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LOCAL APPLICATION OF ERYTHROPOIETIN AND XENOGRAFT FOR ENHANCING BONE REGENERATION

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ABSTRACT

The aim of the present study was to investigate the effects from local co-administration of erythropoietin and cancellous bone granules (bone substitute) on haematological parameters and bone regeneration in a calvarial bone defect model in rats. The study was performed with 12 male Wistar albino rats, 6 months of age. Two symmetrical 5-mm critical-size defects were created in the calvaria of each animal. A combination of bone substitute and physiological saline-soaked collagen membrane was placed in the right defect site, whereas bone substitute + erythropoietin-soaked collagen membrane – in the left defect site. Bone regeneration was monitored by radiography and computed tomography on the 30th and 90th days after the surgery. Blood samples were collected at the same time intervals for determination of erythrocyte counts, haemoglobin content and haematocrit. The independent application of a cancellous bone granules xenograft resulted in bone regeneration of critical-size calvarial bone defects in rats. Its co-administration with erythropoietin enhanced the bone healing process.

Key words: erythropoietin, xenograft, calvaria, bone regeneration, rats.

Introduction

Bone defects could occur consequently to fractures, removal of a tumour growth, congenital malformations or periodontal disease (Develioglu et al., 2009). For many years, the „gold standard“ of their treatment has been the autogenous bone grafts, whereas now, a variety of bone substitutes of natural or synthetic origin are successfully applied at the defect site (Shafiei et al., 2009; Nandi et al., 2010; Bigham et al., 2011; Parizi et al., 2012).

The main disadvantage of bone grafts or bone substitutes is the scarce vascularisation at the defect site, which requires combination with angiogenic factors promoting the formation of blood vessels (Altundal et al., 2005).

Many researchers have confirmed that apart its physiological role for erythropoiesis, the erythropoietin (EPO) possessed additional, pleiotropic functions (Mocini et al., 2007; Zubareva et al., 2019). Expression of EPO receptors in the vascular endothelium and smooth muscles was demonstrated, which stimulates angiogenesis, wound healing and vascular protection (Jaquet et al., 2002; Heeschen et al., 2003; Yaghobee et al., 2018). With respect to bone regeneration, EPO enhanced fracture healing and improved mechanical bone strength (McGee et al., 2012; Wan et al., 2014; Klontzas et al., 2016). The similarity of EPO's angiogenic potential to that of the vascular endothelial growth factor (VEGF) determines its important role in bone healing events (Steinbrech et al., 2000; Jaquet et al., 2002).

The aim of the present study was to investigate the effects from local co-administration of erythropoietin and cancellous bone granules (bone substitute) on haematological parameters and bone regeneration in a calvarial bone defect model in rats.

Materials and methods

Experimental design: Twelve male Wistar albino rats, 6 months of age, with average weight 262±33 g were used in the study. The experiment was performed in accordance with permit No. 241 issued by the Ethics Committee to the Bulgarian Food and Safety Agency. The rearing and housing of experimental animals was fully compliant with 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.

Surgical intervention: The rats were anaesthetised with 80 mg/kg ketamine hydrochloride 10% (Anaket®, Richter Pharma AG, Austria) and 10 mg/kg xylazine hydrochloride 2% (Xylazin®, Bioveta, Czech Republic) applied intramuscularly. After aseptic preparation, standardised 5-mm critical-size calvarial bone defects were created as described by Spicer et al. (2012). Two symmetrical 5-mm critical-size defects were created in the calvaria of each animal. A combination of bone substitute (Bio-Gen cancellous bone granules of equine origin; BiOTECK, Italy) and physiological saline-soaked collagen membrane (Collacone®; Botiss biomaterials GmbH, Germany) was placed in the right defect site, whereas bone substitute + collagen membrane soaked with erythropoietin (epoietin alpha Binocrit® 2000 IU Sandoz GmbH, Austria) – in the left defect site.

Clinical and haematological examinations: Daily physical examination was conducted in the post operative period and a special attention was paid on the operative wound state. Blood samples were collected at the day of surgery as well as on post surgery days 30 and 90 from the lateral tail vein to determine the effect of erythropoietin application. Erythrocyte counts (T/L), haemoglobin content (g/L) and haematocrit (%) were assayed on automated haemanalyzer (BS-5000 Vet, Mindray).

