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IDENTIFICATION AND LOCALIZATION OF NITRIC OXIDE SYNTHASE ISOFORMS IN VARIOUS STAGES OF FRACTURE HEALING

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INTRODUCTION: Although it is known that nitric oxide (NO) upregulates the healing of wounded tissues, its exact biological role in fracture healing is still not clear. We previously reported¹ that nitric oxide synthases (NOS) which are responsible for the generation of NO are induced during fracture healing, inhibition of NOS inhibits this process and addition of NO enhances fracture healing. The purpose of this study was to further quantitatively identify the temporal expression patterns of three NOS isoforms (iNOS, eNOS and bNOS), and localize their cellular distribution during the phases of fracture healing at both mRNA and protein levels.

MATERIALS AND METHODS: Seventy outbred Sprague-Dawley rats weight 250-300g were utilised for evaluating and localizing NOS isoforms mRNA expression by semi-quantitative competitive RT-PCR (n=28) and in situ hybridization (n=16), and for protein expression by western blot (n=24) and immunohistochemistry (n=16). Healing callus was investigated at day 4, 7, 14, 21 post right femur fracture. The unfractured left femur was used as an internal control. Paraffin embedded serial sections for in situ hybridization and immunohistochemistry studies were evaluated with rat macrophage (ED+1) and fibroblast (anti-rPH beta) cell markers.

RESULTS: There was minimal iNOS and low expression of eNOS and bNOS mRNA and protein in unfractured bone. Expression of iNOS and eNOS mRNAs was found to be maximal at 4 days and 7 days after fracture, rising 35 times and 5 times the control levels and then decreased to control levels at day 21. In contrast, bNOS mRNA elevated steadily and reached its peak amount, 16.5 times control, 21 days after fracture (Fig 1). The temporal expression of NOS isoforms at the protein level was consistent with the results of those at the mRNA level, in that they showed similar patterns except that the protein level for eNOS took slightly longer to reach its peak expression at day 14 (Fig 2). In situ hybridization and immunohistochemistry confirmed the NOS isoform temporal expression pattern and revealed that iNOS mRNA and protein expression was predominantly confined to cells with morphological appearances of macrophages, osteoblasts and cells along the edge of periosteal callus. eNOS mRNA and protein were significantly increased in endothelial cells and bNOS was mainly expressed in fibroblasts, spindle cells in connective tissue and large round chondroid-type cells at the junction of perichondrium and the cartilage (Fig 3).

DISCUSSION AND CONCLUSION: This is the first description of the temporal and cellular distribution of NOS isoforms in fracture healing. During the four stages of fracture healing: haematoma, intramembranous ossification, endochondral ossification and bone remodelling, the expression of NOS isoforms was isoform-specific, time-dependent and cellular distinctive. These findings may improve clinical treatment in the future, as administration of different NOS isoform specific inhibitors or stimulators could facilitate regulation of fracture healing at different stages after injury.

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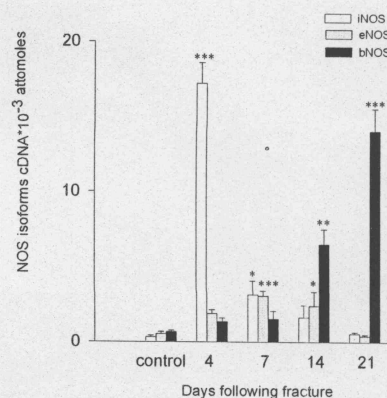


Figure 1: Temporal expression of NOS isoforms mRNA in fracture healing

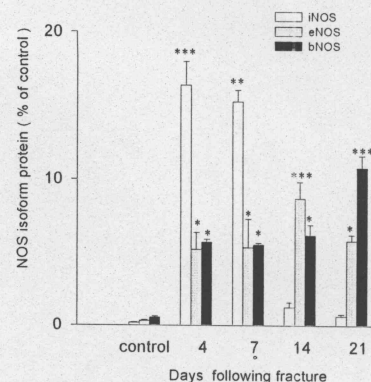


Figure 2: Temporal expression of NOS isoforms protein in fracture healing

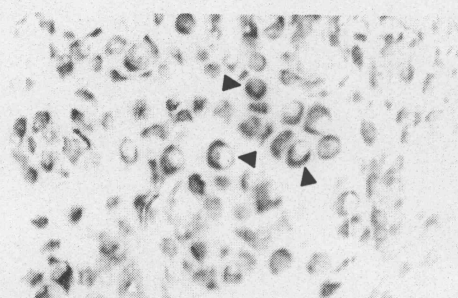


Figure3. Example of in situ hybridization detection for bNOS mRNA in chondroid cells within callus, 21 days following fracture (arrowheads).

