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Evaluation of recombinant human erythropoietin as a promoter of bone healing in cats with femoral fractures

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Abstract

Background: The bone regeneration potential of erythropoietin (EPO) is not yet fully investigated, but some previous experimental studies demonstrated that its application activated the differentiation of osteoblasts and promoted bone formation.

Aim: The aim of the present study was to evaluate the effects of recombinant human erythropoietin (rhEpo) on bone healing in cats with fragmented long bone fractures.

Methods: Twelve cats were divided into two groups—control ($n = 6$) in which physiological saline was applied at the fracture gap site and EPO ($n = 6$) with the application of 1,000 IU rhEpo. The effects of EPO on blood erythrocyte counts, hemoglobin content, and hematocrit were monitored by serial complete blood cell tests, whereas bone formation was evaluated by clinical and radiographic examinations on post-operative weeks 1, 2, 3, 4, 6, and 8.

Results: All tested blood parameters were within the reference range. A faster fracture healing and full limb weight-bearing were observed in the EPO group, with statistically significant differences with respect to the control group.

Conclusion: The obtained results confirmed that the local application of rhEpo promoted bone healing in cats with fragmented femoral fractures and increased bone callus strength without having significant systemic effects.

Keywords: Bone healing, Cat, DEXA, Erythropoietin, Fracture.

Introduction

Erythropoietin (EPO)—a hormone produced by peritubular fibroblasts of the renal cortex, stimulates the production of red blood cells (RBCs) in animal and human bodies (Jelkmann, 2016). EPO-stimulating agents are recombinant versions of EPO synthesized pharmacologically in cell cultures (Schoener and Borger, 2023). Their use is indicated mainly in chronic renal failure patients with anemia syndrome or during chemotherapy of cancer patients (Bruce *et al.*, 2022; Chung *et al.*, 2023).

The additional (pleiotropic) functions of EPO are of interest to regenerative medicine. EPO possesses an angiogenic, anti-inflammatory, cytoprotective, and anti-apoptotic effect (Trassanidou-Chatzifotiadou, 2014). Studies in the field of bone pathology demonstrated that the recombinant human erythropoietin (rhEpo) improved bone healing and may serve as a therapeutic agent promoting bone regeneration (Wan *et al.*, 2014). There is evidence that EPO stimulates bone healing and improves bone mechanical strength (McGee *et al.*, 2012). Its direct influence consists in the activation of the differentiation of osteoblasts, whereas the indirect effect involves stimulation of the expression

of vascular endothelial growth factor (Holstein *et al.*, 2011; Singbrant *et al.*, 2011).

Previous experimental studies on calvarial and long bones in rats showed that EPO had an osteoinductive potential and that its local application resulted in healing of critical bone defects (Chaprazov *et al.*, 2022; Vasileva and Chaprazov, 2023). Similar studies were performed also in mice and rabbits with promising results regarding the regenerative potential of EPO (Holstein *et al.*, 2011; Rölfing *et al.*, 2012). Data from clinical studies are however scarce. The first research in humans with tibial and fibular fractures has analyzed the effect of rhEpo applied at the fracture site 2 weeks after osteosynthesis. The results confirmed that EPO enhanced bone healing and shortened the restoration period (Bakhshi *et al.*, 2013). A recent preliminary study in men with periodontitis demonstrated that local EPO gel application reduced inflammation and improved the strength of the periodontal ligament (Aslroosta *et al.*, 2021).

To the best of our knowledge, there are no veterinary scientific reports on the effect of rhEpo on bone regeneration in patients. The aim of the present study was to evaluate the effects of rhEpo on bone healing in cats with fragmented long bone fractures.

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Materials and Methods

Erythropoietin

In this study, 2,000 IU Binocrit injection solution containing 2,000 IU/ml epoietin alpha equivalent to 16.8 µg/ml (Sandoz, Kundl) was used. It is produced by re-combinant DNA technology in the Chinese Hamster Ovary cell line expression system.

Animals

Twelve cats and patients of the Small Animal Clinic, University Veterinary Hospital were included. The inclusion criterion was a recent fragmented fracture of the femoral diaphysis. Exclusion criteria were animals with systemic diseases, animals with multiple fractures, and animals, which due to the type of fracture, needed a combination of different techniques of bone fixation at the same time.

