Effects of DNA/protamine and DNA/gelatin Paste on Bone Formation at Tooth Extraction Wound Sites

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Abstract: DNA/protamine complex has been reported to enhance bone formation in animal experiments. Another notable property of the complex is that it becomes a paste after being kneaded with water. Conventionally, DNA/protamine paste is prepared by kneading the DNA/protamine complex with water; however, conventional DNA paste has problems with regards to the preparation method, handling, moldability, etc. In the present study, we developed a new method for preparing DNA pastes, called the on-the-spot preparation method. DNA/protamine paste was prepared by mixing DNA into viscous liquid protamine or mixing gelatin into viscous liquid DNA. The viscosity of the DNA/protamine and DNA/gelatin paste was better than that of the conventional DNA/protamine-complex paste. The DNA/protamine and DNA/gelatin pastes were injected into the extracted sockets of rat maxillary molars. Microfocus X-ray computed tomography (micro-CT) and histological observation of nondecalcified sections after 2 weeks of injection revealed that the DNA/protamine and DNA/gelatin paste enhanced the formation of new bone in the extracted sockets. Quantitative analysis for calcification degree and newly formed bone mass showed there was a significant difference between DNA/protamine-paste- and DNA/gelatin-paste-injected specimens. The bone formation ability of DNA/gelatin paste is better than that of the DNA/protamine paste in the extracted sockets of rat maxillary molars. It is suggested that DNA paste prepared by on-the-spot preparation method will be useful candidate materials for bone regeneration.

Key words: DNA, Protamine, Gelatin, Paste, Tooth Socket.

Introduction

DNA is a unique biomacromolecule that is known as a source of biological information. DNA also has the characteristics of biomaterials. For example, DNA can intercalate and/or bind cytokines such as bone morphogenetic protein (BMP) or antibiotics between base pairs in the grooves of its strands; additionally, DNA is less antigenic than other biomacromolecules. DNA can also be a bone-guiding scaffold, because it contains numerous phosphate groups, which have the ability to bind to calcium compounds such as hydroxyapatite.

Fukushima et al. attempted to utilize DNA as a bone reconstruction material and developed several DNA complexes with polycations such as chitosan, polyamino acids, and protamine. They found that among all the complexes tested, the DNA/protamine complexes were useful for enhancing bone formation after implantation into rat calvarial defect sites. Toda et al. implanted DNA/protamine complex solid disks into the critical size calvarial bone defects of 10-week-old rats and found that these disks nearly healed the bone defects 3 months after their implantation. DNA is also used for coating materials used for making dental titanium implants. It is reported that a multilayer coating of DNA with bis-ureido-surfactants favors early bone responses after implantation into the femoral condyles of rats. Sakurai et al. and Miyamoto et al. reported that multilayered DNA/protamine-coated titanium implants promoted the formation of new bone at the early stage of bone healing after their implantation into the extracted sockets of the maxillary molars of rats.

Another notable property of the DNA/protamine complex is that it becomes a paste after being kneaded with water. The paste form has the following advantages: i) it can be easily injected into defect sites or cavities of any shape and size, and ii) it can be easily molded into the desired shape. Moreover, it is easy for pastes to be mixed with inorganic or organic compounds, such as hydroxyapatite or cytokines, to change their viscosity or facilitate tissue formation. Fukushima et al. evaluated the viscosity of DNA/protamine-complex paste and the histopathological and antibacterial activities of DNA/protamine solid disks that were prepared from the DNA/protamine paste. It was found that these solid disks showed a mild soft tissue response and antibacterial effects against gram-positive bacteria. Shinozaki et al. also reported that solid disks of DNA/protamine complex prepared from conventional DNA/protamine-complex paste induced the formation of new bone after their implantation into rat cranial defect sites. However, there are no reports regarding the bone response towards the DNA/protamine paste itself.

