The Angiogenic Effects of Local Ultralente Insulin on Periosteal Bone Formation

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ABSTRACT INTRODUCTION:
While the effect of local insulin delivery upon osseous healing in diabetic animal models has been previously investigated [1, 2, 3], no studies have been performed on normal, non-diabetic animal models. The purpose of this study was to quantify the effects of local delivery of insulin (10 units intramedullary (IM)) on fracture healing compared to untreated saline controls.

METHODS:

Animal Model
Healthy, diabetic-resistant BB Wistar rats were used in this study. All research protocols were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey - New Jersey Medical School. A closed mid-diaphyseal fracture surgery was performed whereby Ultralente insulin was injected into the right femoral intramedullary canal and insertion of a stainless steel wire to stabilize the impending fracture. A closed, midshaft fracture was then created using a three-point bending fracture machine.

Insulin and Glucose Quantification
Local insulin levels were measured from the fractured and contralateral femora at 12, 24, 48, and 96 hours after fracture by Insulin rat/ mouse specific insulin ELISA Kits using a bicinchoninic acid (BCA) protein assay method.

Histomorphometry
The fractured femora resected at 4 and 7 days post-fracture were decalcified with Immumocal, dehydrated, and embedded in paraffin. The fractured femora resected at 21 days after fracture were dehydrated and embedded in polymethylmethacrylate (PMMA). All analysis was performed using Image Pro software.

Immunohistochemistry

Rats were euthanized 2 and 4 days after fracture and paraffin sections were analyzed for cellular proliferation with an antibody specific for proliferating cell nuclear antigen (PCNA, EPOS Clone PCIO, DAKO, Denmark). Vascularity was measured at 7-days post-fracture with the staining of an antibody specific for Platelet endothelial cell adhesion molecule-1 (PECAM-1, BD Pharmingen, San Jose, CA). Vascular density was measured in the area of the periosteum-cartilage junction and excluded avascular cartilage within the gap callus region.

Gene Expression Analysis
Total RNA was isolated from fracture calluses at 4 and 7 days post-fracture and cDNA was obtained by reverse transcription. Relative gene expression was calculated by normalizing to GAPDH mRNA values.

Statistical Analysis
Statistical analysis was performed using Student’s t-test to identify differences between the insulin treated and saline control groups using SigmaStat software.

RESULTS SECTION:
Local insulin levels showed no significant increase between the fractured and contralateral femora within the first 48 hours demonstrating complete release at 2 days. Histomorphometry at day 21 showed a significant increase in percent mineralized tissue for insulin-treated animals compared to saline controls (p=0.02) (Fig 1). Early histologic and histomorphometric analysis showed no difference in the density of proliferating cells or percent osteoid within the fracture callus, but did detect significant differences in vascularity (p=0.03) (Fig 2). RT-PCR analysis revealed that local insulin treatment significantly enhanced the expression of osteopontin at 4 days (p<0.02), and Colla2 at 7 days (p<0.002), compared to the untreated saline group. Osteopontin expression at day 7 was found to be significantly lower than control animals than with insulin (p=0.03), suggesting expression peaks at day 4 in animals treated with Ultralente insulin (Table 1).

DISCUSSION:
The remarkable effectiveness of insulin therapy to ameliorate the diminished fracture healing capacity seen in diabetic patients and experimental animals, led us to hypothesize that local insulin treatment could enhance fracture healing in normal animals by increasing cell proliferation at the fracture site. The mitogenic effects of insulin are well documented. It was therefore surprising that cell proliferation results at 2 and 4 days post-fracture, were not significantly different between saline and insulin treated animals. It is possible that the amount of insulin present in normoglycemic animals is sufficient to support early callus cell proliferation, despite how this differs for diabetic animals. Histomorphometric results from 7 and 21 days demonstrate the ability of insulin to enhance endochondral ossification and percent mineralized tissue within the periosteal region of the fracture callus (Figure 1). Bone formation in the fracture callus was significantly higher with local insulin treatment. Early, acute insulin therapy, while dissipating within 96 hours of administration, may thus promote the early osteogenic stages of fracture healing (Table 1), by altering the response of callus cells to other growth factors, or by inducing the expression of additional growth factors, such as IGF-1. Alternatively, application of the insulin may support greater angiogenesis as demonstrated by enhanced blood vessel density 7 days post-fracture in insulin treated animals (Figure 2).

Figure 1: Histological Comparison Between Ultralente Insulin and Saline Control at Week 3:
(A) 10 Units Ultralente Insulin at 1.67 X (B) Saline Control at 1.67 X (C) 10 Units Ultralente Insulin at 2.5 X (D) Saline Control at 2.5 X as visualized under stereomicroscope

Figure 2: Early Immunohistochemistry of PECAM at Day 7

Early Immunohistochemistry (PECAM)

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<tr>
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<th>Number of Blood Vessels Per Unit Area (cells/mm^2)</th>
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<tr>
<td>Saline</td>
<td>10±0.000</td>
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<tr>
<td>Local Insulin</td>
<td>15±0.000</td>
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The data represents average values ± standard deviation
* Represent values statistically higher than control, p<0.05
** Represent values statistically lower than control, p<0.05
# Represent values statistically higher than control, p<0.05

REFERENCES: