Dental Implants with Electrochemical Nanopattern Formation to Increase Osseointegration.

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Abstract

Nowadays, The coming acceleration of global population ageing. When people get older, the importance of implants is emphasized. Dental implants have a crucial effect on life satisfaction. Many techniques have already been developed it. However, those still required many procedures. Shin et al suggested the new model based on MA. The electrochemical nanopattern formation (ENF) model takes out lots of steps when it is made. It is very simple because it only removes TiO₂ NT layers. Interestingly, we investigated many studies, this simple model did not distinguish the other commercial models. Surface modification by ENF process improved the osteogenic differentiation in cells. It did not limit the cells. In vivo study, ENF also promoted the bone to implant connectivity. The ENF technique used for the new substitute.

1. Introduction

In recent years, numbers of people are choosing dental implantation, because of higher chewing function and more satisfied orofacial esthetics to repair their dentition defect [1]. The dental implants have been widely used for replacing the lost natural teeth, with evidence of long-term success and improvement of patient quality of life [2]. However, some patients feel physical and psychological discomfort. Because of the functional limitation occurring during the healing after implant placement [3]. Dental extraction can also cause big changes in bone tissues characterized by the resorption of alveolar bone. Additionally, multiple-tooth extractions can be generated to the dental papilla [4, 5]. Another problem is periodontal tissue can cause the loss of the periodontal ligament after extraction [6, 7]. Therefore, implant restorations are essential for preserving and maintaining the bone anatomy [8]. Nowadays, many techniques and materials are reported for the implant for the bone resorption. The recommended technique is the immediate dental implant placement [9]. Osseointegration is the essential factor for the long-term success of dental implants. It is defined as the direct structural and functional connection between the implant and the bone, which is often measured by the bone-to-implant contact (BIC) value under an optical microscope [10].

In osseointegration, the critical factor is the biocompatibility of the implant surface. The high hydrophilicity and suitable roughness surfaces support more bone deposition than other surfaces. In histologic sections, osseointegration is validated as alternative parameters including resonance frequency analysis and removing the torque or direct mineralized bone-implant connection. For the high level of clinical success, direct bone-implant connection is important. The surface microstructure helped to increase the rate of bone adaptation to the implant surface. Therefore, new implant surface technique is key to develop bone to implant adaptation and evaluate the clinical protocol.

There are four kinds of dental implants used for the clinical trials such as Osseotite, TiUnite, SLA and SLActive. Among those implants, TiUnite is a new-generation surface in the field of dental implants [11]. Titanium (Ti) oral implants can be successfully helped for the partially edentulous subjects and rehabilitation of completely [12]. It and its alloys are used for alternative materials for hard tissues. These materials are considered a suitable substrate, but still have the problems with conductivity [13]. To

increase the bioinert Ti-based surfaces, modification techniques are developed such as alkali-heat treatment, sol-gel-coating, H₂O₂ oxidation, and anodization(ref). Anodization with regards to convenience, reproducibility, and efficient technique to prepare microstructure on metal surface, which is broadly applied to titanium [14, 15]. This process can be accelerated on the long-term performance of dental implants. However, there is still a problem between dental implants and bone. The vertically aligned TiO₂ nanotube (NT) arrays are broadly applied because of the high biocompatibility and nanotopographical surface. It also improved differentiation, vasodilation, and proliferation [16, 17]. However, it has been very limitedly used, because of poor connection and unexpected effect on the local tissue [18].

Shin et al. [17] reported the additional technique for nanopattern Ti surface name as ENF overcoming those limitations. They applied the simple techniques that removed the TiO₂ NT layers.

Here, we targeted ENF to investigate the functional study. ENF led the cell proliferation and dramatically developed the ALP activity in vitro study. In vivo study, most experiments indicated ENF did not functionally differ compared to SLA. It means ENF is a new candidate technique and can use substitute technique.