Conventional DNA/protamine-complex paste could be prepared by kneading the complex with water, but an extra amount of water is needed for preparing the paste. Careful and skillful kneading is necessary for preparing the complex paste, and the handleability and moldability of...
the DNA/protamine-complex paste are insufficient for clinical use. Moreover, it is difficult to make DNA/protamine paste with suitable consistency in a small scale of the mg order. It is necessary to overcome these shortcomings for the dental clinical application of DNA pastes. In the present study, we aimed to improve the preparation, handleability, and moldability of DNA pastes. We tried to prepare DNA paste by the on-the-spot method, and not by using the DNA/protamine complex. We first prepared viscous liquid DNA, and then added protamine to form a paste. We also tried to prepare a DNA/gelatin paste. We called this preparation method ‘on-the-spot preparation’. For evaluating the biological response of the DNA paste, we injected the DNA paste into the extracted sockets of rat maxillary molars. Extracted sockets are a suitable model for evaluating the biological response of the injectable DNA paste, and the bone formation behavior of the extracted sockets of the rat maxillary molars was histologically observed.

Materials and Methods

Materials

DNase-cleaved DNA (300 bp, Maruha-Nichiro Corp., Tokyo, Japan) and protamine (protamine sulfate, average molecular weight, approximately 4,500, Maruha-Nichiro Corp., Tokyo, Japan), which were obtained from salmon testes, were used (Fig. 1). RM-Gelatin (RM-100, average molecular weight, approximately 800,000–1,000,000 Isoelectric point = approximately 9, Jellice Co., Ltd. Miyagi, Japan) from pig skin was used.

DNA/protamine and DNA/gelatin paste preparation

The scheme for preparing the DNA/protamine and DNA/gelatin pastes is shown in Figs. 2 and 3. First, to prepare viscous liquid DNA, 50 mg of DNA was taken in a mortar and mixed with 200 µl of double distilled water using a pestle. Then, 50 mg of protamine was added to the viscous liquid DNA, followed by gentle mixing with the pestle, to obtain the DNA/protamine paste (Fig. 3).

It was not possible to prepare DNA/gelatin paste by adding gelatin to viscous liquid DNA. Thus, we changed the order of mixing. First, viscous liquid gelatin was prepared by adding 50 mg of gelatin to 400 ml of double distilled water using a pestle. Then, 50 mg of protamine was added into the viscous liquid DNA, followed by gentle mixing with the pestle, to obtain the DNA/gelatin paste (Fig. 3).

A 1.5-cm tip of the Nozzle for Dentject (Showa Yakuhin Kako Co. Ltd., Tokyo) was cut with scissors and then placed into the CR syringe nozzle (J. Morita Corp., Tokyo, Japan). The obtained paste was filled into a CR syringe nozzle and injected from the top of the Nozzle for Dentject through a gray plug using a root canal plugger (Fig. 3). The diameter of the Nozzle for Dentject is 0.6 mm.

Measuring the consistency of the DNA paste

The consistency of the DNA paste was measured according to the
methods described in previous reports, with a slight modification\(^{19,20}\). DNA/protamine or DNA/gelatin paste was filled into a silicone mold (internal diameter of 5.0 mm and height of 0.8 mm) on a polyethylene terephthalate (PTFE) plate. The top surface of the DNA/protamine or DNA/gelatin paste was covered using a PTFE plate to flatten it, and the covered PTFE plate was then removed. After carefully removing the bottom PTFE plate and silicone mold, DNA paste disks were used to measure the consistency of the pastes. The DNA/protamine or DNA/gelatin paste disk was placed on a glass plate, and another glass plate was then carefully placed on another surface of the DNA paste disk. The DNA paste disk was continuously subjected to a load of 1 MPa at 20°C for 10 min by using a constant load compression testing machine (A-001 Japan Mecc Co. Ltd., Tokyo, Japan). The diameter of the spread disk was then measured using calipers.

For comparison, the diameter of the spread disks of the conventional DNA/protamine-complex paste was also measured. The conventional DNA/protamine-complex paste was prepared from DNA/protamine according to a previous report\(^{19}\).

