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Review

PRECLINICAL STUDIES ON PLEIOTROPIC FUNCTIONS OF ERYTHROPOIETIN ON BONE HEALING

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Summary

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Erythropoietin (EPO) is a glycoprotein hormone, mainly known for its haemopoietic function. For orthopaedics, its pleiotropic effects – osteogenic and angiogenic potential, are of primary interest. The exact mechanism of EPO action is still unclear. The effects of EPO on bone healing were investigated through experiments with rats, mice, rabbits and pigs. Each of used models for experimental bone defects (calvarial models, long bone segmental defects, posterolateral spinal fusion and corticosteroid-induced femoral head osteonecrosis) has specific advantages and flaws. Obtaining specific and correct results is largely dependent on the used model. The brief evaluation of models could serve for standardisation of preclinical studies on bone regeneration.

Key words: bone defect, bone healing, erythropoietin (EPO), experimental models

INTRODUCTION

Erythropoietin (EpO) is a glycoprotein hormone, mainly known for its haemopoietic function (Zubareva *et al.,* 2019). EPO promotes the growth and development of erythrocyte precursors in bone marrow and increases erythrocyte counts (Coleman & Brines, 2004; Debeljak *et al.,* 2014; Uversky & Redwan, 2017; Ganz, 2018; Perreault & Venters, 2018). Therefore, the treatment with erythropoietin is a standard for management of EPO-deficient anaemia, seen in chronic renal failure patients (Hayat *et al.*, 2008; Jelkmann, 2013).

The main source of EPO in adults is the peritubular renal cortex (Jelkmann, 2011). EPO gene expression was also detected in liver, lungs, spleen, brain, testes (Jelkmann & Wagner, 2004), heart (Obara, 2008), uterus, placenta, ovaries (Yasuda, 2001) and hair follicles (Bodo, 2007). The secretion of EPO is controlled by hypoxia-inducible factors 1 and 2 (HIF-1 and HIF-2) in response to reduced oxygen supply of tissues (Jones & Bergeron, 2001; Rankin *et al.*, 2007; Keswani *et al.,* 2011; Maiese *et al.,* 2012). During the intrauterine development of foetuses, the main source of EPO is the liver (Zanjani

et al., 1977; Dame *et al.*, 1998; Jelkmann, 2001).

EFFECTS OF ERYTHROPOIETIN ON MESENCHYMAL STEM CELLS AND BONE FORMATION

Apart its physiological role for erythropoiesis control, EPO fulfills also a number of non-haemopoietic functions (Mocini *et al.*, 2007). During the last decades, the so-called pleiotropic functions of EPO were subject of research. Evidence for expression of EPO-receptors in the endothelium and smooth vascular muscles which promoted angiogenesis, wound healing and vascular protection was provided (Jaquet *et al.*, 2002; Heeschen *et al.*, 2003; Yaghobee *et al.,* 2018). The protective effects of EPO on brain, heart and kidney tissues manifested by protection from stroke (Tsai *et al.*, 2006), myocardial infarction (Cai *et al.*, 2003; Calvillo *et al.*, 2003; Moon *et al.*, 2003; Parsa *et al.*, 2003; Wright *et al.*, 2004) and renal ischaemia (Ates *et al.*, 2005) were reported. Its anti-inflammatory effect, attributed to the fact that EPO counteracts the action of some proinflammatory cytokines TNFα and IL-6 was also reported (Cervellini *et al.*, 2013).

The osteogenic and angiogenic potential of erythropoietin are of particular interest for orthopaedics (Rölfing, 2014). Studies in this field have proved that EPO improved bone healing and could serve as a therapeutic means enhancing bone regeneration (Wan *et al.*, 2014; Klontzas *et al.*, 2016). The exact mechanism of action is still unknown. It is hypothesised that two pathways could be involved: direct and indirect. In the latter EPO binds to EPO-receptors on the surface of haemopoietic stem cells (HSCs), which activates a signalling pathway for synthesis of bone morphogenetic proteins (BMPs) (Kim *et al.*, 2012). The formation of BMPs, mainly BMP2 and BMP6, and their interaction with BMP-receptors, results in differentiation of bone progenitor cells into osteoblasts and stimulates callus formation (Sun *et al.*, 2012). In the former mechanism, EPO exerts it effect on bone healing directly via bone marrow stromal cells (BMSCs), inducing their differentiation into osteoblasts (Suresh *et al.,* 2019). Additionally, increased expression of the runt-related transcription factor 2 (Runx2) necessary for the growth of osteoblasts, accretion of minerals and increased activity of alkaline phosphatase, osteocalcin and bone sialoprotein is established (Shiozawa *et al.*, 2010).

