TIBIAL FRACTURE DECREASES OXYGEN LEVELS AT THE SITE OF INJURY

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ABSTRACT

Objectives: Oxygen is an essential component for many aspects of tissue repair. However, the effect of oxygen levels on differentiation of stem cells into osteoblasts and chondrocytes during fracture healing is unknown, in part because of the difficulty in measuring oxygen during fracture healing. In this study we tested the feasibility of using electron paramagnetic resonance (EPR) oximetry to assess tissue oxygen partial pressure (pO_2) after tibial fractures in mice.

Methods: Transverse tibia fractures were created by three-point bending in adult mice. Paramagnetic material, lithium phthalocyanine (LiPc), was implanted into the fracture site or adjacent to the periosteum in the contralateral leg immediately after fracture. Tissue pO_2 was assessed by EPR 90-110 minutes after implantation of the crystals. In a second experiment, LiPc was implanted into the fracture site and fracture repair and the biocompatibility of LiPc were assessed at 14 and 28 days after injury.

Results: At the very early stage after fracture, injury significantly decreased tissue oxygenation at the fracture site. When animals were breathing 21% oxygen, pO_2 at the fracture site (30.6 ± 12.7mmHg, n=7) was lower than that in contralateral legs (45.5 ± 15.3mmHg, n=7, $p<0.01$). Breathing 100% inspired oxygen increased the pO_2 in both the fractured (72.8 ± 28.2mmHg; n=7) and contralateral legs (148.4 ± 59.2mmHg; n=7, $p<0.01$). In addition, LiPc crystals implanted into fracture sites did not interfere with normal fracture healing at 10 and 28 days post-injury.

Conclusions: EPR oximetry is a valuable tool for monitoring oxygen levels during fracture repair in mice.

INTRODUCTION

Fractures are usually accompanied by local tissue hypoxia due to the disruption of vasculature. Oxygen levels at fracture sites are initially very low, and they gradually increase as re-vascularization occurs.[1,2,3] During wound repair, oxygen is required for cell survival, collagen and extracellular matrix deposition,[4,5] angiogenesis,[6,7] epithelialization,[8] and production of the oxidative burst used for intracellular bacterial killing.[9,10] However, the role of oxygen in fracture healing has not been extensively studied. Previous studies have shown that chronic systemic hypoxia inhibits[11] and oxygen supplementation enhances bone repair.[12,13] These studies suggest that oxygen is important for fracture healing. Therefore studying the role of oxygen during bone repair could greatly increase our understanding of mechanisms that regulate skeletal regeneration.

In order to examine the role of oxygen during fracture healing in detail a reliable method of measuring tissue oxygen levels in vivo is required. Oxygen-sensing electrodes are widely used to measure tissue PO_2 directly.[1,2,14] However, this technique has several disadvantages when it is used to measure oxygenation at fracture sites. First, inserting an electrode into tissue is invasive and affects O_2 measurement immediately after injury. Second, microelectrodes are fragile and may be damaged during insertion into the fracture callus. Last, measuring tissue O_2 repeatedly at the same location in the same animal is not possible. An alternate approach of measuring oxygen concentration *in vivo* utilizes spectroscopic relaxation methods (reviewed by Vanderkooi et al.[15]). Oxygen affects the relaxation times of excited species such as luminescent or magnetic probes. These relaxation times can be measured optically or magnetically and used to determine pO_2 directly. Among all spectroscopic relaxation methods, electron paramagnetic resonance (EPR)[15,16,17,18] is a particularly promising method to use for studying tissue oxygenation in murine fracture models. *In vivo* EPR oximetry is non-invasive after the initial implantation of the oxygen-sensitive paramagnetic probe

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into the tissue of interest.[16,17,18] This procedure causes only minimal injury as materials such as lithium phthalocyanine (LiPc) can be placed with a 25-gauge needle, and the amount deposited is less than 50 micrograms. Furthermore, these paramagnetic probes are biocompatible and induce minimal or no tissue reaction.[18] In the brain, repetitive and reproducible measurements of pO_2 using EPR have been achieved for more than 30 days.[18] Importantly, the location of paramagnetic material in the tissue can be determined on histological sections, allowing the exact site of pO_2 measurement to be determined. In this study, we tested the feasibility of using EPR oximetry to assess tissue oxygenation at the fracture site in mice.

