Controlled Release of Fibroblast Growth Factor 2 Stimulates Bone Healing in an Animal Model of Diabetes Mellitus

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Purpose: *Bone formation and the healing of calvarial defects in mice is diminished in chemically induced type 1 diabetes. The present study investigated whether controlled local release of fibroblast growth factor 2 (FGF-2) stimulates bone defect healing in this model of diabetes.* ***Materials and Methods:*** *First, in vitro release kinetics of different doses of recombinant human FGF-2 (rhFGF-2) from polyglycolate:polylactide membranes was determined over a 14-day period by incubating loaded membranes in PBS with constant shaking. The amount of FGF-2 was measured by enzyme-linked immunosorbent assay. Then, the effects of rhFGF-2–loaded and control membranes on calvarial defect healing over a 14-day healing period were determined in diabetic and nondiabetic mice. The degree of healing was determined by histomorphometric analyses of bone area percentage and by area measurements. The significance of the data was determined by statistical analyses, including analysis of variance.* ***Results:*** *Kinetic release data in vitro showed that membranes loaded with 5 μg FGF-2 released measurable levels of growth factor for more than 14 days. Data from the in vivo study supported the previous finding that diabetes inhibits bone formation. Membranes containing rhFGF-2 significantly (*$P < .05$*) stimulated bone formation in diabetic animals to near normal levels during the healing period.* ***Conclusion:*** *FGF-2–loaded membranes may be useful in further studies aimed at developing therapeutic strategies for correcting deficient bone healing in patients with diabetes.* (More than 50 references) Int J Oral Maxillofac Implants 2006;21:711–718

Key words: *bone regeneration, controlled release, diabetes, fibroblast growth factors, growth factors*

Osseointegration represents a direct connection between bone and implant without interposed soft tissue layers[1] and is a fundamental prerequisite for long-term survival of dental implants.[2] Inadequate amounts of bone at the time of implantation have been associated with decreased success rates.[3] Methods to increase host bone volume have been developed to obtain a larger area of bone-to-implant contact. Osteopromotion via guided bone regeneration (GBR) involves the creation of a protected environment for the blood clot and competent regenerative cells. A physical barrier is placed between the soft tissues of the gingival flap and the osseous defect to form a protected environment for regeneration.[4–8] The goals of the procedure are to improve bone healing, bring about complete bone restitution, improve bone grafting results, and create new bone (bone neogenesis).[9] GBR has been used clinically for the treatment of a variety of intraoral bone deformities, including periodontal defects,[10–13] extraction site bone defects,[14,15] and defects around implants[16,17] and in conjunction with other types of oral surgeries.[18–22] Barrier membranes used in these GBR techniques should possess tissue integration capacity, cell occlusion properties, clinical manageability, space-making ability, and biocompatibility.[23] The membrane, however, acts as a passive element in bone regeneration and does not possess an active stimulatory effect on osteogenesis. Thus, the effectiveness of GBR is dependent on the intrinsic healing potential of the host.

Osteopenia is a complication of type 1 diabetes in humans.[24] Decreased bone mineral content[25,26] and delayed fracture healing[27] are common in type 1 diabetes. In addition, reduced osteoblastic activity occurs in humans and in animal models of type 1 diabetes.[28–32] Osteopenia is likely to result in diminished

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bone formation, and studies have demonstrated diminished bone formation in experimental bone defects[33] as well as delayed bone regeneration in extraction sockets[34] and inhibited osseointegration of implants in type 1 diabetic animals.[35] Such negative effects of diabetes on bone metabolism may influence normal as well as GBR-mediated bone regeneration.

Fibroblast growth factors (FGFs) play important roles in morphogenesis and wound healing[36] and are potent stimulators of osteoblastic proliferation in vitro.[37] FGF-2 regulates extracellular matrix production by osteoblastic cells in vitro.[38–40] FGFs systemically administered in vivo have increased endosteal bone formation in rats.[41,42] The goal of the current study was to assess whether the controlled local application of recombinant human FGF-2 (rhFGF-2) from an absorbable GBR membrane restores intramembranous bone healing to normal levels in an animal model of type 1 diabetes.

