The effects of local insulin delivery on diabetic fracture healing

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Abstract

Several studies have documented that diabetes impairs bone healing clinically and experimentally. Systemic insulin treatment has been shown to ameliorate impaired diabetic bone healing. However, these studies failed to distinguish between a direct and a systemic effect of insulin upon bone healing. A novel intramedullary insulin delivery system was used in the diabetic BB Wistar femur fracture model to investigate the potential direct effects of insulin on bone healing. Insulin delivery at the fracture site normalized the early (cellular proliferation and chondrogenesis) and late (mineralized tissue, cartilage content and mechanical strength) parameters of diabetic fracture healing without affecting the systemic parameters of blood glucose. These results suggest a critical role for insulin in directly mediating fracture healing and that decreased systemic insulin levels in the diabetic state lead to reduced localized insulin levels at fracture site with concomitant increases in diabetic fracture healing time.

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Keywords: Diabetes mellitus; Fracture; BB Wistar rat; Insulin; Drug delivery

Introduction

The association between diabetes mellitus and impaired osseous healing has been documented in clinical and experimental settings. Several clinical series have noted that the healing time for diabetic patients is approximately twice as long as for non-diabetic patients [7,19]. Chemically-induced and spontaneous diabetic animal models have demonstrable impairments in fracture healing. In the various fracture healing models, diabetes has led to reduced biomechanical properties of the healing fracture, reduced cellular proliferation in the early callus and reduced collagen synthesis and content compared to non-diabetic control animals [2,11,14,21,31,33].

The mechanism by which diabetes impairs fracture healing is unknown. However, previous studies have demonstrated that high glucose concentrations impair the proliferative response of osteoblast-like cells to insulin like growth factor-I (IGF-I) and delay osteoblast differentiation indicating that defective osteoblast function might contribute to impaired bone formation [1,32]. Sustained hyperglycemia increases formation of advanced glycation endproducts (AGEs) that can signal through a cell-surface receptor, receptor for AGE (RAGE) [6,29]. Previous studies have demonstrated that osteoblasts express RAGE and that RAGE is expressed at significantly higher levels during diabetic vs. non-diabetic bone healing [6,29]. In non-diabetic animals, AGE treatment caused a dose-dependent reduction in bone healing [6,29]. These studies suggest that elevated AGE levels caused by systemic hyperglycemia contribute to diminished bone healing in diabetes, possibly mediated through RAGE.

Systemic insulin treatment reverses impaired bone healing in diabetic animals, possibly through enhancement of bone formation and inhibition of bone resorption [2,14,15,21]. However, these studies did not discriminate between an indirect systemic effect on skeletal metabolism or a direct action of insulin on bone healing. It is difficult to ascertain whether the amelioration of impaired healing in the diabetic state through insulin therapy is a result of its
direct and local effect on the fracture repair process or is a secondary effect due to systemic alterations in the metabolic state, such as by reducing hyperglycemia or formation of advanced glycation end products [29].

To isolate the effects of local vs. systemic insulin on bone repair, a diabetic animal model was used to evaluate the direct effects of insulin on bone repair, in the absence of systemic metabolic alterations. A local intramedullary insulin delivery depot was evaluated in a rat femur fracture model. We hypothesize that local insulin delivery to the fracture site directly ameliorates impaired diabetic fracture healing independent of the systemic metabolic state.

Materials and methods

Animal model

The onset of insulin-dependent diabetes mellitus in the BB Wistar rat occurs through the development of insulitis accompanied by a selective, autoimmune destruction of pancreatic β cells and is associated with genetic and immune factors [25]. Within 4 to 7 days after the onset of glycosuria, the beta cells are destroyed completely and if insulin is not administered, muscle wasting, dehydration and ketoacidosis leading to animal death occur. The BB Wistar rat represents an animal model with a close homology to human type I diabetes mellitus.

Male BB Wistar rats were purchased from Health Canada Animal Research Division (Ottawa, Canada) as either diabetic resistant (non-diabetic) or diabetic prone at 60 days of age. The rats were housed under pathogen-free conditions and fed ad libitum. All research protocols were approved by the Institutional Animal Care and Use Committee at UMDNJ-New Jersey Medical School. Urine from the diabetic prone rats was checked for glycosuria three times a week. Once glycosuria was detected, blood obtained from the tail vein was tested for blood glucose levels (ACCU-CHEK Advantage, Roche Diagnostics, Indianapolis, IN). When blood glucose levels became greater than 250 mg/dl, a bovine insulin-releasing palmitic acid implant (Linplant, LinShin Canada, Ontario, Canada) was aseptically placed subcutaneously in the dorsal neck which provides constant insulin release for approximately 30 days (14% bovine insulin, 86% palmitic acid; weight 26 ± 2 mg/implant; 2 U/day release rate). Non-diabetic rats received a similar subcutaneous sham implant which does not release insulin. The amount of subcutaneous insulin implant each diabetic rat received was adjusted accordingly to achieve the appropriate experimental systemic blood glucose level of 300–400 mg/dl. Blood glucose levels were measured three times a week after initial treatment.