MATERIALS AND METHODS

Animals and creation of tibia fractures

All procedures in this work were approved by IACUC (Institutional Animal Care and Use Committee) at Dartmouth Medical School, Hanover, NH or at the University of California at San Francisco, San Francisco, CA. Male C57B6 mice (8 weeks old) were purchased from Charles River Laboratories, Inc. and accommodated in a conventional animal facility for 1 week before initiating experiments. Animals were anesthetized with 2% isoflurane in 100% oxygen. A transverse closed fracture was created by three-point bending at the mid-shaft of right tibia.[19]

To create ischemic tibial fractures, the right femoral artery and its branches were ligated and removed immediately prior to fracture.[20] Briefly, using aseptic technique, a small incision was made over the right groin to expose the femoral vessels. The femoral nerve and vein were separated from the femoral artery, and the femoral artery and its branches between the inguinal ligament and the popliteal bifurcation were ligated. Then the femoral artery was removed. Immediately after resection of the femoral artery, a transverse closed fracture was created in the mid-shaft of the right tibia.

EPR oximetry

Immediately after the creation of tibia fractures, LiPc crystals (20 -30 micrograms) were implanted into the fracture site and, as controls, adjacent to the periosteum in the contralateral legs using a 25 gauge needle. Tissue pO_2 was then sequentially assessed by EPR oximetry using an *in vivo* EPR spectrometer, with a custom made low-frequency microwave bridge operating at 1.1 GHz and an extended loop resonator (11 mm diameter).[18,21] Animals were placed in the magnetic field and maintained under general anesthesia (1.8% inspired isoflurane). Euthermia (37.5°C ± 0.5°C) was monitored with a rectal temperature probe and maintained with a circulating water blanket. The leg of interest was positioned under the extended loop resonator. The EPR spectrum was then acquired with the spectrometer. Although spectrometer settings varied slightly from scan to scan, typical settings were: incident microwave power-10mW, magnetic field center-425G, scan range 1G. Modulation amplitude was set less than one third of the EPR line width. Scan time was approximately 2 to 3 minutes and 3 to 5 individual scans were averaged to achieve a better signal to noise ratio. The line-width was determined from the EPR spectra, and the partial pressure of tissue oxygen was calculated from an existing calibration curve specific to the given batch of LiPc.[16,18]

Determining the time course of tissue pO_2 change after injury

The length of time required to obtain a steady state of pO_2 after fracture was determined in 4 animals with tibia fracture and intact femoral artery, 1 animal with femoral artery resection without fracture (ischemia only), and 1 animal with femoral artery resection with a tibia fracture (ischemic fracture). Animals were administered 100% oxygen and pO_2 at the fracture sites was measured every 15 minutes after injury for 90 minutes. The time required to reach pO_2 equilibration after changing the inspired oxygen level was also determined using one animal with a tibia fracture and an intact femoral artery. This animal was switched to 21% oxygen and pO_2 was measured every 5 minutes for 20 minutes. Inspired oxygen for this same animal was again changed back to 100% and pO_2 was monitored for another 20 minutes.

Determining the effects of fracture on tissue pO_2

Tibia fractures were created in 7 animals and LiPc crystals were implanted into the fracture sites and adjacent to the periosteum in the contralateral legs. Following randomization, animals were initially administered either 100% or 21% oxygen for 90 minutes and pO_2 at the fracture sites or periosteum in the contralateral legs was measured. The inspired oxygen level was then changed to 21% or 100% respectively and pO_2 measurements were performed again 20 minutes later.

Determining the effects of femoral artery resection on tissue pO_2 at fracture sites

Tibia fractures with femoral artery resection were created in 4 animals and LiPc crystals were implanted into the fracture sites. Animals were administered 100% O_2 and oxygen concentration was measured at 90 minutes after injury. No measurements were made in 21% oxygen.