Injection procedure of the DNA/protamine and DNA/gelatin paste

The Animal Experimental Ethics Committee of the Tsurumi University School of Dental Medicine approved this study (Certificate No. 28A057, 28A071, 30A018). Eighteen male Wistar rats (180 g, 6 weeks old) were housed, two per cage, at 20°C to 25°C under a 12-h circadian light rhythm and fed a powdered diet and tap water ad libitum during the experiment. The rats were divided into three groups, namely, the sham group (control) \((n = 6)\), DNA/protamine paste-injected group \((n = 6)\), and the DNA/gelatin paste-injected group \((n = 6)\). Half of the rats from each group were considered for non-decalcified evaluation, and the other half, for decalcified evaluation.

Surgery was performed under general anesthesia induced using an intraperitoneal injection of ketamine hydrochloride (0.8 mg/kg) and medetomidine hydrochloride (0.4 mg/kg). As shown in Fig. 4, maxillary first molar was extracted using forceps. Afterwards, the DNA/protamine or DNA/gelatin paste was injected into the extracted sockets from the top of the Nozzle for Dentject through a gray plug using a root canal plugger. Incisions into the periodontal tissue were closed using 7-0 polyamide non-absorbable sutures (BioFit-D, WASHIESU, Tokyo, Japan). After surgery, the rats were subcutaneously injected with benzyl penicillin G procaine (3,000,000 U/kg) and awakened with an intraperitoneal injection of atipamezole hydrochloride (0.2 mg/kg).

**Micro-CT observation**

Two weeks after the DNA paste injection, the rats were euthanized using a lethal dose of carbon dioxide. Each tissue specimen was dissected using a diamond saw (Cutting Grinding System, BS-300CP band system, EXAKT, Apparatebau GmbH & Co., KG, Norderstedt, Germany). The specimens were fixed in 10% neutral buffered formalin for 7 days, and bone formation in the extracted sockets of the rat maxillary molars was first observed using a high-resolution microfocus X-ray computed tomography system (micro-CT, inspeXio, Shimadzu, Kyoto, Japan) at a voltage of 115 kV and a current of 70 μA. The slice thickness was 0.053 mm. An isotropic resolution of 32 μm/voxel was selected, which displayed the microstructure of the rat’s calvarial bone. After micro-CT observation, the CT values were converted to brightness values. The degree of calcification, namely new-bone formation, was determined based on the brightness histogram. The region of interest (ROI) for the quantification of the degree of calcification for micro-CT evaluation is a rectangular box \((0.170 \text{ mm } \times 0.170 \text{ mm } \times 0.159 \text{ mm})\) in the distal root of the extracted tooth socket. The CT values were converted to tissue mineral density (TMD, mg/mm\(^3\)) values by using hydroxyapatite phantoms. Quantification of the degree of calcification was determined by the TMD value.

**Histological and histomorphometrical observations**

After micro-CT observation, non-decalcified and decalcified sections were prepared for each group. For the preparation of non-decalcified sections, the specimens were dehydrated using a series of ethanol con-
centrations (70%, 80%, 90%, 96%, and 100%) after the formalin fixation, and then embedded in methyl methacrylate resin. After polymerization, the non-decalcified sections were prepared using a cutting grinding technique (Cutting Grinding system, BS-300CP band system EXAKT; 400CS micro-grinding system; Apparatebau GmbH & Co., KG, Norderstedt, Germany). The thicknesses of the specimens were adjusted to approximately 50–70 µm. The non-decalcified thin sections were stained with methylene blue and basic fuchsin and histologically observed using a light microscope (200×; BX51; OLYMPUS, Tokyo, Japan).

For the preparation of decalcified sections, the specimens were decalcified with 4% ethylenediaminetetraacetic acid (EDTA) for three weeks after the formalin fixation and were dehydrated using a series of ethanol concentrations (70%, 80%, 90%, 96%, and 100%). Then, the specimens were immersed in xylene and were embedded in paraffin blocks in a routine manner. Then, 6-mm-thick histological decalcified sections were prepared. After the deparaffinization of the paraffin-embedded sections with xylene, each decalcified thin section was stained with hematoxylin and eosin (HE). The stained sections were histologically observed using a light microscope at a magnification of 100×. In addition to a descriptive evaluation, histomorphometrical analysis of the formation of new bone was performed in an extracted tooth socket. The area of newly formed bone in the extracted tooth socket was analyzed as newly formed bone mass (BM) using an image analysis system (WinROOF, Visual System Division, Mitani Corp., Tokyo, Japan). The ROI was determined as a rectangle (0.5 mm x 0.8 mm) in the distal root of the extracted tooth socket. BM was defined as the percentage of the area of newly formed bone relative to the area of the ROI.