Some research reports suggest that EPO stimulates directly the precursors of osteoclasts and monocytes and induces bone resorption (Hiram-Bab *et al.*, 2015; 2017; Orth *et al.,* 2019). Nevertheless, the enhanced formation of osteoclasts triggered by EPO does not result in their activation, meaning that EPO induces osteoclastogenesis but did not influence osteoclastic function (Shiozawa *et al.*, 2010).

The information presented so far allowed suggesting that EPO combines haemopoiesis and bone formation (McGee *et al.,* 2012). Tens of experimental research studies have been conducted for more detailed investigation of EPO effects on bone healing. One group of studies have analysed the effect of systemic application of EPO through intraperitoneal (Holstein *et al.*, 2007; Garcia *et al.*, 2011; Yan *et al.*, 2018) or subcutaneous injections (Bozlar *et al.*, 2006; Rölfing *et al.*, 2012). A second group has analysed the effect of its local application through EPO-soaked collagen or gelatin sponges (Rölfing *et al.,* 2014) or its inclusion along with hyaluronic acid in microhydrogel (Hahn *et al.,* 2007). A third group

of researchers have investigated the effects of both the local and systemic application of EPO (Omlor *et al.*, 2016; Li *et al.*, 2018). The information for the possible regenerative effect of EPO applied on bone graft is scarce (Diker *et al.*, 2018).

ANIMAL MODELS USED IN THE STUDY OF ERYHTROPOIETIC EFFECTS ON BONE HEALING – ADVANTAGES AND SHORTCOMINGS

The effects of local or systemic erythropoietin treatment on bone healing have been evaluated in models with various animal species: mice (Holstein *et al.,* 2007; Holstein *et al*., 2011; Garcia *et al***.,** 2011), rats (Diker *et al.*, 2018), rabbits (Rölfing *et al.*, 2012; Omlor *et al.*, 2016) and pigs (Rölfing *et al.*, 2012).

Rodents

Rodents (mice and rats) are a preferred animal model in preclinical studies on bone regeneration (Gomes & Fernandes, 2011). Their most important advantage is the possibility for using inbred lines, e.g. genetically identical laboratory animals from both sexes. The short duration of gestation (18-21 days) and rapid growth rate provide the necessary number of animals for experimental designs (Ostergaard *et al.*, 2010).

Rodents are not a suitable alternative in long-term studies and for experimental designs with repeated collection of blood samples or biopsies due to the short life span and relatively small blood and tissue volume. The limitations of using these species as compared to rabbits and pigs are due to small size and the thin and frail cortex of their long bones (An & Freidman, 1998).

Rabbits

Rabbits are used in research studies because of their small size, from one part, and due to similar bone mineral density of rabbits and humans from the other (Wang *et al.*, 1998). It should be noted that there are substantial differences between these two species as anatomy (size and shape of bones) is concerned (Pearce *et al.*, 2007). Another advantage of rabbits is that they attain skeletal maturity parallelly to sexual maturity at about 6 months of age (Gilsanz *et al.*, 1988). In comparison to primates or rodents, in rabbits these changes in the skeleton and bone transformation occur faster (Castaneda *et al.*, 2006).

Pigs

It is believed that pigs resemble humans as bone anatomy, morphology, remodelling, mineral density, concentration and bone regeneration events are concerned (Aerssens *et al.*, 1998; Thorwarth *et al.*, 2005). Pigs are appropriate animal models for critical size bone defects as their bone regeneration rate is comparable to that of men (Schlegel *et al.*, 2006). Disadvantages of using pigs in orthopaedic research comprise rapid growth rate and high body weight (Pearce *et al.*, 2007).