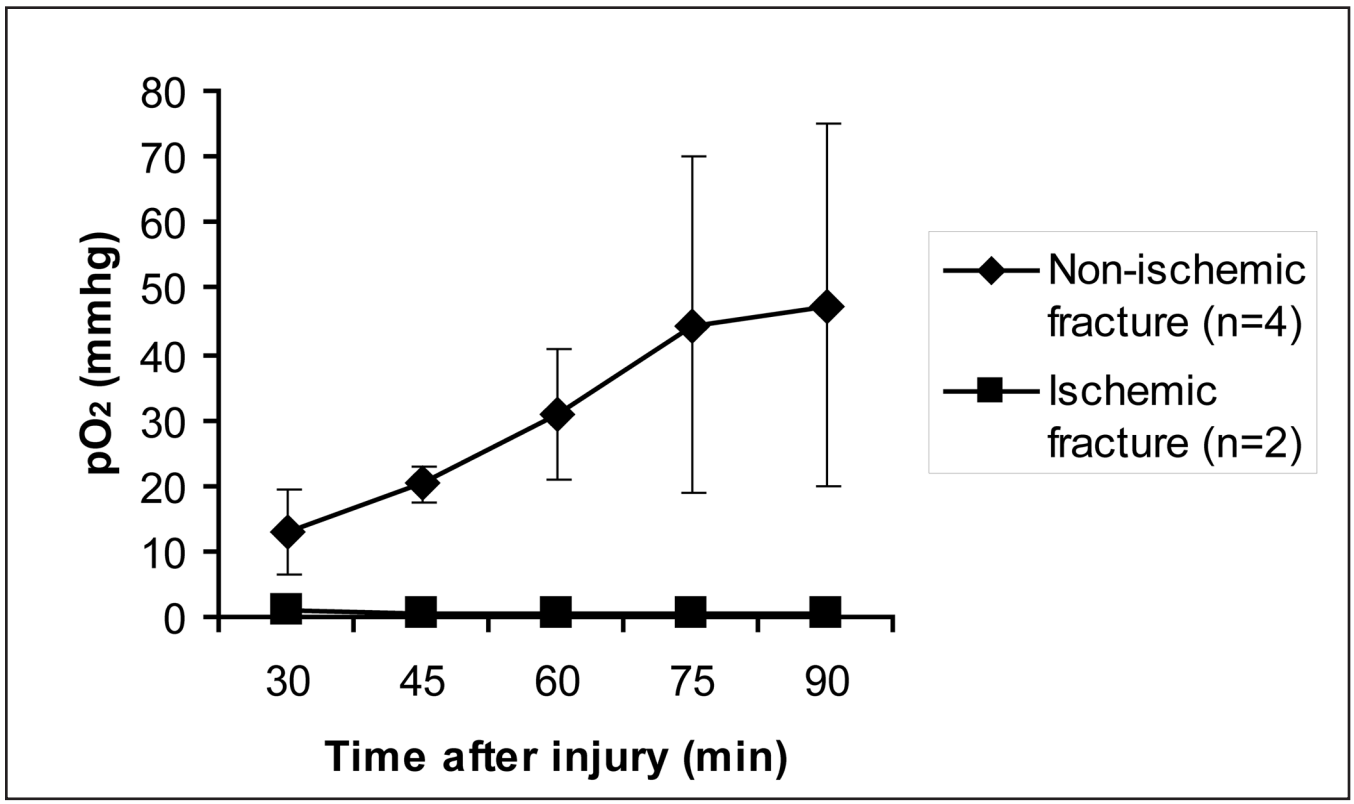

Figure 1. Time course of pO_2 change at the fracture site. When animals breathed 100% oxygen, tissue oxygen equilibrium was achieved in 90 minutes in fractures with intact femoral artery. After femoral artery resection, very low tissue pO_2 was detected at fracture sites, which remained low even after 90 minutes of inspiring 100% oxygen.

Histological analysis of crystal placement

To correlate the position of crystals with measurements of pO_2, animals from the above experiments were sacrificed immediately after the completion of oximetry and both the fractured and contralateral tibiae were collected. Tissues were processed to decalcified paraffin sections as described above. Sections were stained with Hemotoxylin and eosin.

