Effect of Valproic acid on bone healing in rat

by

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Preface

This thesis titled “Effect of Valproic acid on bone healing in rat” is submitted to the Niigata University Graduate School of Medical and Dental Sciences, Japan, in partial fulfillment of the requirements for the PhD degree.

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The undersigned certify that they have recommended to the Niigata University Graduate School of Medical and Dental Sciences for the acceptance of this thesis titled "Effect of Valproic acid on bone healing in rat" submitted by Md. Mamunur Rashid in partial fulfillment of the requirements for the PhD degree carried out at the department of Bio-Prosthosthodontics.

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Abstract

Objectives: Sufficient bone quality and quantity before placement of dental implant are necessary to achieve successful result. Although numerous materials and methods have been reported regarding bone augmentation, more reliable and feasible ones are still required in this field. Valproic acid (VPA) regulates osteoblast differentiation through Runx2-dependent transcriptional activation. The aim of the present study was to evaluate the effect of systemic administration of VPA on bone regeneration in rat maxillary bone cavity.

Materials and Methods: Forty two Wistar rats were divided into control and experimental groups. Upper first and second molars were extracted at the age of 4 weeks. Three weeks after extraction, experimental group received intraperitoneal injection of VPA for 7 days prior to the preparation of bone cavity at the first molar area. Rats were sacrificed on day 3, 7, 14, and 21 and samples were prepared for micro-CT and histological analyses. Serum was used for alkaline phosphatase (ALP) activity measurement.

Results: Micro-CT analysis showed higher amount of newly formed bone, bone volume fraction (BV/TV), trabecular thickness (Tb.Th) and less trabecular separation (Tb.Sp) in

experimental group than control group. VPA treated animals showed significantly higher ALP activities at 7, 14 and 21 days than control group. According to the histological observations, the amount of newly formed bone was clearly larger in experimental group at 7, 14, and 21 days than control group.

Conclusion: Since systemic administration of VPA accelerated bone regeneration in rat maxillary bone cavity, it might be useful for the bone augmentation prior to, or upon implant therapy.

Key words

bone regeneration, histone deacetylase inhibitors (HDACi), valproic acid (VPA), bone cavity, osteoblast

Introduction

The concept of ‘Top-down treatment’ or ‘Prosthetic driven implant’ is essential for appropriate replacement of the lost teeth by dental implants. In this regard, sufficient bone quality and quantity to support implant are prerequisites. Loss of teeth often results in complex horizontal and vertical alveolar ridge defects. Therefore, alveolar bone augmentation before placement of dental implant is often required.

Various bone augmentation techniques using autogenous bone grafts alone or combined with bone substitutes have been achieving certain degree of success. They possess osteogenic, osteoconductive and osteoinductive properties (Becker et al. 1994; Egusa et al. 2012). However, even though autologous bone grafts exhibit high variability in their osteogenic potential among harvest sites and individual (Crepsi et al. 2007), it could result in a less-than desirable clinical outcome. In addition, they have limitations because of mainly donor site morbidity, infection and/or, delayed healing (van Steenberghe et al. 1997; Haas et al. 1998; Lee 2006; Babiker et al. 2012). Bone substitutes mostly used as hydroxyapatite or β-tricalcium phosphate matrices have been shown to be osteoconductive (Dahlin et al. 1998; Haas et al. 1998). However, no reliable long-term alternatives to autogenous bone grafts have been established so far (Payer et al. 2013). Growth-factor-based regenerative therapies and/or multipotent *ex vivo* expanded cells for tissue engineering could not yet be realized with satisfying and predictable outcomes (Schimming & Schmelzeisen 2004; Ivannovski 2009; Sahrman et

al. 2011). Even though existing bone augmentation techniques have limited problems clinically, improved and more reliable procedures for bone regeneration are necessary to optimize the treatment outcomes.