EXPERIMENTAL BONE DEFECT MODELS FOR INVESTIGATION OF ERYTHROPOIETIN EFFECTS ON BONE HEALING

In order to imitate orthopaedic states, defects have been created in various bones – calvaria (Rölfing *et al*., 2012; Diker *et al.,* 2018), radius (Omlor *et al.,* 2016), femur (Holstein *et al.,* 2007; Garcia *et al.,* 2011), tibia (Bozlar *et al.,* 2006).

Four primary bone defects models have been used in experiments evaluating

bone healing after erythropoietin application. Every one has its advantages and drawbacks which should be considered when selecting a specific model of bone defect.

Long bone defects

The creation of critical size segmental long bone defect is the standard in studies on the osteogenetic potential of various materials. This bone defect should not heal spontaneously during the lifetime of the animal (Horner *et al.*, 2010). By means of osteotomy, a bone segment is removed from a predetermined site. It is accepted that bone defect size should be 2–2.5 times larger than the bone diameter (ASTM, 2014). Its size is also influenced by other factors e.g. animal age and species, defect location, bone structure, presence of periosteum, mechanical loading of bone and bone metabolism, the condition of the animals, as well as bone fixation device used for stabilisation of the defect (Reichert *et al.*, 2009; Glatt & Matthys, 2014).

To evaluate the effect of EPO application on the healing of long tubular bones, models with closed fracture, osteotomy or ostectomy with creation of segmental bone defect have been used. When a rodent radius model is selected, the fracture may remain non-stabilised, whereas osteotomies in femoral or tibial diaphyses require additional bone fixation (Muschler *et al.,* 2010). Studies are presented in Table 1.

Calvarial bone defect

Calvarial defects are popular among researchers because the bone structure allows creating a standardised defect that could be then submitted to histological, radiological or computed tomography evaluation (Gomes & Fernandes, 2011).

Table 1. Long bone models used in investigation on EPO effects on bone healing

Animal species	Defect	EPO application	Effect on bone healing	References
Rats	Tibia, osteotomy	Systemic s.c. treatment with EPO 200 UI/kg for 7 days	↑ periosteal reaction ↑ bone union ↑ bone remodelling	Bozlar *et al.*, 2006
Mice	Femur, closed fracture	Systemic i.p. treatment with 5000 UI/kg EPO for 5 days	↑ expression of EPOR ↑ callus density ↑ callus mineralisation ↑ torsional stiffness	Holstein *et al.,* 2007
Mice	Femur, segmental defect	Systemic i.p. treatment with 500 UI/kg EPO for 2 or 10 weeks	↑ bone formation ↑ bone volume ↑ vascularisation	Holstein *et al.,* 2011
Mice	Femur, osteotomy	Systemic i.p. treatment with 500 UI/kg EPO for 2 or 10 weeks	↑ callus diameter ↑ callus mineralisation ↑ bone density	Garcia *et al.,* 2011
Rabbits	Radius, segmental defect	Single s.c. treatment with EPO 4900 UI/kg	↑ bone formation ↑ bone volume ↑ vascularisation	Omlor *et al.,* 2016
		Local application of EPO-soaked gelatin sponges (4900 UI/kg)		

The creation of calvarial defects is an easy procedure. It comprises a sagittal incision along the scalp surface and lifting of the skin flap. A critical size circular defect, most commonly on the parietal bone and comprising all layers is then made by trephination. Critical size bone defects are the smallest intraosseous defects that could not heal spontaneously during the life of an animal (Schmitz & Hollinger, 1986). The critical size in mice is 3–5 mm, in rats: 5–8 mm and in rabbits: 15 mm (Cooper *et al.,* 2010). Two openings are usually created, one serving as control. The surgical procedure is terminated with removal of the bone fragment in order to preserve dura mater from injury. The periosteum is returned to its place and the skin is sutured (Nakamura *et al.*, 2017).

The main advantage of the method is the possibility to introduce simultaneously the scaffold and EPO with a single approach without need from external fixation due to the pressure exerted by dura mater and skin (Gomes & Fernandes, 2011). A flaw of this approach is the impossibility to evaluate the efficacy of EPO in mechanical loads, important in regeneration of load-bearing bones.

