RESEARCH REPORTS
Biological

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*J Dent Res* 92(3):253-259, 2013

# Occlusal Effects on Longitudinal Bone Alterations of the Temporomandibular Joint

## ABSTRACT

The pathological changes of subchondral bone during osteoarthritis (OA) development in the temporomandibular joint (TMJ) are poorly understood. In the present study, we investigated the longitudinal alterations of subchondral bone using a rat TMJ-OA model developed in our laboratory. Changes in bone mass were examined by micro-CT, and changes in osteoblast and osteoclast activities were analyzed by real-time PCR, immunohistochemistry, and TRAP staining. Subchondral bone loss was detected from 8 weeks after dental occlusion alteration and reached the maximum at 12 weeks, followed by a repair phase until 32 weeks. Although bone mass increased at late stages, poor mechanical structure and lower bone mineral density (BMD) were found in these rats. The numbers of TRAP-positive cells were increased at 12 weeks, while the numbers of osteocalcin-expressing cells were increased at both 12 and 32 weeks. Levels of mRNA expression of TRAP and cathepsin K were increased at 12 weeks, while levels of ALP and osteocalcin were increased at both 12 and 32 weeks. These findings demonstrated that there is an active bone remodeling in subchondral bone in TMJs in response to alteration in occlusion, although new bone was formed with lower BMD and poor mechanical properties.

**KEY WORDS:** temporomandibular joint (TMJ), osteoarthritis (OA), bone remodeling, temporomandibular disorders (TMD).

DOI: 10.1177/0022034512473482

Received August 6, 2012; Last revision November 11, 2012; Accepted December 10, 2012

A supplemental appendix to this article is published electronically only at http://jdr.sagepub.com/supplemental.



## INTRODUCTION

It has been reported that up to 10 million Americans suffer from temporomandibular disorders (TMDs) each year (Slavkin, 1996). Temporomandibular joint (TMJ) osteoarthritis (OA), characterized as progressive cartilage degradation and subchondral bone changes (XD Wang *et al*., 2012), is one of the most serious subgroups of TMD. One percent of the Chinese in Hong Kong was reported to have TMD-related frequent jaw pain with moderate or severe intensity (Pow *et al*., 2001), and 14.56% of mainland Chinese patients with TMD had radiographic signs of OA (Zhao *et al.*, 2011).

In the early stages of OA of the knee, there is pathological remodeling with low bone mineral density (BMD) (Pelletier *et al*., 2004; Bouchgua *et al*., 2009). RANKL and OPG are critical molecules regulating bone resorption (Teitelbaum, 2000). Cathepsin K, a cystein protease mainly expressed by osteoclasts, is involved in osteoclast function and bone resorption *via* the cleavage of type I collagen (Col1), the major component of bone matrix (Kafienah *et al*., 1998). During bone formation, osteoblast secretes a range of proteins, including Col1, ALP, osteocalcin, and DMP-1, to form bone matrix. VEGF is also involved in subchondral bone remodeling through the regulation of angiogenesis (Dai and Rabie, 2007).

The role of occlusion in the development of TMD is still under debate (Türp and Schindler, 2012). Based on clinical observations (Wang *et al.*, 2009), we developed a rat TMJ-OA model by creating posterior disordered occlusion. Histological changes within 12 wks were observed in this animal model, including reduced chondrocyte proliferation, increased chondrocyte apoptosis (Jiao *et al*., 2009), changes in aggrecan and matrix metalloproteases (MMPs) expression, and degradation of mandibular condylar cartilage (Jiao *et al*., 2010; Wang *et al*., 2010). Subchondral bone changes were also noted, such as resorption of the subchondral bone (Jiao *et al*., 2011) and increased osteochondral angiogenesis 24 wks after creation of the disordered occlusion (QY Wang *et al*., 2012). Long-term longitudinal changes in subchondral bone in response to this disordered occlusion and changes in osteoclast and osteoblast marker gene expression correlated to the morphological changes are worthy of investigation.

