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Research Article

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Determination of the critical diabetes duration in a streptozotocin-induced diabetic rat calvarial defect model for experimentation regarding bone regeneration

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ABSTRACT

Purpose: The purpose of this study was to determine the critical diabetes duration in a streptozotocin (STZ)-induced diabetic rat calvarial defect model for experimentation regarding bone regeneration by evaluating the association between diabetes duration and bone healing capacity through histological and radiographic analyses.
Methods: Experimental diabetes was induced in 50 of 60 rats by an STZ injection. The rats were divided into 5 groups, including a control group (group 1), according to diabetes durations of 0, 2, 4, 6, and 8 weeks, respectively. Eighteen rats survived: 4 in group 1, 4 in group 2, 4 in group 3, 5 in group 4, and 1 in group 5. Calvarial defects were created at 0, 2, 4, 6, and 8 weeks after STZ injection in groups 1–5. Cone-beam computed tomography scanning was performed at baseline and at 5 and 7 weeks after surgery. The rats were sacrificed 7 weeks after surgery, followed by histological evaluation.
Results: The voxel gray values (VGVs) of group 1 and group 2 increased, whereas the VGVs of group 3 and group 4 decreased starting 5 weeks after surgery, although this trend did not reach statistical significance between groups. On the reconstructed 3-dimensional images and based on an analysis of histological features, groups 1 and 2 showed apparent bone regeneration, while groups 3–5 showed very limited bone regeneration.
Conclusions: The critical diabetes duration in an STZ-induced diabetic rat calvarial defect model for experimentation regarding bone regeneration was between 2 and 4 weeks. It is suggested that researchers who use STZ-induced diabetic rats wait for more than 2 weeks following diabetes induction before placing implants or conducting bone regeneration studies to allow definite disturbances in bone healing to emerge.

Keywords: Bone regeneration; Cone-beam computed tomography; Diabetes mellitus; Streptozocin

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

INTRODUCTION

Several previous studies have established that titanium dental implants can be healed by direct bone-to-implant contact (BIC) or osseointegration [1,2]. However, a careful consideration of the systemic condition of the patients, including metabolic or pharmacological interactions, is required for treatment planning to ensure successful implant therapy with well-maintained osseointegration [3]. Once osseointegration is successfully established, any disturbance of the biological process or metabolic alteration may negatively affect implant treatment outcomes. As a significant systemic condition involving metabolic changes, diabetes mellitus (DM) is known to increase the risk of dental implant failure and to interfere with proper osseointegration [4].

DM, a metabolic disease characterized by increased blood glucose levels, occurs when the pancreas cannot produce sufficient insulin or when the produced insulin is not effectively utilized. It has been reported that the prevalence of periodontitis in diabetic patients is 60%, whereas it ranges from 20% to 50% in the general population [5,6]. The number of people with diabetes worldwide doubled from 153 million in 1980 to 347 million in 2008 [7]. It was reported that approximately 13.7% of a Korean population aged 30 years or older had diabetes in 2014, and nearly 25% of Korean adults had prediabetes [8]. Individuals with diabetes have a high frequency of periodontitis or tooth loss [9]. In addition to periodontal problems, these patients also have delayed wound healing [10], an impaired response to infection [11], and a high prevalence of cardiovascular diseases and stroke [12,13].

The fact that the increased blood glucose levels in diabetic patients can influence the process of bone modeling and remodeling has been well supported by several studies [14,15]. Schwartz and Sellmeyer [16] reported that diabetic patients showed a higher incidence of hip fracture. Persistent hyperglycemia in diabetic patients can result in disorders of the vascular system, which are divided into a microvascular and macrovascular complications. The former category consists of retinopathy, nephropathy, neuropathy, and periodontal disease, and the latter category consists of ischemic heart disease, peripheral vascular disease, and cardiovascular disease. Many studies have established that poorly controlled diabetes can be a substantial risk factor for implant failure or complications because of those adverse biological responses. Siqueira et al. [17] and de Morais et al. [18] reported that hyperglycemic animals simulating untreated type 1 diabetes showed decreased levels of implant osseointegration. Hasegawa et al. [19] reported that type 2 diabetes also showed the same consequences.

