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Original

Osteogenic Evaluation of DNA/Protamine Complex Paste in Rat Cranial Defects

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Abstract: A DNA/protamine complex powder, prepared from the reaction between DNA and protamine sulfate solutions, was mixed with water to make a paste. The ALP activity of MC 3T3 E1 cultured on a thin film of the complex paste was higher than that cultured on a plastic well. It was found that DNA/protamine complex induced new bone formation from the results of micro-computed tomography (μ-CT) images and conventional histological sections with hematoxylin and eosin staining in rat cranial defect tests. μ-CT image analyses showed that newly formed bone areas in defects for DNA/protamine complex were significantly higher than those for sham operation (controls) two and three months after surgery. Although the area of red-appearing osteoids reduced with increasing times after surgery, stimulated new bone formation areas were observed around newly formed bone in histological sections stained with Villanuva osteochrome bone stain. Therefore, DNA/protamine complex paste with a biodegradable property will be a useful injectable biomaterial for the repair of bone defects.

Key words: DNA/protamine complex paste, Micro-computed tomography, Osteogenic evaluation, Rat cranial defect

Introduction

Biodegradable polymers are more desirable than non-biodegradable polymers for procedures that require the temporary presence of biomaterials in the human body because they obviate the need for a second surgical procedure[1)]. Some natural and synthetic biodegradable polymers have become commercially available as biomaterials[1,2)]. Moreover, other biodegradable polymers have been recommended as candidates for biomaterials[3)].

Nair and Laurencin[3)] reported that the major advantage of natural polymers compared to synthetic polymers is their excellent biocompatibility. Thus, we have also developed new biodegradable biomaterials composed of natural polymers, such as salmon serum deoxyribonucleic acid (DNA), chitosan and protamine, as a scaffold for tissue engineering or drug delivery systems (DDS)[4-7)]. In particular, we have focused our interest on DNA because DNA has same favorable characteristic properties for biodegradable biomaterials. For example, DNA can specifically interact with antibiotics such as daunomycin or proteins such as protamine[8-10)]. DNA is less antigenic than other macromolecules such as proteins or polysaccharides[11)]. In particular, we expect that DNA will be an interesting candidate for novel bone substitution material because it has many phosphate groups. There are some reports indicating that phosphate in organic compounds such as methacryloyloxyethyl phosphate[12)] and DNA[13)] can induce bone formation. For example, it has been reported that a polymer modified by surface graft polymerization of a phosphate-containing monomer promoted the deposition of hydroxyapatite in in vitro[12)] and in vivo[14)] tests. Beck et al.[15)] described an in vitro study of MC3T3-E1 cells in which phosphate cleaved from β-glycerol phosphate by alkaline phosphates could induce osteopontin gene expression. Beucken et al.[13)] reported an in vitro study of titanium multilayered with poly-D-lysine and DNA or poly (allyamine hydrochloride) and DNA in which pre-treatment with DNA-based simulated body fluid affected rat bone marrow-derived osteoblast-like cell differentiation, evidenced by increased deposition of osteocalcin.

However, its water solubility is a disadvantage for application to biomaterials because DNA spreads rapidly from the implantation site in the body. In previous studies, we described that water-insoluble DNA/polycation complexes were obtained from the reaction of DNA with polycation materials such as chitosan[4,5)], polyamino acids[16)] and protamine[6,7)]. Of the complexes, DNA/protamine complex powder prepared from DNA with a mean 300 base-pairs and protamine became a paste by kneading with water and that the paste had suitable viscosity for clinical use[7)]. Moreover, this paste showed good cell viability, mild soft tissue responses, or

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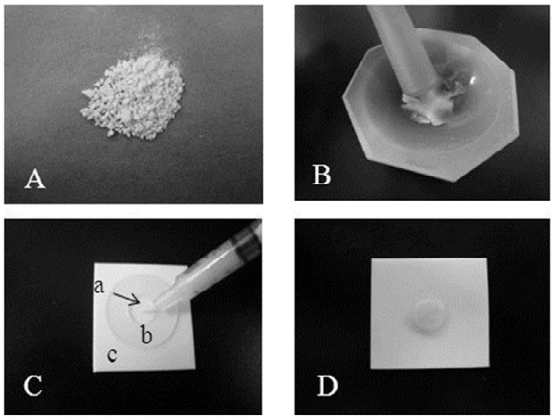

Figure 1. Preparation paste of DNA/protamine complex and preparation disk of DNA/protamine complex paste. A: DNA/protamine complex powder; B: DNA/protamine complex paste; C: Injection of paste (a) into silicon mold (b) on Teflon plate (c); D: Fabrication disk.

