Hypoxia Enhances the Tissue Protective Effect of Erythropoietin and Its Analogues in an Endothelial Cell Injury Model

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Introduction

- Injury of the vascular endothelium represents a critical feature in the early stages of vascular disease[1-3].
- Erythropoietin (EPO) is a cytokine which is induced by hypoxia and promotes erythropoiesis (red blood cell formation), *via* ligation to a homo -dimeric EPO receptor (EPOR)[4,5] .
- In recent years it became clear that EPO is expressed in several tissues and has multiple tissue-protective and reparative activities mediated by a distinct heterodimeric receptor (EPOR and a common beta-subunit) [6,7]
- A new generation of EPO derivatives that are tissue protective but not erythropoietic have therefore been developed. These compounds bind to the EPOR-βCR complex but not the EPOR homo-dimer [8].
- EPO and its non-erythropoietic derivatives have been studied in models of cardiovascular injury and have been reported to promote wound healing in the skin [9].

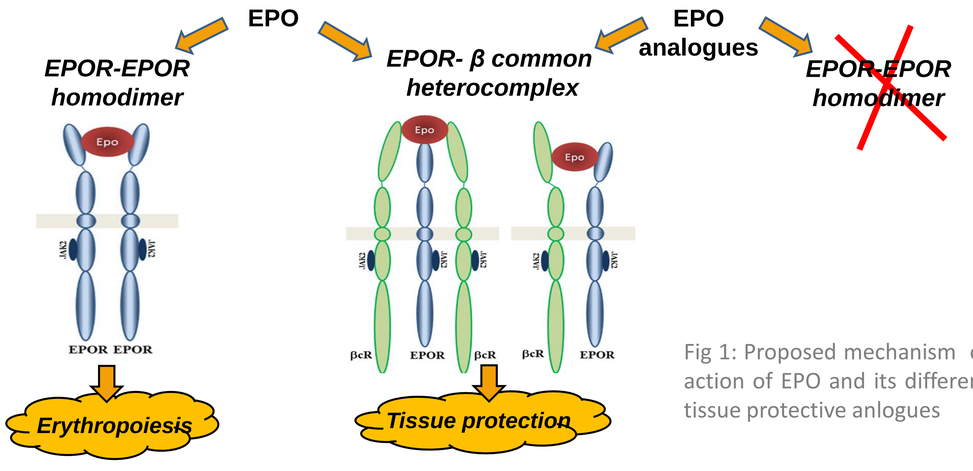


Fig 1: Proposed mechanism of action of EPO and its different tissue protective anlogues

Aim and Hypothesis

Aim: Investigate the tissue protective effect of EPO and its analogues in an *in vitro* model of wound healing under hypoxia and normoxia.

Hypothesis: 1. EPO and its analogue exert similar tissue protective effects

2. Oxygen level has an effect on the activity of EPO and its analogues

Methods and Results

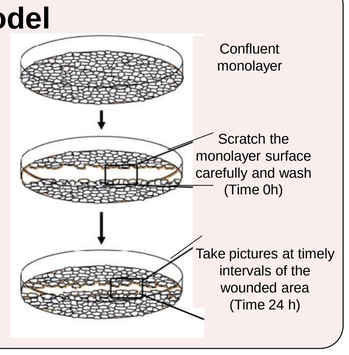
1. *In vitro* wound healing model

Wound healing model: Scratch assay

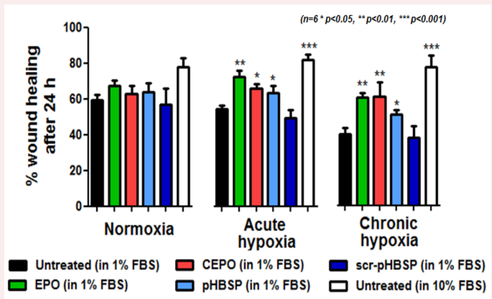
Cells: Bovine aortic endothelial cells (BAECs)