Assessing the long term biocompatibility of LiPc

The long-term biocompatibility of LiPc was firstly assessed by implanting LiPc crystals (20-30 micrograms) with a 25 gauge needle into fracture sites right after injury and analyzing fracture healing at 10 (n=4) and 28 (n=3) days post-fracture. Fractured tibiae were collected, fixed in 4% paraformaldehyde, decalcified in 19% EDTA, and then dehydrated and embedded in paraffin. Longitudinal sections (10μm) were prepared. Sections were stained with Hall and Brunt's Quadruple stain (HBQ[22,23]) to stain cartilage blue and bone red.

Statistical analyses

A one-sided paired (one-sample) t-test was used to determine whether fracture and inspired oxygen altered the pO_2 *in vivo*. The effect of femoral artery resection on the pO_2 level at fracture sites was determined using a t-test. Data are presented as mean ± one standard deviation. A power analysis on current data demonstrated that a sample size of 7 in each group is sufficient to detect a 30% decrease in pO_2 in fractured legs compared to contralateral legs, assuming a power of 80% and a significance level of 5%.

RESULTS

Assessing equilibration of pO_2 after fracture and after changing inspired oxygen

The length of time required to reach a steady state of pO_2 after fracture and after changing the inspired oxygen level was determined in a small group of animals. In fractures without femoral artery resection (n=4), tissue pO_2 equilibrium was achieved in 90 minutes after fracture with animals breathing 100% oxygen (Figure 1). After changes in the inspired oxygen concentration, pO_2 equilibrated within 20 minutes (data not shown). In the animals with femoral artery resection (n=2), pO_2 in the hind limb remained low and unchanged during the first 90 minutes after injury (Figure 1). Therefore all measurements throughout this work were made at 90 minutes after fracture with constant administration of