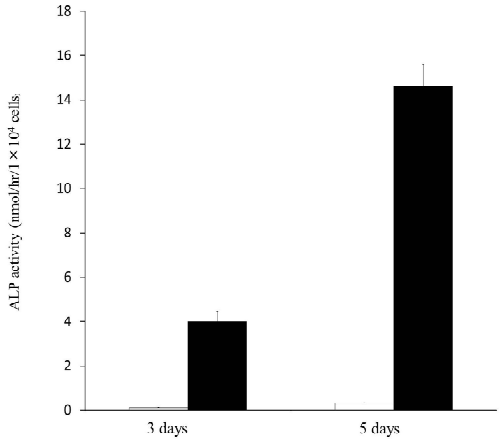

Figure 2. Alkaline phosphatase (ALP) activity of MC 3T3 E1 after culture for 3 and 5 days (black bars) on DNA/protamine complex paste (black bars) and plastic dish (control: white bars).

antibacterial effects against Gram-positive bacteria[7]. Further, Zhou et al.[17] reported that protamine alone can induce bone sialoprotein transcription. Thus, we expect that DNA/protamine complex paste can induce osteogenesis in bone defects.

The purpose of this study was to prepare DNA/protamine complex paste and to investigate its effect on new bone formation in rat cranial defects by micro-computed tomography (μ-CT) and histopathological examination.

Materials and Methods

Preparation of DNA/protamine complex

Sterilized salmon testis DNA provided by Maruha-Nichiro (Maruha-Nichiro Holdings, Ltd., Tokyo, Japan), which was cleaved by nuclease into 300-bp fragments, and 2 % sterilized salmon testis protamine sulfate (mol. wt. 4500) solution (Maruha-Nichiro Holdings, Ltd., Tokyo, Japan) were used in the present study.

DNA (500 mg) was dissolved in 100 ml distilled water. The distilled water was added to 2 % sterilized salmon testis protamine sulfate solution to prepare 0.5 % protamine sulfate solution. DNA in 100 ml distilled water was added to protamine sulfate solution (100 ml) and the mixture was stirred at 20°C for 1 h. The DNA/protamine complex was collected by centrifugation at 9000 rpm for 10 min and washed with distilled water. This process was repeated two times, after which the DNA/protamine complex was frozen in liquid nitrogen and then dried for 24 h in an FD-5N freeze-dryer (Eyela, Tokyo, Japan). All procedures were carried out under sterile conditions and with sterilized instruments and materials.

Preparation of DNA/protamine complex paste and paste disks

The freeze-dried DNA/protamine complex powder is shown in Figure 1A. The complex powder (1.5 g) and 1.2 ml distilled water were kneaded in a mortar with a pestle and then any extra water was absorbed with paper. Kneading followed by absorption was repeated two more times. The DNA/protamine complex paste (Fig. 1B) was use to fill a syringe with a nozzle of 1-mm internal diameter (Fig. 1C).

For disk preparation of DNA/protamine complex paste, the DNA/protamine complex paste was injected into a silicone mold (8-mm internal diameter and 0.8-mm height) on a Teflon plate (Fig. 1C). The top surface of the complex was covered with a Teflon plate to flatten it and then the covered Teflon plate was removed. Fabricated complex disks (40 mg) were immediately and carefully removed from the Teflon plate and silicone mold (Fig. 1D).

All procedures were carried out under sterile conditions and with sterilized instruments and materials.

Alkaline phosphatase assay

Teflon plate was placed on the surface of DNA/protamine paste on a glass disk (14φ mm × 1 mm) and then the Teflon plate was pressed with a finger to make a DNA/protamine thin film on glass disk for an alkaline phosphatase assay. The glass disk with paste was placed in a well of a 24-well multiplate.