At sacrifice, 2 ml of whole blood was collected and glycosylated hemoglobin (HbA1c) levels were determined using the Glyc-AffinGHb kit (Perkin Elmer Life Sciences, Norton, OH). HbA1c is a time-averaged measure of blood glucose control and can be twice as high in patients with poor blood glucose control when compared to normal patients [4,18].

The total number of BB Wistar rats utilized for this study (n = 177) included animals sacrificed due to unstable femur fracture pattern (n = 8), loss of fracture reduction secondary to rod migration (n = 9) and post-operative infection (n = 15). The remaining animals were utilized for insulin quantitation (n = 30), cellular proliferation analysis (n = 51), histomorphometric analysis (n = 30) and biomechanical testing (n = 34).

The rationale for the early time points (days 2, 4 and 7 post-fracture) was to look at the inflammatory/early proliferative phase of healing. These time points were chosen based on our previously published data demonstrating reduced platelet-derived growth factor (PDGF) expression in the diabetic fracture callus possibly indicating impaired platelet function/aggregation and a corresponding decrease in cell proliferation [34]. The rationale for the later time points was to determine whether a correlation exists between impaired early inflammation/proliferation and the histomorphometric and mechanical properties of the fracture callus at 6 and 8 weeks. Previous studies have documented that rats will heal by 6 weeks and that diabetic rats will show delayed healing that is not resolved by 8 weeks [2,12].

Intramedullary insulin delivery depot

Insulin was delivered directly to the fracture site using an insulin-palmitic acid implant placed within a hollow rod that was inserted into the femoral canal in order to stabilize the fracture. The intramedullary insulin-palmitic acid implant consisted of 95% micro-recrystallized palmitic acid and 5% bovine insulin (1 mm diameter, 7 mm long, 8 ± 1 mg; Linshin Canada, Ontario, Canada). Degradation of the intramedullary implant and release of insulin begins immediately upon implantation. As per the manufacturer, the intramedullary insulin implant has a release rate of 0.5 U/day for a minimum of 10 days. An intramedullary sham implant consisting entirely of palmitic acid was used for the non-diabetic and diabetic without intramedullary insulin treatment groups.

The fixation device consisted of a stainless-steel hollow rod, with an outer diameter of 1.25 mm and an inner diameter of 1.05 mm. The rod was laser machined to produce a regular array of 100 micron diameter holes around the circumference. This array was placed to coincide with the mid-diaphyseal region of the femur upon implantation. The appropriate implant (sham vs. insulin-releasing) was placed within the intramedullary rod prior to stabilization of the femur fracture allowing immediate release directly to the fracture site.

Three experimental groups were used: (i) control, non-diabetic rats that received a sham subcutaneous implant and a sham intramedullary implant, (ii) diabetic rats that received an insulin releasing subcutaneous implant that...
only poorly controlled systemic blood glucose levels, and a sham intramedullary implant and (iii) diabetic rats that received an insulin releasing subcutaneous implant that only poorly controlled systemic blood glucose levels, and an insulin-releasing intramedullary implant.

**Fracture model**

Fourteen days after the onset of diabetes, a closed mid-diaphyseal fracture was created in the right femur using a modification of the method described by Bonnarens and Einhorn [3]. Only animals that exhibited non-committed, transverse fractures, confirmed by radiographs, were used in this study. The animals were allowed to ambulate freely immediately post-fracture. All animals were age-matched (106 ± 7 days) at the time of fracture.

**Insulin quantitation**

Systemic insulin levels (plasma) were measured prior to fracture and on days 2, 4 and 7 post-fracture in the non-diabetic, diabetic and diabetic treated with intramedullary insulin groups. Prior to fracture, blood was collected by tail bleed and the plasma was isolated by centrifugation (1000 rpm for 30 min) and stored at −80°C until testing. After fracture, blood was collected by cardiac puncture from rats euthanized at days 2, 4 and 7 post-fracture. Plasma was isolated and stored as above.