*In vivo* micro-CT scanning is a non-invasive imaging technique and facilitates a continuous and dynamic observation of the three-dimensional subchondral bone morphology of joints (Zhao *et al*., 2010; Chiba *et al*., 2011). In this study, a combined approach of *in vivo* micro-CT technique and histological analysis was used to evaluate the morphological changes in the

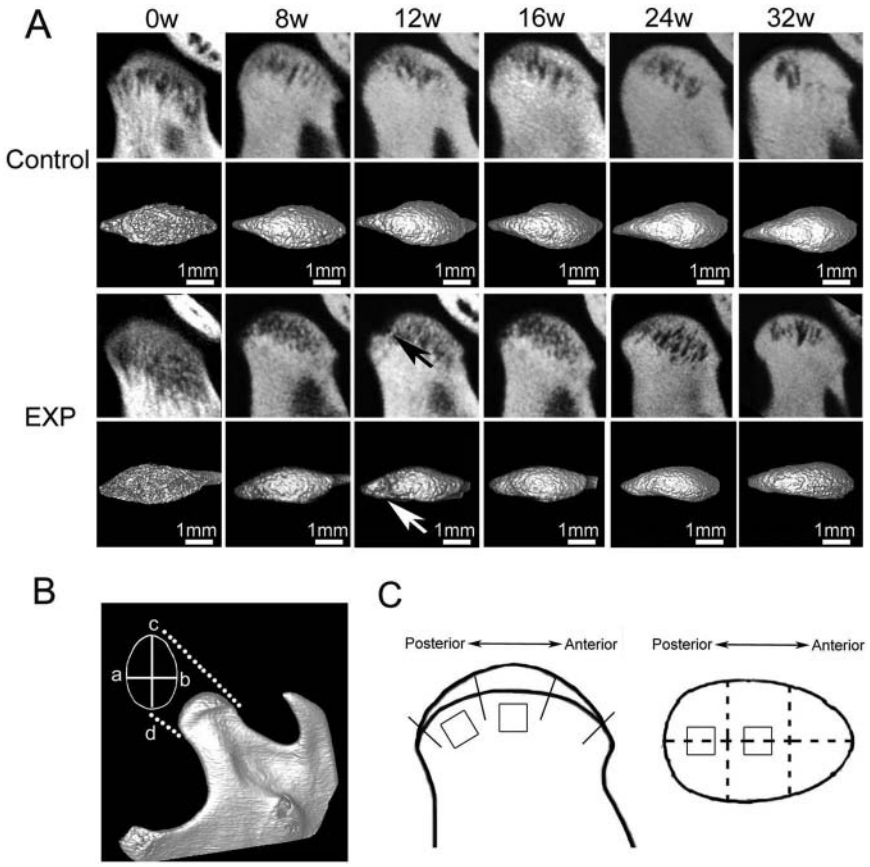


**Figure 1.** *In vivo* micro-CT images of the TMJ condylar head from the control and experimental (EXP) groups. **(A)** The images were arranged from left to right according to the time sequence. Local subchondral bone lesions were observed in the experimental group. In general, the lesion regions (indicated by arrows) were larger at 12 wks after surgery than those at the other time-points. **(B)** Illustration of the methods for measuring the width (a–b) and length (c–d) of the condylar head. (a) The outermost point of the ventral contour of the condyle, (b) the outermost point of the dorsal contour of the condyle, (c) the most anterior point of the condyle, and (d) the most posterior point of the condyle. **(C)** Illustration of the method for locating the region of interest (ROI) for micro-CT analysis. The surface of the condylar head was equally divided into 3 parts, and the ROIs were located at the center of the middle and posterior parts.

condylar subchondral bone in the TMJ-OA rat model. Expression of osteoclast and osteoblast marker genes was examined by real-time PCR assay. Changes in mRNA expression of bone resorption-related genes, such as *RANKL, OPG, cathepsin K*, and *TRAP*, and bone-formation-related genes, such as *Col1a1, Col1a2, ALP, osteocalcin, VEGF*, and *DMP-1*, were examined to evaluate osteoclastic and osteoblastic activities. The goal of this study was to investigate longitudinal changes in the subchondral bone of TMJ-OA rats induced by experimental disordered occlusion.

## MATERIALS & METHODS

### Animals

Thirty-six 8-week-old female Sprague-Dawley rats (weight, 180-200 g) were used. All orthodontic and animal care procedures were approved by the university ethics committee and performed according to institutional guidelines. The rats were randomly assigned to 2 control and 2 experimental groups. Each group had 9 animals (n = 9). Sixteen of the 36 rats used for *in vivo* micro-CT scanning were randomly assigned to control and experimental groups (n = 8) (Appendix Table 1). Rats in experimental groups were randomly paired with those in control groups. In experimental groups, disordered occlusion was created as described previously (QY Wang *et al*., 2012). Briefly, an elastic rubber band (1/8#; 3M Unitek, Saint Paul, MN, USA), about 1 mm in diameter, was inserted between the first and second molars of the left side of the maxillary dentition and the right side of the mandibular dentition. In this way, the first molars were gradually moved medially by the elastic force of the rubber bands. One wk later, the rubber bands were replaced with self-curing resin (Zhangjiang Biomaterial, Shanghai, China) to maintain the gaps (about 0.8 mm) between the first and second molars until the end of the treatment. Ten days after the beginning of the experiment, the same method was used to push the left maxillary and right mandibular third molars distally. In this way, 2 first and 2 third molars were moved away from their original positions and no longer intercuspated their opposite molars. In control groups, rats underwent the same procedure, but the rubber bands were immediately pulled out so that the molars did not receive elastic force from the bands, and no resin remained between molars. Rats in experimental groups were monitored every other day. If a rubber band or self-curing resin was lost, the procedure was repeated. At the same time, a repeated sham operation was performed to the matched control rat(s). In this way, the numbers of operating times between experimental and control groups were similar, and the effects of repeated operations were balanced between the experimental and control groups. The photos showing the disordered occlusion *post mortem* were presented in previous reports (Wang *et al*., 2010; QY Wang *et al*., 2012).