2. Methods/experimental

2.1 Ethics approval and animal preparation

In in vivo animal model, all experimental protocols including animal care, surgical procedures, and etc. were performed in accordance with the Korea Laboratory Animal Use and Breeding Management Regulation. Our animal protocol and procedure were approved by the Seoul National University Institutional Animal Care and Use Committee (IACUC). In addition, our study was carried out in compliance with the ARRIVE guidelines. New Zealand White Rabbit (2.9~3.2 kg, male, KNOTUS, Incheon, Republic of Korea) and Beagle Dog (9~10 kg, male, KNOTUS, Incheon, Republic of Korea) were used in this study (IACUC No.: Rabbit SNU-210730-2, Beagle SNU-210813-2).

2.2 Specimen preparation

The electrochemical nanopattern formation (ENF) production methods reported by Shin et al., 2019 was used, and the ENF surface was treated with a micropore size of 10nm and a dimple size of 150-200nm [17].

Among the implants used in the experiment, Straumann SLActive (SS, Straumann, Basel, Swiss) was purchased, Machined (MA) implants, ENF surface-treated implants based on MA (MA+ENF, ME), resorbable blasting media (RBM) with surface treatment (MA+RBM, MR), implants with overlapping ENF on MR (MR+ENF, MRE), sandblasted with large grit and acid etched (SLA) implants with surface treatment (MA+SLA, MS), and an implant with overlapping surface treatment of ENF on MS (MS+ENF, MSE) was provided by DENTIS (Daegu, Republic of Korea).

2.3 in vitro cellular assay

hMSCs were purchased from Lonza (Walkersville, MD, USA), and all experiments were conducted using hMSCs between passages 3 and 5. For maintenance of undifferentiated state, hMSCs were cultured in MSC basal medium (Lonza) containing 10% of MSC growth supplement (Lonza), 2% of l-glutamine, 0.1% of GA-1000, and 1% of antibiotic-antimycotic solution (10,000 units of penicillin, 10 mg of streptomycin, and 25 µg of amphotericin B per mL, Sigma-Aldrich, St. Louis, MO, USA) under 5% CO₂-containing 37°C humidified incubator. Culture media were routinely replaced every 48 h and the cells were sub-cultured at 80% of confluency. For induction of osteogenic differentiation, hMSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10⁻⁸ M dexamethasone (Abcam, Cambridge, UK), 0.2 mM ascorbic acid (Sigma-Aldrich), and 10 mM β-glycerolphosphate (Sigma-Aldrich) containing 1% of antibiotic-antimycotic solution. hMSCs were initially spot-seeded at a density of 1 x 10⁴ cells on each cylindrical implant sample with 2 cm in diameter and cultured for predetermined periods.

The proliferation rates of hMSCs were evaluated by cell counting kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan), which was performed according to the manufacturer's protocol. After 1, 4, and 7 days of incubation, each sample was washed twice with Dulbecco's phosphate buffered saline (DPBS, welgene, Kyungsan, Republic of Korea) and cultured in CCK-8 solution diluted in a medium (1/10) for 2 h at 37°C in the dark under 5% CO₂ in a humidified incubator. The absorbance at 450 nm was assessed at each time point by using a SpectraMax® 340 plate reader (Molecular Devices Co., Sunnyvale, CA, USA). For evaluation of early osteogenic differentiation of hMSCs, cellular alkaline phosphatase (ALP) activity assay was conducted. After 1, 7, and 14 days of culture, ALP activity of hMSCs was determined by measuring the conversion of p-nitrophenyl-phosphate to p-nitrophenol by an ALP assay kit (Abcam) as per the manufacturer's protocol. The absorbance at 405 nm was assessed at each time point by using a SpectraMax® 340 plate reader. ALP activity was estimated by calculating the total amount of p-nitrophenol formation (µmol) divided by the reaction time (min) and volume of sample (mL) (OD value*1000/60/0.24). To estimate the rate early osteogenic differentiation of hMSCs, extracellular calcium nodule deposition was measured. After 1, 14, and 21 days of culture, hMSCs were washed twice with DPBS solution, fixed with 3.7% of formaldehyde for 10 min, and stained with 40 mM of alizarin red S (ARS, pH 4.2, Sigma-Aldrich) in DPBS solution. The hMSC-cultured samples were imaged with a digital camera (Olympus Optical Co., Tokyo, Japan). Quantitative analysis was performed by extracting ARS in stained hMSCs. To extract ARS from stained hMSCs, 10% of acetic acid solution was added, and hMSC-cultured substrates were incubated for 30 min with constant shaking at 80 rpm. Consequently, 10% of ammonium hydroxide solution was added to neutralize the aqueous solution of the ARS extracts, and the absorbance values were measured using a SpectraMax® 340 plate reader at 405 nm.