Rölfing *et al.* (2012) performed a research to evaluate the effect of single local application of EPO on collagen carrier at the site of defects created by trephination in porcine skull bones. The results were assessed by the 5th post treatment week and demonstrated enhanced healing of skull bones, increased bone volume and enhanced vascularisation. Using a similar experimental design in rats, Diker *et al.* (2018) studied the effect from systemic intraperitoneal application of EPO at 500 UI/kg after creation of 5-mm trephination openings and placements of a heterologous xenograft in one of defects. The results showed that the treatment with EPO alone has no effects on angiogenesis and bone formation in minimally critical bone defects by the end of the 4th week. The authors concluded that the systemic application of EPO could be successful as complementary therapy in xenografting.

Posterolateral spinal fusion

Rabbit spinal fusion model is widely used in research aiming to evaluate the effect of various biomaterials on bone formation at the site of bone defects. It consists in making a skin incision along the median line of the back, dissection of m. multifidus and m. longissimus dorsi to reach the transverse processes of L5 and L6. By means of a high-speed burr, transverse processes are decorticated at a distance of 10 mm from their base (Walsh *et al.*, 2009). The tested material is placed in thus created defect. By the end of the intervention, tissues are sutured layer by layer (Virk *et al.,* 2016).

This model is applied in an attempt to demonstrate the effect of systemic treatment with EPO on bone healing. Rölfing *et al.* (2012) used a experimental design with rabbits submitted to posterolateral spinal fusion and placement of autograft from the iliac crest at the site of the defect. Over 20 days, rabbits were subcutaneously injected with low doses of erythropoietin – 250 UI/kg. By the 6th week post surgery, computed tomography and radiography demonstrated increased bone volume, whereas histomorphometry provided evidence for enhanced vascularisation. Haematopoietic potential of EPO was confirmed by blood samples collected on post operative weeks 2, 4 and 6 for measurement of haemoglobin and haematocrit values.

Corticosteroid-induced femoral head osteonecrosis

Continuous therapy with corticosteroids could result in osteonecrosis of the femoral head. The most popular hypothesis is that the hypertrophy of bone marrow adipocytes increases the pressure in bone marrow, thus reducing the blood flow in sinusoids and causing ischaemia. Glucocorticoids may have a direct toxic effect on bone cells and thus, play an important role in apoptosis (He *et al.*, 2016).

Corticosteroid-induced femoral head osteonecrosis in rodents could be reproduced by adding dexamethasone to drinking water over 15 weeks: 4 mg/L during the first week and 2 mg/L during the remaining period (Yang *et al.*, 2009), through intramuscular injection of 20 mg/kg methylprednisolone twice a week over 6 weeks (Yan *et al.*, 2018) or using slow-release prednisolone implants of 2.1 mg/kg/day (Weinstein *et al.*, 2017).

Several studies were performed to show the therapeutic effect of erythropoietin applied simultaneously with corticosteroids to induce femoral head osteonecrosis. Li et al. (2018) used the model in rats. By the 8th week after osteonecrosis reproduction, animals were euthanised and submitted to radiological, histological and histometrical examinations as well as Western Blot analysis. The obtained results proved that EPO application increased bone volume, the number of trabeculae, their thickness and separation. Also, enhanced expression of alkaline phosphatase and Runx2, HIF-1, and vascular endothelial growth factor (VEGF) at the site of osteonecrosis was demonstrated. The researchers concluded that local injection of EPO was more successful in mediating HIF-1 controlled osteogenic and angiogenic expression of factors and improved substantially the condition of animals.

These results were also confirmed by Yan et al. (2018) who reported that daily intraperitoneal injection of EPO resulted in enhanced VEGF expression and that the morphology of EPO-treated group was very similar to that of controls. These facts affirmed the protective effect of erythropoietin in corticosteroid-induced femoral head osteonecrosis (Yan *et al.*, 2018).

CONCLUSION

The present literature overview is focused on most important models of bone defects used in studies evaluating the effect of local or systemic erythropoietin application on bone healing. Preclinical studies on laboratory animals are a prerequisite before proceeding with EPO application tests in clinical patients.

In vivo experimental studies allowed evaluating the effects, safety and possible unwanted responses after using a specific biomaterial for stimulation of bone formation. Experimental designs using small-size animals have a number of advantages, therefore rats are a preferred animal species. The standardisation of surgical protocols in the study on various bone defects in animals would make the results from studies fully comparable.

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