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Bone response around titanium alloy implants in osteoporosis

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Few studies have investigated bone healing properties of the extracted tooth socket in osteoporosis. We evaluated the bone's response to mandibular incisor extraction and titanium alloy screw placement in a rat model of osteoporosis both *in vitro* and *in vivo.* Ovariectomy (OVX) or a sham operation (control group) was performed at 9 weeks of age. For the *in vitro* study, alkaline phosphate activity at 7 and 14 days, osteocalcin production at 21 and 28 days, and calcium deposition at 21 and 28 days were measured on titanium alloy disks. For the *in vivo* study, animal body weights were measured every 3 weeks to observe changes. Titanium alloy screws were placed into the the mandibular incisor extraction sockets. At 3, 6 and 9 weeks post-insertion, histological analyses were performed. At all measured time points, cells of the control group showed significantly more alkaline phosphate activity, osteocalcin production, and calcium deposition than cells of the OVX group. Healing processes of the extraction socket after insertion of a titanium screw differed between the control and OVX groups. Histopathologically, ovariectomized rats showed a marked inhibition of the formation of new bone in the extraction socket. (J Osaka Dent Univ 2015 ; 49(1) : 11–19)

Key words : Implant ; Osteoporosis ; Osseointegration

INTRODUCTION

There has been a concerted effort among material scientists and clinicians worldwide to improve the performance of dental implants, with the aim of accelerating and maintaining their integration into hard and soft tissues and/or extend their range of application. The use of osseointegrated implants in the treatment of edentulous patients has become a common alternative to conventional prosthetic dentistry. The success of osseointegration depends in part on the state of the host bone and its healing capacity, and concerns have been raised about various conditions affecting its quality and quantity.

Osteoporosis, which is a major health problem for the elderly population, is a reduction in the volume of bony tissue relative to the entire bone volume. The underlying clinical problem is a deficiency of bone mass. Because age and sex are reported to be important risk factors for osteoporosis,[1–3] the rate of implant loss caused by the failure of osseointegration may also be expected to increase correspondingly.[4] Thus, a large proportion of the target population for dental implants may be at high risk of implant failure. If so, the choice between conventional treatment and implant-supported prostheses in the prosthodontic treatment of older patients would need to be reassessed. For that reason, systems of dental care and plans for regenerative medicine need to be established based on an understanding of osteoporosis. Various experiments are being undertaken using osteoporotic models to obtain findings for that purpose.

Effects such as delays in new bone formation in the maxilla and mandible and incomplete bone repair in the extraction socket in rat models with osteoporosis have been reported.[5–11] In contrast, Mori *et al.* has reported that good osseointegration was obtained even in groups with decreased bone mineral content following ovariectomy.[12] Shoji *et al.* reported that the effect of ovariectomy (OVX) on osteoprogenitors is either

small or develops later in the mandible relative to the femur, and OVX increases the turnover of alveolar bone in the healed extraction socket of the rat mandibular incisor, resulting in a decrease in cancellous bone volume over time.[13, 14] Findings have thus differed among researchers.

In this study, we investigated bone responses around titanium alloy implants in a rat model of osteoporosis both *in vitro* and *in vivo.*

MATERIALS AND METHODS

Experimental animals

The experimental animals were 66 nine-week-old female Wistar rats (Shimizu Laboratory Supplies, Kyoto, Japan). The rats were divided into two groups : an experimental group subjected to complete bilateral OVX (OVX group, n = 33) ; and a control group subjected to a sham operation (control group, n = 33). At 15 weeks, the mandibular left lateral incisor was extracted, and the course of healing was observed every 3 weeks thereafter. *Ad libitum* access to solid feed and water was provided throughout the experimental period. Rats were weighed every 3 weeks to observe growth until the time of sacrifice.

Study protocols were approved by the Animal Experiment Committee of Osaka Dental University (Approval No.13–02039), and conducted in accordance with the University guidelines for animal experiments.

