Increased bone formation in a rabbit long-bone defect model after single local and single systemic application of erythropoietin

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Background and purpose — Delayed bone healing with non-union is a common problem. Further options to increase bone healing together with surgery are needed. We therefore evaluated a 1-dose single application of erythropoietin (EPO), applied either locally to the defect or systemically during surgery, in a critical-size rabbit long-bone defect.

Material and methods — 19 New Zealand White rabbits received a 15-mm defect in the radius diaphysis. An absorbable gelatin sponge was soaked with saline (control group and systemic treatment group) or EPO (local treatment group) and implanted into the gap. The systemic treatment group received EPO subcutaneously. In vivo micro-CT analysis was performed 4, 8, and 12 weeks postoperatively. Vascularization was evaluated histologically.

Results — Semiquantitative histomorphometric and radiological evaluation showed increased bone formation (2.3- to 2.5-fold) in both treatment groups after 12 weeks compared to the controls. Quantitative determination of bone volume and tissue volume showed superior bone healing after EPO treatment at all follow-up time points, with the highest values after 12 weeks in locally treated animals (3.0- to 3.4-fold). More vascularization was found in both EPO treatment groups.

Interpretation — Initial single dosing with EPO was sufficient to increase bone healing substantially after 12 weeks of follow-up. Local application inside the defect was most effective, and it can be administered directly during surgery. Apart from effects on ossification, systemic and local EPO treatment leads to increased callus vascularization.
be sufficient to improve bone healing is the subject of debate (Rölling et al. 2014a).

We therefore analyzed bone healing after single local or systemic EPO application in an established in vivo rabbit long-bone model based on a 15-mm-radius critical-size defect.

Material and methods

Animals and surgical procedure

19 skeletally mature 6-month-old female New Zealand White rabbits with closed epiphyseal plates were randomly assigned to 1 control group and 2 treatment groups. Mean animal weight was 4.2 (3.6–4.8) kg. A detailed description of the animal model has already been published (Kleinschmidt et al. 2013, 2014). Briefly, a 15-mm defect was prepared in the middle of the diaphysis of the radius using a handsaw. Due to physiological membrane fixation of the proximal and distal endings of the radius to the ulna, no additional fixation of the defect was needed to stabilize the radius. The resulting gap in the radius was filled with an absorbable gelatin sponge (Gelita-Spon; Gelita Medical BV, Amsterdam, the Netherlands). Gelita-Spon is a common, regularly available hemostat without intrinsic hemostatic action, which induces hemostasis through its intensely porous structure. It is pH-neutral, and therefore suitable as a local drug carrier. Depending on application and environment, it is completely biodegraded in less than 4 weeks (Goncalves et al. 2015). The sponge was cut into pieces of 2 × 0.5 cm and soaked in sterile saline (control group and systemic treatment group) or EPO (local treatment group).

6 rabbits served as controls that did not receive EPO. The remaining 13 rabbits were divided into 2 EPO treatment groups. 6 animals received 1 single high dose (4,900 IU per metabolic body weight in kg (metkg)) of EPO systemically (subcutaneously; injection site: upper back) during surgery after the defect was prepared and the saline-soaked gelatin sponge was placed. 7 animals received 1 single high dose (4,900 IU/metkg) of EPO locally. Here, the gelatin sponge was manually soaked with the individual animal EPO dose during surgery, immediately before the sponge was placed in the defect. The rationale for this setup was that it was an easy-to-use application process that could be directly transferred to the clinical situation, since all components and substances are routinely available without further modifications.

The rabbits were kept in single cages in the institutional animal laboratory according to animal care regulations (automatic light-dark cycle, pain medication with carprofen (4 mg/ kg body weight), with water and food ad libitum). Follow-up was done by in vivo micro-CT analysis 4, 8, and 12 weeks postoperatively. The rabbits were then killed bypentobarbital injection and specimens were resected for additional histological evaluation.