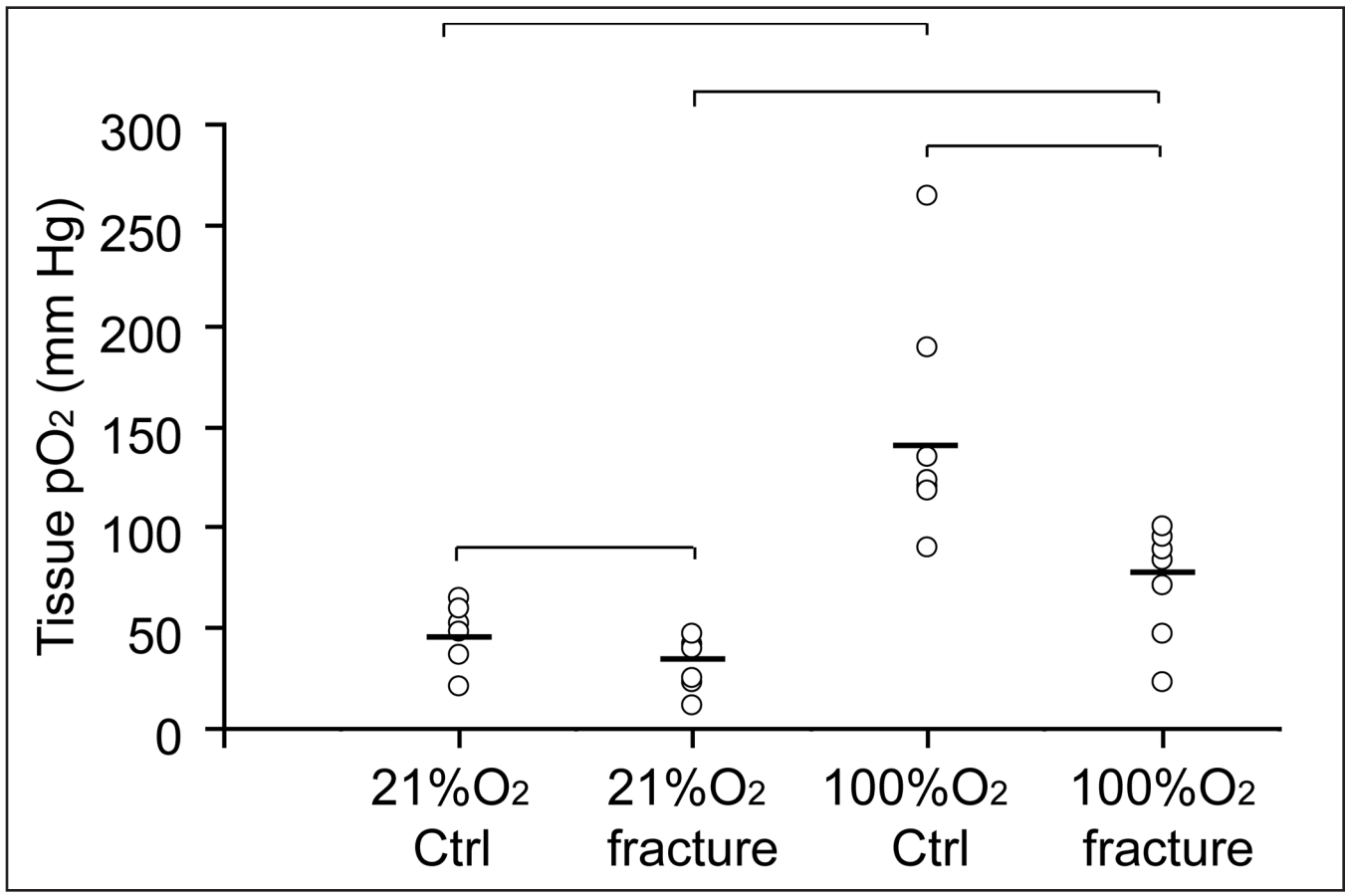

Figure 2. Fracture and inspired oxygen level significantly affect tissue pO_2. When animals (n=7) were breathing 21% oxygen, the mean pO_2 was 45.5 ± 15.3 mmHg in contralateral limbs and 30.6 ± 12.7 mmHg at fracture sites. When animals were breathing 100% oxygen, the mean pO_2 was 148.4 ± 59.2 mmHg in contralateral legs and 72.8 ± 28.2 mm Hg in fractured limbs. Ctrl = contralateral limbs.——mean of tissue pO_2. ⌜⌝ p<0.01.

the same concentration of oxygen and 20 minutes after changing the inspired oxygen level (i.e., 110 minutes after injury).

Injury decreases tissue oxygenation at fracture sites

Our next objective was to determine the immediate effects of traumatic injury on tissue oxygen levels at the fracture site. At the fracture site in animals breathing 21% oxygen, the mean pO_2 (30.6 ± 12.7 mmHg; n=7) was significantly lower than that at the periosteum in contralateral limbs (45.5 ± 15.3 mmHg; n=7; p<0.01. Figure 2). pO_2 was significantly increased in both the fractured limbs (72.8 ± 28.2 mm Hg, p<0.01) and contralateral legs (148.4 ± 59.2 mmHg, p<0.01) during 100% oxygen administration (Figure 2). However, the pO_2 at fracture sites still remained significantly lower than that in the control tibial periosteum (Figure 2, p<0.01), suggesting that disruptions to the vasculature had occurred that limited the functional capacity of the blood supply. To further assess the ability of EPR to measure vascular oxygen delivery after skeletal injury, we measured fracture and periosteal pO_2 levels in animals breathing 100% oxygen, that had undergone femoral artery resection as an additional ischemic insult; this injury is sufficiently severe to delay fracture healing.[20] Femoral artery resection significantly reduced pO_2 at the fracture site (1.4 ± 0.7 mmHg; n=4) compared to 72.8 ± 28.2 mm Hg in fractures with intact femoral artery (n=7; p<0.01).

Crystal placement

In the majority of limbs with skeletal injury, LiPc crystals were located within the injured muscle near the fracture site (Figure 3). The location of LiPc crystals does not appear to correlate with the variation of pO_2 measurements, suggesting that extensive muscle and vascular injury was present around fracture sites.