MC-3T3-E1 mouse osteoblast-like cells (Riken BioResource Center, Riken Tsukuba Institute, Tsukuba, Ibaragi, Japan) were cultured in α minimum essential medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen Corp.). A 1-ml suspension of cells (1 x 104 cells/ml) was added to each well of the 24-well multiplate and incubated for 3 and 5 days at 37°C in a humidified 5% CO2 atmosphere. The medium contained 50 μg/ml ascorbic acid and 10mM β-glycerophosphate.

After the 3- and 5-day incubation, the medium was removed and cells were washed with Dulbecco's phosphate-buffered saline (PBS). One milliliter of medium containing 0.1 ml Cell Counting Kit-8 Aqueous (Dojindo Laboratories, Masuki, Kumamoto, Japan) was added to each well, and the plate was incubated for 2 h at 7 °C in a humidified atmosphere containing 5 % CO2. Optical density

was measured using a plate reader (Multiskan JX; Labsystems Oy, Helsinki, Finland) at 450 nm as the detection wavelength and at 650 nm as the reference wavelength. Cell numbers were counted from the regression line between cells and absorbance was measured at 450 nm as the detection wavelength and at 650 nm as the reference wavelength.

The activity of intracellular alkaline phosphatase (ALP) was measured with a commercial phosphatase substrate kit (AnaSpec, Inc., Fremont, CA). After counting the cell number, the medium was removed and cells were washed with PBS. One millilter of medium was added to each well, and the plate was incubated for 5 h. After incubation, 200 μl Triton X-100 was added to each well and then cells were removed with a cellscraper. Subsequently, aliquots of 50μl cell lysates were obtained by centrifugation and mixed with an equal amount of substrate solution (p-nitrophenyl phosphate). Mixtures were then incubated for 30 min at 37 °C in a 96-well multiplate. The reaction was stopped by the addition of 50 μl stop solution to each well and the resulting optical densities were measured on a plate reader at 405 nm as the detection wavelength.

All experiments were performed in quintuplicate. ALP acidity was analyzed statistically using two-way analysis of variance (ANOVA) and Scheffe's multiple comparison tests to determine statistical differences in cell viability among the samples at the 5% level of significance.

Implantation of DNA/protamine complex paste disk

Animal experiments were performed in accordance with the ethics guidelines for animal experiments at Fukuoka Dental College (No. 10017). Ten-week-old male Sprague-Dawley rats weighing approximately 300 g were used in the present study. Surgery was performed under general anesthesia induced by 2 % isoflurane (Abbott Laboratories, Abbott Park, IL, USA) and an air mixture gas flow of 1.0 L/min using an anesthesia gas machine (Anesthesia machine SF-B01; MR Technology, Inc., Tsukuba, Ibaraki, Japan). An incision was made in the cranial of the rats, and the exposed periosteum was semicircularly incised and carefully separated from cranial bone. The 8-mm calvarial defects were created in the central parietal bone with a trephine drill with meticulous care to avoid damaging the underlying dura mater. The defects were treated with an 8-mm diameter paste disk. Defects with sham treatment were used as controls. After the insertion of samples, periosteum and scalp tissues were each closed in separate layers by suturing with intracutaneously resorbable Vicryl 3-0 (Ethicon Inc., Somerville, NJ, USA). The rats (18) were divided into two groups (experimental group and control group) for different observation times (1, 2 and 3 months). Three rats were used for each group in each period.

Micro-computed tomography analyses

Image analyses of new bone formation were performed using an *in vivo* μ-CT system (Skyscan-1176; Bruker microCT, Belgium) at 50 kVp and 500 μA. The thickness of one μ-CT slice is 35μm. The analyses were performed under general anesthesia induced by 2% isoflurane (Abbott Laboratories) and air mixture gas flow to 1.0 l/min using an anesthesia gas machine (Anesthesia machine SF-B01; MR Technology, Inc.).