Local insulin levels were measured from the fractured and contralateral femora on days 2, 4 and 7 post-fracture in the non-diabetic and diabetic treated with intramedullary insulin groups. The fractured and contralateral femora were resected and the fracture callus and mid-diaphyseal region corresponding to the fracture callus were isolated. The bone diaphysis or callus was flash frozen in liquid nitrogen, pulverized and total protein extracted using an acid extraction protocol as described [8]. After extraction, the supernatant was isolated by centrifugation (12,000 rpm for 10 min) and stored at −80°C until testing.

Insulin quantitation was performed in non-diabetic rats using a rat specific ELISA assay (ALPCO Diagnostics, Windham, NH) and in diabetic rats using bovine specific ELISA assays (ALPCO Diagnostics, Windham, NH), since endogenous rat insulin is not detectable in the diabetic rats [25]. The assay and analyses were performed according to the instruction of the manufacturer. Systemic insulin levels were expressed as units per milliliter of plasma. Local insulin levels were normalized to total protein concentration measured using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

**Cellular proliferation**

Cellular proliferation was measured by counting cells that had incorporated 5-bromo-2-deoxyuridine (BrdU, a thymidine analog) into replicating DNA. Animals were injected with 30 mg/kg of BrdU (Sigma Chemical Co., St. Louis, MO) 1 h prior to sacrifice at days 2, 4 and 7 post-fracture. The fractured femora were decalcified, embedded and sectioned using standard histological techniques. Cellular proliferation was determined by using monoclonal mouse anti-BrdU (Clone Bu20α, DAKO Corp., Carpinteria, California) primary antibody. A rectangular area of 0.8 mm × 4.0 mm adjacent to the cortex was used to count positively stained cells on the anterior and posterior, exterior callus. Areas of proliferation were separated into regions representing either periosteal (intramembranous bone formation) or gap (cartilage formation) callus. In order to isolate the effect of callus area on cell proliferation, total cell proliferation (periosteal + gap callus proliferation) was normalized to callus area.

**Early histomorphometry**

The fractured femora were resected at days 2, 4 and 7, decalcified, embedded and sectioned using standard histological techniques. To identify cartilage formation, the sections were stained with Weigert’s iron hematoxylin, biebrich scarlet and aniline blue. Mineralized tissue appears red-blue and cartilage appears dark blue. Histomorphometric analysis was performed to determine areas of cartilage formation as a percentage of total fracture callus area using Scion Image software (Scion Corp, Frederick, MD). The fracture callus was defined as the region located on either side of the cortices, external to the intramedullary marrow cavity. The samples were viewed and digital photomicrographs were taken using an Olympus BH2-RFCA microscope (Olympus Optical Co., Ltd., Shinjuku-ku, Tokyo, Japan) and a Polaroid DMC1e digital camera (Polaroid, Waltham, MA).

**Late histomorphometry**

Fractured femora were resected at 6 and 8 weeks and embedded in polymethylmethacrylate, as described [28]. Sections were cut sagitally through the fracture callus using an Isomet diamond saw (Buehler, Ltd., Lake Bluff, IL), mounted on plexiglass slides and polished to a thickness of 100 um. The slides were stained with Stevenel’s blue and Van Gieson picro-fuchsin to identify cartilage formation and new bone growth [22]. Mineralized tissue appears orange-red and cartilage appears dark blue. The fracture callus was defined as the region located on either side of the cortices, external to the intramedullary marrow cavity. Histomorphometric analysis was performed to determine areas of cartilage formation and new bone growth as a percentage of total fracture callus area using Scion Image software (Scion Corp, Frederick, MD).

**Mechanical testing**

Fractured and contralateral femora were resected at 6 and 8 weeks and cleaned of soft tissue. The intramedullary rod
was removed from the fractured femur. The proximal and distal ends of the fractured and contralateral femora were embedded in Wood’s metal (Alfa Aesar, Ward Hills, MA). Torsional testing was conducted using a servohydraulics 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. The peak torque (T), torsional rigidity (TR) and the maximum shear stress (τ) were determined through standard equations modeling each femur as a hollow ellipse \[9,10\]. Normalized data were obtained by dividing each fractured femur value by its corresponding contralateral femur value.

Statistical analysis

All statistical analyses were performed using SigmaStat 3.0 (SPSS Inc., Chicago, Illinois). One-way analyses of variance (ANOVA) were performed followed by Holm–Sidak post-hoc tests to determine specific differences. A \( P \) value < 0.05 was considered statistically significant. The power for a one-way ANOVA was determined by specifying in this study, the difference between the group means of the non-diabetic and diabetic groups, the standard deviation of the population, the number of groups and corresponding sample sizes and an alpha of 0.05.