### Longitudinal Micro-CT Analysis

The rats were scanned with an *in vivo* micro-CT system (Inveon, Siemens, MUC, Bavaria, Germany) before and 8, 12, 16, 24, and 32 wks after surgery. Scanning was performed at 80 kV and 500 mAs. The x-ray beam was collimated to irradiate only the head of the rat and reconstructed with an isotropic voxel size of 30 μm. The micro-CT volume was calibrated in the conventional linear scale of CT numbers, otherwise known as Hounsfield units (HU),

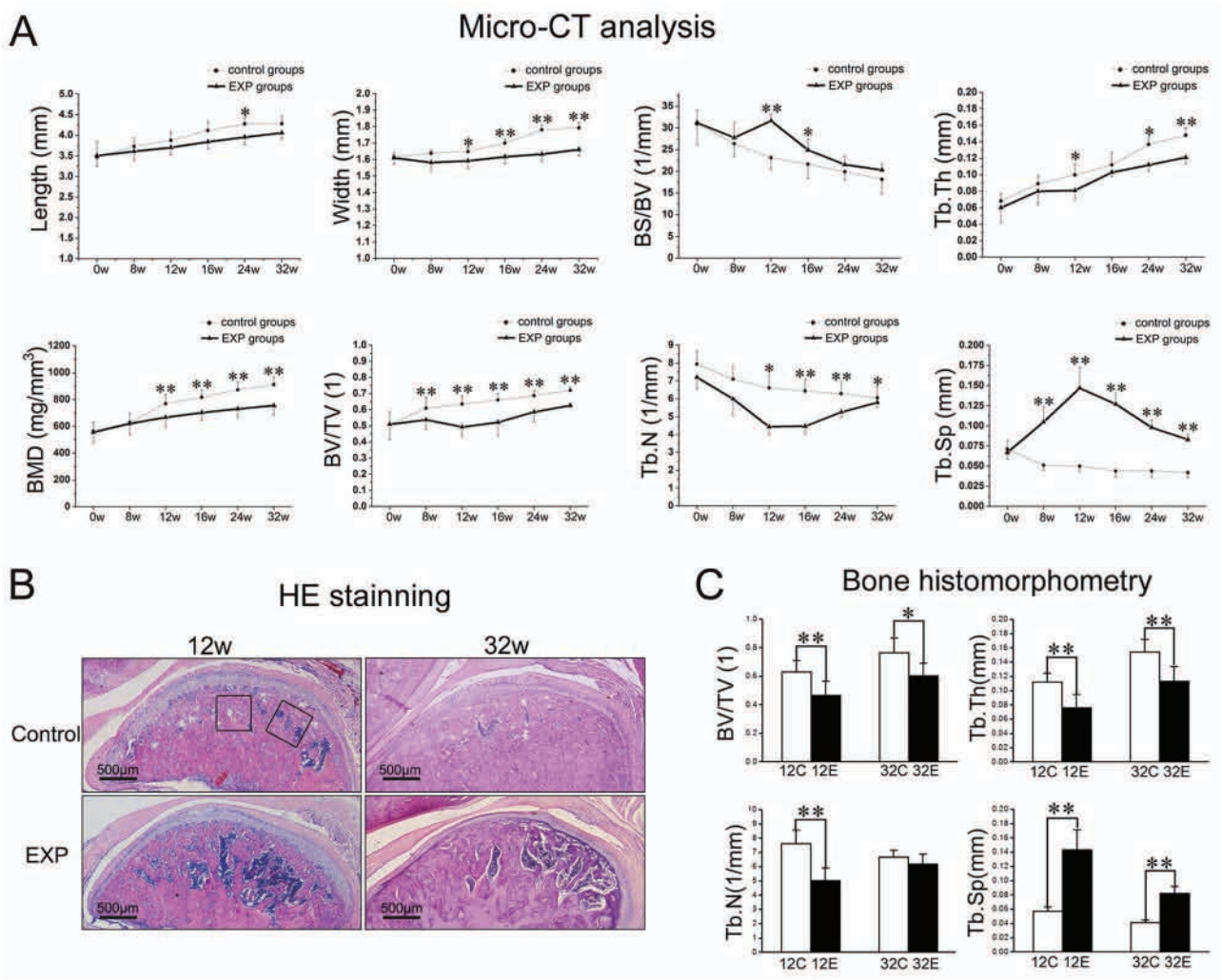


**Figure 2.** Subchondral bone loss in the TMJ condyle. **(A)** Micro-CT analysis showed that significant bone loss started from the end of 8 wks and reached the peak at around 12 wks. **(B)** Hematoxylin-eosin (HE) staining of sections showed larger trabecular spacing in both the 12- and 32-week experimental groups. **(C)** Histomorphological parameters confirmed the significant subchondral bone loss in both the 12- and 32-week experimental groups. The black areas show the selected regions used for analysis of the histomorphological parameters (*$p < 0.05$, **$p < 0.01$).

wherein the values for water and air are 0 and -1,000, respectively. The three-dimensional images acquired from microtomographic slices were utilized for quantitative evaluation. The width and length of the condylar heads were measured directly with software provided by the manufacturer (Fig. 1B). In previous studies, we observed obvious cartilage degradation and subchondral bone resorption at the middle and posterior