The percentage of newly bone formed bone in the defect (New-Bone %) was calculate as the area of newly formed bone/area of the defect originally created by trephination. Firstly, the newly formed bone area on μ-CT slice images in the horizontal direction was quantified two-dimensionally using WinROOF image analysis software (MITANI Corp., Tokyo, Japan). The analyzed cycle area with 8 mm φ was drawn on each μ-CT slice image. Each cycle area was closely adjacent to the location where the defect was created while additionally referring to μ-CT slice images with coronal and sagittal sections and histological images of decalcified sample with HE staining. Sequence ten μ-CT slice images showing the highest newly formed bone areas were used for one sample analysis. The percentage of newly bone formed bone in the defect (New-Bone %) was calculated as the total area of newly formed bone per 10 μ-CT slice images/total area of the defect per 10 μ-CT slice images × 100.

All experiments were performed in quintuplicate. New-Bone % was analyzed statistically using two-way analysis of variance (ANOVA) and Scheffe's multiple comparison tests to determine statistical differences in cell viability among the samples at the 5% level of significance.

Histological evaluation

In each period, the animals were sacrificed by injection with an overdose of isoflurane. After sacrificing the animals, the cranial tissues containing the implanted samples were immediately excised. The same sample groups were divided into two groups. One group was used as a decalcified sample stained with hematoxylin and eosin (HE). Another group was used as a non-decalcified sample stained with Villanueva osteochrome bone staining (VOB).

For HE staining, samples were fixed in 4 % (w/v) paraformaldehyde in phosphate buffer (pH 7.4), decalcified with 0.24 M EDTA•4Na•4H2O solution, dehydrated with graded alcohols, cleared in xylene, and embedded in paraffin by routine procedures. The specimens were sectioned at 3 μm. The sections were stained with HE for histological observation using a Nikon Eclipse 55i light microscope (Nikon, Tokyo, Japan).

For VOB staining, samples were immersed in VOB solution for 3 days and dehydrated with graded alcohols, defatted in acetone and embedded in methyl methacrylate. The specimens were sectioned at 20 μm. The sections were histologically observed with a Nikon Eclipse Ti-u fluorescence microscope (Nikon).

Results

ALP assay

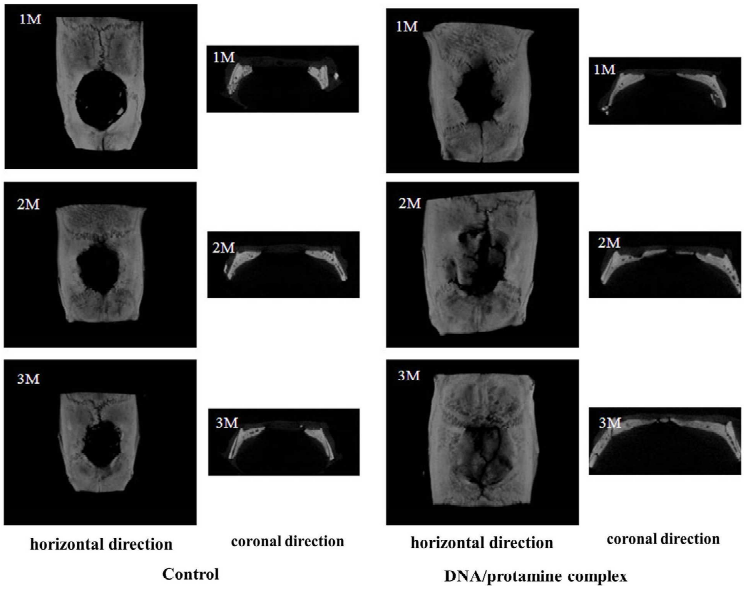

Figure 3. Representative μCT images of parietal defects after one month (1M), two months (2M) and three months (3M).

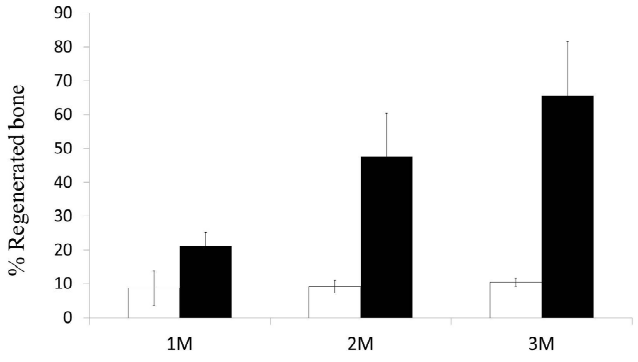

Figure 4. Quantification of percent are bone regeneration in calvarial defects after one month (1M), two months (2M) and three months(3M). Control: white bars; DNA/protamine complex paste: black bars.