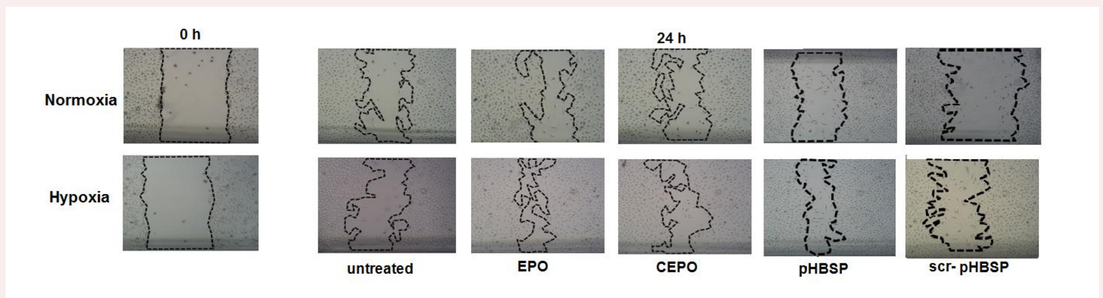
Oxygen level: Normoxia (21% oxygen) and hypoxia (5% oxygen)

EPO analogues tested: CEPO
pHBSP
Scr-pHBSP



• *Hypoxia enhances the wound closure effect of EPO and its analogues*





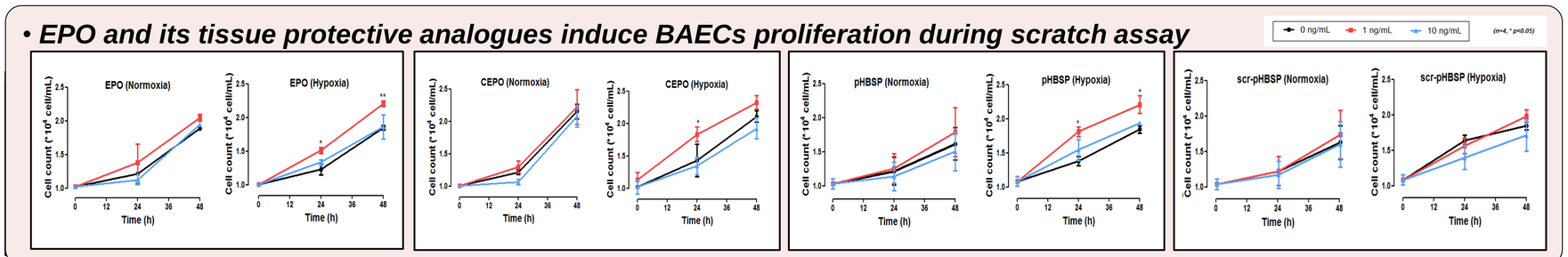
2. Proliferation assay
(Trypan blue viability method)

a. Seed cells at 1 x 10^5 cells/mL in 96 well plate

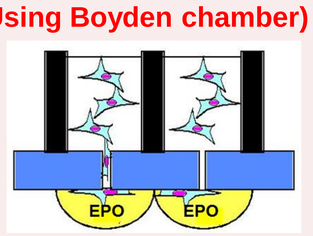
b. Add EPO or EPO analogue at concentrations (0-10 ng/mL)

c. After 0, 24 and 48 h add trypan blue and count viable cells

• *EPO and its tissue protective analogues induce BAECs proliferation during scratch assay*