Cell cultures

The rat cells were obtained from the femurs of OVX and control rats for *in vitro* experiments. OVX and control cells were maintained in growth medium containing minimal essential medium (Nacalai Tesque, Tokyo, Japan), 10% fetal bovine serum (FBS ; Nacalai Tesque), and Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque), and the medium was cultured in a humidified atmosphere with 5% CO_2 at 37 °C. After 3 days, the medium was replaced with the non-adherent cells removed, and thereafter the medium was changed every 3 days. When the culture grew to about 80% confluence, cells from both groups were trypsinized using 0.5 g/L trypsin and 0.53 mmol /L EDTA (Nacalai Tesque), and then seeded on specimens at a density of 4×104 cells/cm^2 with unprocessed titanium alloy disks (Ti6Al4V). The medium was removed and replaced with differentiation medium containing 10% FBS, Antibiotic-Antimycotic Mixed Stock Solution, and osteogenic supplements (10 mM β-glycerophosphate (Wako Pure Chemical Industries, Osaka, Japan), corbic acid (Nacalai Tesque), and 10 nM dexamethasone (Nacalai Tesque)). This differentiation medium was changed every 3 days.

Alkaline phosphatase activity

After 7 and 14 days of culture, cells were washed with PBS, lysed with 200 μL of 0.2% Triton X-100 (Sigma, St. Louis, MO, USA), and the lysate was transferred to a microcentrifuge tube containing a 5-mm hardened steel ball. Tubes were agitated on a shaker (Mixer Mill Type MM 301 ; Retsh Gmbh & Co., Haan, Germany) at 29 Hz for 20 sec to homogenize the sample. Alkaline phosphatase (ALP) activity was measured using the Alkaline Phosphatase Luminometric ELISA Kit (Sigma) according to the manufacturer's protocol. The reaction was terminated with 3 N NaOH to a final concentration of 0.5 N NaOH, and p-nitrophenol production was measured by absorbance at 405 nm using a 96-well microplate reader (SpectraMax® M5 ; Molecular Device, Sunnyvale, CA, USA). DNA content was measured using the PicoGreen dsDNA Assay Kit (Invitrogen) according to the manufacturer's protocol. To normalize ALP activity, the amount of ALP was normalized to the amount of DNA in the cell lysate. All data are shown as mean and standard deviation. Statistical significance was determined by Student's *t*-tests.

Osteocalcin ELISA analysis

The sandwich enzyme immunoassay used in this study is specific for rat osteocalcin (OCN) and can measure its levels directly in cell culture supernatant after 21 and 28 days of culture. The OCN levels in cell culture supernatant were measured using a commercially available ELISA kit (Rat Osteocalcin ELISA Kit DS ; DS Pharma Biomedical, Osaka, Japan) according to the manufacturer's instructions. All data are shown as mean and standard deviation. Statistical significance was determined by Student's *t*-tests.

Mineralization

Calcium deposited in the extracellular matrix was measured after dissolution with 10% formic acid. The amount of calcium was quantified using a Calcium E-test Kit (Wako Pure Chemical Industries). After 21 and 28 days of culture, 1 mL Calcium E-Test reagent and 2 mL kit buffer were added to 50 μL of collected medium, and the absorbance of the reaction products was measured at 610 nm using a 96-well microplate reader (SpectraMax® M5 ; Molecular Devices, Sunnyvale, USA). The concentration of calcium ions was calculated from the absorbance value relative to a standard curve. All data are shown as mean and standard deviation. Statistical significance was determined by Student's *t*-tests.

Extraction of the mandibular left lateral incisor in the rats

Based on the method described by Sato,[15] the rats were lightly anesthetized with isoflurane (Isoflurane Rhodia® ; Nissan Chemical, Tokyo, Japan) a total of four times, at 14, 11, 7, and 4 days before tooth extraction, as a preparatory procedure. After fixing the animal in a prone position in a holding device, the left lower lateral incisor was cut off at the height of the interdental papilla using a dental turbine, taking care not to damage the interdental papilla or adjacent tooth. The incisal edge of the maxillary left lateral incisor was also reduced out of occlusion.