To overcome the numerous adverse outcomes of bone regeneration procedures and implant therapy in diabetic patients, numerous studies have been conducted to elucidate the precise mechanism responsible for impaired bone healing, and rats have been commonly utilized as experimental diabetic models. Streptozotocin (STZ) is one of the materials most commonly used to induce type 1 diabetes in rodent models, and it has been reported that a single dose of STZ is effective for diabetes induction in rats [20]. Despite that finding, relatively little is known regarding the time when bone healing disturbances occur following experimental diabetes induction. Takeshita et al. [21,22] conducted an experiment to evaluate bone formation around titanium implants in diabetic rats, and they performed implant placement immediately after the confirmation of diabetes without consideration of the actual diabetes duration. Nevins et al. [23] also studied the influence of diabetes on the process of implant osseointegration, and they waited for 2 weeks following STZ injection until implant placement. Similarly, Giglio et al. [24] waited 12 days after the STZ injection to conduct

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experiments in diabetic rats. However, the bone healing disturbances analyzed in these studies may not be manifested until a certain period of time has elapsed following diabetes induction. Therefore, the use of rats with unstandardized diabetes duration may create inconsistencies in experimental results, and it is therefore quite important to determine the diabetes duration when bone healing disturbances clearly appear. The purpose of this study was to determine the critical diabetes duration in a STZ-induced diabetic rat calvarial defect model for experimentation regarding bone regeneration by evaluating the association between diabetes duration and bone healing capacity through histological and radiographic analyses using cone-beam computed tomography (CBCT).

MATERIALS AND METHODS

Sixty 8-week-old Sprague-Dawley rats weighing between 300 and 350 g were used in this study. After acclimatization to the experimental conditions for 1 week, rats were divided into 5 groups (group 1 as the controls, groups 2–5 as the diabetic groups). According to the diabetes duration, rats that survived until the end of the experimental period were divided as follows: group 1 (control group; n=4), group 2 (2-week-DM group; n=4), group 3 (4-week-DM group; n=4), group 4 (6-week-DM group; n=5), and group 5 (8-week-DM group; n=1). The protocol for the animal experiments was approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of Gangneung-Wonju National University (Approval No. GWNU-2014-26).

STZ-induced diabetic rats

Type 1 diabetes was induced in 50 of the 60 animals by an intravenous injection of 50 mg/kg of STZ (Sigma-Aldrich, St. Louis, MO, USA) through the tail vein under anesthesia by an intramuscular injection of a 4:1 mixture of Zoletil (Virbac Laboratories, Carros, France) and Rompun (Bayer Korea, Seoul, Korea). DM was confirmed by the measurement of a blood glucose concentration greater than 300 mg/dL (16.7 mmol/L) with a glucometer (Roche, Basel, Switzerland). This day was considered day 0 of diabetes. Daily monitoring of blood glucose levels was performed to verify diabetes during the first half of the experimental period, and weekly monitoring was done during the latter half. Rats that failed to develop diabetes were excluded from the study.

Animals in the control group were injected with citrate buffer only. During the experiment, the animals were individually housed in cages at room temperature (23°C) with controlled humidity (60%), fed *ad libitum* with a standard rodent diet, and exposed to a natural light/dark cycle.

Surgical procedure

Non-critically sized bone defects were artificially created in the calvarial bone 2, 4, 6, and 8 weeks after the STZ injection in groups 1 through 4. Table 1 shows the experimental protocol. After shaving the calvarial skin and swabbing it with a mixture of iodine, the calvarial skin and periosteum were lifted along the sagittal incision. One transosseous circular defect (outer diameter, 5 mm) was made in a one-sided parietal bone area with a stainless-steel trephine bur under copious irrigation with sterile saline. The defect size of 5 mm was selected because it was smaller than the critical size for rat calvarial defects, as reported by Cooper et al. [25] and Schmitz and Hollinger [26]. Bilayered suturing using chromic gut 5-0 (Ethicon, Somerville, NJ, USA) and black silk 4-0 (Ailee, Busan, Korea) sutures was performed to close