MATERIALS AND METHODS

The FGF-2 delivery system was first prepared. The system chosen for rhFGF-2 delivery was a polylactide: polyglycolide copolymer kit (Atrisorb; Atrix Laboratories, Fort Collins, CO). This kit consists of polymer, polymerization buffer, a plastic molding device containing 2 opposite Porex pads (Porex, Fairburn, GA), and plastic spacers and permits preparation of resorbable membranes of 0.8 mm in thickness. A stock solution of rhFGF-2 (Chemicon, Temecula, CA) in phosphate buffer solution (PBS) was prepared, and either 2 or 5 μg of rhFGF-2 in a final volume of 5 μL were applied to about 35 μL of the liquid polymer solution on a 5 × 5-mm area (limited by the plastic spacers) of the hydrated Porex pads of the Atrisorb molding device. After closure, the device polymerized the liquid polymer solution into a uniform layer that was 0.8 mm thick. After 4 minutes, the polymerized membrane was removed, and limited excess was trimmed so that the membrane was 5 × 5 × 0.8 mm. Control membranes were prepared the same way, except that vehicle (5 μL PBS without FGF-2) was applied to the polymer mixture.

Release kinetics of rhFGF-2 in vitro was then determined. Four membranes containing either 2 or 5 μg of rhFGF-2 were prepared. After polymerization, the membranes were individually incubated with constant shaking at 32 rpm in 3 mL of PBS at 37°C for 14 days in closed borosilicate glass tubes. The solutions were collected and changed on days 1, 2, 3, 5, 7, and 14. Aliquots of the collected solutions were diluted 1:15 and assayed in triplicate with a competitive enzyme-linked immunosorbent assay (ELISA) system (Millipore/Chemicon, Billerica, MA); the valid range of this assay for FGF-2 is 0.5 ng/mL to 500 ng/mL. Data were expressed as total FGF-2 released ± SD.

Diabetic and nondiabetic control mice were then prepared. Sixteen diabetic and sixteen control animals were used. All procedures involving mice were approved by Boston University Institutional Animal Care and Use Committee and performed as described in a previous publication.[33] Diabetes was experimentally induced in 16 male 8-week-old test animals via 5 daily low-dose intraperitoneal (IP) injections of streptozotocin using a dose of 40 mg/kg body weight.[43] The first day of injection was designated experimental day zero. Normal controls received IP injections of the solvent alone (10 mmol/L sodium citrate, pH 4.0).[44] The diabetic condition was confirmed. Glucose levels were determined in the whole blood or serum twice weekly (Accu-Check Advantage; Roche Diagnostics, Indianapolis, Indiana, and Glucose Trinder; Sigma Diagnostics, St. Louis, MO). Glucose and ketone (acetoacetic acid) levels were evaluated in the urine (Bayer Multisix 10SG reagent strips for urinalysis; Bayer Diagnostics, Tarrytown, NY). Glycated hemoglobin was quantified in whole blood at sacrifice (Glyc-Affin Isolab, Akron, OH).

Surgical procedures were performed 7 days following confirmation of onset of diabetes, on experimental day 19. Animals were anesthetized by IP injection of 0.12 mL/100 g body weight of 62.5 mg/mL ketamine hydrochloride (Ketalar; Parke Davis, Ann Arbor, MI) and 6.25 mg/mL of xylazine (Rompum; Mobay, Pittsburgh, PA), in 0.5 mol/L sodium chloride (NaCl). The animals' heads were shaved and scalps were washed with 1% iodine. Circular craniotomy defects 1.6 mm in diameter were prepared in the parietal bones with cylindric carbide burs at slow speed under constant saline irrigation. The membrane, loaded with 5 μg of rhFGF-2, was applied with the surface containing the growth factor facing the bone defect (n = 5). In a second group of animals, the defects were treated with membranes without rhFGF-2 (n = 5), and a third group of animals did not receive membranes (n = 6). Identical treatment regimens were used for both the control nondiabetic and diabetic animals; thus, there were a total of 6 different groups of animals. Group sizes were determined based on power analyses, assuming normal distributions and with unequal variances[45] and the knowledge that bone healing is inhibited by 50% in diabetic animals in this model.[33] The flaps were sutured with interrupted resorbable 4-0 expanded polytetrafluoroethylene (ePTFE) sutures. After the surgical procedures, the animals were given IP analgesic injections of buprenorphine (0.3mg/mL Temgesic; Reckitt & Collman, Hull, United Kingdom) at a dose of 3 mg/kg twice a day. All the animals were sacrificed 14 days after the surgical procedure in a