Subsequently, 15-week-old rats under isoflurane anesthesia were placed under general anesthesia by intraperitoneal injection of pentobarbital sodium (Nembutal® ; Dainippon Pharmaceutical, Tokyo, Japan). Then, after completely luxating the tooth with an elevator modified for rats, the mandibular left lateral incisor was pulled along the long axis using a needle holder for extraction, taking care not to rotate the tooth. The mandibular right lateral incisor was not extracted, to allow the rat to continue ingesting food. After tooth extraction, all rats continued to eat normal solid feed (Fig. 1).

Titanium alloy screw implants

Soon after extraction of the mandibular left lateral incisor in both the OVX and control groups, a titanium al-

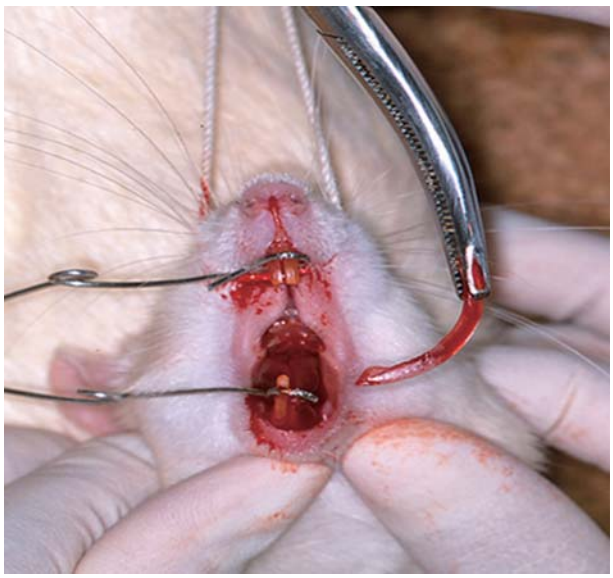

Fig. 1 Tooth extraction.

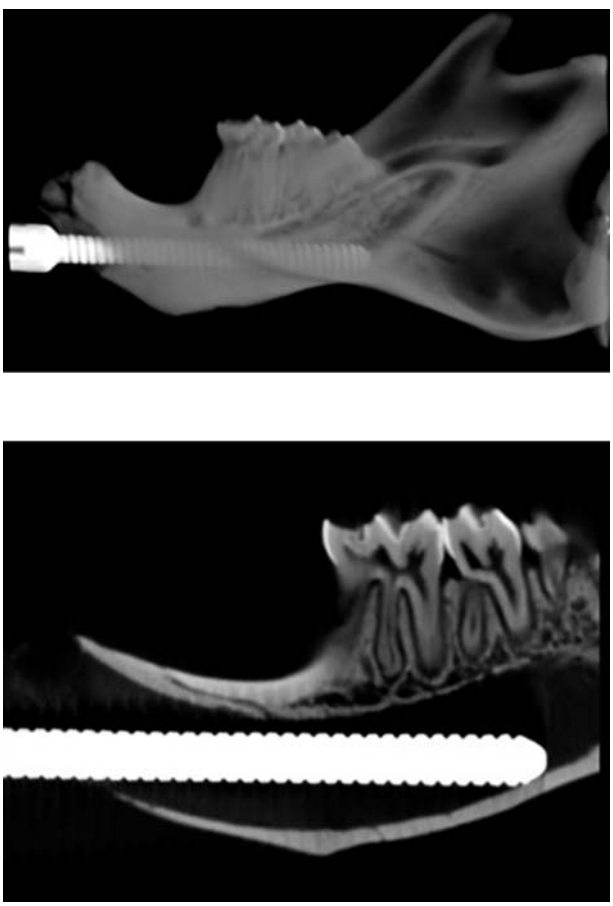

Fig. 2 X-rays showing insertion of the titanium alloy screw.

loy (Ti-6Al-4V) screw (SNK Screw Post Titanium®; Dentsply Sankin, Tokyo, Japan) with a diameter of 1.2 mm and length of 17.0 mm was implanted. At this time, the screw was set so as to obtain early fixation. Several locations, including the screw tip, were thus placed in contact with the wall of the extraction socket. The position of the screw in the mandibular incisor extraction socket was confirmed using a three-dimensional X-ray analyzer[16] (Fig. 2).