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Table 1. Experimental protocol

	0 wk	2 wk	4 wk	5 wk	6 wk	7 wk	8 wk	9 wk	11 wk	13 wk	15 wk
Group 1 (n=4)	DC CBCT			CBCT		CBCT HA					
Group 2 (n=4)	STZ	DC CBCT				CBCT		CBCT HA			
Group 3 (n=4)	STZ		DC CBCT					CBCT	CBCT HA		
Group 4 (n=5)	STZ				DC CBCT				CBCT	CBCT HA	
Group 5 (n=1)	STZ						DC CBCT			CBCT	CBCT HA

DC: defect creation, CBCT: cone-beam computed tomography, HA: histological analysis, STZ: streptozotocin

the surgical site. All animals received an intramuscular injection of cefazolin (30 mg/kg) for 2 days to prevent postsurgical infection.

CBCT scanning and voxel gray value (VGV) measurement

All calvarial defects in the experimental animals were scanned 3 times under isoflurane anesthesia using CBCT (Alphard Vega 3030, Asahi, Kyoto, Japan): immediately after surgery, 5 weeks later, and 7 weeks later. The exposure settings for the scans were as follows: voxel size resolution of 100 μm, tube current of 5 mA, tube voltage of 80 kV, and a scan time of 17.0 seconds. The field of view was 51×51 mm. The obtained data were exported into the Digital Imaging and Communication in Medicine (DICOM) format for analysis, and an experienced examiner segmented all CBCT scans. The VGV of a region of interest (ROI) set by a circular area 6 mm in diameter containing a previously created complete defect 5 mm in diameter was measured using a DICOM viewer (OnDemand3D, Cybermed, Seoul, Korea) (Figure 1A). Considering the calvarial bone thickness, the mean VGV was calculated from the uppermost, middle, and lowermost reference planes (Figure 1B). Using 3-dimensional (3D) reconstructed images enabled the extent of newly formed bone to be assessed.

Histological analysis

Seven weeks after surgery, all animals were sacrificed for histological analyses. The surgical field on the parietal bone was resected and fixed in 10% neutral formalin for 2 weeks and decalcified with hydrochloride solution (KC-X, FALMA, Tokyo, Japan) for 5 days, dehydrated

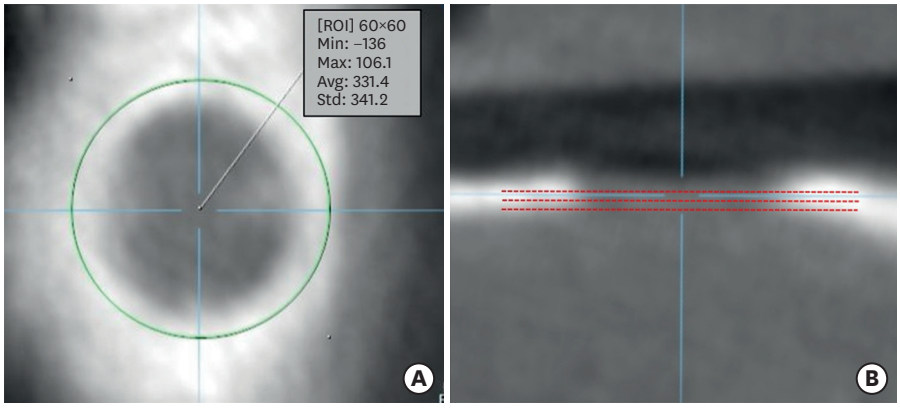

Figure 1. (A) Axial view of CBCT showing the VGV of the ROI. (B) Sagittal view of CBCT indicating 3 different reference planes (red lines) for measurements.
CBCT: cone-beam computed tomography, VGV: voxel gray value, ROI: region of interest, Min: minimum, Max: maximum, Avg: average, Std: standard deviation.

