INTRODUCTION: Nitric oxide (NO) is a messenger molecule which plays a role in many physiological processes. A group of enzymes called the nitric oxide synthases (NOS) catalyze the conversion of L-arginine to nitric oxide and L-citrulline. Two of the NOS isoforms are constitutive in nature, are labeled as eNOS and nNOS, and are Ca++ dependent. The inducible form (iNOS) is Ca++ independent and is induced by proinflammatory mediators (e.g., IL-1, TNF-α) and is of a high output type. Following redox reactions the endproducts of NO metabolism are nitrate (NO₃⁻) and nitrite (NO₂⁻). The role of NO in fracture repair has not been evaluated. The overall aim of this paper is to study the role of NO in fracture healing. The first part of the study aimed to identify in a temporal fashion the presence of nitrates and NOS in rat femoral fracture repair. The second part of the study investigated the presence of NO in healing fractures of humans.

METHODS: Study I: 27 male Sprague Dawley rats had a right femoral fracture created by three point bending. Rats were sacrificed on day 4(n=7), 7(n=6), 10(n=1), 15(n=6), and day 30(n=7). The control group consisted of weight matched (n=6) rats without any fracture. Nitrate was measured in the serum of these rats by gas chromatography mass spectrometry. Fracture callus NOS activity was quantified using a ³H L-arginine to ³H L-citrulline conversion assay. Polymerase Chain Reaction (PCR) and Western Blotting (n=9) and immunoperoxidase staining (n=7) using a polyclonal antibody specific for human iNOS. The samples were evaluated by RT-PCR (n=6) using human specific primers designed for the three NOS isoforms, NOS activity assay (n=6), Western Blotting (n=9) and immunoperoxidase staining (n=7) using a polyclonal antibody specific for human iNOS.

RESULTS: Study I: The mean(SD) serum nitrite concentration prefracture was 21(3)nmol/ml. Serum nitrate post fracture was: day four 26(9), day seven 36(4) (p<0.001), day ten 30(7), day fifteen 20(6) and day thirty 38(17)nmol/ml (p<0.001). Ca++ independent NOS activity was absent in the unfractured group but peaked on day 15 (Figure 1). Ca++ dependent constitutive NOS activity was absent prior to fracture and gradually increased to 26(10) (p<.05) on day 15 and was 50(11) cpm/mg protein/min (p<0.01) on day 30. A strong signal for mRNA of iNOS was detected by RT-PCR on days 7 and 15. The protein for iNOS was detected at all stages of fracture healing but not in the controls (Figure 2). Immunoperoxidase staining for iNOS on day 10 of fracture showed staining of cartilage cells and cells in the periosteal callus. Study II: Rats fed the NOS inhibitor L-NAME, an inactive enantiomer of L-NAME, added to their drinking water ad lib. The control group had D-NAME, an inactive enantiomer of L-NAME, added to their drinking water in the same dose. Treatment was administered preoperatively and then for 24 days, at the end of which all rats were sacrificed. Callus diameter was measured by observers blinded to the groups. Bone mineral density was measured using DEXA scan. Three point bending was carried out using a Shimadzu materials testing machine. This study was repeated two more times with end points at day 17 and day 21 post fracture. Study III: Fracture callus was collected from 14 patients undergoing open reduction and internal fixation of a fracture more than four days following trauma. The samples were evaluated by RT-PCR (n=6) using human specific primers designed for the three NOS isoforms, NOS activity assay (n=6), Western Blotting (n=9) and immunoperoxidase staining (n=7) using a polyclonal antibody specific for human iNOS.

DISCUSSION: These results show for the first time that NO is present and functioning in fracture healing in rat models. The results are consistent with a high early (day 7 to 15) expression of iNOS and a gradual increase in eNOS expression up to day 30. The study also establishes a role for NO in modulating rat fracture healing.

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