Epigenetic regulation of gene expression is recognized as a central mechanism that governs cell stemness, determination, commitment, and differentiation (Oreffo et al. 2005; Lotem & Sachs 2006; Wu & Sun 2006; Alexanian 2007; Feinberg 2007; Delcuve et al. 2009). Histone acetyl-transferases (HATs) and Histone deacetylases (HDACs) are enzymes involved in remodeling of chromatin structure and epigenetic integrity. HATs are responsible for acetylation of histone which promotes a more relaxed chromatin structure, allowing transcriptional activation. On the other hand, HDACs promote chromatin condensation and act as transcription repressors (Lehrmann et al. 2002). Eighteen HDACs have been identified in human, and they are divided into four subclasses: class I HDACs (1, 2, 3, and 8), class IIa HDACs (4, 5, 7, and 9), class IIb HDACs (6 and 10), class III HDACs (SIRT1 to 7), and class IV HDACs (HDAC11). Runx2 activity has been implicated in the inhibitory action of HDACs on osteoblast differentiation. Several class I HDACs (HDAC1 and 3) and class II HDACs (HDAC4, 5, 6, and 7) interact with Runx2 and repress its transcriptional activity (Westendorf et al. 2002; Schroeder et al. 2004; Lee et al. 2006; Jensen et al. 2008). Histone deacetylase inhibitors (HDACi) regulate osteoblast differentiation by enhancing Runx2-dependent transcriptional activation and accelerate osteogenesis through up-regulating osteoblast marker genes in mesenchymal stem cells (MSCs) of bone marrow (Cho et al. 2005),

osteogenic cell lines (Schroeder & Westendorf 2005), and murine calvarial organ cultures (Jeon et al. 2006).

Valproic acid (2-n-propylpentanoic acid, VPA) is an effective and widely used antiepileptic drug for more than 30 years. HDACi activity of VPA was observed in 2001 (Gottlicher et al. 2001; Phiel et al. 2001). It suppresses class I and class II HDACs. VPA promotes cell proliferation of pre-osteoblast cell line and activates Runx2 transcriptional activity in MC3T3-E1 (Schroeder & Westendorf 2005). VPA induced differentiation and accelerated mineralization of human mesenchymal stem cells (Hyun et al. 2005) and pulp derived cells (Duncan et al. 2012). Although previous *in vitro* studies have shown the beneficial effects of VPA on osteoblast differentiation and mineralization, it remains unknown whether systemic administration of VPA could improve bone regeneration *in vivo*. The objective of this study was to evaluate the effect of systemically administrated VPA on bone healing of maxillary bone defect in rats. In this study, bone cavity healing was assessed and acquired results will be applied to establish a novel bone augmentation therapy using epigenetic theory.

Materials and methods

Animals and experimental procedure

Forty two 4-week-old male Wistar rats (Charles River, Japan) were divided control and

experimental groups. At the age of 4 weeks, 1st and 2nd maxillary molars on both sides were extracted. At 3 weeks after extraction, experimental group received intraperitoneal (i.p) injection of VPA at a dose of 300 mg/kg 2 times daily (Feng et al. 2008) and control group received saline injection for consecutive 7 days before cavity preparation. At the age of 8 weeks, full thickness flaps were elevated at the recipient sites (maxillary 1st molar area on both sides) and bone cavities were prepared by drilling with a slow speed dental hand piece at 500 rpm equipped with a Peeso reamer (diameter 1.7 mm) in both groups. Profuse irrigation with sterilized physiological saline was maintained throughout the drilling. Flaps were repositioned and sutured with nylon.

All animal experiments in this study were approved by the ethical committee of Niigata University and conducted according to the Niigata University Guidelines for Animal Experimentation.

Body weight measurement

Body weight of all rats (both experimental and control groups) was measured once a day for consecutive 7 days during the period of VPA and saline injection.

Micro-CT images and bone analysis

In the present study, 7, 14 and 21 days samples were scanned in same manner using a

micro-CT scanner (Elescan). Briefly maxilla was placed on a custom made jig with axial direction and palatal area facing towards scanner. The scan was performed at 53kV, 100μA, 900 projections and 0.5mm aluminum filter. Based on the serial scanned images, 3D images were reconstructed using TRI/3D-BON software (RATOC, Japan).

Since main purpose was to observe and analyze newly formed bone, the region of interest (ROI) was selected at the area of bone defect (Fig. 1). Bone volume fraction (BV/TV), Trabecular thickness (Tb.Th) and Trabecular separation (Tb.Sp) within ROI were calculated which represent percentage of mineralized bone volume in volume of total bone tissue within the area of bone cavity, thickness and organization of trabeculae respectively.