EPO dosage

We used Epoetin alfa (Erypo FS; Centocor BV, Leiden, the Netherlands). 1 IU contained 0.0084 µg Epoetin alfa, which is genetically engineered from ovarian cells of the Chinese hamster (cell-line CHO-K1). The clinically available, ready-to-use standard injection fluid also contained polysorbate 80, glycine, sodium chloride, disodium phosphate dihydrate, sodium dihydrogen phosphate dihydrate, and water.

For dosage, the metabolic body weight was applied and calculated as follows: metabolic body weight of the animal = individual body weight of the animal to the power of 0.75. Based on our own unpublished pilot experiments on the New

Figure 1. Bone formation score from 0–3 at endpoint. No relevant bone growth (bone formation thickness < 200% of ulna corticalis; bone formation width < 50% of the 15-mm-long defect) was assigned a score value of 0 (panel A). Bone formation thickness > 200% and width > 50% without significant separation from the ulna was assigned a score value of 1 (B). Bone growth around the radial aspect of the ulna with significant separation from the ulna over a distance of > 50% of the 15-mm-long callus was assigned a score value of 2 (C). Bone growth with radius corticalis and marrow cavity formation was assigned a score value of 3 (D). Here, long bone corticalis and marrow cavity should be visible over a distance of > 50% of the 15-mm-long callus.
Zealand White rabbit and clinical data on anemic cancer patients (Shasha et al. 2003; Gabrilove et al. 2001), a single EPO dosage of 4,900 IU/metkg was chosen as a feasible single high dose, avoiding any side effects related to increase in hematocrit.

**Semiquantitative histomorphometric and radiological evaluation of bone formation after 12 weeks**

Micro-CT scan reconstructions in all planes and sagittal histological slides from each rabbit were manually analyzed independently by 2 observers (GO and AS) who were blinded regarding the treatment group. They judged bone growth semiquantitatively according to a scoring system ranging from 0 to 3 points (Figure 1), where 0 points meant no relevant bone growth (bone formation thickness less than 200% of ulna corticalis; width of bone growth less than 50% of the 15-mm-long defect); where 1 point meant isolated bone growth around the radial aspect of the ulna (more than 20% thickness; more than 50% width); where 2 points meant bone growth around the radial aspect of the ulna with separation from the ulna (separation should be visible in more than 50% of the 15-mm-long callus); and 3 points meant bone growth with radius corticalis and marrow cavity formation (new long-bone corticalis and marrow cavity should be visible in more than 50% of the 15-mm-long callus). As measures of reliability, Cohen’s kappa indicated a good (control animals: \( \kappa = 0.7, p = 0.4 \)) to very good (systemically or locally treated animals: \( \kappa = 1, p = 0.01 \)) level of agreement between the 2 raters.

**Quantitative micro-CT analysis**

In vivo scanning of the rabbit forelegs was done 4, 8, and 12 weeks postoperatively. The procedure was performed as previously reported (Kleinschmidt et al. 2013). Briefly, a micro-CT scanner (1076; Skyscan, Antwerp, Belgium) was used together with a rabbit bedding device customized by RJL Micro & Analytic (Karlsdorf-Neuthard, Germany). This allowed positioning of the animal beyond the system while only the foreleg was fixed in an aluminum tube that moved the region of interest horizontally into the course of the X-ray beam. Proximate scans were performed using a 0.5-mm aluminum filter with the following settings: 48 kV, 200 mA, 320 ms, spatial resolution 17.7 mm³/voxel, and rotation step 0.6°. Parameters were analyzed (using Skyscan software) with a lower gray level set at 40 and an upper gray level set at 255 to define callus tissue. The volume of interest (VOI) was determined in its proximal and distal dimensions by choosing the middle of the defect and taking the same number of 200 slices, which corresponded to 7 mm in each direction. Then interpolated VOIs that circumscribed the radius and whole callus excluding the ulna were drawn manually and automatically shrunk to the outer boundaries of the callus using the shrink-wrap function of the software. As objective parameters for quantitative evaluation of new bone formation inside the radius defect, volume of the whole callus tissue including non-mineralized marrow areas (tissue volume (TV)) and volume of the mineralized callus tissue (bone volume (BV)) were calculated.

