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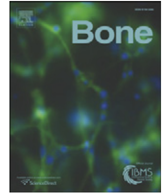

Original Full Length Article

The role of oxygen during fracture healing

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ABSTRACT

Oxygen affects the activity of multiple skeletogenic cells and is involved in many processes that are important for fracture healing. However, the role of oxygen in fracture healing has not been fully studied. Here we systematically examine the effects of oxygen tension on fracture healing and test the ability of hyperoxia to rescue healing defects in a mouse model of ischemic fracture healing. Mice with tibia fracture were housed in custom-built gas chambers and groups breathed a constant atmosphere of 13% oxygen (hypoxia), 21% oxygen (normoxia), or 50% oxygen (hyperoxia). The influx of inflammatory cells to the fracture site, stem cell differentiation, tissue vascularization, and fracture healing were analyzed. In addition, the efficacy of hyperoxia (50% oxygen) as a treatment regimen for fracture nonunion was tested. Hypoxic animals had decreased tissue vascularity, decreased bone formation, and delayed callus remodeling. Hyperoxia increased tissue vascularization, altered fracture healing in un-complicated fractures, and improved bone repair in ischemia-induced delayed fracture union. However, neither hypoxia nor hyperoxia significantly altered chondrogenesis or osteogenesis during early stages of fracture healing, and infiltration of macrophages and neutrophils was not affected by environmental oxygen after bone injury. In conclusion, our results indicate that environmental oxygen levels affect tissue vascularization and fracture healing, and that providing oxygen when fractures are accompanied by ischemia may be beneficial.

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Introduction

Inadequate blood supply is a significant contributing factor for delayed fracture healing or nonunion [1,2]. In order to understand the mechanisms that impair fracture healing after disrupted blood flow and to explore new therapies to stimulate healing in an ischemic environment, we previously developed an animal model of ischemic fracture by resecting the femoral artery before creating a fracture in the tibia [3]. We found that ischemia created a hypoxic environment at the site of fracture [4], which led to cell death, delayed chondrocyte and osteoblast differentiation, and impaired fracture healing [3]. These findings inspired us to study the role of oxygen in fracture healing.

Oxygen is involved in multiple basic cellular processes that are important for normal fracture healing. First, oxygen is required for aerobic metabolism. Second, oxygen is required for activity of many enzymes, including hydroxylases, oxygenases, and cycloxygenases, which are involved in fracture healing. For example, lack of cycloxygenase-2 activity impairs bone repair by affecting osteoblast differentiation [5,6]. Third, reduction in tissue oxygen interferes with the process of collagen synthesis, because oxygen is required for hydroxylation of lysine and proline during collagen cross-linking and bundle formation [7]. Fourth, oxygen is an important signaling molecule, which regulates the expression of several angiogenic genes through the hypoxia inducible factor (Hif) pathway [8].

Additionally, tissue oxygen levels may modulate stem cell responses after fracture. Oxygen has been implicated in stem cell maintenance, mobilization, and recruitment to sites of injury [9–13]. *In vitro* experiments have demonstrated that oxygen tension has profound effects on skeletogenic cells, including osteoblasts, chondrocytes, and osteoclasts. Hyperbaric oxygen increases cell proliferation and mineralization of alveolar osteoblasts [14]. Under normobaric conditions, 2% oxygen applied to cells in the early phase of osteoblast differentiation decreases collagen production and mineralization compared to 20% oxygen [15]. Compared to 21% oxygen, 5% oxygen increases the differentiation of osteoblasts and their transformation to osteocytes [16]. Hypoxia also influences the expression of genes in cultured osteoblasts. Hypoxia

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decreases sclerostin expression [17], increases Wnt signaling [17], and increases BMP2 [18], IGF [19], and VEGF expression [20]. Similar to osteoblasts, chondrocytes in culture are also affected by oxygen levels. Hypoxia (2–5% oxygen) increases the expression of VEGF [21], collagen type II, glycosaminoglycan, and aggrecan [22–24]. Cultured chondrocytes tend to dedifferentiate and hypoxia can induce their redifferentiation [23]. Compared to osteoblasts, chondrocytes normally reside in avascular cartilage and have been speculated to be well-adapted to low oxygen tension [25], and these *in vitro* data have been used to support this idea. However, the growth plate is well perfused suggesting that oxygen may not be limiting for chondrocyte function in the growth plates [26]. Hypoxia also affects osteoclast activity. Changing culture conditions from 20% oxygen to 2% oxygen significantly stimulates osteoclast formation and bone resorption [27,28].

While the effects of oxygen tension on skeletal cells have been extensively studied *in vitro*, little is known about the relevance of these studies to the situation *in vivo*. Most *in vitro* studies use 2–5% oxygen as the hypoxic conditions and results are compared to cultures in 20–21% oxygen, which is well-above the physiological state of tissues and cells *in vivo*. Further, the *in vivo* environment is much more complex. There are multiple cell types that have different metabolic demands. These cells are responding to a variety of growth factors and cytokines that interact to regulate the process of repair, and this complexity is not recapitulated in the *in vitro* experiments. Normally, Hif1α protein and VEGF increase when cells are hypoxic, but in the presence of inflammation and lactate, as in wounds, the effects are different, and oxygen promotes VEGF expression and angiogenesis [8,29–31]. The goal of the current study was to determine the role of oxygen in bone repair *in vivo* and to explore the efficacy of non-hyperbaric hyperoxia on enhancement of fracture healing. We hypothesized that environmental oxygen alters fracture healing by regulating stem cell differentiation, angiogenesis, and inflammation during early fracture healing. We tested this hypothesis in a mouse model of tibia fracture healing.