Serum alkaline phosphatase (ALP) activity measurement

Blood samples were collected during euthanasia at 3, 7, 14 and 21 days after cavity preparation. The blood was allowed to clot and centrifuged at 3000 rpm for 10 minutes (Ayalogu et al. 2001). Serum was harvested and stored at −20 °C until bio-chemical assays. ALP activity was measured using ALP kit (Takara, Japan). Briefly, serum samples were diluted with extraction solution as manufacturer's instruction. At first, 50 μl diluted serum from each sample was taken in each well of 96 well plate. Then 50 μl of substrate solution was added and plate kept in 37 °C for 60 minutes. After that 50 μl of stop solution (0.5N NaOH) was added to each well. Absorbance was measured at 405

nm using micro plate reader. Each experimental sample was assayed in triplicate.

Histological observation

Animals were sacrificed at 3, 7, 14 and 21 days after cavity formation. At the appointed time, they were anesthetized and fixed with a transcardiac perfusion with a fixative containing 4% paraformaldehyde (pH 7.4). The specimens were decalcified in a 10% EDTA solution for 4 weeks at 4 °C. Serial paraffin sections were prepared sagittally at 5-μm thickness; sections from the most central part of the defect were chosen and stained with Hematoxylin & Eosin for histological observation.

Statistical analysis

All numerical data are represented as the mean ± SD ($n \geq 3$). Two-group comparisons were performed by using Student's *t* test. Statistical significance was defined at $p < 0.05$.

Results

Body weight

No significant difference in body weight was observed between control and experimental group during the period of saline and VPA injection (Fig. 2)

Micro-CT images and bone analysis

Micro-CT images showed greater amount of newly formed bone in the defect cavities of experimental animals compared to the controls at 7 and 14 days after defect formation (Fig. 3a-h). After 21days, defect seemed to be completely healed up in experimental group (Fig. 3i-l). Quantitative analysis of newly formed bone showed a gradual inclination of bone volume fraction (BV/TV) in both groups from day 7 to 21. However, experimental group showed significantly higher BV/TV than control at 14 and 21days (Fig. 4a). Trabecular thickness (Tb.Th) was also gradually increased in both groups; however, experimental group showed higher Tb.Th than control group at 14 and 21 days after defect preparation (Fig. 4b). A gradual declination of trabecular separation (Tb.Sp) was observed in both experimental and control group from day 7 to 21. Nevertheless, at 14 and 21days after defect preparation, experimental group showed significantly less Tb.Sp than control group (Fig. 4c).

Serum ALP activity

Serum ALP activity was increased gradually in both groups after bone cavity preparation. However, VPA treated animals showed significantly higher ALP activities at 7, 14 and 21 days than control group (Fig. 4d).

Histological observation

Three days after cavity preparation

Control group showed numerous red blood cells and inflammatory cells at the defect area (Fig. 5a and b). On the other hand, defect area in experimental group was mainly occupied by inflammatory cells (Fig. 5c and d). In the centre of the defect, cell debris and bone fragments were observed in both groups. Preexisting bone facing cutting edge contained empty osteocytic lacunae (Fig. 5b and d).

Seven days after cavity preparation

At 7 days after defect preparation, no new bone formation was observed in control group (Fig. 5e and f); on the other hand, new bone formation was observed in experimental group at the periphery of the defect. Newly formed bone was continuous to the preexisting bone (Fig. 5g). Cuboidal or conical shaped osteoblast like cells were arranged uniformly on the surface of the newly formed bone (Fig. 5h). Bone with empty osteocytic lacunae still existed at the lateral wall of the cavity in both groups. The volume of cellular elements was observed to be equally distributed in all the defect area. No remarkable changes in histological features of the preexisting bone were found at this stage (Fig. 5f and h).

Fourteen days after cavity preparation

New bone formation was observed in both control and experimental group; however, the amount of newly formed bone was greater in experimental animals than the control group (Fig. 5i-l). Newly formed bone was extended from the surface of the parent bone into the bone defect in both groups. The osteoblast like cells, which had appeared cuboidal in shape at day 7 after defect formation, had become flattened. Several wide bone marrow areas surrounded by osteoblasts were observed in the newly formed bone in both groups (Fig. 5j and l).

Twenty one days after cavity preparation

The amount of newly formed bone was still greater in experimental group than control group (Fig. 5m-p). In the newly formed bone, an irregular arrangement of woven structure was observed in control group (Fig. 5n and p). On the other hand, lamella like structure was observed in experimental group (Fig. 5n and p). Bone marrow areas in newly formed bone became narrower than 14 days in both groups.