The study included 12 mixed-breed cats. The average body weight of patients was 2.05 ± 0.96 kg (control group) and 2.17 ± 1.2 kg (EPO group). The respective average age of both groups was 7.0 ± 3.7 and 7.3 ± 4.3 months. There were no statistically significant between-group differences in body weight and age. The signalment data of patients are listed in Table 1.

The cats were assigned to one of two groups depending on the applied treatment.

- EPO group: local application of 1,000 IU epoietin alpha at the fracture site, corresponding to 0.5 ml of solution;
- Control group: local application of the same amount of physiological saline at the fracture site.

Anesthesiological and operative protocol

The cats were premedicated with atropine sulfate (0.1% Atropin, Sopharma) at a dose of 0.02 mg/kg, and

applied subcutaneously. The induction of anesthesia was done by intravenous application of the combination zolazepam/tiletamine (Zoletil 50, Virbac) at a dose of 15 mg/kg, and the maintenance through inhalational anesthesia with 2.5% isoflurane with oxygen (AErrane, Baxter).

After hair clipping, the field was disinfected with 4% chlorhexidine digluconate (HMI Scrub Classic) and 10% povidone-iodine (Braunol, B. Braun Melsungen). After a routine lateral approach to the femur, bone fragments were aligned and fixed with a metal plate and screws. Fixation elements were selected according to the patient's size. The skin incision was closed in layers through a simple continuous suture. Fasciae and subcutaneous tissue were closed with absorbable suture material (PGA 2-0, Kruuse), and the skin—with nonabsorbable material (Nylon 3-0, Kruuse).

During the post-operative period, pain was controlled with meloxicam (Meloxidolor, Produlab Pharma) at a dose of 0.3 mg/kg, applied subcutaneously. Amoxicillin/clavulanic acid (Synulox RTU, Pfizer) at 25 mg/kg was also subcutaneously applied for 6 days.

Complete blood count tests

Complete blood count analyses were conducted before the surgery and at post-operative weeks 1, 2, 3, 4, 6, and 8. The following parameters were determined: total white blood cell count (WBC, G/l), RBC, T/l, hemoglobin (Hb, g/l), hematocrit (Hct, %), and platelet counts (PLT, G/l) on an automated hematological analyzer BS-5000 Vet (Mindray).

Clinical examinations

The operated limb function was evaluated before the surgery and at post-operative weeks 1, 2, 3, 4, 6, and 8. The day of limb loading and lameness disappearance was recorded by inspection, and the day without swelling and pain was by palpation. The day of removal of metal implants was also registered.

Radiological examinations

Serial craniocaudal radiographs of the femoral region were obtained before the surgery and at post-operative weeks 1, 2, 3, 4, 6, and 8 with PHILIPS SUPER 50 CP-D equipment (Hamburg, Germany) and exposure data 50 kV/10 mAs for monitoring of early bone healing stages. Radiographs were processed by the iQ-VIEW/PRO version 2.7.0 software. The researcher who scored all radiographs with 1–6 points (0: Presence of fracture line with no bone formation; 1: Visible fracture line, no callus formation; 2: Visible fracture line with initial callus formation; 3: Visible fracture line with bridging callus formation; 4: Exuberant osseous callus in evolution and discrete radiolucent line at the gap between the fracture fragments; 5: Exuberant osseous callus and absence of radiolucent line; 6: Bone remodeling) was blind to the given treatment (EPO or control).

Osteodensitometry

The femoral bone density of all cats was measured after the removal of metal implants by means of dual-energy

Table 1. Signalment data of cats from the control and EPO groups.

Control group	Age (months)	Gender	Weight (kg)
1	4	F	1.5
2	8	F	2
3	2	F	0,7
4	12	M	3.2
5	6	M	1.8
6	10	M	3.1
Control group	Age (months)	Gender	Weight (kg)
1	5	F	1.2
2	2	F	0.6
3	12	M	3.4
4	7	M	2.5
5	6	F	1.7
6	12	F	3.1

X-ray absorptiometry (DEXA). During the procedure, cats were anesthetized with intravenous application of 7 mg/kg 1% Propofol (Propofol, Fresenius Kabi Deutschland).

Scans were done with OsteoSys SMART FAN-BEAM densitometer (OsteoSys, Seoul, Korea). Bone mineral density data were generated by the Excellus software (OsteoSys, Seoul, Korea) and presented as g/cm².