Results

Animal model—general health

The blood glucose and HbA1c levels for the non-diabetic group were significantly lower compared to the diabetic and diabetic treated with intramedullary insulin groups (Table 1). The blood glucose and HbA1c levels between the diabetic and diabetic treated with intramedullary insulin groups were not different indicating that insulin from the intramedullary depot had no effect on systemic blood glucose (power = 0.99) or HbA1c levels (power = 0.99). Percent weight gain from the time of surgery in both diabetic groups was comparable and significantly lower than in the non-diabetic groups at 6 (power = 0.94) and 8 weeks (power = 0.92) post-fracture (Table 1). Based on X-rays taken at the time of sacrifice in the AP and lateral planes, the intramedullary rods showed no evidence of bending. In addition, there was no angulation of the fractured femora evident in either the X-rays or the resected bone.

Insulin quantitation

Diabetic BB rats do not produce detectable levels of endogenous rat insulin and therefore the only source of insulin is from a subcutaneous bovine insulin-releasing implant \[25\]. Systemic rat insulin levels in the non-diabetic group were significantly greater than the systemic bovine insulin levels in the diabetic and diabetic treated with intramedullary insulin groups indicating the presence of a hypoinsulinemic state in both diabetic groups (Table 2). Systemic rat insulin levels in the non-diabetic group and systemic bovine insulin levels in both diabetic groups remained relatively constant through day 7 post-fracture (Table 2). Systemic bovine insulin levels in both diabetic groups were comparable indicating that insulin from the intramedullary insulin delivery system did not have a significant systemic effect (power = 0.99).

In the diabetic rats treated with intramedullary insulin, bovine insulin levels in the fractured femora were approximately fivefold higher than in the contralateral femora demonstrating the efficacy of the intramedullary insulin delivery system (Table 3). Bovine insulin levels in the fractured femora remained relatively constant through day 7 post-fracture indicative of a constant release profile. In the non-diabetic group, rat insulin levels in the fractured femur and contralateral femur were comparable and constant through day 7 post-fracture. The intramedullary insulin delivery system attained bovine insulin levels in the diabetic fractured femur that were significantly lower (approximately 70%) than rat insulin levels in the non-diabetic fractured femur (power = 0.99).

Cellular proliferation

At day 2, periosteal and gap callus proliferation in the diabetic callus was significantly reduced compared to the...
non-diabetic and diabetic treated with intramedullary insulin callus (Table 4). The intramedullary insulin depot normalized periosteal and gap callus cell proliferation in the diabetic treated with intramedullary insulin group to levels not statistically different from non-diabetic group (power = 0.92). At day 4, all three groups showed reduced periosteal and gap callus proliferation compared to day 2. Periosteal and gap callus proliferation in the diabetic fracture calluses also was significantly reduced at day 4, compared to periosteal and gap callus proliferation in the non-diabetic and diabetic treated with intramedullary insulin group. The intramedullary insulin depot normalized cell proliferation in the diabetic treated with intramedullary insulin group to levels not statistically different from non-diabetic group (power = 0.89). At day 7, cell proliferation for all three groups was not significantly different. Total cell proliferation normalized to the fracture callus area in the diabetic group was significantly decreased compared to the non-diabetic and diabetic treated with intramedullary insulin at days 2 and 4 post-fracture (Table 4; power = 0.86). No differences between the three groups were demonstrated at day 7 post-fracture.

**Early histomorphometry**

At day 2, hematoma was adjacent to the fracture site with an elevated periosteum above the cortical bone with no significant difference in callus area between the three groups (Table 4). At day 4, the callus area was not significantly different between the three groups but there was more significantly more percent cartilage in the non-diabetic compared to the diabetic callus (Table 5). The diabetic treated with intramedullary insulin also showed increased percent cartilage compared to the diabetic group but the difference was not significant (power = 0.85). At day 7, the periosteal callus was comparable in all three groups but the gap callus in non-diabetic and diabetic treated with intramedullary insulin animals was more advanced with increased percent cartilage compared to the diabetic group (power = 0.87). The cartilage in the non-diabetic and diabetic treated with intramedullary insulin consisted of more proliferating chondrocytes and hypertrophic chondrocytes as compared to the diabetic fracture callus.

**Late histomorphometry**

At week 6, histologic analysis showed bridging of the cortege/fracture callus in the non-diabetic and diabetic treated with intramedullary insulin groups whereas the diabetic group showed incomplete bridging (Fig. 1). The diabetic group also showed no evidence of remodeling resulting in the continued presence of the fracture callus. The diabetic fracture callus also contains a greater proportion of fibrous tissue. The diabetic treated with intramedullary insulin showed some remodeling resulting in a reduced fracture callus size. At week 6, the diabetic group exhibited significantly decreased percent mineralized area (power = 0.97) and significantly increased percent cartilage area (power = 0.96) compared to the non-diabetic and diabetic treated with intramedullary insulin groups (Table 6).