Statistical analysis
Data for paste consistency, TMD values from the micro-CT evaluation, and BMs from the histological evaluation of DNA pastes were analyzed using one-way analysis of variance and the post-hoc Tukey’s test for multiple comparisons among means. Statistical analyses were conducted using Origin Pro 9.0 J (OriginLab Corp., Northampton, MA, USA). p<0.05 was considered significant, and the data are expressed as the mean ± standard deviation (SD).

Results
Table 1 shows the results of the diameter of each paste disk after be-
ing subjected to a constant load. Both DNA/protamine and DNA/gelatin paste showed a significantly larger diameter than the conventional DNA/protamine-complex paste ($p<0.05$). No significant difference existed in case of the diameter between the DNA/protamine and DNA/gelatin pastes ($p>0.05$).

The experimental animals remained in good health during the test period and no clinical signs of inflammation or adverse tissue reactions were macroscopically seen when the animals were sacrificed. Fig. 5 shows the micro-CT images of the extracted socket of rat maxillary molars after DNA paste injection. The dotted box shows the area of the extracted tooth sockets. In the control specimen, presence of the tooth socket was still identified and the shape of the existing tooth root was visible. On the other hand, the tooth socket was filled with newly formed bone in case of the DNA/protamine paste- and DNA/gelatin paste-injected specimens, and homogeneous radiopacity in the extracted tooth socket was observed.

Quantification of the degree of calcification, i.e. the TMD values obtained from micro-CT images are shown in Table 2. The TMD values of the DNA/protamine paste- and DNA/gelatin paste-injected specimens were higher than those of the control specimens ($p<0.05$), and there was a significant difference in the calcification degree between the DNA/protamine paste- and DNA/gelatin paste-injected specimens ($p<0.05$).

| Table 1. Diameter of the disks of each DNA paste |
|-----------------------------------------------|
| Paste | Conventional DNA/protamine-complex paste | DNA/protamine paste | DNA/gelatin paste |
|-------|-------------------------------------------|-------------------|-------------------|
| Diameter (mm) | 10.2 (0.4)$^a$ | 21.3 (0.6)$^b$ | 201.0 (1.0)$^b$ |

Different letters indicate significant differences ($p<0.05$).

Fig. 6 shows the non-decalcified histological appearances of the extracted socket of rat maxillary molars after DNA paste injection. Pictures d), e) and f) are higher magnification appearances of extracted socket areas in pictures a), b) and c), respectively. The control specimens showed the formation of new bone partly in the extracted tooth socket, and the presence of connective tissue (asterisk) was recognized around the top of the extracted tooth socket. Formation of new bone in the extracted tooth socket was higher and the amount of connective tissue was lower in the DNA/protamine paste- and DNA/gelatin paste-injected specimens than in the control specimen. Arrangement of bone trabeculae was random in the control specimen, but the trabeculae in the DNA/protamine paste- and DNA/gelatin paste-injected specimens were regularly oriented in the vertical direction (arrow head). Haversian lamellae were identified in the DNA/gelatin paste-injected specimens (arrow), and bone formation was more clearly observed in the DNA/
Table 2. Calculated TMD values (mg/mm³) from the micro-CT observations

| paste                        | Control (sham) | DNA/protamine paste | DNA/gelatin paste |
|------------------------------|----------------|---------------------|------------------|
| TMD value (mg/mm³)           | 4.2 (3.5)*     | 13.1 (1.0)*         | 44.3 (9.6)*      |

( ) : SD, n = 3
Different letters indicate significant differences (p < 0.05)

Table 3. Percentage of measured bone mass (BM) from the calcified histological sections

| paste                        | Control (sham) | DNA/protamine paste | DNA/gelatin paste |
|------------------------------|----------------|---------------------|------------------|
| BM (%)                       | 58.9 (1.7)*    | 74.3 (2.4)*         | 79.9 (1.8)*      |