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through a graded ethanol series, cleared in xylene, and embedded in paraffin. Sections with a 5-μm thickness were obtained from each block and stained with Masson trichrome (Trichrome Stain Kit, Abcam, Cambridge, UK). The trichrome staining procedure was as follows. First, a slide was placed in preheated Bouin's fluid for 60 minutes, followed by a 10-minute cooling period. After rinsing, it was stained with Weigert's iron hematoxylin for 5 minutes, followed by the application of Biebrich scarlet/acid fuchsin solution. Then, it was differentiated in phosphomolybdic/phosphotungstic acid solution, and an aniline blue and acetic acid solution was applied (1%). Finally, it was dehydrated very quickly through graded ethanol and cleared in xylene. Histological evaluations were performed by observing the stained sections under a microscope (Axio Imager 2, Carl Zeiss, Göttingen, Germany). Images were captured using a digital camera (EOS 100D, Canon, Tokyo, Japan) attached to the microscope.

Statistical analysis

Statistical analyses were performed using commercially available software (SPSS version 23, IBM Corporation, Armonk, NY, USA; Microsoft Excel, Microsoft Corporation, Redmond, WA, USA). Repeated-measures analysis of variance was carried out to evaluate differences in VGV among the groups, and the Friedman test was also conducted. The Wilcoxon signed-rank test was also performed for the pairwise comparison of time points within each group. Data are presented as the mean±standard deviation (SD). *P* values less than 0.05 were considered to indicate statistical significance.

RESULTS

STZ-induced diabetic rats

Among the 60 experimental rats, 39 rats died, either from complications of diabetes or the surgical trauma of bone defect creation. Three rats failed to show elevated blood glucose levels. A total of 18 rats were ultimately included in this experimental study. The control group (group 1), the 2-week-DM group (group 2), and the 4-week-DM group (group 3) consisted of 4 rats each, and the 6-week-DM group (group 4) consisted of 5 rats. Unfortunately, only 1 rat in the 8-week-DM group (group 5) survived until the end of the experiment, and this group was therefore not included in the intergroup comparison. The blood glucose concentration increased over time after STZ injection, and it reached greater than 300 mg/dL a week after injection (Figure 2). The diabetic state of all diabetic rats was well maintained throughout the experimental period.

CBCT scanning and VGV measurements

Table 2 shows the VGVs of the groups. Group 5 (the 8-week-DM group) was excluded from statistical analysis due to the small sample size (n=1) that resulted from a high mortality rate, and no further experiments to ensure an adequate sample size were conducted, since bone healing disturbance was apparent even in the groups with a shorter DM duration. Changes in VGVs were significantly related to the healing time, and the VGVs were significantly different between the baseline and 5 weeks after surgery in all groups. However, no significant difference was found between the VGVs at 5 and 7 weeks after surgery. In groups 1 and 2, the VGVs increased continuously over the whole experimental period, indicating ongoing bone healing. However, there was a tendency for bone healing to be disturbed, with decreased VGVs from 5 to 7 weeks after surgery in groups 3 and 4, although the differences were not statistically significant.

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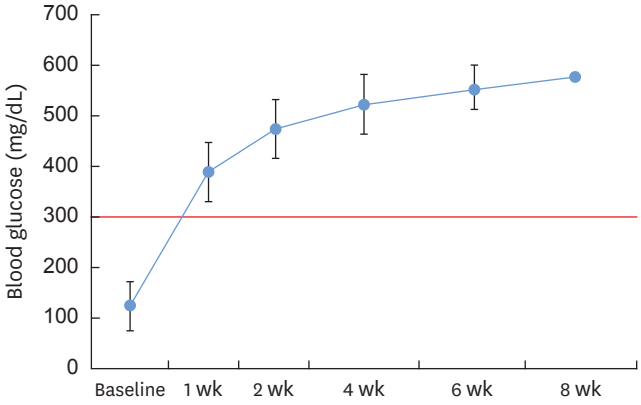

Figure 2. Changes in blood glucose levels after STZ injection in the surviving experimental rats. Diabetes induction was confirmed by the measurement of a blood glucose concentration greater than 300 mg/dL (red line). The diabetic state of all diabetic rats was well maintained throughout the experimental period.
STZ: streptozotocin.