At week 8, the non-diabetic rats and the diabetic rats treated with intramedullary insulin showed almost complete restoration of the cortical bone whereas the diabetic group showed incomplete bridging (Fig. 1). The diabetic group

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**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>Prior to fracture (pg/ml)</th>
<th>Day 2 post-fracture (pg/ml)</th>
<th>Day 4 post-fracture (pg/ml)</th>
<th>Day 7 post-fracture (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic (n = 6)</td>
<td>343 ± 12σ</td>
<td>398 ± 14σ</td>
<td>381 ± 15σ</td>
<td>352 ± 11σ</td>
</tr>
<tr>
<td>Diabetic (n = 6)</td>
<td>20 ± 7</td>
<td>24 ± 7</td>
<td>29 ± 8</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>Diabetic treated with intramedullary insulin (n = 6)</td>
<td>24 ± 8</td>
<td>27 ± 6</td>
<td>25 ± 7</td>
<td>25 ± 8</td>
</tr>
</tbody>
</table>

The data represent average value ± standard deviation. Plasma insulin levels were measured in the non-diabetic group using a rat insulin ELISA and in the diabetic and diabetic treated with intramedullary insulin groups using a bovine insulin ELISA.

σ Represents values statistically higher than contralateral femur values, *P* < 0.001.

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**Table 3**

<table>
<thead>
<tr>
<th></th>
<th>Insulin levels normalized to total protein levels (pg/mg)</th>
<th>Day 2 post-fracture (pg/mg)</th>
<th>Day 4 post-fracture (pg/mg)</th>
<th>Day 7 post-fracture (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic (n = 6)</td>
<td>Fractured femur 1.23 ± 0.35</td>
<td>1.08 ± 0.26</td>
<td>1.11 ± 0.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Contralateral femur 0.96 ± 0.24</td>
<td>0.84 ± 0.28</td>
<td>0.92 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>Diabetic treated with intramedullary insulin (n = 6)</td>
<td>Fractured femur 0.76 ± 0.19σ</td>
<td>0.69 ± 0.21σ</td>
<td>0.78 ± 0.23σ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Contralateral femur 0.12 ± 0.03</td>
<td>0.10 ± 0.02</td>
<td>0.14 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

The data represent average value ± standard deviation. Local insulin levels were measured in the non-diabetic group using a rat insulin ELISA and in the diabetic treated with intramedullary insulin group using a bovine insulin ELISA.

σ Represents values statistically higher than contralateral femur values, *P* < 0.01.
continued to exhibit delayed remodeling resulting in the presence of the fracture callus. The diabetic fracture callus also contains fibrous tissue. At week 8, the diabetic group exhibited significantly decreased percent mineralized area (power = 0.98) and significantly increased percent cartilage area (power = 0.97) compared to the non-diabetic and diabetic treated with intramedullary insulin groups (Table 6). All three groups had increased percent mineralized area at week 8 compared to week 6 but the difference was not statistically significant.

**Mechanical testing**

The fractured femora, exhibiting complete or partial union, and the contralateral, intact femora failed spirally in the mid-diaphyseal region following torsional mechanical testing to failure. Femora that did not heal failed transversely through the non-union site (fracture gap). The diabetic femur showed no significant difference between the three groups. The maximum shear stress was significantly reduced in the diabetic animals compared to the non-diabetic animals (power = 0.91). When these values were normalized to the contralateral limb, the percent torque to failure (power = 0.98) and percent torsional rigidity (power = 0.81) were significantly reduced in the diabetic animals compared to the non-diabetic and diabetic treated with intramedullary insulin animals.

At week 8 post-fracture, torque to failure (power = 0.97), torsional rigidity (power = 0.94) and maximum shear stress (power = 0.95) were significantly reduced in the diabetic fractured femora compared to the non-diabetic and diabetic treated with intramedullary insulin groups. When the data were normalized to the contralateral limb, percent torque to failure (power = 0.69), percent torsional rigidity (power = 0.80) and percent maximum shear stress (power = 0.51) were all significantly reduced in the diabetic animals compared to the non-diabetic (Table 7).