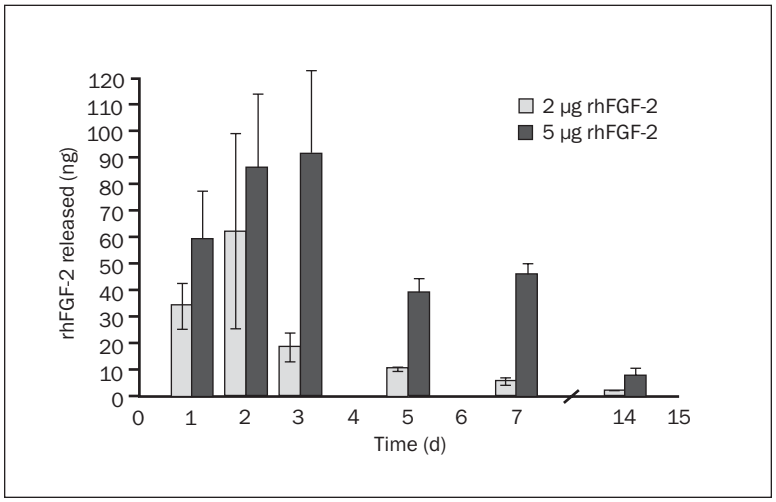

Fig 1 In vitro release kinetics of rhFGF-2 from Atrisorb membranes. Atrisorb membranes (n = 4) (size, 25 mm^2) containing 2 µg or 5 µg of FGF-2 were prepared. After polymerization, the membranes were incubated under constant shaking at 32 rpm in 3 mL of PBS at 37°C for 14 days. The solution was collected and changed at intervals (days 1, 2, 3, 5, 7, and 14). Aliquots of the collected solutions were assayed in triplicate with a competitive ELISA system. White bars represent the membranes loaded with 2 µg of rhFGF-2 and the black bars represent the membranes loaded with 5 µg of rhFGF-2. Data are means ± SDs.

carbon dioxide chamber, and the heads were processed for histology. The lesion size (1.6 mm diameter) was selected based on the authors' previous studies showing partial bone healing of defects in a 2-week healing period in normal animals,[33] as required by the experimental design.

Tissues were then prepared for histology and histomorphometric analysis. Block biopsies were fixed in 10% buffered formalin at 4°C and decalcified in EDTA for 5 to 7 days, dehydrated, and embedded in paraffin. Serial sections (4 µm) were stained with hematoxylin and eosin (H&E) or Masson trichrome, and the 3 most central sections of each defect were analyzed. Linear measurements were performed with an image analysis system (ImagePro 3.1; Media Cybernetics, Silver Spring, MD). Bone ingrowth from the border of the each initial defect toward the center was measured, and bone bridging was expressed as a percentage of the total defect width.[46] The results of the histomorphometric measurements for bone bridging were analyzed with 1-way analysis of variance (ANOVA). Post-hoc analyses were carried out with the Wilcoxon sign rank test. Post-hoc statistical testing was performed using the Bonferroni method. Alpha values of 95% or higher were considered statistically significant.

RESULTS

rhFGF-2 Release Kinetics In Vitro

The release kinetics of FGF-2 from a resorbable membrane in vitro was first determined. Two doses of rhFGF-2 (2 and 5 µg) were loaded into membranes, and the release of the growth factor was measured by ELISA. The results demonstrate that rhFGF-2 was released for the entire experimental period in a dose-dependent manner (Fig 1). Membranes containing 2 µg of rhFGF-2 released peak amounts of the growth factor at 48 hours and then released growth factor at lower levels until day 14, when virtually undetectable amounts were liberated. In contrast, membranes loaded with 5 µg of rhFGF-2 released continuously higher amounts of the growth factor until 72 hours, when the released amounts peaked. A lower constant release rate was then maintained until day 7, and lower levels of released growth factor were still being observed by day 14. Thus, membranes loaded with 5 µg of rhFGF-2 exhibited clear sustained release of the growth factor throughout the 14-day experimental period, whereas the lower-dose membranes released low amounts of FGF-2 after day 3 (Fig 1). The devices made with 5 µg of rhFGF-2 were, therefore, selected for the in vivo studies.

Table 1 Biochemical and Biometric Measurements of Diabetic (n = 16) and Nondiabetic (n = 16) Animals

Parameter	Normal	Diabetic
Glucose (mg/dL)	116.2 ± 18.1	383.6 ± 48.2*
Glycated hemoglobin (%)	5.3 ± 0.4	10.25 ± 0.9*
Food (g/d/animal)	6.6 ± 3.6	10.25 ± 2.4*
Weight (g)	29.3 ± 0.4	26.12 ± 0.9*

Data shown for glucose and glycated hemoglobin levels were obtained at sacrifice from whole blood preparations. Average food consumption and animal body weight were derived from twice weekly measurements of food consumption and body weight. Data presented are means ± SD (*$P < .05$; unpaired t test or Mann-Whitney test).

