present experiment with rats.

The effect of local EPO application in the defect site was investigated in the first experimental group. The obtained results agreed with previous reports about increased bone tissue and blood vessels formation as compared to untreated defects[31-34]. Experiments with long tubular bones also demonstrated that EPO application improved mechanical strength of bones[16-17,35].

The information about the use of erythropoietin in combination with various grafts is scarce. Kharkova *et al*.[22] investigated tricalcium phosphate and EPO complex scaffolds and reported that it was promising for bone healing. To check this, cancellous bone granules only were applied in the right defect, whereas they were co-administered with EPO in the left defect. As anticipated, regeneration of created defects was complete, yet histologically, the effect of combined application was better. It turned out that EPO potentiated the effect of bone substitute. It was probably due to the fact that it improved considerably vascularization through promotion of VEGF synthesis and angiogenesis. Increased number of blood vessels was reported by Diker *et al*.[21] in a rat calvarial bone defect model after application of xenograft and systemic treatment with EPO at 500 IU/kg throughout 28 days. Their results demonstrated that independent EPO application had no effect on angiogenesis and bone formation in minimally critical bone defects at the end of the fourth week, as confirmed by our experiments. Our findings established that systemic application of EPO did not result in new bone formation. The causes could be attributed to inadequate treatment schedule: we have chosen a single intraperitoneal EPO application at a high dose, whereas other studies have used lower doses with more prolonged application – from 5 to 7 days or from 2 to 10 weeks[16-17,33,35-36].

Future studies should be aimed to elucidate some aspects of erythropoietin application for bone healing promotion, namely dose, route of application, duration of application. Possible side effects from both local and systemic injection should be also addressed.

CONCLUSION

Local erythropoietin application resulted in bone formation and could be successfully used for bone regeneration. Combined with bone substitute, EPO potentiated its effect and improved bone healing. Contrary to expectations, the effect of EPO systemic application was found to be unsatisfactory.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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