2.4 Surgical Procedures

Rabbit: A mixture of Xylazine hydrochloride (Rompun® 5mg/kg, BAYER Korea, Seoul, Republic of Korea) and Ketamine hydrochloride (Ketara®, 35mg/kg, Yuhan, Seoul, Republic of Korea) was injected

intramuscularly for general anesthesia. All the hair on the left and right hind legs was removed, and the skin at the surgical site was wiped with Povidone-iodine (Green Pharmaceutical Co., Ltd., Seoul, Republic of Korea). After removed the muscle and periosteum and exposed the implant site, used the low-speed handpiece 10 mm below the cancellous bone of the tibial head so that the distance between the implants was 15 mm after the initial implant implantation, and the second implant was placed (Fig. 1). An implant with a diameter of 3.3 mm and a length of 6.0 mm was used, and the following surface-treated implants were used as experimental materials - □ MA, □ ME, □ MR, □ MS, □ MRE, □ MSE. A total of 28 animals, 6 types of implant groups, were sacrificed at 3 weeks (3wks) and 6 weeks (6wks), respectively.

Beagle: For general anesthesia, Xylazine hydrochloride (Rompun® 2.3mg/kg, BAYER Korea, Seoul, Republic of Korea) and Ketamin hydrochloride (Ketara®, 5mg/kg, Yuhan, Seoul, Republic of Korea) were mixed and injected intravenously. Tooth extract was performed after local anesthesia using 2% lidocaine HCL (containing 1:100000 epinephrine) on the beagle dog mandibular gingiva that had been anesthetized. After a recovery period of 3 months after tooth extraction, anesthesia was performed using the same anesthesia method as for tooth extraction, drilling was performed at 1110 rpm / 55 Ncm, and implants were placed at 30 rpm / 55 Ncm (Fig. 2). The implants used were DENTIS implant with a diameter of 3.5 mm and a length of 8.0 mm and Straumann implant with a diameter of 3.3 mm and a length of 8.0 mm. The following surface-treated implants were used as experimental materials - □ SS, □ MA, □ MS, □ ME. A total of 10 animals, 4 types of implant groups were sacrificed for 6 weeks (6wks) and 12 weeks (12wks), respectively. MA, MS and MR used commercial products, and ME, MSE, and MRE used implants with nanopatterns processed into commercial products. Both Rabbit and beagle dogs were sacrificed after induction of cardiac arrest using Potassium Chloride (KCL, 0.5ml/kg, Huons, seongnam, Republic of Korea) after anesthesia with a mixture of Xylazine hydrochloride and Ketamin.

2.5 Implant Stability Quotient measurement

All implants were measured for implant stability quotient (ISQ) using Osstell Mentor (Integration Diagnostics AB, Sweden). Measurement were taken immediately after rabbit implant placement (immy), 3wks and 6wks, and immediately after beagle dogs implant placement (immy), 6wks and 12wks. Rabbit (3/6 weeks) and beagle dog (6/12 weeks) ISQ measurements were performed on a mixture of Xylazine hydrochloride and Ketamin immediately before sacrificed after anesthesia.

2.6 Removal Torque measurement

For all implants placed in rabbits and beagle dogs, the implant mount was connected to the implant body for removal torque (RT) measure and the RT was measured with the Series TT03 digital torque meter (MTT03-100, MARK-10, NY, USA).

2.7 Histomorphometric Analysis

The samples were fixed in a neutral 10% formalin solution for 48 hours, the gross samples were placed in a cassette or a brown glass bottle, washed with water for 3 ~ 6 hour, dehydrated with 80 ~ 100% alcohol, and the samples were placed in a Technovit® 7200 VLC basic solution resin (KULZER GmbH, Hanau,

Deutschland). Stirred and embedded for 3 ~ 5 days. The embedding block was manually timed with sandpaper, and the block was attached to the acrylic slide for block middle cutting. The middle cutting slide was grinded, and samples were prepared by gliding the cut slide to about 40 ~ 50 μ m. Samples for histomorphological analysis were taken at a resolution of 3.4 per pixel using a microscope (Nikon Microphot-FXA, Nikon Corporation, Tokyo, Japan) equipped with a digital camera (Kodak Professional DCS 420, Eastman Kodak Company, Rochester, NY, USA). Using an optical microscope, 20x magnification image was taken, and the part showing the structure between the implant and the implant was digitized and the bone site connected to the implant was measured to measure the contact between the bone and the implant. The bone to implant contact ratio (BIC) was calculated, and the bone contact rate was calculated at three sites of the spiral with good bone fusion to measure the bone volume (BV).