Table 2. VGVs obtained from CBCT

Group	Baseline	5 weeks later	7 weeks later	*P* value
Group 1 (n=4)	65.6±122.0[a)]	286.9±38.1[b)]	356.1±101.9[b)]	0.018
Group 2 (n=4)	−61.7±148.7[a)]	285.9±108.6[b)]	356.5±95.2[b)]	0.018
Group 3 (n=4)	−25.1±86.3[a)]	295.4±135.9[b)]	267.8±144.5[b)]	0.018
Group 4 (n=5)	63.8±106.8[a)]	246.9±74.7[b)]	215.8±26.2[b)]	0.018

Data are shown as mean±standard deviation.
VGV: voxel gray value, CBCT: cone-beam computed tomography.
[a,b)]The same superscript letters indicate values that are not significantly different. (P>0.05).

Reconstructed 3D images

On the reconstructed 3D images based on CBCT scan data, groups 1 and 2 showed apparent bone regeneration interconnecting the bone defect margin, whereas groups 3–5 showed a reduced bone healing capacity, limited to the defect margin area (Figure 3).

Histological evaluations

Histological sections showing the whole defect area were obtained (Figure 4; Masson trichrome staining, ×200 original magnification). Group 1 showed notable bone regeneration filling most of the defect area (Figure 4A). There was also an apparent bone regeneration pattern into the central part of the defect in group 2 (Figure 4B). However, groups 3–5 showed very limited bone regeneration (Figure 4C-E). In groups 3–5, most areas of the defect were stained blue (collagen) by Masson trichrome, suggesting disturbed bone healing [27].

DISCUSSION

In most previous studies investigating the influence of diabetes on bone regeneration or implant therapy utilizing STZ-induced diabetic rats, substantial inconsistency or uncertainty was present regarding the diabetes duration, or the exact period from the start of diabetes induction to certain surgical procedures, such as defect creation or implant placement [18,23,24]. Even if the experimental rats were defined as having diabetes based on the monitoring of blood glucose concentration, these studies have mainly assessed bone healing disturbances in rats with a short diabetes duration. Therefore, the main purpose of this study was to determine the critical diabetes duration needed to show consequent bone healing disturbances through histological and radiographic evaluations and to suggest a standardized diabetic rat model with an optimal