**Discussion**

Diabetes impairs the fracture healing process beginning with a reduction in early cellular proliferation, continuing with a delay in endochondral ossification and ending with a decrease in the biomechanical properties of the fracture

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### Table 4

**Cellular proliferation analysis**

<table>
<thead>
<tr>
<th></th>
<th>Callus area (mm²)</th>
<th>Gap callus (positive cells)</th>
<th>Periosteal callus (positive cells)</th>
<th>Positive cells per unit area (cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Two days</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Non-diabetic (n = 5)</td>
<td>0.96 ± 0.24</td>
<td>499 ± 84</td>
<td>1400 ± 384</td>
<td>1980 ± 601</td>
</tr>
<tr>
<td>Diabetic (n = 5)</td>
<td>0.84 ± 0.31</td>
<td>305 ± 41&lt;sub&gt;a,b&lt;/sub&gt;</td>
<td>722 ± 168&lt;sub&gt;a,b&lt;/sub&gt;</td>
<td>1130 ± 512&lt;sub&gt;a,b&lt;/sub&gt;</td>
</tr>
<tr>
<td>Diabetic treated with intramedullary insulin (n = 6)</td>
<td>0.92 ± 0.34</td>
<td>404 ± 155</td>
<td>1290 ± 346</td>
<td>1840 ± 624</td>
</tr>
<tr>
<td><strong>Four days</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-diabetic (n = 6)</td>
<td>4.05 ± 1.13</td>
<td>388 ± 127</td>
<td>1155 ± 231</td>
<td>381 ± 125</td>
</tr>
<tr>
<td>Diabetic (n = 6)</td>
<td>3.89 ± 1.01</td>
<td>204 ± 35&lt;sub&gt;a,b&lt;/sub&gt;</td>
<td>604 ± 212&lt;sub&gt;a,b&lt;/sub&gt;</td>
<td>208 ± 91&lt;sub&gt;a,b&lt;/sub&gt;</td>
</tr>
<tr>
<td>Diabetic treated with intramedullary insulin (n = 6)</td>
<td>3.94 ± 1.15</td>
<td>328 ± 157</td>
<td>1010 ± 287</td>
<td>339 ± 134</td>
</tr>
<tr>
<td><strong>Seven days</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Non-diabetic (n = 6)</td>
<td>12.51 ± 3.02</td>
<td>216 ± 148</td>
<td>599 ± 304</td>
<td>65 ± 21</td>
</tr>
<tr>
<td>Diabetic (n = 6)</td>
<td>11.94 ± 4.15</td>
<td>141 ± 84</td>
<td>445 ± 152</td>
<td>46 ± 13</td>
</tr>
<tr>
<td>Diabetic treated with intramedullary insulin (n = 5)</td>
<td>12.32 ± 3.45</td>
<td>189 ± 75</td>
<td>505 ± 234</td>
<td>57 ± 25</td>
</tr>
</tbody>
</table>

The data represent average value ± standard deviation.

<sup>a</sup> Represents values statistically lower than non-diabetic values, P < 0.01.

<sup>b</sup> Represents values statistically lower than diabetic treated with intramedullary insulin values, P < 0.01.

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### Table 5

**Early histomorphometry**

<table>
<thead>
<tr>
<th></th>
<th>Percent cartilage content (cartilage / total callus area)</th>
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<tbody>
<tr>
<td></td>
<td>Four days</td>
</tr>
<tr>
<td>Non-diabetic (n = 6)</td>
<td>1.57 ± 1.02</td>
</tr>
<tr>
<td>Diabetic (n = 6)</td>
<td>0.33 ± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic treated with intramedullary insulin (n = 6)</td>
<td>0.83 ± 0.42</td>
</tr>
</tbody>
</table>

The data represent average value ± standard deviation.

<sup>a</sup> Represents values statistically lower than non-diabetic values, P < 0.05.

<sup>b</sup> Represents values statistically lower than diabetic treated with intramedullary insulin values, P < 0.05.
callus. However, the mechanism through which diabetes impairs bone healing is currently unknown. In the present study, the role of local insulin delivery upon fracture healing was investigated in rats that spontaneously develop insulin-dependent diabetes mellitus.

Few studies have isolated the individual effects of diabetes (i.e., hypoinsulinemia, hyperglycemia or AGE production) upon the healing process [1,24,29,32]. However, insulin treatment has been shown to ameliorate impaired bone healing in vitro and in vivo. In a diabetic bone explant model, insulin treatment sufficient to achieve physiologic blood glucose control resulted in normalization of impaired thymidine uptake reflective of increased DNA synthesis and proliferation. This model also demonstrated that early insulin treatment restored proteoglycan production and chondrogenesis [15]. In a diabetic femur fracture model, tight systemic blood glucose control achieved through increased systemic insulin supplementation normalized cell proliferation, chondrogenesis and biomechanical parameters of the diabetic fracture callus [2]. However, these studies demonstrate a failure to distinguish between the direct and indirect effects of insulin upon bone healing.