2.8 Statistical Analysis

Each data was processed using SigmaPlot 14 statistical program, expressed as Mean $\pm$ Standard deviation. It was evaluated by One-way ANOVA and verified by Student-Newman-Keuls. It was considered significant that the P value was less than 0.05 for all statistics.

3. Results

3.1 Implant stability Quotient measurement

ISQ was quantitative analysis of the degree of fixation to the bone after implant placement and was measured using an Osstell Mentor. There were 6 types of implants placed in rabbit tibia (3wks each group n=8, and 6wks each group n=8), and the measured value average were distributed between 74 and 79 (Fig. 3A, Additional file 1: Figure S1). Statistical significance of ISQ measurements in rabbit was observed in immy MS and 6wks MS ($p<0.001$), immy ME and 3wks MS ($p<0.05$), immy ME and 6wks MS ($p<0.05$), immy MA and 3wks MS ($p<0.05$), immy MA and 6wks MS ($p<0.01$), 3wks MA and 6wks MS ($p<0.01$), 3wks MA and 3wks MS ($p<0.01$), 3wks ME and 6wks MS ($p<0.001$), 3wks MS and 6wks MS ($p<0.001$), 6wks MA and 6wks MS ($p<0.001$), and 6wks MS and 6wks ME ($p<0.01$) (Fig. 3A).

There were a total of 4 types of implants placed in the beagle dog (6wks each group n=8, 12wks each group n=8), and the measured value average were distributed between 65 and 81 (Fig. 3B). Compared with immy MA, Statistical significance of ISQ measurements was observed with immy MS, immy ME, 6wks MA, 6wks MS, 6wks ME, 12wks MS and 12wks ME ($p<0.001$), and 12wks MA ($p<0.01$). Compared with immy MS, it was observed in 6wks MA, 6wks ME, 12wks MA and 12wks ME ($p<0.001$), 12wks MS ($p<0.01$), and 6wks MS ($p<0.05$). Compared with immy ME, it was observed in 6wks MA, 12wks MA and 12wks ME ($p<0.001$), 6wks ME and 12wks MS ($p<0.01$), and 6wks MS ($p<0.05$). Compared with 6wks MA, it was observed in 6wks MS, 6wks ME, 12wks MA, 12wks MS and 12wks ME ($p<0.001$). Compared with 12wks MA, it was observed in 6wks MA and 6wks ME ($p<0.001$) (Fig. 3B).

3.2 In vitro cellular study

The effects of ENF surface on the growth and proliferation of hMSCs were evaluated. The cell proliferation was assessed by CCK-8 assay, which determines cell population by quantification of reaction between metabolic products of cells and the assay solution. During 7 days of incubation, every group did not hinder the proliferation of hMSCs compared to the TCP group. Until the 4 days of incubation, the proliferation rates of MA, MR, MS, and their ENF counterparts were comparable to that of the TCP group. After 7 days of incubation, cell proliferation of ME, MRE, and MSE groups were significantly ($p < 0.05$) increased compared to their non-ENF counterparts, while there were no significant differences between ENF groups (Fig. 4). It was observed that the surface treatment through a single technique (MA, MR, and MS alone) did not facilitate cell proliferation compared to TCPs, while the additional ENF coating was significantly enhanced the cell proliferation after 7 days of culture. These results suggest that the synergistic effect of ENF with conventional functionalization methods can enhance the cytocompatibility of implant surfaces. These findings are mostly in accordance with those of previous studies demonstrating nanopatterned surface facilitates cell proliferation and cytocompatibility [19–22]. This is mainly because the transcriptional response of stem cells concerning cell proliferation is affected by cytoskeletal rearrangement by nanotopography sensing [23–24]. Furthermore, nanopattern can mediate the regulation of microRNA for maintenance of population growth and phenotype of stem cells [25]. Because the adhesion and early proliferation of surrounding cells on implant surface are essential factors for successful osteogenesis, ENF dual-modification is considered to be advantageous for osseointegration of implant at early post-transplant state [26–28].