Specimen acquisition

At 3, 6 and 9 weeks after extraction, heparin sodium anticoagulant (Novo-Heparin Injection 1000®; Mochida Pharmaceutical, Tokyo, Japan) was injected intraperitoneally. Rats were killed 30 min later with an overdose of pentobarbital sodium injected intraperitoneally. Following perfusion fixation with 10% neutral-buffered formalin according to standard protocol, the mandible was removed in a single piece and cut into right and left halves at the midline using a scalpel. Only the left side was used as a specimen.

Preparation of pathological specimens

The resected left mandibular specimens fixed in formalin were decalcified with Plank-Rychlo solution[23] at 4°C for 3 days. The titanium alloy screw was removed and neutralized with 5% sodium sulfate for 24 h, dehydrated with an ascending alcohol series by routine methods, and embedded in paraffin. Mandibular sections were prepared at about 20 μm in thickness in the sagittal plane along the screw insertion area and stained with hematoxylin-eosin.

Histological observation

In addition to histological observation, grade evaluation ([1] slight or a small range, [2] moderate, and [3] marked or a wide range) was performed for various items by the same observer to facilitate evaluation of histological changes. When there were no applicable findings, the grade was classified as [–].

Measurement of removal torque of the titanium alloy screw

After formalin fixation following standard protocol, the removed left mandible was held carefully so as to avoid touching the screw and a hand-held torque gauge (HTG2-50N; Imada, Toyohashi, Aichi, Japan) was attached in line with the long axis of the implant. Removal torque was measured once for each specimen.[17]

RESULTS

Changes in weight

Although the control group was heavier than the OVX group at each age (weeks), both groups gained weight over time. Weight gains were more marked in the OVX group than in the controls at 12 weeks of age and in the OVX group at 15 weeks (Fig. 3).

Alkaline phosphatase activity

Cell differentiation was assessed by measuring the activity of the differentiation marker, ALP, in the OVX and control groups at 7 and 14 days. At both time points, ALP activity was significantly lower in cells of the OVX group than in the controls (Fig. 4).

Osteocalcin production

Figure 5 shows the production of OCN in the OVX and control groups at 21 and 28 days. The presence of OCN in the supernatant of the OVX group was significantly lower than that of the controls.

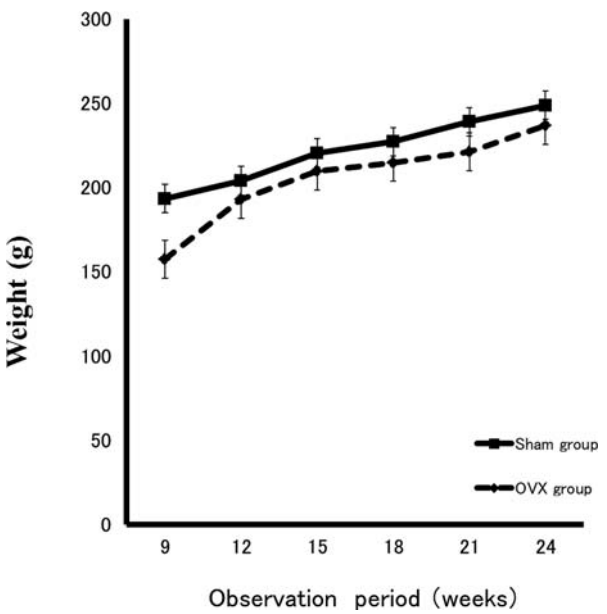

Fig. 3 Changes in weight.