( ) : SD, n = 3
Different letters indicate significant differences (p < 0.05)

The on-the-spot preparation method made it possible to prepare a small quantity of DNA pastes; in this case, it is easy to adjust the amounts of DNA required. Another advantage of on-the-spot preparation method is its simplicity; there is no need for the preparation of DNA/protamine or DNA/gelatin complexes. Some experimental techniques such as centrifugation and freeze-drying are necessary for preparing the conventional DNA/protamine complex.6-7 On-the-spot paste preparation does not require such experimental procedures. It will also be possible to adjust the viscosity of the pastes by changing the mixing ratio of DNA and protamine or gelatin.

Animal experiments have confirmed the acceleration of bone formation in extracted tooth sockets by DNA/protamine and DNA/gelatin paste injection. There are a few reports regarding the mechanism of bone formation by DNA/protamine complex. Toda et al. cultured mesenchymal-like cell outgrowths from DNA/protamine complex-engrafted defect sites. They found that these cells showed high expression levels of osteogenic genes, including those for Runt-related transcription factor 2 (Runx-2), Alkali phosphatase (ALP), osteopontin, and osteocalcin, and speculated that DNA/protamine complexes may recruit osteocompetent cells and induce their differentiation into osteogenic cells. Sato et al. reported that the addition of 20,000-bp salmon DNA to cell culture of preosteoblast MG63 cells upregulated the mRNA and protein expression of osteogenic factors such as RUNX2, osterix, and osteopontin. ALP activity was also upregulated in MG63 cells cultured with DNA. The present study could reveal the superior bone formation behaviors by DNA/protamine and DNA/gelatin paste injection in the limited conditions. Another animal experiments should be elucidating the mechanism of bone formation by DNA/protamine and DNA/gelatin paste as a next series of our study.

Fukushima et al. prepared DNA/chitosan, DNA/arginine, DNA/histidine, and DNA/lysine complexes, besides DNA/protamine complex. They concluded that these complexes have problems with respect to degradation, handleability, and moldability, and that the DNA/protamine complexes are effective for bone regeneration. In the present study, we selected basic gelatin for making DNA/gelatin paste in addition to the DNA/protamine paste. Gelatin is natural polymer and is called as heat-denatured collagen. Gelatin is widely used as a scaffold material for bone regeneration. Tabata et al. and their group utilized gelatin hydrogel as a controlled-release system for biologically active substances such as growth factors. It is known that basic gelatin reacts with DNA. In the present study, we used low-endotoxin basic gelatin obtained from fresh pig skin.

Protamine is a known antibiotic protein, and DNA/protamine-complex paste can delay the growth of some bacteria such as Porphrymonas gingivalis. It is expected that the DNA/protamine paste will also have an antibacterial property.

The DNA/gelatin paste showed a greater amount of new-bone formation than the DNA/protamine paste. The reason for this is not still clear, but it is speculated that the degradation rate of the DNA/protamine and DNA/gelatin pastes is one of the factors for controlling their bone-formation ability. It was reported that the DNA/protamine complex degraded and disappeared 10 days after its implantation into the soft tissues of the skin of rats. Wada et al. evaluated the bone response of gelatin/calcium phosphate paste (CaP) with regards to different degradation rates of gelatin. They implanted the gelatin/CaP paste into the superioisteal pocket of rat calvaria and found that gelatin/CaP paste with a lower degree of gelatin cross-linking showed greater amounts of new-bone formation. As mentioned above, gelatin is used as a degradable scaffold material for bone regeneration. It is speculated that gela-
tin is easier to degrade than protamine. The in vivo degradation rates of the DNA/protamine and DNA/gelatin pastes will be further investigated.

In the present study, extracted tooth sockets of rats were used as a model for new-bone formation to study the characteristics of DNA-based pastes. The present DNA/protamine and DNA/gelatin pastes are applicable to such small cavities. It is expected that such DNA pastes will also be applicable for curing infrabony defects such as vertical bone loss or wedge-shaped defects by adjusting the mixing ratio or the amounts of different components. Shinozaki et al added fibroblast growth factor-2 (FGF-2) to DNA/protamine-complex paste by kneading. The addition of specific amounts of cytokines to pastes is easier and more accurate when the paste is prepared by the present on-the-spot preparation method.