Statistical analysis

Data from clinical and radiographic examinations were presented as median (minimum-maximum), and those from complete blood count tests: as mean and standard deviation (SD). The differences between the groups were evaluated by the Mann-Whitney test or one-way ANOVA. For all analyses, the MedCalc v.15.8 (Belgium) statistical software was used.

Ethical approval

All owners were informed about the purpose of the experiments and gave informed written consent for the inclusion of their pets.

Results

Control cats began to bear weight on the operated limb 3 (2–4) days after the surgery, whereas those with local EPO application—significantly earlier: 1.5 (1–2) days after the osteosynthesis ($p < 0.01$). The swelling at the fracture region was visible until post-operative day 5 (4–7) in control cats and day 3 (2–5) in cats from the EPO group ($p < 0.05$). In cats from the control group, the lameness disappeared as late as the 10th day after the surgery (8–10) which was statistically significantly different ($p < 0.01$) from the EPO group: 6.5 (5–7) day. The pain upon palpation of the limb disappeared on post-operative day 3 (2–4) in the EPO-treated group compared to day 7 (6–9) in the control group ($p < 0.01$). The time until removal of the implant in cats from the EPO group was significantly shorter (median 61; range 60–70 days) than in cats from the control group (median 72.5; range 65–80) ($p < 0.05$).

The results from hematological analysis confirmed that all studied parameters were within the reference ranges for the species. The RBC counts, Hb content, and Hct increased during the different post-operative periods only in patients from the EPO group. By the end of the 8th post-operative week, they were statistically significantly higher than the respective average values of control cats (Table 2).

During the first post-operative week, the fracture line was visible and both groups exhibited decreased radiopacity at the fracture site corresponding to bone resorption occurring at the ends of bone fragments. Initial bone callus formation was visualized on radiographs during the second week after the osteosynthesis at the time of calcification, which was more evident in the EPO group. Bridging callus formation was manifested during the 3rd and the 4th week, and again, was more expressed in the EPO group. Signs of bone remodeling were observed during the 8th week when the bone regained its initial shape. The radiological scores of both groups are presented in Table 3.

The results from DEXA scans confirmed that the bone mineral density of operated femurs exceeds that of healthy contralateral limbs, with a statistically significant difference in the EPO group (Fig. 1).

Discussion

In cats, the proportion of femoral bone fractures is reported to be 20%–25% (Beale, 2004; DeCAMP *et al.*, 2016). According to a more recent study, the percentage of femoral fractures from all long tubular fractures was 52% (Vasileva and Chaprazov, 2022). The commonest causes are road accidents and high-rise syndrome injuries (Denny and Butterworth, 2000; Piermattei *et al.*, 2006; Scott and McLaughlin, 2007). The patients included in this study were with fragmented femoral fractures, associated with bone defects requiring a longer healing period. The effects of the local application of EPO on the healing of fragmented fractures were assessed by

Table 2. Results from studied hematological parameters (mean ± SD, $n = 6$).

		0 hour	1 week	2 weeks	3 weeks	4 weeks	6 weeks	8 weeks
WBC,	Control	17.2 ± 6.6	16.1 ± 3.4	13.2 ± 6.2	11.7 ± 3.1	10.8 ± 2.8	10.9 ± 2.1	11.9 ± 2.9
G/l	EPO	18.1 ± 7.9	15.3 ± 7.3	13.3 ± 2.7	8.8 ± 4.2	15.3 ± 6.6	11.8 ± 4.3	9.4 ± 2.2
RBC,	Control	5.9 ± 1.1	5.6 ± 1.3	6 ± 0.9	5.5 ± 0.5	5.7 ± 0.7	5.5 ± 0.9	5.9 ± 0.3
T/l	EPO	6.2 ± 1.3	6.3 ± 1.1	7.7 ± 1.4*	7.6 ± 1.3**	7.5 ± 2	7.8 ± 1.6*	9.2 ± 1.4***
Hb, g/l	Control	94.2 ± 18.6	90.3 ± 17.7	90.5 ± 9.9	92.5 ± 6.5	95.5 ± 11.1	94 ± 9.8	97 ± 4.1
	EPO	90.2 ± 15.6	93 ± 18.4	109.3 ± 19.1	106 ± 10.2	106.8 ± 20.3	106.8 ± 20.3	122.2 ± 12.8*
Hct, %	Control	28.4 ± 2.7	27.8 ± 5.1	28.5 ± 2.7	29.2 ± 2.5	28.9 ± 2.4	29.1 ± 2.4	28.1 ± 2.7
	EPO	26.9 ± 3.9	27.7 ± 6	33.7 ± 5.9	31.9 ± 3.5	30.8 ± 6.8	32.6 ± 4.9	36 ± 3.6**
PLT,	Control	238.8 ± 144.4	220.2 ± 100.4	264.2 ± 66.5	283.3 ± 82.9	260.5 ± 111.4	273.2 ± 118.2	245.3 ± 102.8
G/l	EPO	243.7 ± 150.7	262.8 ± 162.8	367.3 ± 115.9	316.8 ± 143.2	322 ± 179.8	261.3 ± 212.2	206.1 ± 118.2