For further investigation of the osteogenic capability of ENF surface, ALP activities of hMSCs on MA, MR, MS, and their ENF counterparts were assessed. The ALP activity is known as a marker for osteogenesis and is expressed in an early stage of osteogenic differentiation of stem cells [29]. On the first day of incubation, there was no significant difference between groups. On 7 and 14 days of incubation, ALP activities of ME, MRE, and MSE groups were significantly increased compared to non-ENF and TCP groups. Especially, the ME group significantly facilitated ALP activity of hMSCs at 7 and 14 days indicating the topography of ME is advantageous for early osteogenesis of hMSCs (Fig. 5). In addition to ALP activity, mineralized calcium nodule deposition of hMSCs, regarded as a marker for the late stage of osteogenesis and bone regeneration, was stained with ARS solution and quantified. On the first day of culture, MA group showed a slight decrease of mineralization nodule while MS, ME, and MSE groups showed a modest increase of that. On 14 and 21 days of culture, mineralization nodules of MS, ME, and SLA groups significantly increased while MA groups still maintained a similar level to the TCP group. Especially, ME induced the highest degree of mineralization nodule formation of hMSCs at 14 and 21 days of culture, suggesting the osteogenic differentiation and bone regeneration of hMSCs are most promoted on ME group (Fig. 6A, B). Considering the previous CCK-8 and ALP assay, the ENF double coating can promote the proliferation and osteogenesis of hMSCs. Especially, the ME group did not hamper proliferation rate and significantly increased early and late stage of osteogenesis of hMSCs compared to MRE and MSE groups.

Many studies emphasize that surface roughness and morphology have crucial effects on cell-matrix that regulate integrin-mediated signal cascade [30–32]. In a mechanotransductional interpretation,

osteoblasts have larger cell morphology than other lineages, including adipocytes, fibroblasts, and MSCs, hence need large adhesion to support the tensile cytoskeletal scaffolding [33]. Interestingly, the cytoskeletal tension of MSCs can regulate differentiation lineages. McBeath et al. demonstrated that small fibronectin microcontact ($1,000 \mu\text{m}^2$) led MSCs to differentiate into adipose cells, while large fibronectin contact ($10,000 \mu\text{m}^2$) promoted spreading of MSCs leading to differentiation into osteoblasts [34]. Therefore, ENF-functionalization supported nanotopographical cues on hMSCs that increase the area of focal adhesion and tensile stress of cytoskeletons, hence, could have facilitated osteogenic differentiation of hMSCs. Moreover, microporous surfaces are known to promote cells to secrete osteotropic factors including $1\alpha,25(\text{OH})_2\text{D}_3$, PGE_2 , and $\text{TGF-}\beta 1$ [35]. On the other hand, nanopatterned substrates often enhance protein adsorption and intracellular protein delivery by forming ionic bonding and electrical conductivity [36]. These lead to the anchoring of proteins in osteogenic media including dexamethasone, β -glycerolphosphate, and ascorbic acid. It is known that dexamethasone could upregulate many proteins and enzyme levels concerning osteogenesis, hence enhances calcium deposition [37]. Dexamethasone synergistically acts with β -glycerolphosphate to enhance the ALP activity level in the cells and ascorbic acid favorably affects the maturation of osteoblasts [38]. Therefore, concerning in vitro cellular assays, it is suggested that ME is considered to be the most effective method for coating the implant surface to promote osseointegration and bone regeneration.

3.3 Histomorphometric analysis

After implant placement in rabbit and beagle dog, hematoxyline and eosin (H&E) staining was performed after sacrificed at each experimental schedule. Based on this, BV, new bone area (NB), and BIC was quantitatively analyzed.