The results of the ALP activity measurements are shown in Fig. 2. Two-way ANOVA, followed by Scheffe's multiple comparison tests showed significant differences in ALP activity between DNA/protamine complex and control ($p < 0.05$), and significant differences in ALP activity between incubation periods ($p < 0.05$). A two-way interaction was not found for the sample presence or incubation period ($p < 0.05$). All ALP activities significantly increased with time for DNA/protamine complex and the control ($p < 0.05$). DNA/protamine complex showed significantly higher ALP activity than the control on 3- and 5-day incubations ($p < 0.05$).

Micro-computed tomography analyses

The μ-CT images and the new bone formation (New-Bone %) obtained from the μ-CT images are shown in Figs. 3 and 4, respectively. As the control, the μ-CT images in horizontal and coronal directions showed a little new bone formation in defect areas. In contrast, for DNA/protamine complex paste, newly formed bone was observed in defect areas and the areas of newly formed bone increased with increasing recovery periods. Newly formed bone was generated in most of the defect areas 3 months after implantation. Two-way ANOVA, followed by Scheffe's multiple comparison tests, showed significant differences in the total areas of newly formed bone between DNA/protamine complex and the control ($p < 0.05$), and significant differences in the total area of newly formed bone between recovery periods ($p < 0.05$). A two-way interaction was found for sample presence or recovery period ($p < 0.05$). The area of newly formed bone significantly increased with recovery periods for DNA/protamine complex and each newly formed bone area at one month, two months and three months after implantation is 21.2 %, 47.6 % and 65.5 %, respectively. For the control, all areas of newly formed bone were very similar and were approximately 10 %. DNA/protamine complex showed significantly higher areas of newly formed bone than the control two and three months after implantation ($p < 0.05$).

Histological evaluation

Figs. 5 and 6 show representative histopathological images of calvarial tissues in defects with sham treatment (Control) and DNP/protamine complex paste treatment.

As shown in Fig. 5, for the control, new bone was formed as approximately 1 mm length inward from cleavage sites, regardless of the elapsed time. The most central region in defects was occupied by connective tissues. Moreover, as shown in Fig. 5 (3M-e), existing bone (b) showed a lamellar structure while newly formed bone (n) showed a disordered structure.

For DNA/protamine complex paste, new bone formation

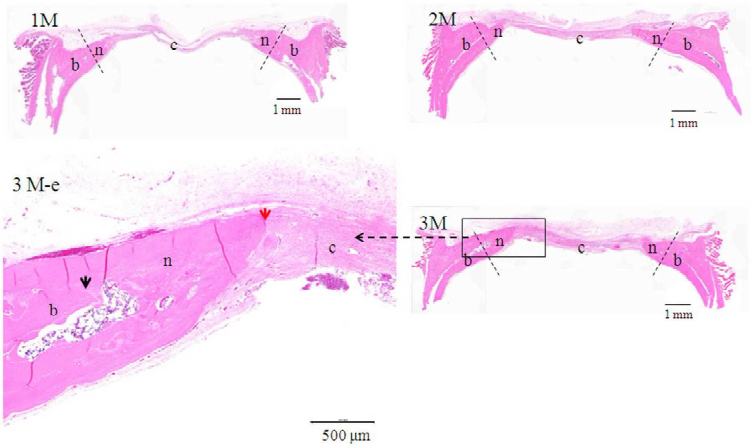

Figure 5. Histology of calvarial tissue in defects treated with sham operating (control) 1 month (1 M), two months (2 M) and three months (3 M and 3 M-e) after sham operation (rod lines: cleavage site; b: existing bone; n: new bone; c: connective tissue; black arrow with a solid line: end of existing bone; red arrow: end of new bone) .