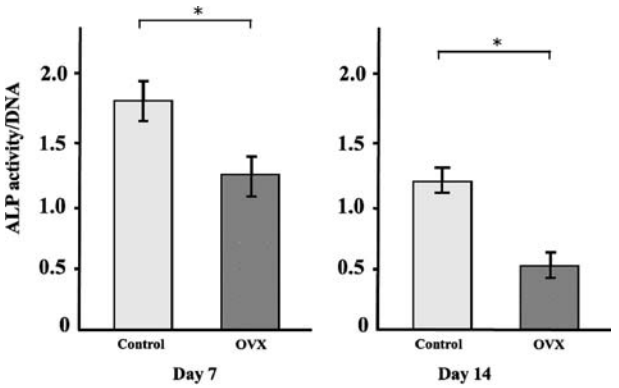

Fig. 4 ALP activity (*$p < 0.05$).

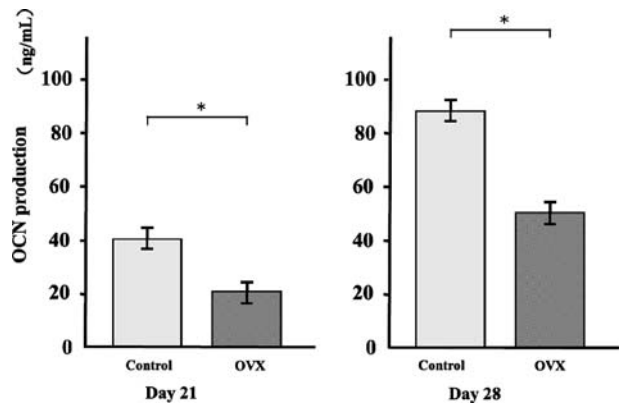

Fig. 5 OCN production.

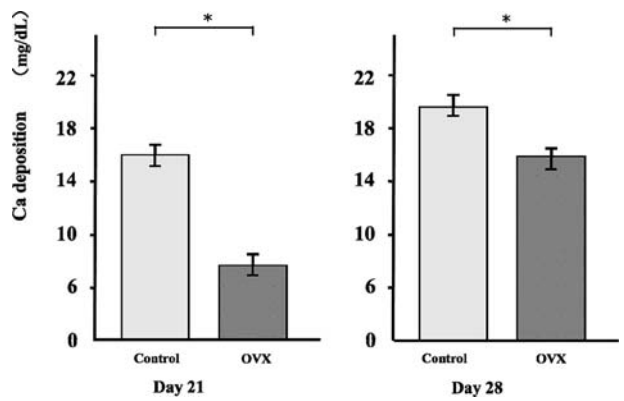

Fig. 6 Ca deposition.

Mineralization

Figure 6 shows calcium deposition in the extracellular matrix of cells in the OVX and control groups at 21 and 28 days. Calcium deposition was cumulative in the culture wells ; measured levels increased normally with exposure time. As shown in the figure, significantly more calcium was deposited by cells in the controls at 28 days than by cells in the OVX group.

Histological findings

Findings in the healing process of the extraction socket after insertion of a titanium alloy screw differed between the control and OVX groups. Although, osteoblast proliferation was observed 3 weeks after tooth extraction in the controls, bone formation was negligible. Six weeks after tooth extraction, there were increases in the continuity of bone tissues around the screw, bone formation in the concave area of the screw, and mature bone (histologically completed bone tissue). Nine weeks after tooth extraction, a further increase in mature bone was observed.

Although, findings 3 weeks after tooth extraction in the OVX group were similar to those in the control group, bone formation 6 weeks after tooth extraction was delayed compared with the controls. This delay became more marked after 9 weeks. In particular, bone formation in the concave area of the screw and mature bone formation were markedly delayed in this group.