In conclusion, we prepared DNA/protamine and DNA/gelatin pastes by on-the-spot preparation method. The viscosity of the DNA/protamine and DNA/gelatin pastes was improved compared to that of the conventional DNA/protamine-complex paste. After the injection of each DNA paste into the extracted sockets of rat maxillary molars, we found that the DNA/protamine and DNA/gelatin pastes enhanced new-bone formation in the extracted sockets. The bone-formation ability of the DNA/gelatin paste is better than that of the DNA/protamine paste in the extracted sockets of rat maxillary molars. We suggest that the on-the-spot method for the preparation of DNA pastes will be useful in the field of bone regeneration.

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Conflict of Interest

The authors have declared that no COI exists.

References

1. Wilson WD. Reversible interactions of nucleic acids with small molecules. In: Nucleic acids in chemistry and biology, ed by Blackburn GM and Gait MJ, Oxford University Press, Oxford, 1996, pp 329–374.
2. Goldman A and Glumoff T. Interaction of proteins with nucleic acids. In: Nucleic acids in chemistry and biology, ed by Blackburn GM and Gait MJ, Oxford University Press, Oxford, 1996, pp 375–441.
3. Werner MH, Gronenborn AM and Clore GM. Intercalation, DNA kinking, and the control of transcription. Science 271: 778–784, 1996
4. Fukushima T, Hayakawa T, Okamura K, Inoue Y, Miyazaki K and Okahata Y. Buffer solution can control the porosity of DNA/chitosan complexes. J Biomed Mater Res B Appl Biomater 76: 121-139, 2006
5. Fukushima T, Ohno J, Hayakawa T, Kawaguchi M, Inoue Y, Takeda S, Toyoda M and Okahata Y. Mold fabrication and biological assessment of porous DNA-chitosan complexes. J Biomed Mater Res B Appl Biomater 91: 746-754, 2009
6. Fukushima T, Kawaguchi M, Hayakawa T, Ohno J, Iwashashi T, Taniguchi K, Inoue Y and Takeda S. Complexation of DNA with cationic polyamino acid for biomaterial purposes. J Oral Tissue Eng, 6: 24-32, 2008
7. Fukushima T, Ohno J, Hayakawa T, Imayoshi R, Kawaguchi M, Doi Y, Kanaya K and Mitarai M. Polycation protamine for water-insoluble complex formation with DNA. Dent Mater J 29: 529-535, 2010
8. Fukushima T, Ohno J, Imayoshi R, Mori N, Sakagami R, Mitarai M and Hayakawa T. DNA/protamine complex paste for an injectable dental material. J Mater Sci Mater Med 22: 2607-2615, 2011
9. Mori N, Ohno J, Sakagami R, Hayakawa T and Fukushima T. Cell viabilities and biodegradation rates of DNA/protamine complexes with two different molecular weights of DNA. J Biomed Mater Res B Appl Biomater 101: 743-751, 2013
10. Shinozaki Y, Toda M, Ohno J, Kawaguchi M, Kido H and Fukushima T. Osteogenic evaluation of DNA/protamine complex paste in rat cranial defects. J Hard Tissue Biology 22: 401-408, 2013
11. Shinozaki Y, Toda M, Ohno J, Kawaguchi M, Kido H and Fukushima T. Evaluation of bone formation guided by DNA/protamine complex with FGF-2 in an adult rat calvarial defect model. J Biomed Mater Res B Appl Biomater 102: 1669-1676, 2014
12. Toda M, Ohno J, Shinozaki Y, Ozaki M and Fukushima T. Osteogenic potential for replacing cells in rat cranial defects implanted with a DNA/protamine complex paste. Bone 67: 237-245, 2014
13. van den Beucken JJJP, Vos MR, Thune PC, Hayakawa T, Fukushima T, Okahata Y, Walboomers XF, Sommerdijk N, Nolte RJ and Jansen JA. Fabrication, characterization, and biological assessment of multilayered DNA-coatings for biomaterial purposes. Biomaterials 27: 691–701, 2006