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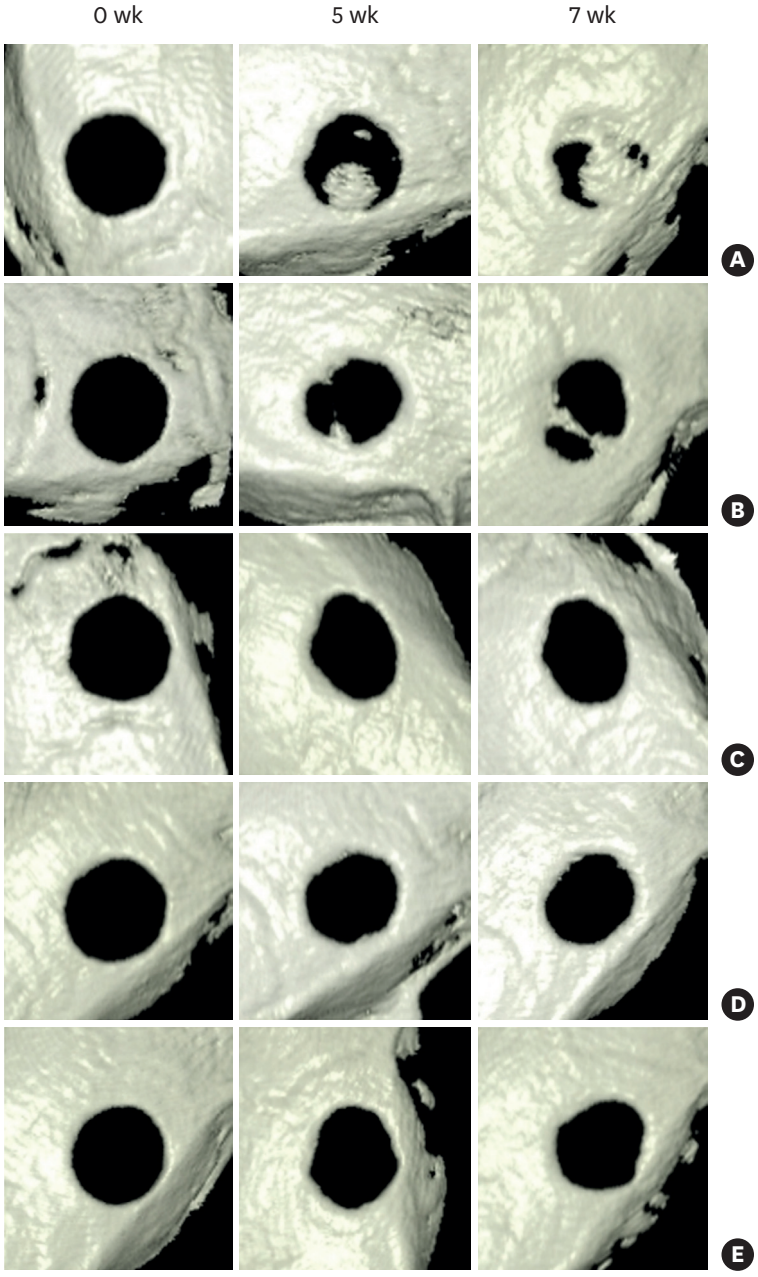

Figure 3. Reconstructed 3D images based on CBCT. Group 1 (A) and group 2 (B) showed apparent bone regeneration interconnecting the bone defect margin, while group 3 (C), group 4 (D), and group 5 (E) showed reduced bone healing capacity, limited to the defect margin area.
3D: 3-dimensional, CBCT: cone-beam computed tomography, W: week.

diabetes duration for experimentation regarding bone regeneration or other surgical therapies related to bone tissue. In the present study, rats were divided into a non-diabetic control group and 4 diabetic groups, according to 4 different diabetes durations at 2-week intervals.

Type 1 diabetic animal models can be induced by compounds such as alloxan, STZ, or Vacor, and it is therefore possible to compare the results of many previous experiments. STZ is a nitrosourea compound derived from *Streptomyces achromogenes* that is particularly toxic to the insulin-producing beta cells of the pancreas in mammals [28]. In rat models, a single dose

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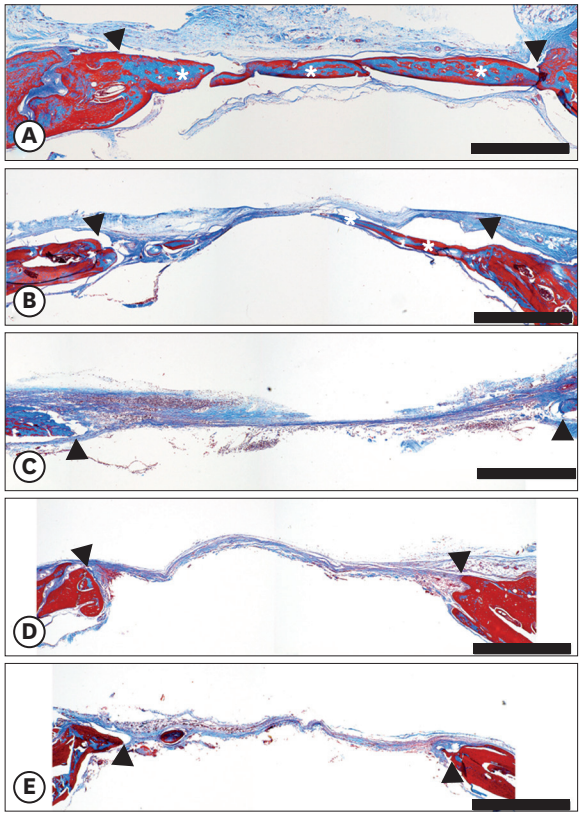