Experimental Diabetes

Animals injected with streptozotocin became diabetic by experimental day 12, with blood glucose levels of more than 250 mg/dl, whereas control animals had no more than 120 mg/dl (Table 1). Diabetic animals exhibited glucosuria at levels similar to blood glucose levels at all time points. Glycated hemoglobin levels were higher than in nondiabetic animals, indicating consistent and intransient hyper-

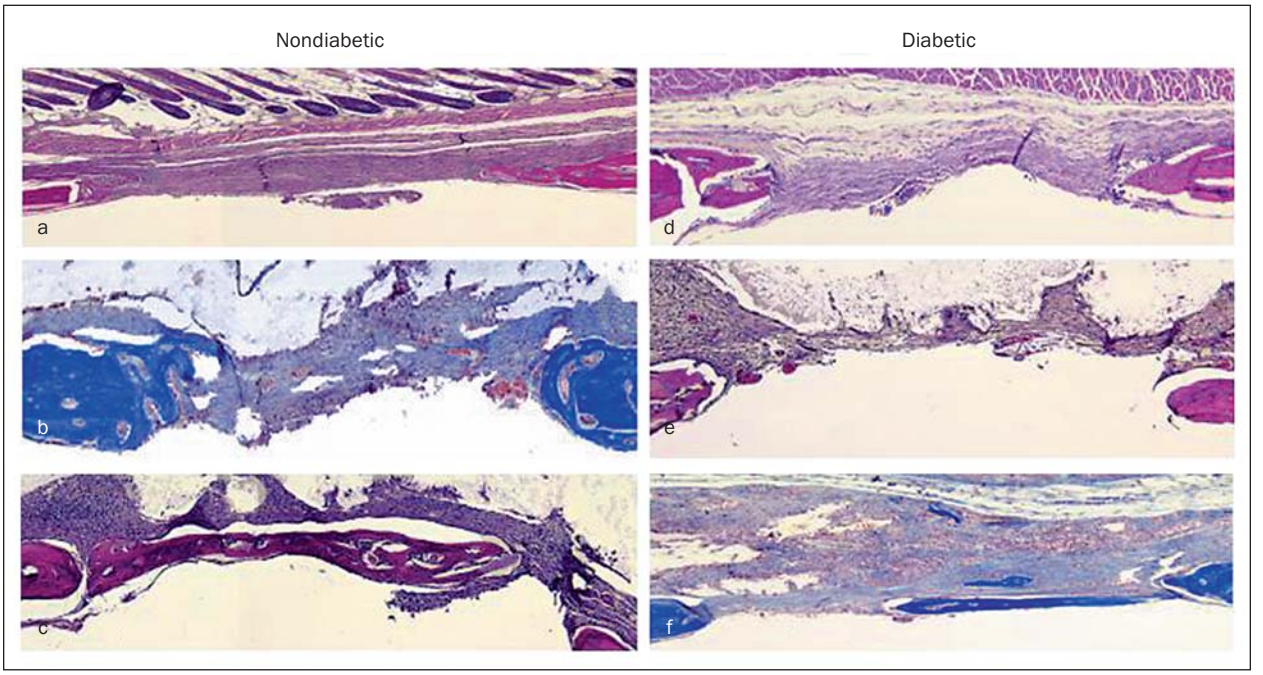

Fig 2 Histologic evaluation of the effects of rhFGF-2 application. Samples from standardized bone defects were harvested after 2 weeks of healing. All defects were 1.6 mm wide. Representative slides from decalcified specimens were stained with H&E (a,c,d,e) or alternatively with Masson trichrome (b,f). *(a)* Nondiabetic animal, no membrane; *(b)* nondiabetic animal, membrane + PBS; *(c)* nondiabetic animal, membrane + rhFGF-2; *(d)* diabetic animal, 1.6-mm bone defect, no membrane; *(e)* diabetic animal, 1.6-mm bone defect, membrane + PBS; *(f)* diabetic animal, 1.6-mm bone defect, membrane + rhFGF-2 (Original magnification 100×).

glycemia in the diabetic animals (Table 1). Ketones and protein were not detected in the urine of any animals. Diabetic animals were hyperphagic and slightly lighter, as expected for this model of diabetes (Table 1). No unexpected metabolic dysregulation occurred in diabetic animals.