14. van den Beucken JJJP, Walboomers XF, Leeuwenburgh SC, Vos MR, Sommerdijk NA, Nolte RJ and Jansen JA. Multilayered DNA coatings: In vitro bioactivity studies and effects on osteoblast like cell behavior. Acta Biomater 3: 587–596, 2007
15. Schouten C, van den Beucken JJJP, Meijer GJ, Sommerdijk NA, Spauwen, PH and Jansen JA. In vivo bioactivity of DNA-based coatings: an experimental study in rats. J Biomed Mater Res A 92: 931-941, 2010
16. Sakurai T, Yoshinari M, Toyama T, Hayakawa T and Ohkubo C. Effects of a multilayered DNA/protamine coating on titanium implants on bone responses. J Biomed Mater Res A 104: 1500-1509, 2016
17. Miyamoto N, Yamachika R, Sakurai T, Hayakawa T and Hosoya N. Bone response to titanium implants coated with double- or single-stranded DNA. Biomed Res Int 13; 2018:9204391, 2018: doi: 10.1155/2018/9204391.
18. Fukushima T, Ohno J, Hayakawa T, Imayoshi R, Mori N, Sakagami R, Mitarai M and Hayakawa T. DNA/protamine complex paste for an injectable dental material. J Mater Sci Mater Med 22: 2607-2615, 2011
19. Shinozaki Y, Yanagi T, Yamaguchi Y, Kido H and Fukushima T. Osteogenic evaluation of DNA/protamine complex paste in rat cranial defects. J Hard Tissue Biology 27: 147-154, 2018
20. Waki T, Mochizuki C, Sato M, Sakurai T, Hayakawa T and Ohkubo C. Effects of a multilayered DNA/protamine coating on titanium implants on bone responses. J Biomed Mater Res A 104: 1500-1509, 2016
21. Donath K and Breuner G. A method for study of undecalcified bones and teeth with attached soft tissues: The Sage-Schliff (sawing and grinding) technique. J Oral Pathol Med 11: 318–326, 1982
22. Sato A, Kajiya H, Mori N, Sato H, Fukushima T, Kido H and Ohno J. Salmon DNA accelerates bone regeneration by inducing osteoblast migration. PLoS One 12: e0169522, 2017: doi: 10.1371/journal.pone.0169522
23. Gibbs D.M, Black CR, Dawson JI and Oreffo RO. A review of hydrogel use in fracture healing and bone regeneration. J Tissue Eng Regen Med 10: 187-198, 2016
24. Garg T, Singh O, Arora S and Murthy R. Scaffold: a novel carrier for cell and drug delivery. Crit Rev Ther Drug Carrier Syst 29: 1-63, 2012
25. Tabata Y. Nanomaterials of drug delivery systems for tissue regeneration. Methods Mol Biol 300: 81-100, 2005
26. Young S, Wong M, Tabata Y and Mikos AG. Gelatin as a delivery vehicle for the controlled release of bioactive molecules. J Control Release 109: 256-274, 2005
27. Shibata F, Uchiyama T and Ogita Z. Gelatin as a new electrophoretic gel medium. The Physico-Chemical Biology 32: 29-32, 1988
28. Kanayama Y, Aoki C and Sakai Y. Development of low endotoxin gelatin for regenerative medicine. Biol Pharm Bull 30: 237-241, 2007
29. Tsukiyama R, Katsura H and Tokuriki N. Antibacterial activity of licochalcone A against spore-forming bacteria. Antimicrob Agents Chemother 46: 1226-1230, 2002
30. Wada T, Amemiya T, Hirota M and Hayakawa T. Bone formation in gelatin/calcium phosphate paste in a subperiosteal pocket of rat calvaria. J Hard Tissue Biology 25: 305-312, 2016
31. Shinozaki Y, Toda M, Ohno J, Kawaguchi M, Kido H and Fukushima T. Evaluation of bone formation guided by DNA/protamine complex with FGF-2 in an adult rat calvarial defect model. J Biomed Mater Res B Appl Biomater 102: 1669-1676, 2014