Discussion

The clinical outcome of dental implant is strongly depends upon the regeneration of bone tissue, which should be quali-quantitatively adequate and rapidly produced. Since

several studies have shown that HDACi have successfully enhanced osteogenic differentiation in different cells and/or cell lines through transcriptional regulation (Hyun et al. 2005; Schroeder & Westendorf 2005; Maroni et al. 2012), in our current strategy, we attempted to regenerate bone tissue through systemic administration of VPA in animal model.

One of the possible effects of VPA treatment is body weight change which in turn is thought to influence bone health (Senn et al. 2010). However, weight effects might not completely explain the VPA-treatment on bone healing observed in this study. Because none of the VPA-treated animal actually lost weight during the injection period and among individual VPA-treated animals there was no correlation between total body mass measurements and the newly formed bone measurements.

Micro-CT images showed greater amount of new bone formation in VPA treated animals than control group. Higher BV/TV and Tb.Th suggested that the osteoblastic activity was increased in experimental group. Increased distance between individual trabeculae (Tb.Sp) in control group suggested looser trabecular structure than experimental group.

VPA is widely used as an antiepileptic drug. Interestingly, patients with epilepsy show an increased fracture risk (Ecevit et al. 2004). Also, long-term oral VPA administration demonstrated a negative effect on bone growth and density (Souverein et al. 2006;

Nissen-Meyer et al. 2007). Thus, it has been extensively studied how antiepileptic drug affect bone turn over; however, no correlation between valporate medication and loss in bone mineral density (BMD) was observed (Triantafyllou et al. 2010; Aksoy et al. 2011). The changes in BMD do not seem to be caused by VPA, but rather are attributed to decreased physical activity levels, vitamin D deficiency, and secondary hyperparathyroidism (Schroeder & Westendorf 2005). In addition, it is likely that the relatively high dose and long time therapy with VPA might be responsible for these adverse effects (Sato et al. 2001; Cho et al. 2005; Niseen-Meyer et al. 2007). Therefore, in our study, we used short period of VPA systemic injection. Our micro-CT bone analysis of femur also showed no statistical difference in BV/TV, Tb.Th and Tb.Sp measurement between control and experimental group (data not shown).

Many studies have reported that HDACi can stimulate *in vitro* osteogenesis by increasing the expression of osteogenic related genes such as osteopontin, ALP, collagen- 1α, osteocalcin, and bone sialoprotein (Cho et al. 2005; Schroeder & Westendorf 2005; de Boer et al. 2006). To our knowledge, until now no *in vivo* experiment was conducted for assessing bone regeneration by systemic injection of VPA. Results from serum ALP measurement indicated that VPA might have stimulated osteoblast bone formation ability through acceleration of osteoblast differentiation or increased number of osteoblast and/or activity. As a result, bone defect healing was accelerated. No ectopic bone formation was observed in experimental group. Thus, it indicated that new bone formation only occurred at the healing site of bone cavity.

Results from histological analysis also showed a marked bone defect healing by the systemic injection of VPA in experimental group. Earlier bone formation at 7 days after cavity preparation and greater amount of newly formed bone at later stages of healing period in experimental group than control group indicated that osteoblast activity and /or number might have increased in rat body by VPA systemic injection. Although the underlying mechanism how VPA accelerated new bone formation was not clear in our study, HDACi demonstrated osteoblastic differentiation of mesenchymal stem cells (MSCs) through Runx2 activation (Cho et al. 2005; de Boer et al. 2006; Huangfu et al. 2008; Shakibaei et al. 2012). Furthermore, several *in vivo* studies stated that bone marrow stem cells or MSCs migrated out of the marrow space and differentiated into osteoblasts at the site of fracture repair (Devine et al. 2002; Kazuhiro et al. 2005) or, ectopic bone formation (Otsuru et al. 2007). Taking these into consideration, we can speculate that in our study, VPA injection might have activated bone marrow stromal cells which contain bone marrow stem cells and osteoblast precursors. Those cells come to the defect healing site through blood stream and differentiated into osteoblast. VPA might also stimulated local precursors around the healing site. As a result, osteoblast number and/or activity increased and bone formation was accelerated. Further investigation regarding healing mechanism is still needed to prove our speculation.