parts of the mandibular condyle using the same animal model (Jiao *et al*., 2009, 2011). In the present studies, 2 cubic regions of interest (0.5 x 0.5 x 0.5 mm) at the middle points of the center and posterior condyle were selected for examination of the longitudinal changes in subchondral bone (Fig. 1C). The following parameters—BV/TV (the ratio of bone volume to tissue volume), BS/BV (the ratio of bone surface area to bone volume), Tb.Th (trabecular thickness), Tb.Sp (trabecular separation), Tb.N (trabecular number), and BMD (bone mineral density) (Appendix Table 2)—were measured and compared between experimental and control groups at different time points.

## Sample Preparation

Animals were sacrificed at 12 and 32 wks after surgery. Because no side differences were found in our previous work (Jiao *et al.*, 2010), TMJ blocks on the right side of the 9 rats in each group were removed and embedded in paraffin. Five-μm-thick sections were used for HE, TRAP, and IHC staining. The subchondral bones of the condyle on the left side of the rats in each group were preserved at -70°C for mRNA extraction.

## Histomorphometric Measurements

The central sagittal sections of each joint stained with HE were imaged with a Leica DFC490 system (Leica, Solms, Hessen, Germany). Two square frames (0.5 x 0.5 mm) under the interface of cartilage and subchondral bone were located at the middle of the center and posterior third of the mandibular condylar (Fig. 2B, black frames). Within the selected area, BV/TV,