3. Migration assay (Chemotaxis assay)
(Using Boyden chamber)

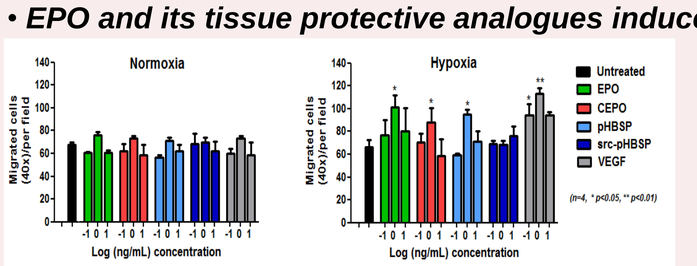


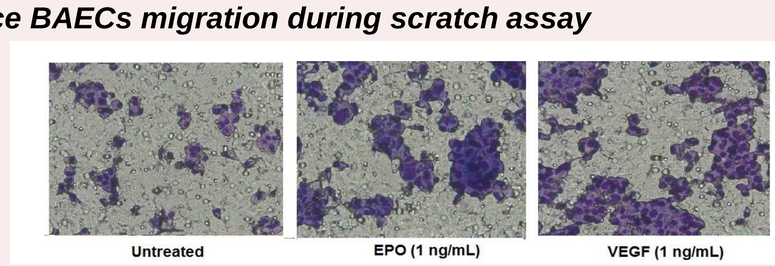
Incubate for 4 h

Fix and stain filter paper with Diff Quick Stain

Microscope (40x)

• *EPO and its tissue protective analogues induce BAECs migration during scratch assay*





Representative examples of Migrated cells (40X) /per field under hypoxia

4. Expression of EPOR and βCR

***a. Gene expression* (Real time qPCR)**

Difference in expression of EPOR and β CR gene were quantified and compared under normoxia and hypoxia using ΔΔCt method

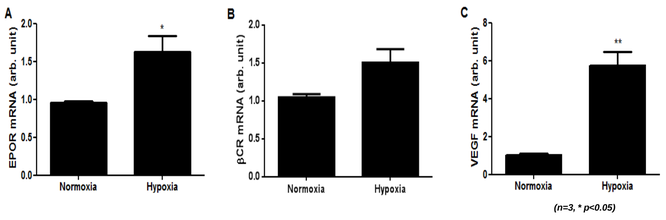
b. Protein expression

➢ **Western blot** ➢ **Immuno fluorescence**

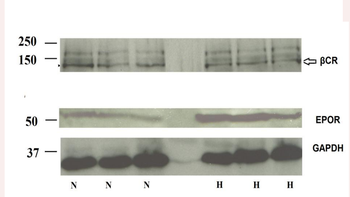
In both methods EPOR and βCR expression on the protein level were compared under normoxic and hypoxic conditions using antibodies specific for each protein

• *EPOR expression increased in BAECs under hypoxic conditions while βCR expression was not changed*

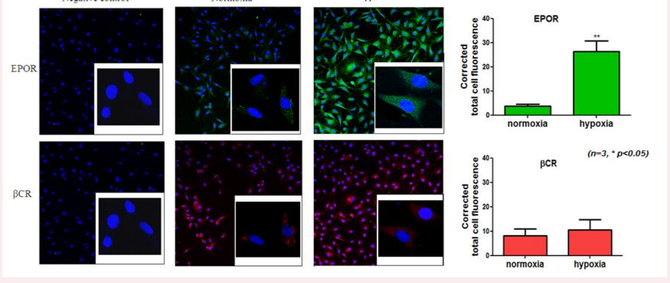
Real time qPCR



Western blot



Immunofluorescence

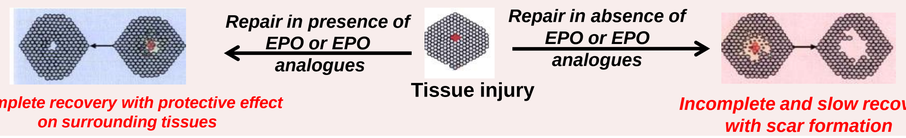


Conclusion and Clinical significance

Conclusion :

- Non erythropoietic analogues of EPO showed similar reparative effect to EPO.
- The effects of EPO and its analogues were enhanced by hypoxia and probably mediated by effects on cell migration and proliferation.
- Expression of EPOR is increased under hypoxia while expression of βCR is not affected.
- This requires further investigation in *in vivo* models of vascular injury, including atherogenesis and vascular re-stensosis.

Clinical significance: Non erythropoietic EPO derivatives may represent a potentially safer and more effective intervention for the treatment of cardiovascular disease as atherosclerosis..



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