Osteoblast proliferation was observed in all samples. Proliferation was dense in new bone around the screw and the surrounding bone tissue. In the control group, osteoblast proliferation tended to decrease with time from 3 to 9 weeks after tooth extraction. In the OVX group, osteoblast proliferation was similar to that in the controls 3 weeks after tooth extraction, more marked than in the controls after 6 weeks, and similar to the controls after 9 weeks, despite no progression in bone formation. In the control group, there were increases in the volume of bone tissue around the screw with time, and in the continuity of bone tissue. In the OVX group, the continuity of bone tissue was poorer than that of the controls even after 6 weeks and markedly poorer after 9 weeks because of a lack of progress in subsequent osteogenesis.

In the control group, bone tissue formation in the screw concave area was negligible 3 weeks after tooth extraction and became marked over time between 6 and 9 weeks after extraction, showing an increase in mature bone. Although, bone tissue formation in the same area of the OVX group was similar to

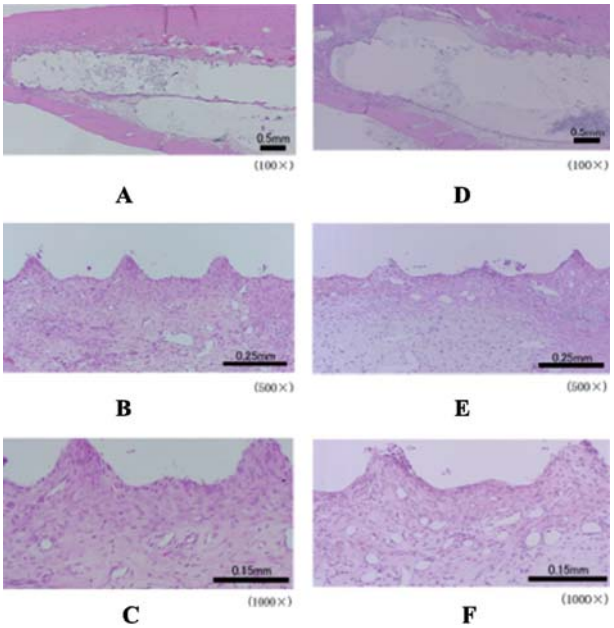

Fig. 7 Three weeks after titanium screw insertion following tooth extraction (hematoxylin-eosin staining). (A–C) There was marked bleeding in the socket of the control group, and many osteoblasts were present around pieces of bone tissue. (D–F) There was marked bleeding in the socket, and the concave area of the screw was unclear.

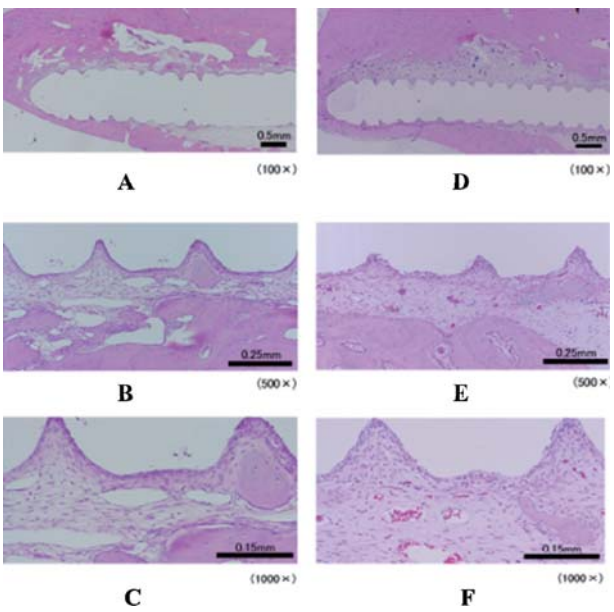

Fig. 8 Six weeks after titanium screw insertion following tooth extraction (hematoxylin-eosin staining). (A–C) Although bone formation was infrequently observed in the controls in the concave area of the titanium screw concave area as a whole, mature bone increased in areas where there was bone formation. (D–F) Although many osteoblasts were present around pieces of bone tissue in the OVX group, no bone tissue formation was observed in the concave area of the titanium screw.