Effects of FGF- 2 on Bone Regeneration

Evaluation of histologic sections made from lesions treated with the membranes containing no rhFGF-2 revealed the presence of wovenlike bone within the lesion, with limited extensions toward the center of the defects (Fig 2). Lesions that received no membranes had slightly more bone ingrowth compared to lesions treated with vehicle-loaded control membranes (Fig 2). Marrow cavities were observed, and osteocytes were seen embedded in the bone matrix. Osteoblastic activity was seen in the growing osteogenic fronts, in both the periosteal and dural surfaces. Most of the bone defect was occupied by connective tissue.

Lesions treated with rhFGF-2–loaded membranes revealed more abundant bone formation than control lesions that did not receive rhFGF-2, both in nondiabetic and diabetic animals (Fig 2). Intense osteoblastic activity was seen in the growing osteogenic fronts in both the periosteal and dural surfaces. Islands of new bone formation could be seen within the connective tissue inside the bone defects. Projections of new bone were present within the lesion extending across the defects in rhFGF-2–treated lesions. The newly formed bone exhibited osteocytes embedded in the bone matrix. A limited amount of connective tissue was seen in the bone defect.

Linear histomorphometric evaluations demonstrated that bone healing was inhibited by diabetes by 40% (Fig 3). Bone area was decreased in diabetic animals as compared with normal controls for all 3 treatment modalities tested (Fig 3). Treatment of the defects with resorbable membranes without rhFGF-2 inhibited bone healing in both diabetic and nondiabetic animals ($P < .05$). Addition of 5 μg of rhFGF-2 to the resorbable membrane significantly enhanced the osteogenic potential of these membranes, since bone area was enhanced 2.6-fold compared with defects treated with membranes not containing rhFGF-2 ($P < .05$). Such enhancement was observed both in normal and diabetic animals. Treatment of bone defects from diabetic animals with membranes containing rhFGF-2 exhibited bridging similar to untreated controls.

DISCUSSION

Undisturbed bone formation and regeneration are fundamental aspects of implant dentistry. Successful osseointegration of metallic implants in native or regenerated bone is intrinsically dependent on normal bone formation. Consistent with previous data,[33] the present study demonstrates that bone healing is significantly reduced by diabetes. Bone area was decreased in diabetic animals compared with nondiabetic controls in each treatment modality tested. Since osseointegration is dependent on de novo bone formation and regeneration,[47] such inhibition of bone formation may have a significant negative impact on osseointegration of implants. Quantitative measurements have consistently shown that implants placed in diabetic animals demonstrate significantly less osseointegration than normal animals.[35,48–53] These differences have been documented for both pure titanium[35,48,50,52,53] and HA-coated implants.[49,51] Reduced areas of formed bone, reduced surface of contact between bone and implant, reduced bone contact thickness, and reduced bone calcification have been observed in these studies. Interestingly, despite positive effects of systemic insulin treatment on total bone formation in diabetic animals, alterations in bone-to-implant contact ratios were not corrected.[35] FGFs play important roles in morphogenesis and wound healing[36] and are potent stimulators of osteoblastic proliferation in vitro.[37] FGF-2 regulates extracellular matrix production by osteoblastic cells in vitro.[38–40] FGFs systemically administered in vivo have been shown to increase endosteal bone formation,[41,42] stimulate the osteoinductive effects of demineralized allogenic femoral diaphysis implanted into intramuscular sites,[54] and increase the volume and mineral content of the fracture callus. They have also improved the mechanical strength of the fractured bone when injected into fractured limbs of diabetic rats.[55]

These studies suggest a positive role of exogenous application of FGF-2 in bone wound healing and regeneration. Optimization of these effects, however, may be obtained through targeted delivery with controlled release. Several approaches have been employed for targeted delivery of FGF to wounded sites, including local injection,[41,42] incorporation into composite bone grafts,[54] incorporation into mini-pellets,[56,57] incorporation into a gelatin sheet,[58] and the use of mini-osmotic pumps.[59] Optimized results appear to be dependent on site of application, delivery mode, dose, and release kinetics of FGF.