**Histology**

After 12 weeks, complete foreleg specimens were fixed, decalcified, and paraffin-embedded. Then, 5-µm-thick longitudinal sections were cut and stained with Masson-Goldner trichrome, hematoxylin and eosin, and Safranin-O, following standard protocols (Kleinschmidt et al. 2013). Aligned overview microphotographs of total slices were made to assess the adequacy of micro-CT-based analysis. For further evaluation, magnified images were taken from the area of the defect.

**Quantitative histomorphometric analysis of blood vessels**

HE-stained tissue sections were analyzed for capillaries and blood vessels that could be easily identified morphologically. For a quantitative evaluation, capillaries and vessels were encircled to calculate the surface area, which was divided by the total area of the tissue section according to Image J software protocols. Examinations were performed by 2 independent and blinded examiners (GO and AS), with good to very good inter-observer reliability (absolute agreement: 0.856–0.999). Data were expressed as the percentage of vascular surface area within the callus.

**Statistics**

The inter-rater reliability for the semiquantitative score (0–3) was tested using Cohen’s kappa. The inter-rater reliability for the quantitative analysis of blood vessels in histological slides was tested using intraclass correlation coefficients. EPO blood concentration was compared across 4 different points in time (day 0, 7, 14, and 21), using non-parametric Friedman tests with post hoc Wilcoxon tests. Differences between the animal groups were assessed with Kruskal-Wallis test and post hoc Mann-Whitney tests. Bonferroni correction for multiple comparisons was also performed, changing the significance level from \( p < 0.05 \) to \( p < 0.017 \), so statistical significance was only assumed for \( p < 0.017 \). All analyses were performed with SPSS version 21.0.

**Ethics**

The study was approved by the committee for animal experimentation of Baden-Wuerttemberg, Germany (reference number AZ35-9185.81/G-109/06).

**Results**

**Animals**

All animals completed the study to the endpoint. EPO therapy in humans has been rarely associated with diarrhea, fever, and local exanthema or edema at the injection site (Henry 2005). None of these effects and no other complications were found.
The evaluation of hematocrit showed no statistically significant change in the control animals (p = 0.7) and in the animals treated locally (p = 0.09), but a relevant increase was seen in systemically treated animals (p = 0.02) until day 14. For all groups, there was a tendency of a decrease in initial hematocrit after 21 days (Table 1).

|                          | Control group | Local group | Systemic group |
|--------------------------|---------------|-------------|----------------|
| Day 0                    | 43 [6.9] (35–52) | 40 [5.3] (33–44) | 38 [5.2] (31–43) |
| Day 7                    | 38 [5.1] (32–45) | 50 [2.8] (46–53) | 48 [5.4] (41–54) |
| Day 14                   | 42 [9.3] (34–57) | 44 [5.2] (39–51) | 48 [6.2] (40–55) |
| Day 21                   | 39 [3.1] (36–44) | 42 [2.7] (39–44) | 43 [3.0] (40–46) |

The control animals showed no bone growth (n = 1) or minor bone growth around the radial aspect of the ulna (n = 5) without any signs of radial bone separation from the ulna, or corticalis and marrow cavity formation (mean score: 0.8).

Locally treated animals showed bone growth around the radial aspect of the ulna (n = 2), bone growth around the radial aspect of the ulna with separation from the ulna (n = 3), and bone growth with radius corticalis and marrow cavity formation (n = 2) (mean score: 2.0).

Systemically treated animals showed bone growth around the radial aspect of the ulna (n = 2), bone growth around the radial aspect of the ulna with separation from the ulna (n = 3), and bone growth with radius corticalis and marrow cavity formation (n = 1) (mean score: 1.8).

**Semiquantitative evaluation of bone formation**
Radiologically increased bone formation (Figure 1) was found in locally treated animals (p = 0.005) and systemically treated animals (p = 0.02) compared to the controls (overall Kruskal-Wallis test, p = 0.01).