As a result of H&E analysis of implant experiments in rabbits, in 3wks MA, bone changes were not evident and inflammatory tissue and connective tissue were observed at the interface, and in 6wks MA, most of the bone defect area was filled with new bone and partial bone remodeling was observed (Fig. 7A, D). In 3wks MS, osteoblasts and osteoclasts were distributed, and continuous bone realignment and differentiation were observed. In 6wks MS, new bone formation was observed to be active, but the contact of the implant interface was observed to be low (Fig. 7B, E). In 3wks ME, osteoblasts were abundant, and bone resorption and bone remodeling of existing bone were observed. In 6wks ME, osteoblasts were aligned and attached between the implant and bone, and most of the bone remodeling was completed, and the appearance of mature bone was also observed (Fig. 7C, F). In 3wks MR, bone resorption proceeded extensively at the implant interface and partial new bone formation was observed. New bone formation was well performed but bone remodeling was not started. In 6wks MR, bone remodeling was mostly completed (Additional file 1: Figure S2A, D). In 3wks MSE, new bone was being formed and contact with the implant interface was observed as an osteoblast lining. Osteogenesis was active but bone density was relatively low. In 6wks MSE, bone remodeling was mostly completed and contact with the implant interface was high (Additional file 1: Figure. S2B, E). In 3wks MRE, new bone formation was actively performed, but bone contact at the implant interface was low, and new bone formation occurred actively but bone density was low. In 6wks MRE, new bone formation and bone

remodeling were in progress, and soft tissue were observed at the implant interface and many immature bones were observed (Additional file 1: Figure. S2C, F).

The implant placed in rabbits were observed separately in the initial period (3wks) and in the recovery period (6wks). Compared with 3wks ME, Statistical significance of BV measurements was observed with 3wks MA, 3wks MS, 6wks MA and 6wks MS ($p<0.001$). Compared with 6wks MS, it was observed with 3wks MA ($p<0.01$), and 3wks MS and 6wks MA ($p<0.05$). Compared with 6wks ME, it was observed with 3wks MA, 3wks MS, 6wks MA and 6wks MS ($p<0.001$) (Fig. 8A, Additional file 1: Figure S3A).

BIC was measured the contact rate between the implant thread and the regenerated bone. Compared with 3wks ME, Statistical significance of BIC measurements was observed with 3wks MA ($p<0.01$), and 6wks MA, 6wks MS and 6wks ME ($p<0.001$). Compared with 3wks MS, it was observed with 3wks MA, 6wks MA, 6wks MS and 6wks ME ($p<0.001$). Compared with 3wks MA, it was observed with 6wks MA and 6wks MS ($p<0.001$), and 6wks ME ($p<0.01$). Compared with 6wks ME, it observed with 6wks MA and 6wks MS ($p<0.001$). Statistical significance was observed because of comparison with 6wks MS and 6wks MA ($p<0.001$). As a result of BIC comparison between implants at each week, statistical significance was observed at both 3wks and 6wks (Fig. 8B, Additional file 1: Figure S3B).

NB measured the new bone area within the ROI after setting the ROI between the implant screw thread and the thread. Compared with 6wks ME, Statistical significance of NB measurements was observed with 3wks MA and 3wks MS ($p<0.01$), and 6wks MS, 6wks MA and 3wks ME ($p<0.01$). As a result of comparing NB between implants at each week, 3wks were not statistically significant, and 6wks were observed to be statistically significant (Fig. 8C, Additional file 1: Figure S3C).

As a result of H&E analysis of implant experiments in a beagle dog, new bone formation was observed in all groups at 6wks, but bone remodeling was not observed clearly, and the 12wks tissue showed the most active bone remodeling of ME (Fig. 9)

Compared with 12wks MA, Statistical significance of BV measurement was observed with 12wks MS ($p<0.01$), and 6wks MA, 6wks MS, 6wks ME and 12wks ME ($p<0.001$) (Fig. 10A).

Compared with 6wks MS, Statistical significance of BIC measurement was observed with 6wks MA and 6wks ME ($p<0.01$). Compared with 12wks MA, it was observed with 6wks MA and 6wks ME ($p<0.001$). Compared with 12wks MS, it was observed with 6wks MA and 6wks ME ($p<0.001$). Compared with 12wks ME, it was observed with 6wks MA and 6wks ME ($p<0.001$), 6wks MS and 12wks MA ($p<0.05$), and 12wks ME ($p<0.01$) (Fig. 10B).