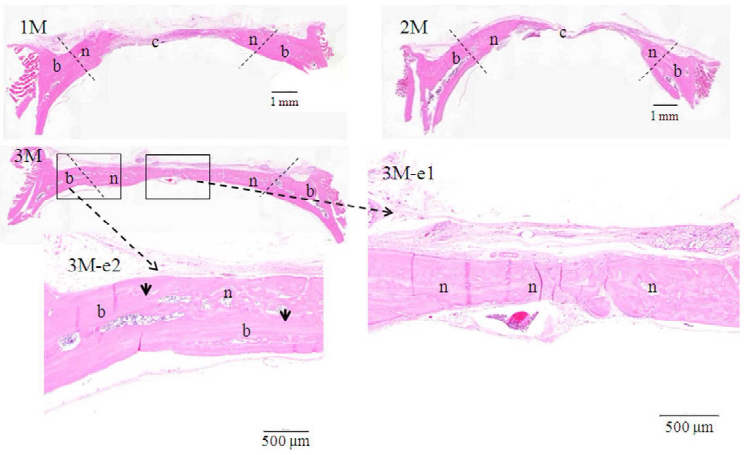

Figure 6. Histology of calvarial tissue in defects treated with DNA/protamine complex paste 1 month (1 M), two months (2 M) and three months (3 M, 3 M-e1 and 3 M-e2) after implantation (rod lines: cleavage site;b: existing bone; n: new bone; c: connective tissue; black arrows with a solid line: the end of existing bone).

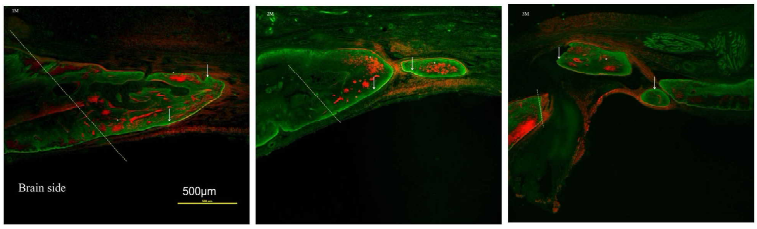

Figure 7. Villanueva osteochrome bone-stained sections of calvarial defects treated with DNA/protamine complex under fluorescent microscope, 1 month (1 M), two months (2 M) and three months (3 M) after implantation (White arrows: stimulated calvarial new bone formation; Asterisks: osteoid).

succeeded with the elapsed time from cleavage sites to the median calvarial suture, as shown in Figure 6. Most regions of defects were occupied by newly formed bone 3 months after implantation. It seemed that the growth of existing bone on the brain side was faster than that on epithelial tissue side because bone with a lamellar structure on the brain side was longer, as shown in Figure 6 (3M-e2).

Fig. 7 shows typical Villanueva osteochrome bone-stained sections of calvarial defects treated with DNA/protamine complex under a fluorescent microscope. Many red-appearing osteoids were observed in newly formed bone and stimulated new bone formation areas were observed around newly formed bone, regardless of the elapsed time.

Discussion

DNA/protamine complex, which was obtained by reaction between 300 bp DNA and protamine solutions[6], has an advantage for use as biomaterial in that it becomes a paste by mixing with water[7]. Paste can be easily injected into defects of bone or soft tissues with any given shape and can be easily mold-fabricated[7]. Moreover, DNA contains a lot of phosphate that can induce osteogenesis[13-15] and protamine alone can up-regulate bone

sialoprotein transcription[17]. Thus, we expect that DNA/protamine complex paste alone will be applied as an injectable bone graft material if it can induce osteogenesis.

Alkaline phosphatase (ALP) is a membrane-bound enzyme that is often used as a marker of osteogenic differentiation[13,14]. It is well known that ALP is one of the first players in the process of osteogenesis[18]. Thus, we first investigated the effect of DNA/protamine complex on ALP activity in an osteoblast-like cell line MC3T3-E1. For the DNA/protamine complex group, ALP activity increased with the incubation time. Statistically significant differences were observed compared to controls (Fig. 4). The result suggested that the complex is capable of inducing osteogenesis.

Next, we evaluated whether DNA/protamine complex can facilitate new bone formation in a rat cranial defect model examination. The quantity and quality of bone that forms in an osseous defect is influenced by the type of animal, age of the animal, stability of defects, size of defect, etc.[19]. In the present study, a calvarial defect with 8 mm φ was created for the animal examination because this bone defect model has been used as a critical size in rat calvarial bone defects[20,21]. Bone formation in the calvarial defect model occurs under unloaded conditions.

