Local Insulin Therapy Affects Fracture Healing in a Rat Model

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ABSTRACT: A significant number of lower extremity fractures result in mal-union necessitating effective treatments to restore ambulation. Prior research in diabetic animal fracture models demonstrated improved healing following local insulin application to the fracture site and indicated that local insulin therapy can aid bone regeneration, at least within an insulin-dependent diabetic animal model. This study tested whether local insulin therapy could accelerate femur fracture repair in normal, non-diabetic rats. High (20 units) and low (10 units) doses of insulin were delivered in a calcium sulfate carrier which provided sustained release of the exogenous insulin for 7 days after fracture. Histomorphometry, radiographic scoring, and torsional mechanical testing were used to measure fracture healing. The fracture calluses from rats treated with high-dose insulin had significantly more cartilage than untreated rats after 7 and 14 days of healing. After 4 weeks of healing, femurs from rats treated with low-dose insulin had significantly higher radiographic scores and mechanical strength (p < 0.05), compared to the no treatment control groups. The results of this study suggest that locally delivered insulin is a potential therapeutic agent for treating bone fractures. Further studies are necessary, such as large animal proof of concepts, prior to the clinical use of insulin for bone fracture treatment. © 2012 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: insulin; calcium sulfate; fracture; rat; carrier

The effect of insulin on diabetic fracture healing has been well documented.1–3 Diabetes leads to reduced cellular proliferation in the early callus, reduced collagen synthesis and content compared to non-diabetic control animals, and reduced biomechanical properties of the healing fracture.1 Administration of systemic insulin to regulate blood glucose within normal levels ameliorates impaired fracture healing in an insulin-dependent diabetic rat model.2 Remarkably though, local insulin treatment at the fracture site in insulin-dependent diabetic rats that were maintained in a severe hyperglycemic state also ameliorates impaired fracture healing associated with diabetes.1,2 Local insulin therapy improved fracture site cell proliferation, cartilage formation, new bone content, and callus strength in hyperglycemic, insulin-dependent, diabetic rats.1,4

The experiments performed in diabetic animals indicate that insulin acts to positively regulate fracture healing at the systemic and local levels. Use of insulin to augment fracture healing or other bone regeneration processes in normal animal models of bone regeneration has not been investigated. Elevating systemic insulin levels would cause hypoglycemia in normal mammals and thus is not a therapeutic option. However, local application of insulin to a fracture site that would provide locally high yet systemically near normal insulin levels could be a therapeutic strategy to enhance fracture healing.

The effects of local insulin therapy on femur fracture healing were measured using a non-diabetic rat model. We hypothesize that in a dose dependent manner, local insulin combined with a calcium sulfate carrier will provide early sustained release within the femoral fracture callus resulting in improved healing while having minimal effect on systemic blood glucose values. To test this therapeutic strategy, healing was measured by histomorphometric analysis of the fractures, by radiographic scoring, and by mechanical testing of the femurs. To our knowledge this is the first study to investigate the effects of local insulin therapy on non-diabetic fracture healing.

MATERIALS AND METHODS
Animal Model
One hundred thirty-one BB Wistar rats were used in the study and 18 of these were excluded because of inadequate tissue processing and staining (five rats), surgical complications (two rats) or because the fracture was not ideally located (11 rats). The rats were obtained from a breeding colony at the University of Medicine and Dentistry of New Jersey New Jersey Medical School (UMDNJ) that was established with rats from BioBreeding (Toronto, Canada). All research protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Different animals were used for histomorphometry (43 rats), mechanical testing (38 rats), and insulin quantification experiments (32 rats). Histomorphometry was performed in female rats. Only male rats were used for biomechanical testing, radiographic analysis, and insulin quantification. Gender was chosen to keep body weights similar for the different outcome measures. We do not expect that female rats would confound the effect of local insulin delivery because of the short 1–2 week time span of...
histomorphometric analysis. Group sizes ranged from four to eleven.