Because deacetylation of core histones by HDACs is associated with a ‘‘closed’’ chromatin conformation and repression of transcription, inhibition of HDACs can be

supposed to lead to activation of transcription. In fact, HDACi have been known to affect gene expression in various cells (Glaser et al. 2003; Tou et al. 2004; Yokota et al. 2004). Multiple HDACs are expressed by osteoblast. They might have specific roles in regulating osteoblast differentiation. Some HDACs (HDAC 3 and HDAC 6) interact with Runx2 and repress Runx-2-dependent transcriptional activity. VPA with other HDACi were showed that they are able to block these HDACs and increased transcriptional activity of the osteoblast differentiation marker genes through Runx2 (Westendrof et al. 2002; Hyun et al. 2005; Schroeder & Westendorf 2005).

In our study, bone defect model was recruited because bone formation would easily occur in comparison with ectopic bone formation models or extra bone formation models, and we thought the model might make it better to compare the effect of HDACi on bone formation. Since VPA turned out to accelerate bone formation in the bone cavity, it could be interpreted that VPA might be effective to improve the bone formation in case of bone augmentation. Of course, we could use this method to accelerate the osseointegration which is the result of bone formation at the interface of bone and implant.

In conclusion, this study clearly demonstrated that the systemic injection of VPA accelerated the healing of maxillary bone cavity. Therefore, systemic administration of VPA could be a potential treatment that might be useful for bone regeneration or augmentation prior to, or upon implant therapy.

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Figure legends:

Fig. 1. Three dimensional construct of micro-CT image of rat maxilla showing third molars and bone cavities. Bone cavities were prepared at the upper first molar areas on both sides at 4 weeks after extraction. Region of interest (ROI) was shown in yellow circle.

Fig. 2. Body weight of control and experimental group during the period of saline and VPA injection.

Fig. 3. Micro-CT images of maxillary bone defect on day 7 (a-d), 14 (e-h) and 21 (i-l). Horizontal (a, c, e, g, i, k) and sagittal views (b, d, f, h, j, l).

Fig. 4. Micro-CT analysis of newly formed bone (a, b, c) and serum ALP activity measurement (d). Bone volume fraction (BV/TV) (a), trabecular thickness (Tb.Th) (b), and Trabecular separation (Tb.Sp) (c). Serum ALP activity was observed on day 3, 7, 14 and 21 (d) after defect preparation.

Fig. 5. Histological specimen on day 3 (a-d), 7 (e-h), 14 (i-l) and 21 (m-p). Lower magnification (5x) images showed healing process of each bone cavity (a, c, e, g, i, k, m and o). High magnification (40x) images (b, d, f, h, j, l, n and p) showed cell contribution for new bone formation. H-E stain.