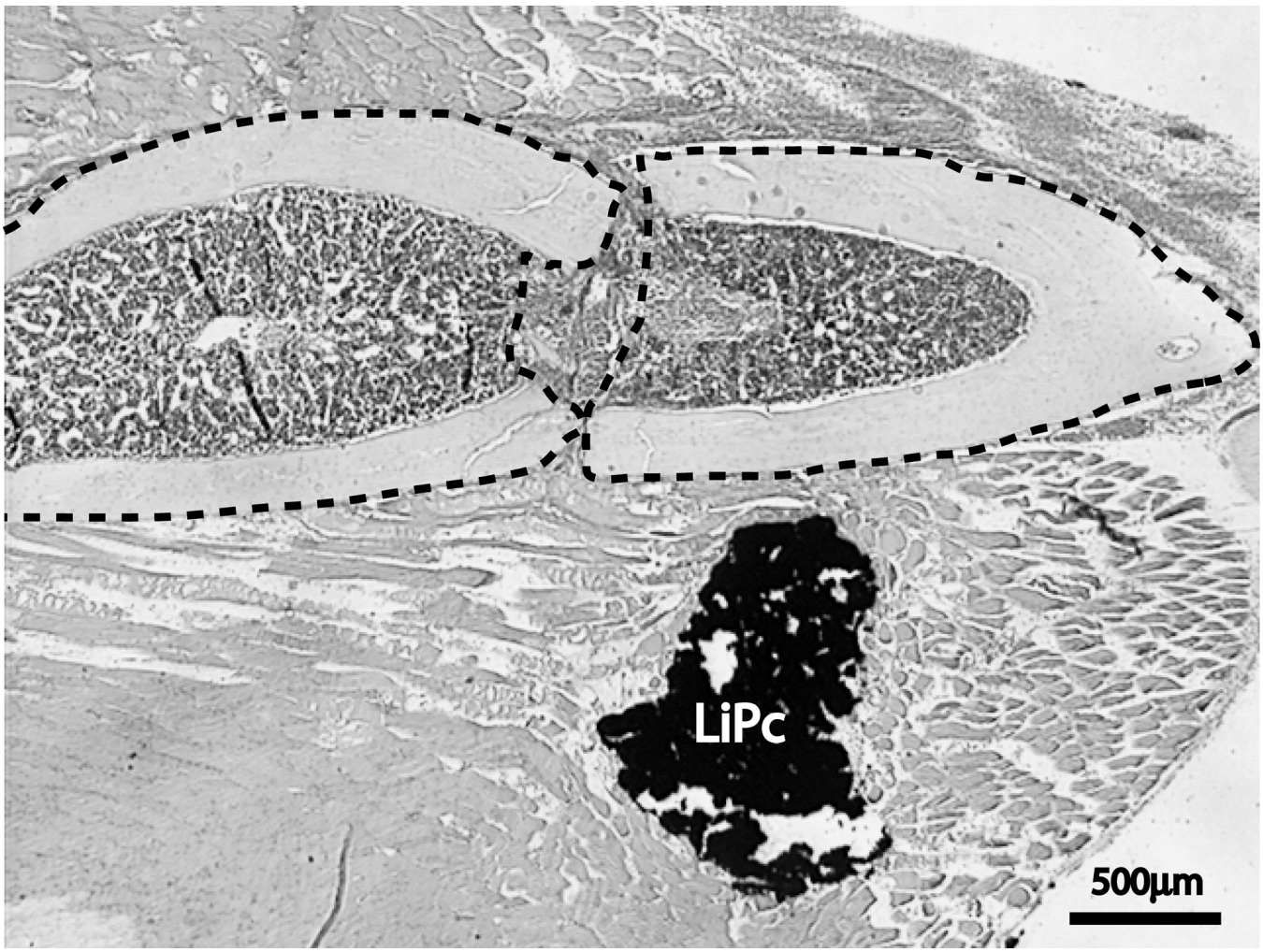

Figure 3. LiPc crystals were located within muscle near the fracture site in the majority of animals. H&E staining. Outlined area = fracture ends.

LiPc does not interfere with normal fracture healing

Our last goal was to examine the biocompatibility of LiPc crystals during fracture repair. LiPc crystals were placed at the fracture site and then the injuries were allowed to heal for 10 days to assess endochondral ossification and for 28 days to assess union of the fractured bone ends. At 10 days after injury (n=4), hypertrophic cartilage and newly formed bone were observed in fracture calluses indicating endochondral ossification was under way (Figure 4A). LiPc crystals were embedded in granulation tissue (Figure 4B) or were in direct contact with newly formed bone (Figure 4C) and cartilage (Figure 4D). At 28 days (data not shown), all fractures (n=3) had healed by bony bridging. LiPc crystals were observed in direct contact with bone or in surrounding soft tissue. A thin layer of fibrous tissue was occasionally observed around LiPc crystals at this time. Compared to previously published data,[19,20,24] LiPc crystals did not significantly alter the normal progress of tibia fracture healing in mice. Mice typically heal experimental tibia fractures by day 28 post-injury and this was the case in our current study. Furthermore, the crystals were in direct contact with cartilage and bone, and there was no evidence of a large population of inflammatory cells in adjacent space. Thus, LiPc crystals do not induce much inflammatory response and they have excellent biocompatibility during fracture healing.