Closed Femur Fracture Procedure and Intramedullary Insulin Delivery

The ultralente (UL) human insulin (Eli Lilly and Company, Indianapolis, IN) solution was 200 IU/ml where 1 IU is equivalent to 45.5 μg of insulin. Four treatment groups were used: (i) saline (0.9% NaCl); (ii) buffer; (iii) low dose (10 IU); and (iv) high dose (20 IU). To prepare the calcium sulfate–insulin mixture two grams of calcium sulfate (CaSO₄) hemihydrate (J.T. Baker) were placed in glass vials. The vials were placed in an autoclave and sterilized for 2 h in a dry cycle. CaSO₄ powder (0.8 g) was mixed for 1 min at room temperature with 400 μl of saline (buffer), 200 μl saline plus 200 μl of UL insulin solution (low dose), or 400 μl of UL insulin solution (high dose). The mixtures were approximately 0.4 cubic centimeters (cm³) in volume.

A closed mid-diaphyseal fracture was created in the right femur using a modification of the method described by Bonnarens and Einhorn. After exposing the distal femur, the intramedullary canal was entered by drilling through the intercondylar notch.

The calcium sulfate or calcium sulfate–insulin mixture (0.4 cm³) was packed into the barrel of a 1 cm³ sterile syringe and 0.1 ml of the mixture was injected into the femoral canal prior to Kirschner wire insertion. The saline group was similarly treated but with 0.1 ml of saline with no CaSO₄. After application of the insulin or control treatment and Kirschner wire insertion, the femurs were fractured and wounds closed.

Insulin quantification was performed using Enzyme-Linked Immunosorbent Assay (ELISA, E2RMI-13K, Linco Research, Inc., St. Charles, MO) using previously described methods. The ELISA kit approached 100% sensitivity to both rat and human insulin. Plasma insulin levels were measured, via cardiac puncture at sacrifice, on days 2, 4, and 7 post-fracture in animals treated with high dose CaSO₄-insulin. Local insulin levels were measured from the fractured (right) and contralateral (left) femora on days 2, 4, and 7 post-fracture. Immediately following sacrifice the fractured and contralateral femora were resected and the callus and mid-diaphyseal region corresponding to the fracture callus were isolated. The fracture callus was flash frozen in liquid nitrogen, pulverized, and total protein extracted. Local insulin values were normalized to total protein concentration measured using a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL).

Histomorphometry

Rats were killed at 7 and 14 days after fracture for histomorphometric analysis of fracture healing. Fractured femora resected at 7 days after fracture were, decalcified, embedded in paraffin, sectioned, and stained with Masson’s trichrome using standard histological techniques as previously described. Tissue corresponding to bone appears blue. Fractured femora resected at 2 weeks after fracture were embedded in polymethylmethacrylate, sectioned, and stained with Stevenel’s blue and Van Gieson picrofuscin as described. Bone appears pink-red and cartilage appears dark blue. The histomorphometric analysis, performed at 25× magnification, measured cartilage area and newly formed bone as a percentage of total fracture callus area using Image Pro software (version 5, Media Cybernetics, Inc., Silver Spring, MD). Specimens at 14 days after fracture were scored to determine the extent of healing using a previously described method in which a score of 1 indicates immature healing and 10 indicates a healed, mature callus.

Radiographic Evaluation

Magnified lateral and anteroposterior (AP) radiographs were obtained of resected femurs 4 weeks post-fracture. These femurs were then used for later mechanical testing. Radiographs were taken using a Packard Faxitron (MX 20–Radiographic Inspection System) and Kodak MinR-2000 mammography film. Exposures were for 30 s at 55 kVp. Qualitative analysis was performed on radiographic samples at 4 weeks post-fracture. Two independent observers individually scored radiographs based on bridging of the lateral and AP femoral orientations. Treatment group averages were computed to estimate healing at 4 weeks post-fracture. The analysis was conducted in a blinded fashion using a validated, five-point radiographic scoring system, 0 = no evident bony bridging, 1 = bony bridging of one cortex, 2 = bony bridging of two cortices, 3 = bony bridging of three cortices, and 4 = bony bridging of all four cortices.