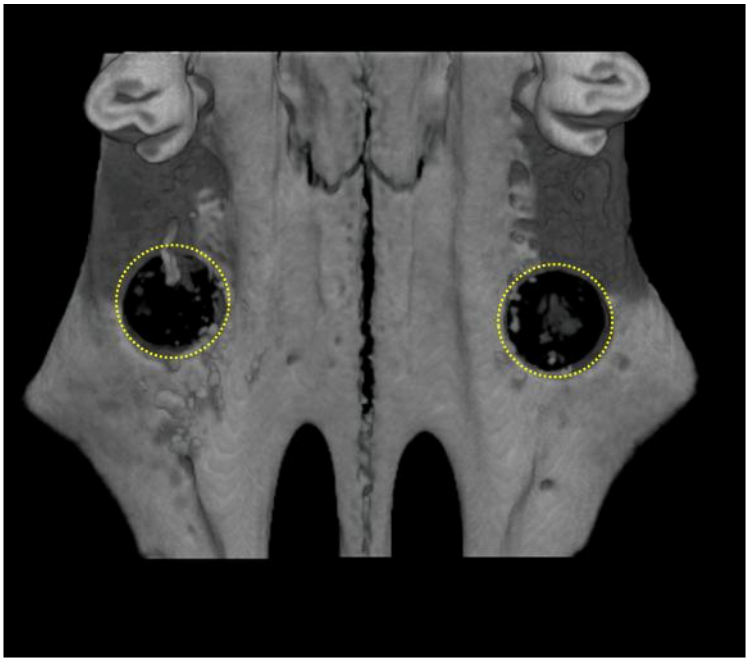


Fig. 1

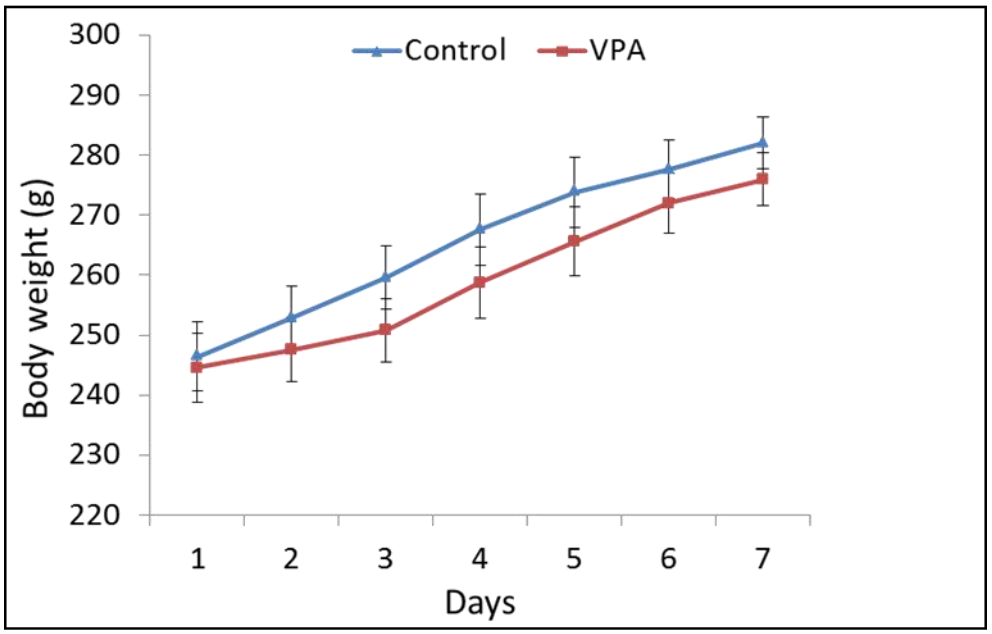

Control
VPA
Body weight (g)
300
290
280
270
260
250
240
230
220
1
2
3
4
5
6
7
Days