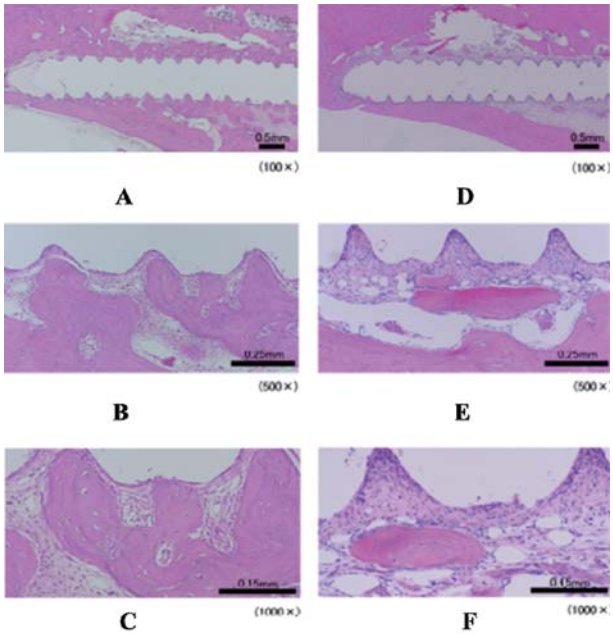

Fig. 9 Nine weeks after titanium screw insertion following tooth extraction (hematoxylin-eosin staining). (A–C) Many bone tissue pieces and continuous bone tissues were present in the controls, and mature bone was mainly observed in the concave area of the titanium screw. (D–F) Although there were only a few continuous bone tissues, there was a moderate number of bone tissue pieces. No mature bone was observed in the concave area of the titanium screw.

that in the controls 3 weeks after tooth extraction, it was delayed compared with the controls after 6 weeks, there was no increase in mature bone, and the difference with the controls further increased after 9 weeks. In most specimens, localized inflammatory cell infiltration was observed on the labial and lingual sides of the extraction socket (Figs. 7–9).

Changes in removal torque of the titanium alloy screw

In the control group, torque increased significantly with the length of the observation period between 3 and 9 weeks ($p<0.05$). In the OVX group, significant differences in torque were seen between 3 and 9 weeks and between 6 and 9 weeks ($p<0.05$). Compared with the controls, removal torque was significantly lower in the OVX group between 6 and 9 weeks ($p<0.05$, Fig. 10).

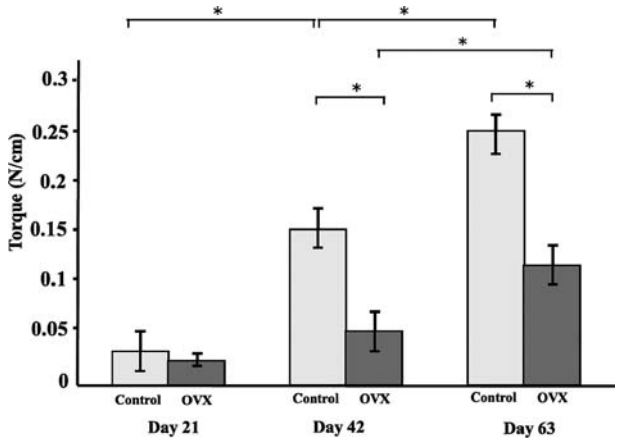

Fig. 10 Removal torque.

DISCUSSION

Contact between implants and surrounding bone is required if useful functioning of dental implants is to be obtained. This process is affected by various systemic and local conditions, including metabolic bone diseases such as osteoporosis. Various authors[18, 19] have suggested on the basis of clinical observations that osteoporosis is not always a risk factor in osseointegration. However, the effects of estrogen deficiency on contact between implants and new bone have not been extensively examined histologically. In this study, we examined the early establishment of implant-bone integration in OVX and control model rats using *in vitro* experiments, histomorphometry, and the biomechanical implant tests. We found that cells of the control group had significantly more ALP activity, OCN production, and calcium deposition than did cells of the OVX group. Findings in the healing process of the extraction socket after insertion of a titanium screw differed between the control and OVX groups.