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ between groups at the same period.

Table 3. Radiological healing scores in cats from control ($n = 6$) and EPO-treated ($n = 6$) groups. Data are presented as median (min-max).

Post-operative week	Control	EPO
1 week	1 (1–2)	2 (1–2)*
2 weeks	2 (2–3)	3 (2–3)*
3 weeks	2.5 (2–3)	4 (3–4)*
4 weeks	3.5 (3–4)	4.5 (4–5)*
6 weeks	4.5 (4–5)	5.5 (5–6)*
8 weeks	6 (5–6)	6 (6–6)

* $p < 0.05$ between groups.

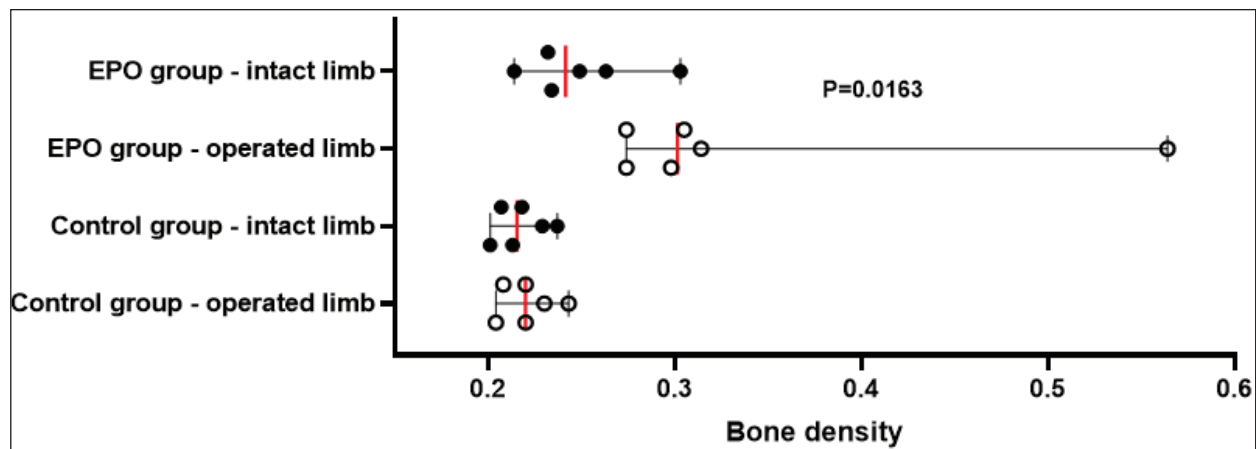


Fig. 1. Bone mineral density (g/cm^2) of the femur in control and EPO-treated cats. The red line corresponds to the median value.

clinical, laboratory, and radiological methods, familiar to every orthopaedist.

The pain from limb loading, pain after palpation at the fracture site, and weight bearing on the operated limb are among the most frequently used clinical criteria for the evaluation of bone healing (Corrales *et al.*, 2008). The last parameter was found to increase with time after the surgery and correlated with fractured bone strength (Joslin *et al.*, 2008). The cats from the EPO group began bearing weight on the operated limb as early as the first post-operative day, whereas control cats—on the third day. This finding indicates stable fixation of bone fragments from one hand and dull pain sensation from the other. EPO pain control (Derakhshan, 2018) is mediated through its regulatory effect on inflammation, as it inhibits the expression of pro-inflammatory factors by reducing of activity of macrophageal nuclear factor kappa B (Nairz *et al.*, 2011).