The normalization of diabetic fracture healing through systemic insulin delivery could be attributed to the indirect actions of insulin on blood glucose levels and AGE production. To isolate the effects of insulin on bone healing, insulin delivery to calvaria of a normoglycemic animal increased the histomorphometric indices of bone formation [5]. This study suggests a direct effect of insulin on bone formation in normoglycemic animals. However, this study does not address the role of insulin on impaired healing associated with hypoinsulinemia and hyperglycemia present in the diabetic condition.

The present study provides new insight into the role of local insulin on fracture healing. Within the early diabetic fracture callus, local insulin normalized impairments in cellular proliferation and chondrogenesis. Local insulin treatment, immediately post-fracture, normalized mineralization, callus bone content and biomechanical properties in the late diabetic callus. These results suggest that insulin is critical to the diabetic fracture healing process through a

### Table 6

<table>
<thead>
<tr>
<th></th>
<th>Percent bone content (bone / total callus area)</th>
<th>Percent cartilage content (cartilage / total callus area)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Six weeks</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-diabetic (n = 5)</td>
<td>84.6 ± 7.3</td>
<td>8.9 ± 4.0</td>
</tr>
<tr>
<td>Diabetic (n = 5)</td>
<td>51.2 ± 8.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>27.7 ± 6.6&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic treated with intramedullary insulin (n = 5)</td>
<td>77.7 ± 7.9</td>
<td>11.5 ± 4.7</td>
</tr>
<tr>
<td><strong>Eight weeks</strong></td>
<td></td>
<td></td>
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<tr>
<td>Non-diabetic (n = 5)</td>
<td>90.4 ± 4.1</td>
<td>4.5 ± 2.5</td>
</tr>
<tr>
<td>Diabetic (n = 5)</td>
<td>61.6 ± 7.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>22.2 ± 5.1&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic treated with intramedullary insulin (n = 5)</td>
<td>84.6 ± 4.2</td>
<td>6.9 ± 3.0</td>
</tr>
</tbody>
</table>

The data represent average value ± standard deviation.

<sup>a</sup> Represents values statistically lower than non-diabetic values, *P* < 0.01.

<sup>b</sup> Represents values statistically lower than diabetic treated with intramedullary insulin values, *P* < 0.01.

<sup>c</sup> Represents values statistically higher than diabetic values, *P* < 0.01.

<sup>d</sup> Represents values statistically higher than diabetic treated with intramedullary insulin values, *P* < 0.01.

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Fig. 1. (A–F) Late fracture callus histology: Histology of gap callus (area of endochondral ossification) at 6 weeks in the (A) non-diabetic, (B) diabetic and (C) diabetic treated with intramedullary insulin groups and 8 weeks in the (D) non-diabetic, (E) diabetic and (F) diabetic treated with intramedullary insulin groups. Slides were stained with Stevenel’s Blue and Van Gieson’s Picrofuchsin (dark blue = cartilage; orange-red = mineralized tissue; 4× magnification).
Table 7
Mechanical testing

<table>
<thead>
<tr>
<th></th>
<th>Torque to failure (Nm)</th>
<th>Percent torque to failure (Nm/rad)</th>
<th>Torsional rigidity</th>
<th>Percent torsional rigidity</th>
<th>Maximum shear stress (Mpa)</th>
<th>Percent maximum shear stress</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Six weeks</strong></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>Non-diabetic (n = 6)</td>
<td>414 ± 50</td>
<td>69 ± 17</td>
<td>20,900 ± 3940</td>
<td>72 ± 26</td>
<td>7.1 ± 1.1</td>
<td>10.7 ± 4.8</td>
</tr>
<tr>
<td>Diabetic (n = 6)</td>
<td>178 ± 84&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>25 ± 10&lt;sup&gt;h&lt;/sup&gt;</td>
<td>9420 ± 3760&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>29 ± 18&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.1 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.1 ± 4.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic treated with intramedullary insulin (n = 5)</td>
<td>360 ± 77</td>
<td>54 ± 13</td>
<td>19,800 ± 6150</td>
<td>80 ± 34</td>
<td>6.2 ± 1.8</td>
<td>15.8 ± 7.5</td>
</tr>
<tr>
<td><strong>Eight weeks</strong></td>
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<td></td>
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</tr>
<tr>
<td>Non-diabetic (n = 5)</td>
<td>500 ± 54</td>
<td>81 ± 21</td>
<td>26,500 ± 4180</td>
<td>89 ± 31</td>
<td>8.1 ± 2.2</td>
<td>40.5 ± 23.2</td>
</tr>
<tr>
<td>Diabetic (n = 6)</td>
<td>250 ± 108&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>42 ± 15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10,700 ± 4120&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>36 ± 19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.6 ± 1.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>16.9 ± 7.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic treated with intramedullary insulin (n = 6)</td>
<td>401 ± 80</td>
<td>65 ± 28</td>
<td>23,100 ± 8880</td>
<td>71 ± 33</td>
<td>8.2 ± 0.9</td>
<td>32.0 ± 21.2</td>
</tr>
</tbody>
</table>