Fig. 2

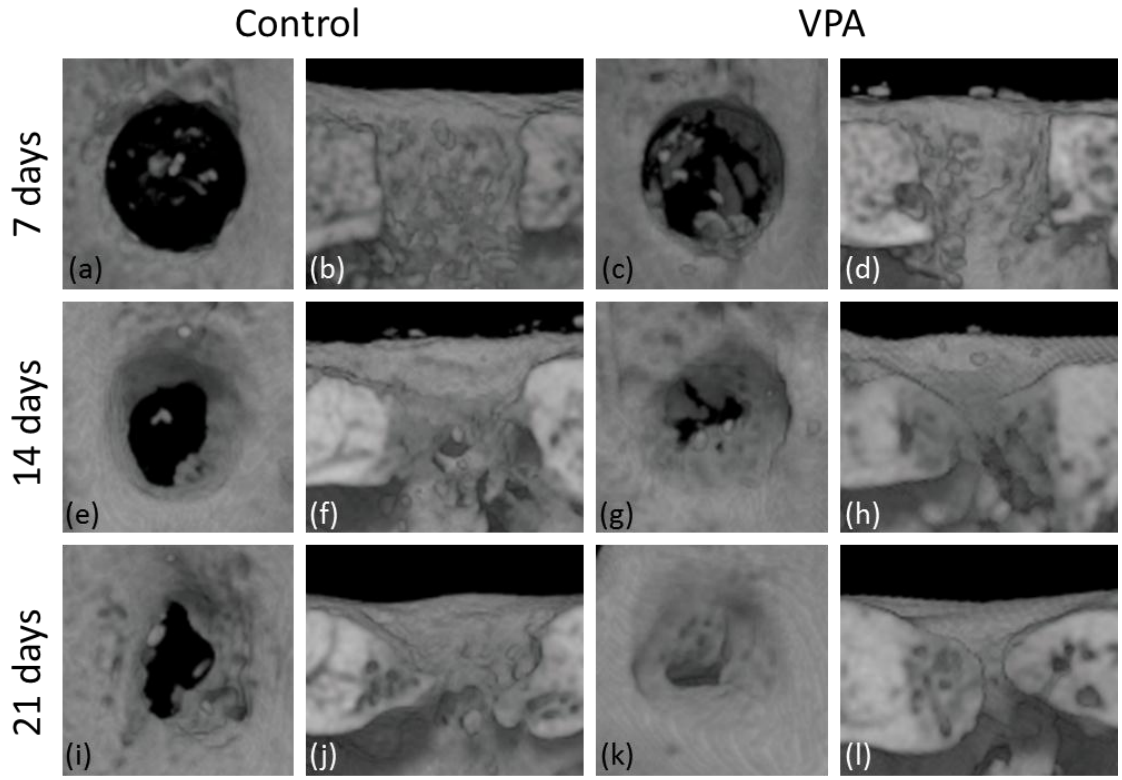
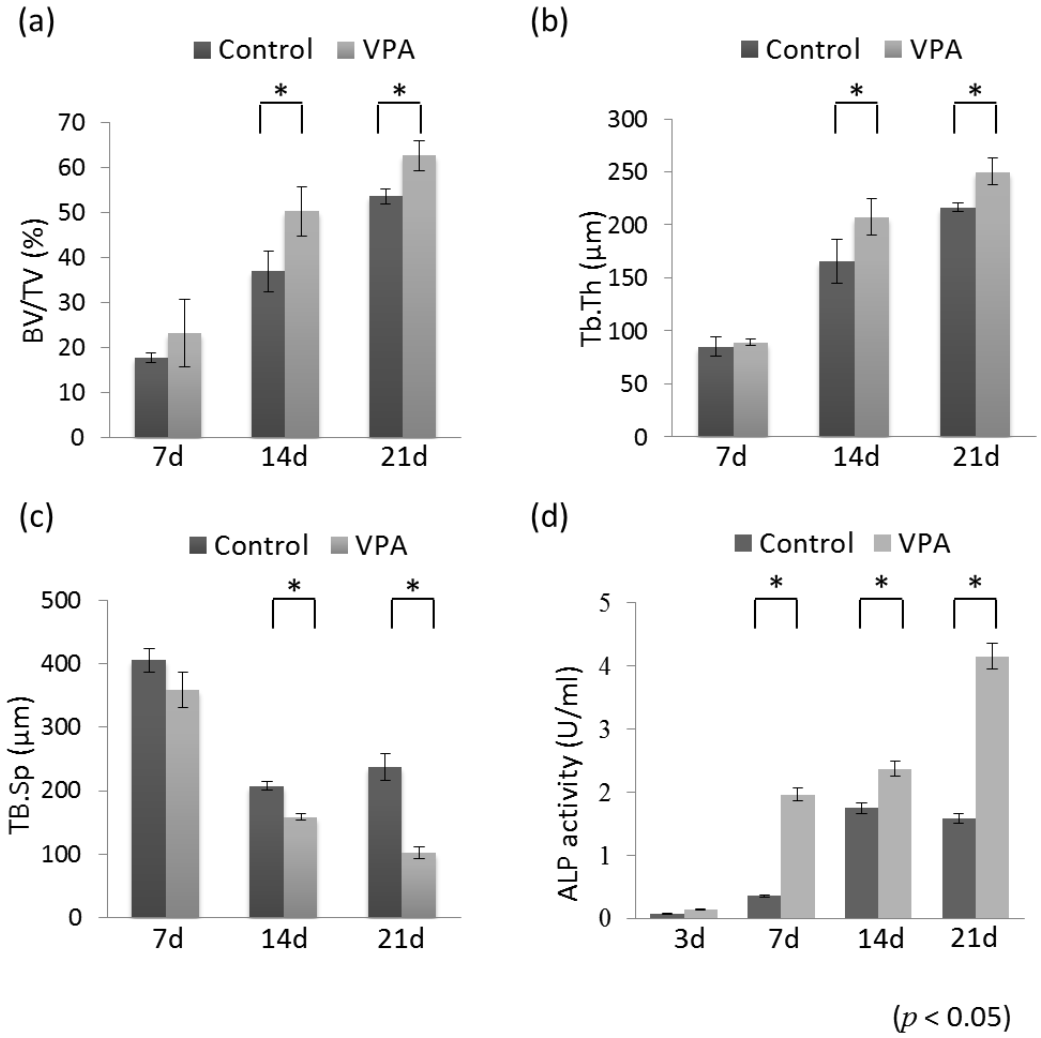


Fig. 3


(a)
Control
VPA
BV/TV (%)
0
10
20
30
40
50
60
70
7d
14d
21d
*
*
(b)
Control
VPA
Tb.Th (μm)
0
50
100
150
200
250
300
7d
14d
21d
*
*
(c)
Control
VPA
TB.Sp (μm)
0
100
200
300
400
500
7d
14d
21d
*
*
(d)
Control
VPA
ALP activity (U/ml)
0
1
2
3
4
5
3d
7d
14d
21d
*
*
*