The control animals showed no bone growth (n = 1) or minor bone growth around the radial aspect of the ulna (n = 5) without any signs of radial bone separation from the ulna, or corticalis and marrow cavity formation (mean score: 0.8).

Locally treated animals showed bone growth around the radial aspect of the ulna (n = 2), bone growth around the radial aspect of the ulna with separation from the ulna (n = 3), and bone growth with radius corticalis and marrow cavity formation (n = 2) (mean score: 2.0).

Systemically treated animals showed bone growth around the radial aspect of the ulna (n = 2), bone growth around the radial aspect of the ulna with separation from the ulna (n = 3), and bone growth with radius corticalis and marrow cavity formation (n = 1) (mean score: 1.8).

**Quantitative micro-CT analysis of bone formation**
Compared to control animals, both locally treated and systemically treated animals showed increased bone formation 4, 8, and 12 weeks after single EPO application (Figure 2). Quantitative analysis of bone formation with TV as a parameter defining the whole new callus volume and BV defining the volume of mineralized parts of the new callus showed higher values compared to control animals without EPO treatment at all time points (Figure 3).

**Quantitative analysis of blood vessels**
Significantly higher callus vascularization was found in both the local group (21.1% vessel surface area; 3.1-fold; p = 0.006) and the systemic group (22.3% vessel surface area; 3.3-fold; p = 0.004) compared to the control group with 6.8% vessel surface area (Figure 4).

**Discussion**
Our study clearly demonstrates the potency of regularly available EPO as an additional treatment option to increase bone healing. However, improvements in the efficiency of EPO on bone formation with less hematopoietic side effects could be achieved by using EPO-derived substances that bind specifically to the heterodimeric EPOR/CD131 receptor for pleiotropic effects instead of EPOR-mediated hematopoietic effects (Brines et al. 2004, Leist et al. 2004, Brines and Cerami 2008, Bohr et al. 2013). Further studies on EPO-derived substances are needed, to find optimal treatment modalities due to possible differences in the effectiveness and potency on bone healing. Longer-lasting EPO molecules especially offer theoretical advantages.

The risk of relevant side effects appears to be low if EPO is only used in a single dose, which was evident in the present study even with a very high single dose of EPO. Mean values of hematocrit, as a main parameter of the side effects of EPO, only increased in systemically treated animals until day 14. The pharmacological and pharmacokinetic characteristics of subcutaneously administered EPO with dosing once a week have been reported (Henry 2005) but these are hardly comparable to the situation in our animal model. Our finding that locally administered EPO resulted in less red blood cell production in the bone marrow may have different explanations. Although we used Gelita-Spon as a carrier substance in the radius defect, local application to the surgically opened defect carries a higher risk of loss of EPO during application,
compared to use of a subcutaneous needle in tissue that has not been surgically opened. Secondly, EPO might be transported more quickly after local application in a bone defect with higher initial peak serum concentration but shorter elimination half-life.

Apart from the direct influence of EPO on bone, EPO-induced neovascularization is another important mechanism for promotion of bone healing. Our study revealed effects on neovascularization with significantly increased blood vessel formation after either local of systemic single-dose EPO treatment, and with a follow-up of 12 weeks. Considering the positive effect on bone formation expressed in the increase in BV and TV in quantitative in vivo micro-CT analysis, EPO treatment appears to have a rather immediate influence on bone formation, which is sustained over time during the 12 weeks of follow-up. Angiogenesis, however, was only evaluated by histology after 12 weeks, at final follow-up. One limitation of the study was that we did not analyze vascularization in the initial phase.

Possible negative effects of EPO on bone healing have been proposed by Singbrant et al. (2011), but this hypothesis has been dismissed by others (Shiozawa et al. 2010, McGee et al. 2012, Sun et al. 2012), and we also found clear evidence of positive effects on bone formation.

It is a matter for debate whether EPO should be given on a daily or a weekly basis to have an effect on bone healing, or whether an initial single dosage is sufficient. The effect of EPO on bone healing appears to be dose-dependent, but the
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