Mechanical Testing

Fractured and contralateral femora were resected at 4 weeks and cleaned of soft tissue with subsequent intramedullary wire removal. The proximal and distal ends of the fractured and contralateral femora were embedded in ¾ in. hex nuts with Field’s metal, leaving an approximate gauge length of 12 mm. After measuring callus and femur dimensions, torsional testing was conducted using a servohydraulic machine (MTS Systems Corp., Eden Prairie, MN) with a 20 Nm reaction torque cell (Interface, Scottsdale, AZ) and tested to failure at a rate of 2.0 deg/s. Data were collected via MTS Test Star software (Test Star II 790.10, Test Ware-SX, Eden Prairie, MN). The peak torque, torsional rigidity, shear modulus, and maximum shear stress were determined through standard equations modeling each femur as a hollow ellipse. Percent normalized data were obtained by dividing each fractured femur value by its corresponding contralateral femur value.

Statistical Analysis

Statistical analyses were performed using SigmaStat 3.0 (SPSS, Inc., Chicago, IL). Parametric data were tested for significant differences by one-way analyses of variance (ANOVA) followed by Holm–Sidak post hoc tests to identify specific differences. Histomorphometric data at 14 days post fracture were tested for significant differences via Kruskal–Wallis one-way analysis of variance followed by Holm–Sidak post hoc tests. The histological scoring data were analyzed using ANOVA on ranks. p-values less than 0.05 were considered statistically significant.

RESULTS

General Health of the Rats

The BB Wistar rats were between 115 and 160 days old at the time of fracture. The percent weight change from time of fracture to euthanization was similar between treatment groups (S-Table I). In addition, no statistical difference in blood glucose levels was found between high dose insulin and control treatment groups indicating that the CaSO₄-insulin treatment
did not release sufficient insulin to cause a systemic decrease in blood glucose (S-Table II).

**Histological Evaluation of Fracture Healing**
At day 7 after fracture, fracture callus percent cartilage in the high dose group was significantly greater \((p < 0.05)\) than the callus percent cartilage in the saline and low dose groups (Table 1, Fig. 1). No statistically different percent cartilage was found between the high dose and buffer groups. Callus area was also significantly greater \((p < 0.05)\) for the buffer and the high dose treatment groups than the low dose group (Table 1).

By day 14 after fracture, callus percent cartilage in the high dose group was significantly higher than the saline \((p = 0.004)\) and low dose \((p = 0.027)\) groups while there was no difference compared to the buffer group (Table 1, Fig. 1). Percent new bone was not significantly different between groups.

Based upon histological scoring, no statistically significant differences were found amongst the treatment groups (Table 2). However, the low dose group had higher trending scores after 2 weeks of healing compared to the other groups.

**Radiographic Evaluation**
Scoring of AP and lateral radiographs at 4 weeks post-fracture is presented in Table 3. The low dose group had significantly higher mean scores compared to saline animals indicating accelerated bridging of cortices (Table 3, Fig. 2). Although not significant, low dose animals’ scores trended higher than high dose and buffer.

**Mechanical Testing**
The effect of local insulin therapy on healing of femur fractures was measured by torsional mechanical testing. At 4 weeks after fracture when the mechanical properties of the fractured femora were normalized to the intact, contralateral femora, the percent torque to failure were significantly greater in the low dose group when compared to the saline, buffer, or high dose groups \((p < 0.05)\). Percent shear stress was significantly greater in the low dose group as compared to the saline groups \((p < 0.05; \text{Table 4})\).

**Local and Plasma Insulin Release**
When the local UL insulin was delivered with a calcium sulfate carrier, tissue insulin levels were significantly greater \((p < 0.05)\) in the fractured femora than the unfractured femora of the rats treated with high dose within the first 4 days (Fig. 3). After 7 days, although tissue insulin levels in the fractured femora were higher than the contralateral femora; this difference was not found to be statistically significant. In addition, no statistically significant difference in plasma insulin levels was detected between saline treated animals and high dose treated animals (Fig. 4).