($p < 0.05$)

Fig. 4

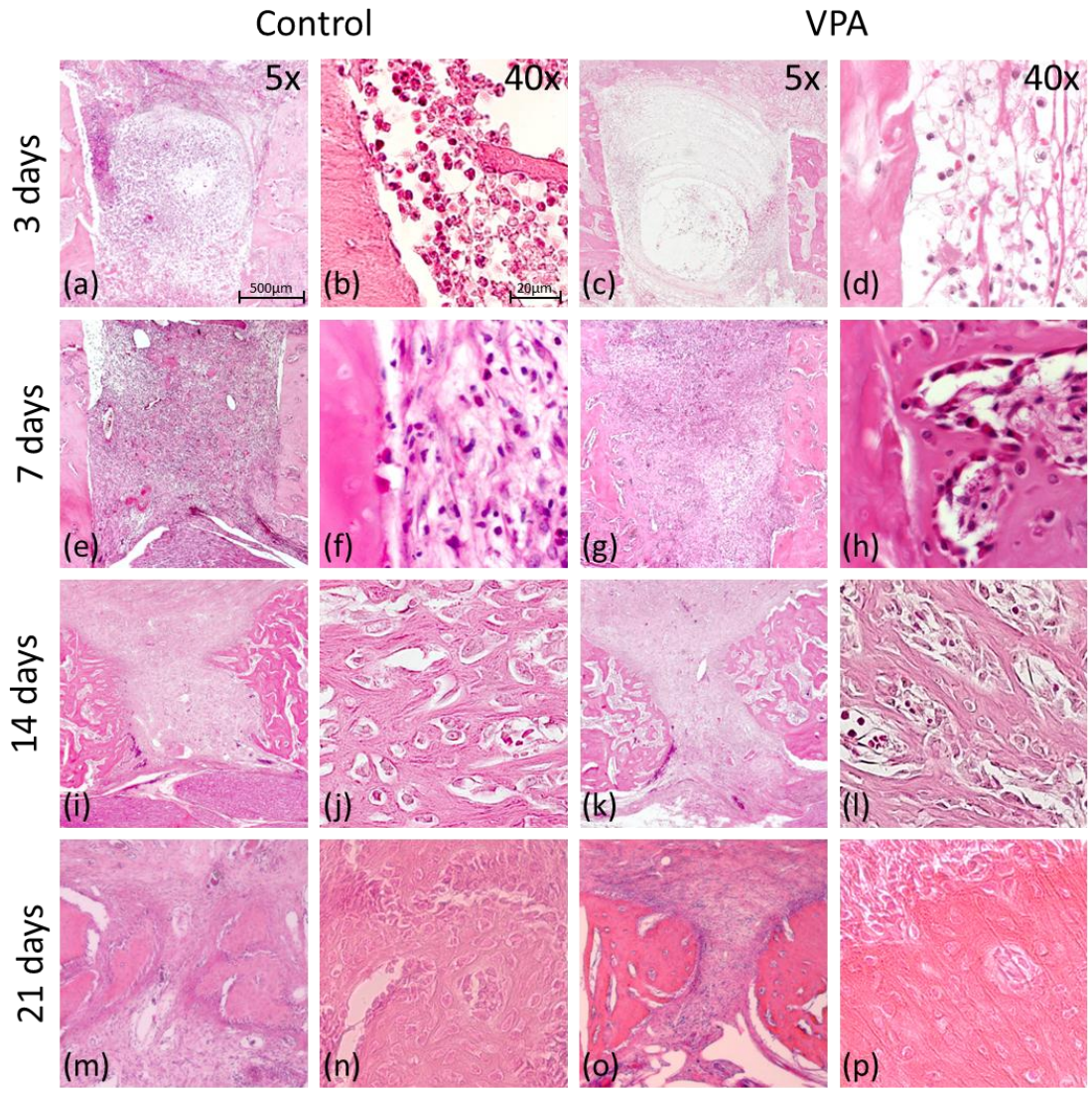

Control
VPA
3 days
7 days
14 days
21 days
5x
40x
5x
40x
(a)
500µm
(b)
20µm
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)
(n)
(o)
(p)

Fig. 5