Radiography: Serial dorsoventral radiographies of the head were done on the same days with stationary X-ray equipment PHILIPS SUPER 50 CP-D (Hamburg, Germany). Relative bone density (RBD) was measured on digital radiographs with ImageJ software as ratio of mean gray values of the defect and mean gray value of surrounding bone.

Computed tomography: Bone regeneration course was also monitored by computed tomography (CT) scans at the same time intervals. For evaluation of the effect of bone substitute + erythropoietin co-administration, 3D images were reconstructed using the ANIMAGE reconstructing software. By means of conventional 3D CT, evaluation of bone formation and bone bridging quality at the defect site was done by the 4-point scoring system of Patel et al. (2008).

Statistical analysis: Relative bone density values are presented as mean and standard deviation (SD), and Patel bone regeneration scores: as median and range. Differences between relative bone density values of left and right defects were assessed by one-way analysis of variance ANOVA, whereas differences between bone regeneration scores and haematological parameters: by the Mann-Whitney test. All analyses were performed with the statistical software MedCalc v.15.8 (Belgium)

Results and Discussion

Clinical and haematological examinations

There were neither complications in the post operative period, nor any side effects from EPO application. Median values of erythrocyte counts, haemoglobin content and haematocrit on post operative days 30 and 90 showed that locally applied EPO had no systemic effect (Fig. 1). Values differed insignificantly and were within the reference ranges for the species.

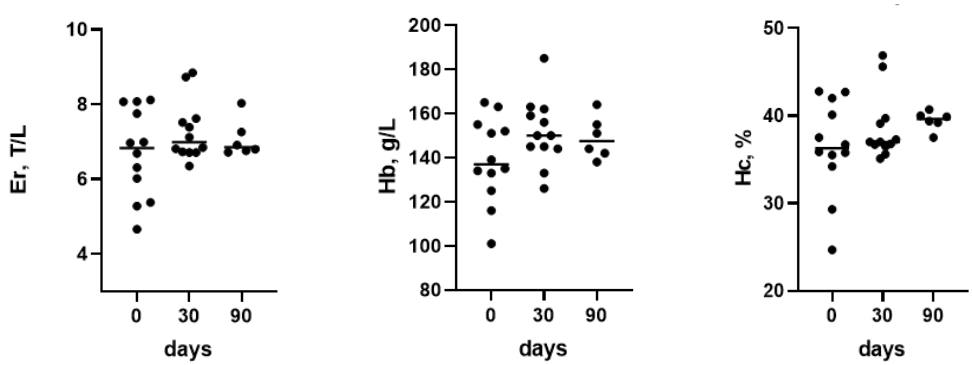


Figure 1: Erythrocyte counts, haemoglobin content and haematocrit in rats (n=12) on day of surgery (day 0) and post operative days 30 and 90. Horizontal lines on graphs are the median values on respective time intervals.

Radiography

Radiographies performed on post operative day 30 revealed organisation of cancellous granules. This was even more pronounces on the 90th day and was accompanied with healing of bone substitute with defect margins (Fig. 2).

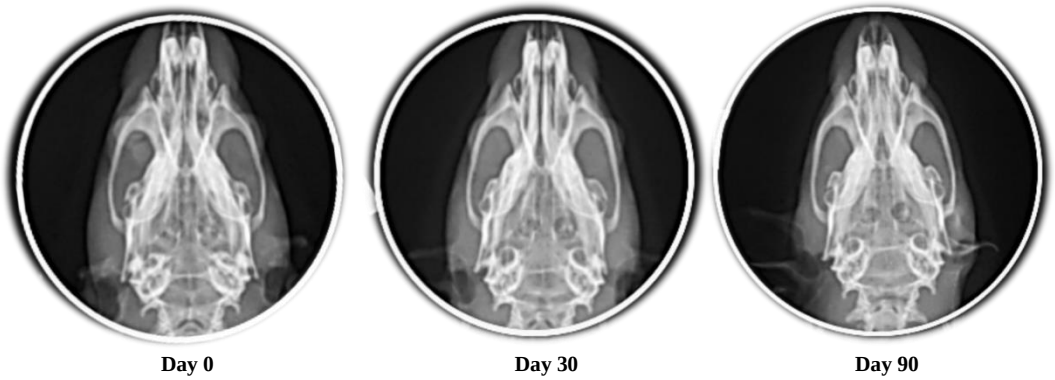


Figure 2: Radiographs of calvarial bones on days 0, 30 and 90.