The data represent average value ± standard deviation.

* Represents values statistically lower than non-diabetic values, *P* < 0.01.

<sup>a</sup> Represents values statistically lower than diabetic treated with intramedullary insulin values, *P* < 0.01.

<sup>b</sup> Represents values statistically lower than non-diabetic values, *P* < 0.05.

direct effect on the fracture callus and indicates that insulin may have a direct effect on fracture healing in normal animals.

Several mechanisms have been proposed, attempting to explain the impaired fracture healing process associated with the diabetic state. One potential mechanism involves the role of insulin receptor substrate-1 (IRS-1), an essential protein in the signaling pathway of insulin and IGF-I, in mediating the fracture healing process. In a tibia fracture model, IRS-1<sup>−/−</sup> mice exhibited increased incidence of non-union and decreased bone mineral content compared to wild-type [30]. Histologically, wild-type mice progressed through normal endochondral ossification at 3 weeks and bony union at 6 weeks. In IRS-1<sup>−/−</sup> mice, fibrous tissue was present at 3 and 6 weeks. Detailed histologic analysis revealed that IRS-1<sup>−/−</sup> fracture site was associated with a decrease in chondrocyte proliferation. These results in conjunction with the results obtained in the present study indicate a central role for insulin signaling through the IRS-1 pathway in mediating the fracture healing process [30].

Another potential mechanism involves the existence of local insulin gradients within the fracture callus that are sufficient to stimulate IGF-1 receptor signaling. IGF-I plays an important role in the anabolic regulation of bone and cartilage metabolism, potentially through the IRS-1 pathway.

Another possible etiology of impaired diabetic fracture healing is the role of insulin signaling on expression of specific factors critical for bone healing. In a model of intramembranous bone formation, diabetes impaired expression of Runx-2, runt domain factor-2, and Dlx5, the human homolog of the *Drosophila* distal-less gene, specific regulators of osteoblast differentiation [20]. The impaired expression of these key regulators was followed by decreased expression of osteocalcin and collagen type I corresponding to decreased bone formation measured through histomorphometric analysis. Insulin treatment ameliorated impaired expression of Runx-2 and Dlx5 as well as bone matrix osteocalcin and collagen type I [20]. A previous fracture healing study demonstrated reductions in bFGF expression in the early diabetic fracture callus which was restored with insulin treatment [17]. bFGF stimulates fracture repair through a mitogenic effect on mesenchymal cells and through enhancement of TGF-β expression which is involved in chondrogenesis and bone formation [16,23,26,27]. Previous studies have theorized that reduced PDGF expression in the diabetic callus led to decreased cellular proliferation and that impaired cellular proliferation in the diabetic fracture callus led to reduced mechanical properties [21,34].

The various stages of fracture healing have been correlated to the temporal expression of three groups of soluble factors (pro-inflammatory cytokines, the TGF-β superfamily and angiogenic factors) that play a critical role in modulating the repair process [13]. The previous study in conjunction with the present study demonstrating impaired cell proliferation and impaired chondrogenesis suggests that diabetes disrupts the regulation and interaction of factors critical for the normal progression of the inflammatory phase and chondrogenesis leading to decreased biomechanical properties of the diabetic femora. While the present study along with previous studies suggests insulin and its signaling pathways initiate a complex cascade of factors, it is difficult to determine the exact mechanism through which insulin signaling plays a role in the normalization of diabetic fracture healing.

To our knowledge, this is the first study looking at the effect of local insulin delivery to the fracture site in diabetic animals. This study confirms the importance of local insulin in ameliorating the impaired early and late parameters of diabetic fracture healing. Local insulin delivery directly to the fracture site could have significant clinical implications. Future work will include examining the relationship between the insulin signaling pathway and the expression of genes critical for the regulation of the fracture healing process.
Acknowledgments

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References