Figure 4. Histological features of defect healing 7 weeks after defect creation (Masson trichrome staining, scale bar=1 mm). Group 1 (A) showed notable bone regeneration filling most of the defect area. There was also an apparent bone regeneration pattern in the central part of the defect in group 2 (B). Group 3 (C), group 4 (D), and group 5 (E) showed very limited bone regeneration. White asterisks indicate newly formed bone. Black arrowheads indicate defect margin.

of STZ is predictably effective for inducing and maintaining type 1 diabetes. This response is known to be similar to the responses in humans during the onset of type 1 diabetes [28]. STZ-induced diabetic rats lose somatomedin activity in the serum because of an advanced glycation end products (AGEs). Somatomedin, which is composed of insulin-like peptides with growth-promoting effects on bone and cartilage, is considered to mediate skeletal growth. AGEs are believed to be one of the mechanisms for the pathogenesis of diabetes, as they cause undesirable alterations in extracellular matrix components [29]. In this study, we used STZ (50 mg/kg) to induce type 1 diabetes in rats [30]. DM induction was confirmed by the measurement of a blood glucose concentration greater than 300 mg/dL (16.7 mmol/L) [31]. Most of the experimental rats successfully reached that level within a week, and their diabetic state was well maintained throughout the experimental period.

It is well known that bone density can be evaluated using Hounsfield units (HUs) derived from multislice computed tomography (MSCT) that are directly related to tissue attenuation coefficients [32]. The HU scale has also been applied to evaluate the bone density of implant sites because it produces site-specific, objective, and quantitative results [33,34]. Parsa et al. [35] demonstrated a strong correlation between VGVs from CBCT and HU from MSCT. Cassetta et al. [36] also reported that these 2 values had a statistically significant linear correlation, so that the lower radiation dose and reduced costs of CBCT could be a useful substitute for MSCT as a practical tool for measuring bone density. In the present study, the

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VGVs of a ROI using CBCT was measured at baseline, 5 weeks after surgery, and 7 weeks after surgery. All ROIs were set with a circular area 6 mm in diameter, containing the entirety of a previously created circular defect measuring 5 mm in diameter and an additional marginal bone area surrounding the defect to include all the areas where active bone modeling and remodeling were in progress. Considering the calvarial bone thickness, the mean VGV was calculated using the uppermost, middle, and lowermost reference planes. The differences of VGVs among groups did not reach statistical significance, although the VGVs of group 3 and group 4 decreased starting 5 weeks after surgery, unlike in groups 1 or 2.

There are several possible reasons why no significant difference was found among the groups despite the definite tendency for bone healing disturbances in groups with a longer diabetes duration. First, it may be difficult to quantify the precise amount of newly formed bone with various densities of VGVs because the bone has a large range of attenuation coefficients. The values of bone in HUs ranges from 400 to 1,000 HU. In addition, an ROI was set to include the host bone of the defect margin, which might also cause errors because each animal had a variety of bone densities. Second, errors could have been made during the surgical operation. Although the surgical procedure was conducted by the same experienced surgeon, damage to the dura mater or the influence of bone heating produced by using the trephine bur could affect the consequent bone healing process. The third possibility is measurement error. Even though 3 sets of repeated measurements were made, each reference plane and ROI position might differ. Finally, it may be possible that the sample size was too small to reach statistical significance.