Compared with 12wks MA, Statistical significance of NB measurement was observed with 6wks ME and 6wks MS ($p<0.001$), and 6wks MA ($p<0.01$). Compared with 12wks MS, it was observed with 6wks MA, 6wks MS, 6wks ME, 12wks MA and 12wks ME ($p<0.001$). Compared with 12wks ME, it was observed with 6wks ME, 6wks MS and 12wks MA ($p<0.001$), and 6wks MA ($p<0.01$) (Fig. 10C).

RT (12wks each group n=4) was measured at 12wks to mechanically check the stability of the implant-bone bonding. MA $32.25 \pm 1.91 \text{ N/cm}^3$; MS $116.77 \pm 24.05 \text{ N/cm}^3$; ME $54.60 \pm 10.02 \text{ N/cm}^3$. MA had the lowest RT among all implant groups, and ME was measured to be about 54% higher than MA but was measured to be about 50% lower than MS (Fig. 10D).

4. Discussion

In this study, we established the ENF treated machine in animal models. ENF technique is modified by the machined model and the unique technique that removes the TiO₂ NT layers by chemical etching. ENF processing is easy and increases the osteogenic differentiation of hMSCs cells. Nowadays, sandblasted and acid-etched (SLA) implants widely use the standard implant model for dental implants. Because this model has a rough surface and reduces the healing period between surgery and prosthesis. We compare those models that established our model is ready to use. First, ISQ study test indicated our ENF model did not differ the implant stability number (ISN) with other models for 6 weeks in rabbits (Fig. 3). In beagle dogs, we clearly confirm ENF models. ENF models immediately have advanced results comparing MA models. ENF models are dramatically different after 6 and 12 weeks (Fig. 3). This means ENF is useful to implant. Second, for bone volume, those models did not have any characteristics after 3 and 6 weeks in rabbits. However, the results are different in beagle dog models. It keeps the higher volume after 12 weeks. ENF models are stable and have a higher bone volume compared to the MA. Third, in rabbit, ENF models did not have any change with MA models after 3 weeks. ENF models increase the BIC percentage comparing the MA models after 6 weeks. This indicated the ENF models is good connectivity with the bones when we implant it. ENF models apparently promoted the value in beagle dogs. After 6 weeks, SLA model evaluated the value, but it did not differ in 12 weeks. Finally, we checked the new bone area in rabbits. The ENF model increased a little when we measured it in 3 weeks. But it did not make any difference in 6 weeks because the healing is complete, that's why we can't see the changes. It is also the same in beagle dogs. We can't see any difference between 6 to 12 weeks. However, we still have limitations. Lastly, we studied the removal torque test. SLA models have higher value compared to ENF models. However, those results indicated ENF model is easy to make and another technique for implant.

5. Conclusion

Our study identified the effectiveness of the new surface treatment method by comparing the ENF method, which is different from the conventional MA and SLA methods in the dental implant fields. Based on our study, the ENF showed superior BV and ISQ results compared to the MA in rabbit and beagle models. Therefore, it is considered to be good for high stability and bone formation. However, the ENF and SLA models were no difference. The ENF is considered to be replaceable because the manufacturing process is easier and the preclinical results are equal to or higher than that of the conventional surface treatment technique. However, it is necessary to obtain additional data through clinical trials and experiments with respect to the physical surface.

Abbreviations

MA: Machined; In this study, ENF: Electrochemical nanopattern formation; NT: nanotube; BIC: Bone-to-implant contact; Ti: Titanium; SLA: sandblasted, Largegrit, Acid-etched; ALP: Alkaline Phosphatase; IACUC: Institutional Animal Care and Use Committee; ME: MA+ENF; RBM: Resorbable blasting media; MR: MA+RBM; MRE: MR+ENF; MS: MA+SLA; MSE: MS+ENF; hMSC: Human mesenchymal stem cells; MSC: Mesenchymal stem cells; DMEM: Dulbecco's modified Eagle's medium; CCK-8: cell counting kit-8; DPBS: Dulbecco's phosphate buffered saline; ARS: Alizarin red S; HCL: Hydrochloric acid; KCL: Potassium Chloride; ISQ: Implant stability quotient; RT: Removal torque; BV: Bone volume; TCP: Tricalcium phosphate; H&E: Hematoxyline and eosin; NB: New bone area; ISN: Implant stability number

Declarations

Acknowledgements

Not applicable.