Complete blood count tests are used for monitoring the clinical status of the patients and occurred post-traumatic changes for the prevention of possible complications. On the 7th day after the osteosynthesis, Kumar *et al.* (2016) reported an insignificant decrease in RBC counts and Hb content, followed by variations

within the reference range for the species, a finding supported by our results in control animals (Kumar *et al.*, 2016). Comparable results were also reported by Tembhurne *et al.* (2010) and Rajhans (2013) in dogs (Tembhurne *et al.*, 2010; Rajhans, 2013), Kumar *et al.* (1999) in calves (Kumar *et al.*, 1999), and Gabriel *et al.* (2014) and Gupta (2015) in goats (Gabriel *et al.*, 2014; Gupta, 2015). The transient reduction of RBC counts in the above-mentioned studies may be attributed to fracture trauma and preoperative and intraoperative blood loss. In the EPO group, the opposite tendency was observed: RBCs, Hb, and Hct increased gradually than the initial levels, with a statistically significant difference versus baseline on the 8th post-operative week. This is attributed to the hematopoietic effect of EPO, which was also observed in experimental studies in rats (Diker *et al.*, 2018). In humans subjected to orthopedic surgery, the perioperative application of EPO reduced the need for blood transfusion (Tamir *et al.*, 2000).

Radiographic changes are characterized by fracture gap widening during the first post-operative week due to the enhanced osteoclastic activity, which usually occurs within 2–4 weeks (Langley-Hobbs, 2003).

After the second week, bone formation events were initiated, which in general are not clearly obvious in primary bone healing and may worry the inexperienced clinician. After the application of EPO, bone healing occurred within a shorter period and even earlier than is usually observed for secondary bone healing. In a feline model of closed femoral fracture fixed with intramedullary osteosynthesis, Emery and Murakami (1967) found no radiological signs of bone formation 2 weeks post surgery, periosteal thickening was observed only by the third week, and during the fourth week—first callus appearance (Emery and Murakami, 1967). For the cats from the EPO group, these processes occurred about 1 week earlier than in the control group: periosteal reaction was visualized by the second week and bone formation—in the third post-operative week. The obtained results confirmed that the radiological assessment of fracture healing was based on the loss of bone fragment sharpness 1–2 weeks after the traumatic injury and the visualization of periosteal callus 3–6 weeks after the fracture (Jeon *et al.*, 2020).

The bone healing rate depends on several factors: the type of fractured bone, the fracture type, the patient's age, the treatment technique, and the presence of other systemic diseases (Claes *et al.*, 2012). Fragmented fractures heal more slowly because of the relative instability of bone fragments and decreased angiogenesis and blood supply of fragments (Dhillon and Dhatt, 2012).

In skeletally immature dogs and cats and in ideal conditions with appropriate vascularisation and stable fixation with plates and screws, the expected time for bone union of a simple diaphyseal fracture is about 4–12 weeks (Menghini *et al.*, 2023). According to Drosos *et al.* (2006), human patients with fragmented fractures needed more time for defect healing whereas out results in the EPO group are entirely close to the normal time for bone healing regardless of the fracture type (Drosos *et al.*, 2006).

The DEXA scans performed after the bone union provided proof for a higher bone mineral density of the operated limb compared to the contralateral intact limb. These results are in line with the only similar study, performed more than 20 years ago in men. Janes *et al.* (1993) affirmed that the increased mineral content 2 weeks after the removal of the metal osteosynthesis plate was due to the amount of formed mineralized bone callus and the still incomplete bone remodeling events (Janes *et al.*, 1993). The authors did not associate these changes with fractured bone strength, whereas a more recent study proved a positive correlation between bone strength and bone mineral density measured by means of DEXA (Seo *et al.*, 2014). It should be, however, remembered that bone strength depends not only on bone mineral content but also on structural and biochemical bone features (Hart *et al.*, 2017). The observed statistically significant differences in bone mineral density between the control and EPO groups

supported the data of Holstein *et al.* (2007) that local EPO application increased the strength of the newly formed bone callus (Holstein *et al.*, 2007).

Conclusion

On the basis of the results, it may be concluded that the local application of EPO in cats with fragmented femoral fractures enhanced bone healing and increased bone callus strength without any significant systemic effects.

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Conflicts of interest

The authors declare no conflict of interest.

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Authors' contributions

Both authors contributed to this study. Both authors read and approved the final manuscript.

Data availability

All data from this study are provided in the manuscript.

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