Using ovariectomized rats, histopathological results showed that the formation of new bone in the extraction socket was markedly inhibited. In experiments on osteoporosis, ovariectomized rats are widely used as model animals for estrogen-deficient-type osteoporosis.[20, 21] In this model, estrogen deficiency artificially induced by ovariectomy promotes both bone resorption and formation. When bone resorption surpasses bone formation there is a reduction in the amount of bone, inducing symptoms resembling those of high turnover osteoporosis.

Estrogen deficiency after ovariectomy has been reported to lead to weight increases.[22] In our experiment, weight markedly increased after ovariectomy up until 3 weeks after ovariectomy (from the age of 9 weeks to 12 weeks) but gradually increased thereafter in both the control and OVX groups. The control group was heavier than the OVX group at all ages. In the OVX group, the degree of weight gain previously reported was not observed. This may be because the difficulty in eating after extraction of the mandibular incisor markedly affected the OVX group.

For the *in vitro* study, we used cells of the same animals as in the *in vivo* study, thus reducing inter-subject variability. ALP activity, OCN production, and calcium deposition were all elevated in the controls. ALP activity is an early marker of osteoblast differentiation, and OCN production and calcium deposition are functional phenotypes expressed in the middle and late stages of culture. It has been reported that using human cells in studies of biomaterials under osteoporotic conditions presents difficulties because of their variability.[23] The histological heterogeneity of osteoblast dysfunction observed in osteoporotic patients indicates that the disease is caused by disturbances at a variety of target sites along osteoblast function pathways.[24, 25] Varying results have been obtained by different authors when comparing healthy osteoblasts with cells from osteoporotic patients. Even if the phenotype characterization did not show variations between normal and osteopenic bone-derived cells, differences have been observed in the rate of proliferation,[26, 27] and in the response to cytokines, hormones, and mechanical stimuli.[28, 29]

Histological observations indicated that virtually no new bone formation was seen at 3 weeks after implantation of the titanium alloy screw, despite an observed increase in osteoblasts around the screw. Given that finding, we predicted that removal torque in the present experiment would be low in both groups. In the controls, a comparison between 3 and 6 weeks after extraction showed that the removal torque had increased significantly at 6 weeks. In the OVX group, no significant difference was seen between 3 and 6

weeks after extraction. However, removal torque was significantly higher in the controls than in the OVX group. This was attributed to the finding of greater increases in continuity of bone tissue around the screw, in the amount of bone formation in the screw thread troughs, and mature bone (histologically complete bone tissue) in the controls compared with the OVX group at 6 weeks.

In the control group, a significant increase in removal torque was seen between 6 and 9 weeks. While the OVX group still displayed a significant difference, it was rather small and markedly different from that of the controls. This was thought to be because of increased levels of mature bone and greater continuity in the control group at 9 weeks, whereas the OVX group showed marked delays in bone formation at 6 and 9 weeks. In particular, bone formation in the screw thread troughs and mature bone formation were delayed. Estrogen was thought to be deficient in the OVX group and normal formation of new bone would thus have been inhibited by the localization of inflammatory cytokines in the extraction socket. As a result, bone union with the surface of the titanium alloy screw was insufficient,[30–33] leading to significant differences in removal torque.

CONCLUSION

The findings of this study suggest that healing of the extraction socket and osseous union immediately following placement of an implant may also be delayed in humans if deficiencies in estrogen are present because of menopause or aging. However, other studies with long-term observations have reported that although sufficient bone union can be expected and that bone union is inhibited around the implant in cancellous bone when estrogen is deficient, but that bone union in cortical bone is unaffected. In addition to histological and biomechanical analyses, future investigations will need to conduct observations over a longer term and evaluate bone mass and density in the extraction socket in greater detail.

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