DISCUSSION

The results of this study indicate that EPR oximetry will be a valuable tool for monitoring oxygen levels during fracture repair in mice. EPR oximetry detected significantly lowered tissue oxygen levels at fracture sites after injury and it also successfully detected the expected changes of tissue oxygen levels after switching the

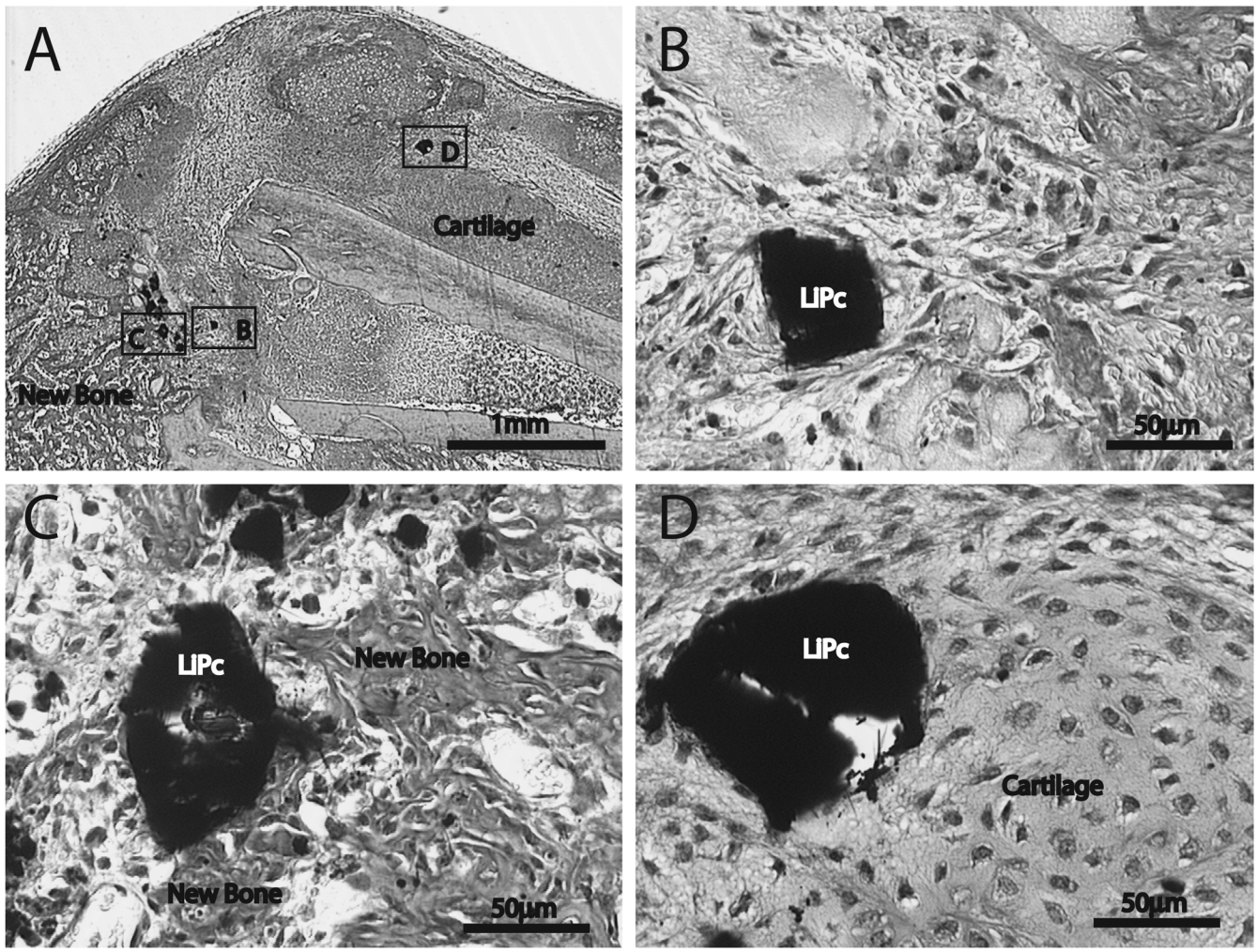

Figure 4. Histological analysis of fracture healing with the presence of LiPc crystals. (A) At 10 days after injury, hypertrophic cartilage and newly formed bone were observed in fracture calluses. (B) High magnification of box B in (A) shows a LiPc crystal is embedded in granulation tissue with minimal fibrosis. (C) High magnification of box C in (A) shows a LiPc crystal is in direct contact with newly formed bone. (D) High magnification of box D in (A) shows a LiPc crystal is in direct contact with cartilage.

breathing oxygen concentration from 21% to 100%. These data suggest that the sensitivity of EPR oximetry is adequate to assess tissue pO_2 during fracture healing. In addition, we confirmed that the paramagnetic probe used in current study, LiPc, is biocompatible. LiPc crystals did not induce obvious inflammatory response and interfere with normal fracture healing. Further, LiPc crystals gave us the ability to make repeated measurements in the same location and to assess tissue formation at each site, providing a unique opportunity to examine the effect that oxygen has on stem cell differentiation and function. For instance, this technique will allow us to assess pO_2 at a particular site and then at a later time point we can examine what type of tissue has formed in this region using histologic methods. Developing and employing these technologies for studying fracture repair in mice is of great importance, because compared to other rodent models, a murine model offers the advantage of using a multitude of genetically engineered strains, and there is an abundance of reagents for molecular and cellular analyses available.

The level of tissue oxygenation may affect fracture repair. Decreasing tissue oxygenation by inducing chronic systemic hypoxia[11] or by ligating femoral artery[20] delays fracture healing. Increasing tissue oxygenation by providing hyperbaric oxygen, on the contrary, can accelerate bone repair.[25] Understanding the mechanisms underlying these effects is of great important. There is evidence suggesting that oxygen levels may have direct effects on stem cells. For example, the function and differentiation of chondrocytes and osteoblasts is affected by oxygen levels in vitro.[26,27,28] Under experimental conditions that induce chronic hypoxia, fractured femurs did not exhibit signs of bone formation. Rather the calluses