In the present study, the degree of hyperglycemia increased over time and with diabetes duration. It was reported in a clinical study that poorer glycemic control resulted in both an increased risk for alveolar bone loss and more severe progression than in individuals without DM or those with better to intermediately controlled DM [37]. Likewise, it can be concluded that the degree of hyperglycemia affected the bone healing capacity differentially in this study based on the fact that group 2, with the lowest blood glucose level among all the diabetic rats, had a much better bone healing capacity than the other diabetic groups, which had higher blood glucose levels. Hajna et al. [38] studied diabetic complications according to 4 different levels of glycemic control in STZ-induced diabetic rats. They reported that rats with medium-scale hyperglycemia corresponding to blood glucose levels between 22 and 25 mmol/L (between 396 and 450 mg/dL) showed significant hyperglycemia-dependent complications such as retinopathy, nephropathy, and neuropathy. In the present study, the mean blood glucose concentrations at 2, 4, and 6 weeks following STZ injection were 487.8±60.1, 544.4±55.7, and 578.0±38.7 mg/dL, respectively. When compared to the study of Hajna et al. [38], bone healing disturbances did not appear until the blood glucose concentration reached a slightly higher level. This difference could be because they studied long-term diabetic complication using rats with a 4-month diabetes duration, and only examined features corresponding to retinopathy, nephropathy, and neuropathy, unlike this study.

When the extent of new bone healing was further evaluated by 3D reconstructed images, groups 1 and 2 showed apparent bone regeneration interconnecting the bone defect margin, whereas groups 3–5 showed bone regeneration limited to areas near the defect margin. The critical size of defects in rats is known to be 8 mm [26]. Even though the outer diameter of the defect created in this study was 5 mm, complete bone healing did not occur in any group, including group 1, at 7 weeks after surgery. An explanation for this was suggested by Cooper et al. [25], in a study in which small (2.3 mm in diameter) defects in the rat calvaria showed approximately 35% healing after 6 weeks.

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In terms of histological features, groups 1 and 2 showed a pattern of apparent bone regeneration, whereas the other groups showed very limited bone regeneration. In groups 2–4, most areas of the defect were stained blue by Masson trichrome stain, indicating collagenous tissue without bone tissue, which suggested a bone healing disturbance.

Based on the radiographic and histologic features documented in this study, the bone healing capacity of all diabetic rats was more limited than that of the control group. The 4-week-DM group and groups with longer diabetes durations were more likely to show apparent bone healing disturbances. Previously, Shyng et al. [39] assessed the extent of bone healing disturbance caused by diabetes depending on the defect created in rat calvaria. The evaluation was performed approximately 3 weeks after STZ injection, and altered bone healing was demonstrated at that time, which is generally consistent with the results of the present study. Kwon et al. [40] evaluated BIC histologically 1, 2, 3, and 4 months after diabetes induction in uncontrolled and insulin-controlled rats following the establishment of osseointegration. The histometric analysis indicated that both groups showed significantly different BIC ratios in the 2-, 3-, and 4-month duration groups, which is somewhat slower than the result of the present study. The difference can be explained by the fact that Kwon et al. [40] observed delayed bone healing in titanium plasma-sprayed implants, while we observed the spontaneous healing pattern of non-critical-sized defects.

Despite several limitations, the results of this study suggest that a diabetes duration of more than 2 weeks is needed for bone healing disturbances to appear following experimental diabetes induction in rats. However, more accurate image analysis than is possible using VGV based on CBCT is necessary to facilitate the quantification of new bone formation. In addition, studies of cellular or molecular factors, such as osteoclasts, osteoblasts, and biological markers, should be conducted to elucidate the mechanism of bone healing disturbance, in addition to tissue-level studies analyzing bone or collagen. It is also important that extensive efforts are made to reduce the mortality rate in animals, and the most suitable experimental period and sample size should be further considered for future studies.

Based on the results of this study, rats with diabetes durations of 4 weeks or longer had a tendency to manifest bone healing disturbances in non-critically-sized rat calvarial defects. However, non-diabetic rats and rats with a diabetes duration of 2 weeks showed apparent bone regeneration.

Altogether, the critical diabetes duration associated with bone healing disturbances in an STZ-induced diabetic rat calvarial defect model for experimentation regarding bone regeneration is between 2 and 4 weeks. Therefore, it is suggested that researchers who study the effect of diabetes on bone healing disturbances using STZ-induced diabetic rats wait for more than 2 weeks following diabetes induction before placing the implants or conducting bone regeneration studies to allow definite bone healing disturbances to emerge.

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