Materials and methods

Generation of tibia fractures

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California at San Francisco and at Dartmouth Medical School, Hanover, NH. Three-month-old male 129J/B6 mice (25–30 g) were anesthetized with a 0.03 ml mixture of ketamine (50 mg/ml) and medetomidine (0.5 mg/ml). Closed transverse mid-diaphyseal fractures of the tibia were created with a three-point bending apparatus. Fractures were either stabilized with an external fixator or left unstabilized. In a second set of animals, the femoral artery was resected before creating tibia fractures, resulting in an ischemic environment that delays fracture healing [3]. After recovery, animals were allowed to ambulate freely and analgesics were provided for the first 72 h (buprenorphine, 0.03 mg/mouse, ZT Sigma, St. Louis, MO). Animals that died during the post-operative period and those with comminuted fractures were excluded from further analyses.

Treatment with different levels of oxygen

After recovery from anesthesia, animals with tibia fractures were transferred into custom-built semi-sealed gas chambers. Oxygen levels in the chambers were maintained at 13% (hypoxia), 21% (normoxia), or 50% (hyperoxia) by infusing compressed nitrogen or oxygen for the duration of the entire experiment. Gas infusion was controlled by ProOx (BioSpherix Ltd, Redfield, NY). The carbon dioxide and humidity in the chambers were maintained at <0.5% and 65–75% respectively. Chambers were opened briefly every other day to change cages, food and water. All animals exhibited excellent tolerance to hypoxia and hyperoxia. No significant change of body weight was observed after surgery and oxygen treatment.

Examining oxygen tension at the fracture site

To determine whether breathing oxygen can alter the oxygen tension at fracture site, non-stabilized tibia fractures were created in 4 adult mice. Lithium phthalocyanine (LiPc), a paramagnetic material, was implanted using a 30 G needle to the fracture site after bone injury. At 24 h after injury, animals were anesthetized again. They were kept euthermic and then were allowed to breathe 13%, 21%, and 50% oxygen. Animals were allowed to breath each oxygen concentration for 20–30 min before oxygen tension at the fracture site was measured using electron paramagnetic resonance (EPR) before switching to the next oxygen concentration [4,32].

To determine the correlation between local oxygen tension and tissue differentiation during fracture healing, LiPc crystals were implanted adjacent to the mid-diaphysis of the tibia immediately after creating non-stabilized tibia fractures. Tissue oxygenation was measured at 1, 3, 5, 7, and 10 days after injury with the animals breathing room air. Animals were sacrificed at 7 and 10 days post-fracture and fractured tissues were collected. Histological analysis was performed to determine the location of LiPc crystal and the types of tissues that the crystals were located in.

Histological, histomorphometric, and molecular analyses of fracture healing

Animals were sacrificed at 5–28 days after injury ($n = 4$–6/group/time) and fractured tibiae were collected, fixed in 4% paraformaldehyde (PFA) at 4 °C overnight, decalcified in 19% EDTA, dehydrated, and embedded in paraffin. Sagittal sections (10 μm) through the entire block were prepared. To analyze fracture healing, every thirtieth slide was stained with HBQ staining, which stains bone red and cartilage blue. Histomorphometry was performed using an Olympus CAST system. The volume of callus (Vcallus) and the volumes of bone (Vbone), and cartilage (Vcartilage) within the callus were estimated using Cavalieri's Principle.

To assess osteoblast and chondrocyte differentiation, *in situ* hybridization using probes that hybridize to markers of osteoblasts (*Osteocalcin* (*Oc*)), chondrocytes (*Collagen type II* (*Col2*)), and of chondrocyte maturation (*Collagen type X* (*Col10*), and *vascular endothelial growth factor* (*VEGF*)), was performed on sections adjacent to those used for histological staining [33].

Examination of fracture callus composition

Since non-stabilized fractures tend to angulate during repair, conventional mechanical testing such as three or four-point-bending is unreliable and insensitive. We turned to Fourier Transform Infrared (FTIR) spectroscopy to study the content and composition of mineral and matrix in the fracture calluses. Animals with non-stabilized tibia fracture were treated with hypoxia, normoxia, or hyperoxia for 21 days and fracture calluses were collected and fixed in 70% ethanol. Chemical composition of the fracture callous tissue was examined with FTIR spectroscopy. Samples were desiccated through an ethanol series followed by exposure in a desiccant chamber. For each sample, a homogenized powder mixture was created of 1% bone by weight in potassium bromide (KBr; Thermo Electron Corporation). The powder mixture was compressed using a manual die to create a solid, clear pellet for FTIR spectroscopy.

Spectroscopy was performed on a bench top interferometer system (Nexus 870, Thermo Electron Corporation). Spectra were acquired using 256 scans at a spectral resolution of 4 cm^{-1}. A background scan was recorded immediately following each sample scan to facilitate background correction. Following acquisition, the spectra were transferred to chemical imaging software (Isys, Spectral

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