The results from measurement of relative bone density of defects showed statistically insignificant increased bone density with time (Table 1). A statistically significant difference between both treatments was found out on post operative day 90 ($p<0.05$).

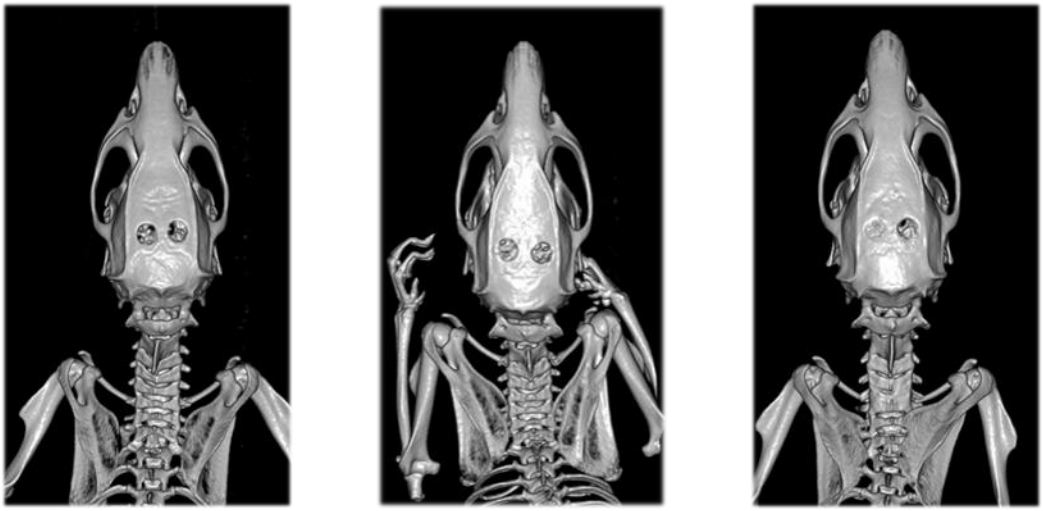
Table 1: Relative bone density of defects (mean ± SD, n=12) on day of surgery (day 0) and post operative days 30 and 90.

	Day 0	**Day 30**	**Day 90**
Right defect	0.95±0.03	0.94±0.05	0.95±0.02
Left defect	0.97±0.02	0.95±0.05	0.98±0.02*

** $p<0.05$ vs the contralateral defect for the same period*

Computed tomography

The evaluation of bone regeneration quality demonstrated bone bridging along the defect length. This was more pronounced for left defects, where bone substitute was combined with EPO (Fig. 3). Difference between regeneration scores on day 30 were statistically significant ($p<0.05$); the same was observed on day 90 ($p<0.05$; Table 2).



Day 0 **Day 30** **Day 90**

Figure 3: 3D CT scans of calvarial bones on days 0, 30 and 90.

Table 2: Bone regeneration scores according to Patel et al., 2008 (median and range, n=12) on day of surgery (day 0) and post operative days 30 and 90.

	Day 0	**Day 30**	**Day 90**
Right defect	1 (1-1)	3 (1-3) ***	3 (3-4) ***
Left defect	1 (1-1)	4 (2-4) *** ##	4 (3-4) *** #

**** $p<0.001$ vs Day 0 within a row; # $p<0.05$; ## $p<0.01$ vs the contralateral defect for the same period.*

The information on the use of erythropoietin in combination with different grafts is scarce. Our results agree with those of Kharkova et al. (2019) who investigated tricalcium phosphate scaffolds with erythropoietin and concluded that they were promising with respect to bone healing promotion.

As anticipated, the created defects healed completely for the experimental period, but the effect of co-administration was better. It turned out that EPO potentiated the effect of bone substitute. This was probably due to the fact that it improved considerable the vascularisation, promoting VEGF synthesis and angiogenesis. An increased number of blood vessels after application of a xenograft and systemic treatment with 500 IU/kg EPO for 28 days was reported by Diker et al. (2018) in a rat model of calvarial bone defects.

Conclusion

The independent application of a cancellous bone granules xenograft resulted in bone regeneration of critical-size calvarial bone defects in rats. Its co-administration with erythropoietin enhanced the bone healing process. Further experimental and clinical investigations are however necessary to determined the appropriate dose and route of EPO application.

Acknowledgements

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