were comprised of abundant cartilage[11] which indicates that chondrocyte differentiation can occur in a more hypoxic environment than osteoblast differentiation. In addition to the effect of hypoxia on stem cells, tissue oxygen levels may play other important roles during bone repair. Studies on wound healing have demonstrated that oxygen is involved in multiple processes, including cell survival, collagen and extracellular matrix deposition,[4,5] angiogenesis,[6,7] epithelialization,[8] and production of the oxidative burst used for intracellular bacterial killing.[9,10] When tissue pO2 falls below 40 mmHg, angiogenesis, extracellular matrix formation, and resistance to infection are all impaired.[29] In current study, we detected that tissue oxygen level at fracture sites dropped below this level after injury, especially after femoral artery ligation, warranting further studies on the effects of oxygen on cell death, angiogenesis, and infection during fracture healing.

In summary, these data illustrate that EPR oximetry is a promising technique to assess tissue oxygenation in murine fracture models. We have used EPR to examine the effect that tibial fracture has on pO_2 during fracture repair. We have demonstrated that these fractures generate vascular damage that significantly reduces tissue oxygen levels to levels that could impair healing. However, mice exhibit exceptional regenerative capacity. Normally no difficulties in skeletal healing are detected without the inclusion of a major traumatic insult such as cautery of the periosteum[30] or resection of the femoral artery.[20] Even in the latter situation the mice salvage their limbs, and eventually the skeletal injury heals.[20] These results suggest that tissue oxygenation returns to normoxic levels rapidly after injury. Thus, understanding this time course and evaluating the role that oxygen plays during fracture repair are important parameters for elucidating and exploiting the mechanisms that underlie the regenerative potential during fracture repair in mice.

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The authors have no conflicts of interest.

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