The present study shows that a commercially available absorbable polymer loaded with rhFGF-2 acts as a suitable material for the incorporation and delivery of rhFGF-2. The amounts of rhFGF-2 released from membranes loaded with 5 μg rhFGF-2 were within the range of previously reported optimal osteogenic doses of FGF-2 in vivo.[60] Surprisingly, control vehicle-loaded membranes diminished bone healing compared to calvarial defects without membranes. Collapse of control membranes into the bone defect may have limited the space available for bone regeneration and may have created a physical barrier against the proliferative osteogenic front prior to membrane resorption. The lack of space-maintenance capacity seems likely to have contributed to the limited bone regeneration observed in the defects treated with membranes without rhFGF-2 incorporated.[61] It seems possible that an absorbable carrier with greater space maintenance capacity might further enhance the osteogenic activity of rhFGF-2.

The amount of bone regenerated with the use of membranes loaded with rhFGF-2 was markedly greater than that regenerated using control vehicle–loaded membranes. Data revealed that the addition of rhFGF-2 to the absorbable membrane resulted in up to 3-fold enhancement of bone area, which is a clear indication of the bioactivity of the growth factor released from the membranes in vivo. The authors speculate that the peak release of rhFGF-2 during the first 48 to 72 hours of healing may have stimulated granulation tissue formation and angiogenesis,[62,63] which, in turn, may have had a significant impact on bone regeneration.[61] The addition of 5 μg of rhFGF-2 to the absorbable membrane

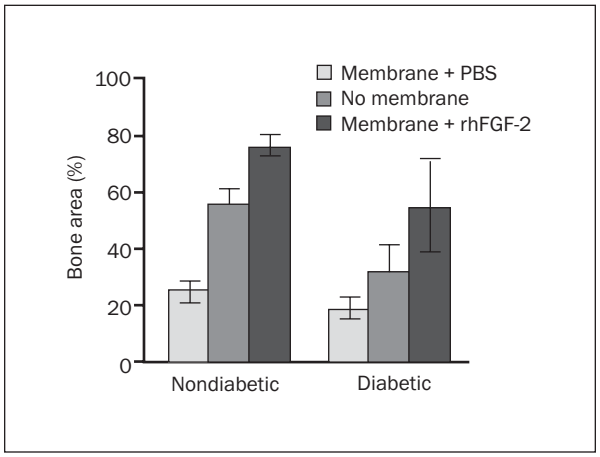

Fig 3 Histomorphometric analyses of bone healing in calvarial defects. Bone area was expressed as a percentage of the total defect width and was measured in 3 slides for each bone defect from each animal. Two defects were made in each animal. The readings were averaged to obtain means for each bone defect, and both defects were averaged to obtain the mean for every animal, which was used as the unit for statistical analyses. Results were presented as means ± standard error.

significantly enhanced the osteogenic potential of these membranes, and such enhancement was observed both in normal and diabetic animals. Therefore, controlled, local application of rhFGF-2 successfully stimulated bone healing and regeneration of bone defects.

The findings of the present study may have important clinical implications. Uncontrolled diabetes results in significantly reduced bone formation, which is manifested as inhibited osseointegration due to reduced area and calcification of formed bone as well as a reduced bone-implant surface contact.[35,48–53] Alterations in bone-to-implant contact are not corrected by metabolic control of diabetes by means of insulin treatment.[35] Therefore, implant placement in diabetic patients should be carefully planned and may carry additional risks in areas of poor bone quality and areas subjected to high occlusal loads. Increased healing times are suggested in order to compensate for the reported inhibited bone formation rates in diabetics. Interestingly, locally applied FGF-2 has been shown to significantly improve bone formation around endosseous titanium implants,[64,65] and it has been suggested that continuous application of FGF-2 may facilitate osseointegration in situations where the bone bed is suboptimal and residual particles and granulomatous tissue are present.[59] Thus, targeted controlled local delivery of biologically-active substances such as growth factors may stimulate bone regeneration and rescue the inhibitory effects of diabetes on bone healing. Biologically-enhanced membranes may offer significant clinical benefits by enhancing the endogenous healing capacity of bone defects and increasing the rate and total amounts of bone formation, and ultimately resulting in significantly improved bone regeneration and endosseous implant osseointegration, especially in locally or systemically inhibited healing sites, such as those observed in diabetes mellitus.

CONCLUSION

In summary, the present study demonstrates that rhFGF-2 released from an absorbable membrane significantly stimulated bone regeneration in vivo and rescued impaired bone healing in diabetic animals to levels similar to those in normal untreated animals. Further studies are needed to show whether these properties of growth factor–enhanced resorbable barriers could have potential clinical use as a biologically enhanced procedure for stimulation of bone regeneration in nondiabetic as well as diabetic patients.

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