Cowan et al.[22] described that recent advances in imaging techniques have been used to assess and characterize fine bony structures such as trabecular bone architecture *in vitro* and *in vivo* and that μ-CT data can accurately substitute for conventional histological sections for bone structure evaluations. Thus, in the present study, μ–CT image analysis was used for new bone formation evaluations.

As shown in Fig. 3, as a control, the contours of defects were clearly observed from μ–CT image analysis although defect sizes 2 and 3 months postoperatively became a little smaller compared one month postoperatively. This result was very similar to that reported by Cowan et al.[22] although the critical size was different from their critical size of 5 mm φ. In contrast, for DNA/protamine complex paste, the formation of new bone was clearly observed and the formation areas increased with time after implantation (Fig. 3). As shown in Fig. 4, treatment with DNA/protamine complex paste showed a significantly higher bone regeneration areas than the sham operation. Messora et al.[20] reported that newly formed bone areas in a calvarial defect of 8 mm φ were approximately 6 % and 12% one month and 3 months after the sham operation, respectively. Their results were very similar to the results obtained from the present study. It is believed that DNA/protamine complex has the ability to induce new bone formation from the results of μ-CT imaging and bone quantification.

Next, we explored new bone regeneration using conventional histological sections with HE and Villanueva osteochrome bone staining to support the results obtained from μCT image analysis. As shown in Figs. 4 and 5, histological sections with HE staining showed more clearly the external and internal surfaces of the original calvarium at the right and left margins of the surgical defect compared to μ-CT images because a difference in structure between existing bone with a lamellar structure and new bone with an amorphous structure was observed. For all controls, most of the defect was replaced with connective tissues (Fig. 5). For the sham operation, these results were very similar to the results reported by Messora et al.[20].

Fukushima et al.[7] reported that DNA/protamine complex paste gradually degraded after implantation into the soft tissue of rats, and deep purple complex fragments were observed 3 days after implantation, and had completely disappeared from the tissues within 10 days after implantation. As shown in Figure 6, deep purple complex fragments were not observed in calvarial bone defects. We suspected that DNA/protamine complex paste completely disappeared at an early stage after implantation. The relationship between degradation speed and bone formation is unclear. Mori et al.[23] reported that the degradation speed of DNA/protamine complex with high molecular weight DNA was slow in the soft tissue of rats compared to 300-bp DNA/protamine complex. We will investigate the effect of degradation speed on new bone formation using DNA/protamine complexes with different molecular weight DNA. As shown in Fig. 7, it seems that the area of red-appearing osteoids[24] reduced with increasing time after surgery. However, stimulated new bone formation areas were observed around newly formed bone[25]. Thus, we consider that new bone forms continuously until the defect has been completely replaced with newly formed bone.

In the present study, we found that DNA/protamine complex can induce new bone formation. However, the detailed mechanism of bone newly formed by DNA/protamine complex is unclear. We expect that DNA/protamine complex can induce the differentiation of mesenchymal-type cells into bone cells such as osteoprogenitor cells or induce osteoblast differentiation in calvarial bone defects. To evaluate our hypothesis, we will examine the expression of genes such as alkaline phosphatase (ALP), osteopontin (OCN) and the production of bone proteins such as bone morphogenetic proteins (BMPs) and transforming growth factor beta (TGF-ß) using biological analyses such as real-time reverse transcription–polymerase chain reaction (RT-PCR) and Western blotting analysis.

In conclusion, DNA/protamine complex increased ALP activity of MC 3T3 E1 in an *in vitro* test. It was found that DNA/protamine complex induced new bone formation from the results of μ-CT images and conventional histological sections with HE staining in an *in vivo* test. DNA/protamine complex showed higher newly formed bone areas than controls two and three months after surgery. Although the area of red-appearing osteoids reduced with increasing time after surgery, stimulated new bone formation areas were observed around newly formed bone in histological sections with VOB staining. Therefore, DNA/protamine complex paste with biodegradable properties will be a useful injectable biomaterial for

the repair of bone defects.

Acknowledgements

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