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**Table 1. Histology: Local Insulin Delivery With CaSO₄ Carrier**

<table>
<thead>
<tr>
<th>Group</th>
<th>7 Days Post-Fracture</th>
<th>14 Days Post-Fracture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% New Bone</td>
<td>% Cartilage</td>
</tr>
<tr>
<td>Saline</td>
<td>19.9±0.4</td>
<td>5.0±1.4</td>
</tr>
<tr>
<td>Buffer</td>
<td>16.0±2.8</td>
<td>7.5±2.7</td>
</tr>
<tr>
<td>Low dose</td>
<td>20.0±4.4</td>
<td>4.4±1.8</td>
</tr>
<tr>
<td>High dose</td>
<td>22.0±7.4</td>
<td>6.6±5.8</td>
</tr>
</tbody>
</table>

The data represent mean values ± standard deviation.

*Represents values statistically higher than saline control, \(p < 0.05\), Represents values statistically higher than low dose, \(p < 0.05\).*
Figure 1. Low dose insulin histology photographs (A) at 7 days post fracture; slides were stained with Masson's trichrome. Low dose insulin histology photographs (B) at 14 days post fracture; slides were stained with Stevenel's blue and Van Gieson picroschin. Staining and histomorphometry were employed to identify cartilage formation and new bone growth. Arrow heads show the fracture sites. Specific regions were labeled as follows: cortical bone (co), intramedullary canal (im), muscle (m), cartilage (c), fibrous tissue (f), trabecular bone (t). Callus area was defined as the region located on either side of the cortices (only one side visualized in this figure), external to the intramedullary canal; callus area encloses all structures labeled except for (co), (im), and (m). New bone within the callus area, mostly (t), stained blue in (A) or pink-red in (B). Scale bar represents 1 mm. Analysis performed at 2.5× magnification as visualized under stereomicroscope.

DISCUSSION
This study examined the effect of local insulin delivery at fracture sites in non-diabetic rats to test the hypothesis that sustained release of exogenous insulin can accelerate the fracture repair process.

Our results show that low dose insulin had a positive effect on biomechanical properties, but did not appear to have a significant effect on any of the histological parameters measured. Animals treated with high dose had significantly more cartilage in the fracture callus at 7 and 14 days (Table 1), consistent with enhanced chondrogenesis, but this did not result in better healing outcomes as determined by mechanical testing at 4 weeks. In contrast, histomorphometry of the healing fractures in the low dose treated rats found no increase in callus cartilage or new bone yet had 2–3-fold better biomechanical testing parameters than the other treatment groups. The elevated biomechanical testing parameters in the low dose group were consistent with qualitative radiographic scoring, which also indicated better healing in the low dose group. Several potential reasons exist for the discrepancy between histomorphometry and mechanical testing results in the low dose group including the early effects of insulin upon cellular proliferation, the chondrogenic phase, and/or osteogenesis.

Weiss and Reddi evaluated the effect of insulin on cartilage and bone differentiation in a demineralized bone matrix implanted subcutaneously in a diabetic rat. Mesenchymal cell proliferation was inhibited in diabetic rats as evidenced by a 65% reduction of ornithine decarboxylase (ODC) activity and a 56% reduction of \[^{3}H\]thymidine incorporation per microgram DNA compared to nondiabetic controls on day 3; the inhibition was prevented by insulin treatment. Chondrogenesis in the diabetic animal on day 7 was reduced by 49% compared to control animals as assessed by \[^{35}S\]S04 incorporation. Exogenous insulin was stimulatory to cartilage development when present during days 0 through 4. Taken together these findings indicate that insulin acts to increase mesenchymal cell proliferation with subsequent cartilage differentiation in the early stages of endochondral bone formation.