Authors' contributions

YCS, MSK, JHL (Jong Ho Lee) analyzed and interpreted the data regarding the cellular assay. WHK, SHL, JW, MSL, JHL (Jong-Ho Lee) analyzed and interpreted the data regarding the animal experiments. All authors analyzed the data and discussed the implications of the results. BK and DWH supervised this project. WHK and YCS have contributed equally to this work. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Animal surgery procedure in Rabbit. Implant surgery in rabbit tibia. A Bone drilling, B Implant placement, C X-ray shooting for implant placement observation, D Implant placement completed in rabbit tibia.

Figure 2

Animal surgery procedure in Beagle dog. Implant surgery in mandibular of beagle dog. A Mandibular of beagle dog drilling for implant placement, B Implant placement, C ISQ measurement after implant placement, D Implant placement completed in mandibular of beagle dog.

(a)

(b)

Figure 3

Implant Stability Quotient measurement. A Observed immediately, 3 weeks, and 6 weeks after implant placement of rabbit tibia, respectively. B Observed immediately, 6 weeks and 12 weeks after implant placement of beagle dog mandibular, respectively.

Figure 4

Proliferation of hMSCs on intact Ti and rGO-Ti substrates. Cells on tissue culture plastics (TCPs) were used as a control. After each day of incubation, the cell proliferation rates of TCPs, RBM, SLA, MA+ENF, RBM+ENF, and SLA+ENF were proportionately compared to the TCP group of 1 day. The data were expressed as the mean $\pm$ SD based on duplicate observations from three independent experiments ($n=6$). An asterisk (*) denotes a significant difference between groups ($p < 0.05$), while ns refers to not significant.

Figure 5

ALP activity of hMSCs on implant samples. Cells on tissue culture plastics (TCPs) were used as a control. After each day of incubation, ARS stains of TCPs, RBM, SLA, MA+ENF, RBM+ENF, and SLA+ENF were proportionately compared to the TCP group of 1 day. The results for RBM and RBM+ENF were not represented owing to the unspecific adsorption of ARS solution on surfaces. The data were expressed as the mean $\pm$ SD based on duplicate observations from three independent experiments ($n=6$). An asterisk (*) denotes a significant difference between groups ($p < 0.05$), while ns refers to not significant.

Figure 6

Mineralization nodule formation of hMSCs on implant samples. A ARS stains and B their corresponding extract in hMSCs on TCPs, SLA, MA+ENF, and SLA+ENF. Cells on tissue culture plastics (TCPs) were used as a control. After each day of incubation, the ALP activity was represented by the total amount of p-nitrophenol formation divided by the reaction time and volume of the sample. The data were expressed

as the mean $\pm$ SD based on duplicate observations from three independent experiments (n= 6). An asterisk (*) denotes a significant difference between groups ($p < 0.05$), while ns refers to not significant.

Figure 7

Bone observation through tissue staining for Rabbit. Observation of the bone morphology of the implant spiral of the rabbit tibia that was sacrificed in 3 weeks (A~C) and 6 weeks (D~F). Implant placement groups were as follow. MA (A), (D); MS (B), (E); ME (C), (F).

(a)

(b)

(c)

Figure 8

Histomorphometric analysis for Rabbit. BV, BIC, and NB were analyzed through histomorphometric analysis. A BV, B BIC, C NB

Figure 9

Bone observation through tissue staining for Beagle dog. Observation of the bone morphology of the implant spiral of the rabbit tibia that was sacrificed in 6 weeks (A~C) and 12 weeks (D~F). Implant placement groups were as follow. MA (A), (D); MS (B), (E); ME (C), (F).

Figure 10

Histomorphometric analysis for Beagle dog. (A) Measure the Bone Volume between the threads. (B) Measurement of contact ratio between screw thread and bone. (C) Measurement of the New bone area created between the threads. (D) Measurement of removal torque 12 weeks after implant placement.

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