During later stages, calcification of cartilage and osteogenesis were reduced by more than 50% in diabetic rats and corrected by insulin as measured by alkaline phosphatase activity and \[^{45}Ca\] incorporation. When insulin was present during the period before cartilage formation (days 0 through 7), \[^{45}Ca\] incorporation at day 11 was still diminished. However, when insulin was absent during days 0 through 3 but administered on days 4 through 11, the amount of \[^{45}Ca\] incorporation at day 11 was not significantly different from control animals. Although Weiss and Reddi’s model is an organ explant in a diabetic rat their results indicate when insulin is present during early cell proliferation (days 0–4) mineralization of tissue is not apparent at day 11, consistent with this study’s findings. Further insight is obtained with the understanding that insulin improves fracture callus strength in diabetic animals and observing this study’s trend of increased percent new bone and lower percentage of cartilage at day 7, which may be indicative of early progression to the remodeling phase of bone healing in the low dose group.

Table 2. Histology: 14 Days Post-Fracture Scoring

<table>
<thead>
<tr>
<th>Group</th>
<th>Score (1–10)</th>
</tr>
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<tbody>
<tr>
<td>Saline (n = 6)</td>
<td>4.7 ± 2.6</td>
</tr>
<tr>
<td>Buffer (n = 5)</td>
<td>5.4 ± 2.3</td>
</tr>
<tr>
<td>Low dose (n = 6)</td>
<td>6.6 ± 1.6</td>
</tr>
<tr>
<td>High dose (n = 4)</td>
<td>4.6 ± 2.1</td>
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The data represent mean values ± standard deviation. Results were not statistically significant.

Table 3. Radiographic Scoring: 4 Weeks Post-Fracture

<table>
<thead>
<tr>
<th>Group</th>
<th>Score (0–4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (n = 9)</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td>Buffer (n = 11)</td>
<td>1.8 ± 1.2</td>
</tr>
<tr>
<td>Low dose (n = 8)*</td>
<td>3.0 ± 1.0*</td>
</tr>
<tr>
<td>High dose (n = 9)</td>
<td>1.6 ± 1.5</td>
</tr>
</tbody>
</table>

The data represent mean values ± standard deviation.

*One animal’s radiograph was not performed. Represents values statistically higher than the saline control, p < 0.05.
Insulin can positively affect chondrogenesis. In the high dose group, the sustained release of the higher dose insulin may have been sufficient to prolong the cartilaginous phase of healing which resulted in delayed, rather than accelerated healing, based upon the biomechanical testing results. Insulin treatment can stimulate cartilage formation in vitro by increasing cell proliferation and differentiation. Independent of insulin, calcium sulfate itself can affect cell proliferation and gene expression. When mouse pre-osteoblasts were cultured on calcium sulfate sub-stratum, expression of genes involved in new bone formation and alkaline phosphatase activity were increased. Additional experiments to test the combined effect of insulin and calcium sulfate on cellular proliferation and gene expression could provide a beneficial link to explain this study’s findings.

Unlike the high dose group, no increase in callus cartilage was observed in the low dose group suggesting that insulin may accelerate fracture healing through a mechanism that does not involve chondrogenesis. For instance, insulin can increase VEGF synthesis by osteoblasts, which in turn would enhance endothelial cell activity and angiogenesis that is essential for fracture healing.

**Table 4. Four Weeks Mechanical Testing: Local Insulin Delivery With CaSO₄ Carrier**

<table>
<thead>
<tr>
<th></th>
<th>Maximum Torque to Failure (Nmm)</th>
<th>Maximum Torsional Rigidity (Nmm²/rad)</th>
<th>Shear Modulus (MPa)</th>
<th>Maximum Shear Stress (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractured femur values</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n = 9)</td>
<td>163 ± 84</td>
<td>2.4 × 10⁴ ± 1.8 × 10⁴</td>
<td>1.1 × 10³ ± 1.2 × 10³</td>
<td>23 ± 14</td>
</tr>
<tr>
<td>Buffer (n = 11)</td>
<td>238 ± 79</td>
<td>2.3 × 10⁴ ± 1.4 × 10⁴</td>
<td>678 ± 507</td>
<td>25 ± 11</td>
</tr>
<tr>
<td>Low dose (n = 9)</td>
<td>460 ± 168*,#,§</td>
<td>4.6 × 10⁴ ± 2.2 × 10⁴</td>
<td>1.9 × 10³ ± 1.4 × 10³</td>
<td>72 ± 70</td>
</tr>
<tr>
<td>High dose (n = 9)</td>
<td>202 ± 89</td>
<td>2.2 × 10⁴ ± 2.1 × 10⁴</td>
<td>1.1 × 10³ ± 1.3 × 10³</td>
<td>30 ± 15</td>
</tr>
<tr>
<td>Fractured femur values normalized to the contralateral (intact) femur</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n = 9)</td>
<td>24 ± 10</td>
<td>42 ± 23</td>
<td>12 ± 11</td>
<td>10 ± 6</td>
</tr>
<tr>
<td>Buffer (n = 11)</td>
<td>42 ± 19</td>
<td>51 ± 33</td>
<td>10 ± 7</td>
<td>14 ± 7</td>
</tr>
<tr>
<td>Low dose (n = 9)</td>
<td>91 ± 54*,#,§</td>
<td>112 ± 105</td>
<td>27 ± 25</td>
<td>37 ± 39*</td>
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<tr>
<td>High dose (n = 9)</td>
<td>38 ± 20</td>
<td>45 ± 48</td>
<td>12 ± 15</td>
<td>13 ± 7</td>
</tr>
</tbody>
</table>

The data represents average mean values ± standard deviation.
*Represents values statistically higher than the saline control, p < 0.05. #Represents values statistically higher than the buffer, p < 0.05. §Represents values statistically higher than high dose, p < 0.05.

Figure 2. Anterior–posterior radiographs taken at 4 weeks post fracture. (A) Saline. (B) Buffer. (C) Low dose. (D) High dose. Scale bar represents 5 mm.
healing. Alternatively, insulin could induce angiogenesis through the phosphatidylinositol-3 kinase/Akt pathway or through increased mTOR (mammalian Target of Rapamycin) signaling.

The insulin signaling pathway can also be stimulated by IGF-I and other studies examining the effects of insulin and IGF-I on fracture healing show complementary findings. Schmidmaier et al. found that locally delivered IGF-I could improve healing of rat tibia fractures. At 4 weeks after fracture, Schmidmaier et al. noted significantly less cartilage in the callus in the IGF-I treated animals as compared to untreated controls. This is similar to our results showing less callus cartilage in the low dose treated animals as compared to the high dose treatment group which had poor biomechanical testing outcomes. Similar to the results shown here, Schmidmaier et al. also did not find a significant difference in the amount of new bone formation following local IGF-I treatment after 4 weeks of healing, even though the IGF-I treated animals had significantly improved biomechanical testing outcomes.

The study results also indicate that insulin dose or duration of insulin treatment are critical variables in controlling the fracture healing process since the high dose did not have a positive effect upon the biomechanical parameters at 4 weeks after fracture. Calcium sulfate was selected as the carrier for UL insulin delivery in the non-diabetic femoral fracture model. Calcium sulfate has a long history of use in medicine and dentistry and is used in bone regeneration as a graft material, to augment surgical procedures, as a delivery vehicle for growth factor and antibiotics. Calcium sulfate is biocompatible, osteoconductive, lacks antigenicity, and is resorbable. As expected, the calcium sulfate carrier provided for at least 7 days of sustained insulin release (Fig. 3). In contrast, insulin could only be detected for 2 days in the fracture site tissue after direct injection without carrier. Other carriers, such as palmitic acid, can provide for sustained insulin release for as long as 4 weeks in a rat model. Thus, the calcium sulfate carrier as used in these experiments appears to have provided an intermediate duration of insulin release of approximately 7 days as compared to 2 days for no carrier and 4 weeks for a palmitic acid carrier. Additional experiments to delineate insulin dose versus treatment length will be needed to optimize this potential therapy.

Although local UL insulin results suggest a promising new therapeutic strategy for treating bone fractures and other skeletal injuries, additional studies are needed to understand how dose and duration of treatment affect bone regeneration as well as to characterize insulin’s mechanism of action. Optimization of methods to deliver insulin via a carrier that would avoid any burst dosing effects and early dissipation of the insulin may increase the effectiveness and safety of this therapeutic approach.

REFERENCES