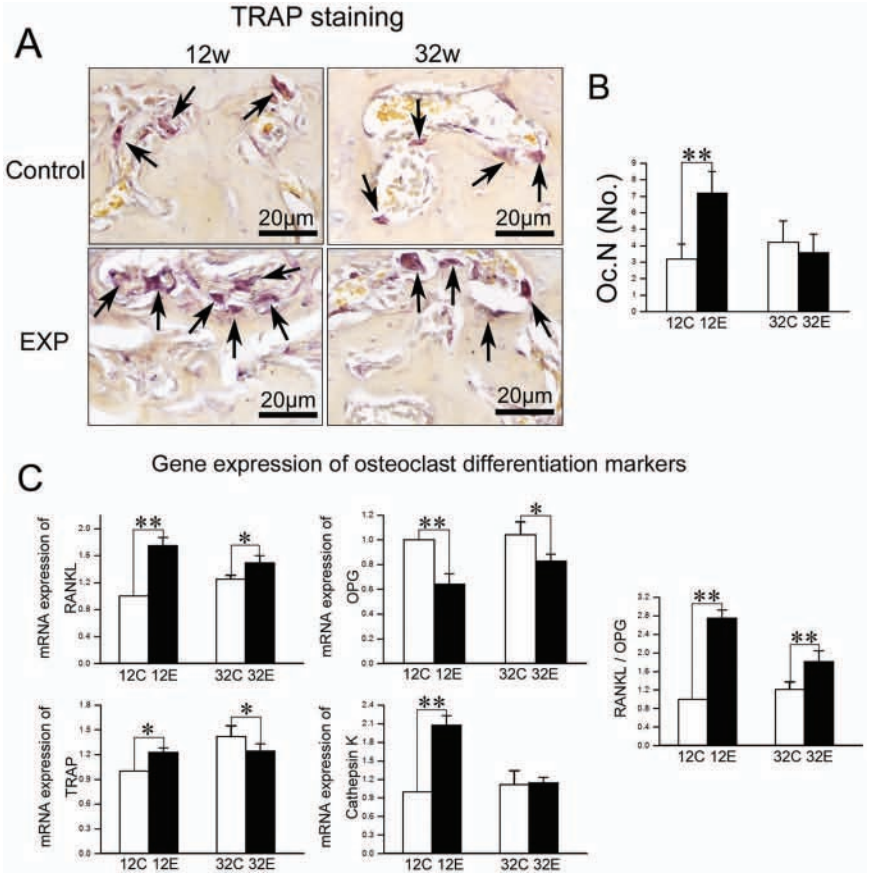


**Figure 3.** Analysis of osteoclast activity in TMJ condylar subchondral bone. **(A)** TRAP staining of TMJ subchondral bone from 12- and 32-week control and experimental groups. **(B)** TRAP-positive cells with more than 3 nuclei were counted as the number of osteoclasts (Oc.N). **(C)** Real-time PCR analysis of expression of *RANKL, OPG, TRAP,* and *cathepsin K* genes from the subchondral bone of mandibular condylar heads in 12- and 32-week control and experimental groups (*$p < 0.05$, **$p < 0.01$).

Tb.Th, Tb.Sp, and Tb.N were measured as described previously (Jiao *et al*., 2011).

### Histochemical and Immunohistochemical Staining

We performed TRAP staining (387-A, Sigma, St. Louis, MO, USA) to examine changes in osteoclast numbers. A standard, three-step, avidin-biotin complex staining procedure was performed with anti-osteocalcin primary antibody (1:100; sc-30044; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for detection of changes in osteoblast numbers. The numbers of TRAP-positive osteoclasts (Oc.N) and osteocalcin-positive (osteocalcin$^+$) cells were counted in 5 randomly selected high-power (400x) fields under light microscopy.

### RNA Extraction and Real-time PCR Assay

In each subgroup, every 3 out of 9 subchondral bone tissues were pooled to create a single sample, and 3 independent pooled samples in both experimental and control groups were prepared for analysis. RNA was isolated by Trizol (Invitrogen, Carlsbad, CA, USA), and purified with an RNeasy Mini Kit (Qiagen, Hilden, USA). Gene expression was analyzed with the Applied Biosystems 7500 Real-time PCR machine (Applied Biosystems, Foster City, CA, USA; Appendix Table 3). Data were collected from a minimum of 3 independent experiments.

### Statistical Analysis

Procedures for quantification of parameters from micro-CT images were performed twice in a blinded fashion, over an interval of 1 wk, by two independent observers with no knowledge of the experimental design. There was a high level of agreement for all the above parameters ($r > 0.9$) between the two observers. The mean of each of the 2 measurements was used for statistical analysis. Two factors, treatment and time, were taken into consideration when 2-way analysis of variance (ANOVA) was applied for statistical analysis. When significant differences were found, a Student-Newman-Keuls post-test was performed to compare changes between groups. The $p$ values $< 0.05$ were considered statistically significant.

## RESULTS

During the experiment, no difference in body weight was found in rats between experimental and age-matched control groups (data not shown).

### Subchondral Trabecular Bone Loss in TMJ Condyle

Local and gradual subchondral bone lesions were observed in the experimental groups. The bone lesion regions reached the maximum at the end of 12 wks but gradually recovered thereafter (Fig. 1A). The sizes, especially the widths, of the condyles in the experimental groups were significantly smaller than those of their age-matched controls (Fig. 2A, $p < 0.05$). Micro-CT analysis revealed that the significantly lower values of BV/TV, but higher values of Tb.Sp, were initially observed in the experimental group at 8 wks after surgery, while lower values of BMD, Tb.N, and Tb.Th became apparent at 12 wks. These changes persisted to the end of the experiment ($p < 0.05$) (Fig. 2A). The BS/BV increased only at 12 and 16 wks compared with their controls (all $p < 0.05$) (Fig. 2A). It seems that the maximal bone loss was reached at 12 wks after surgery.

Larger marrow cavities within subchondral bone were observed in both the 12- and 32-week experimental groups than in the age-matched control groups (Fig. 2B). Histomorphometric analysis showed that BV/TV and Tb.Th significantly decreased

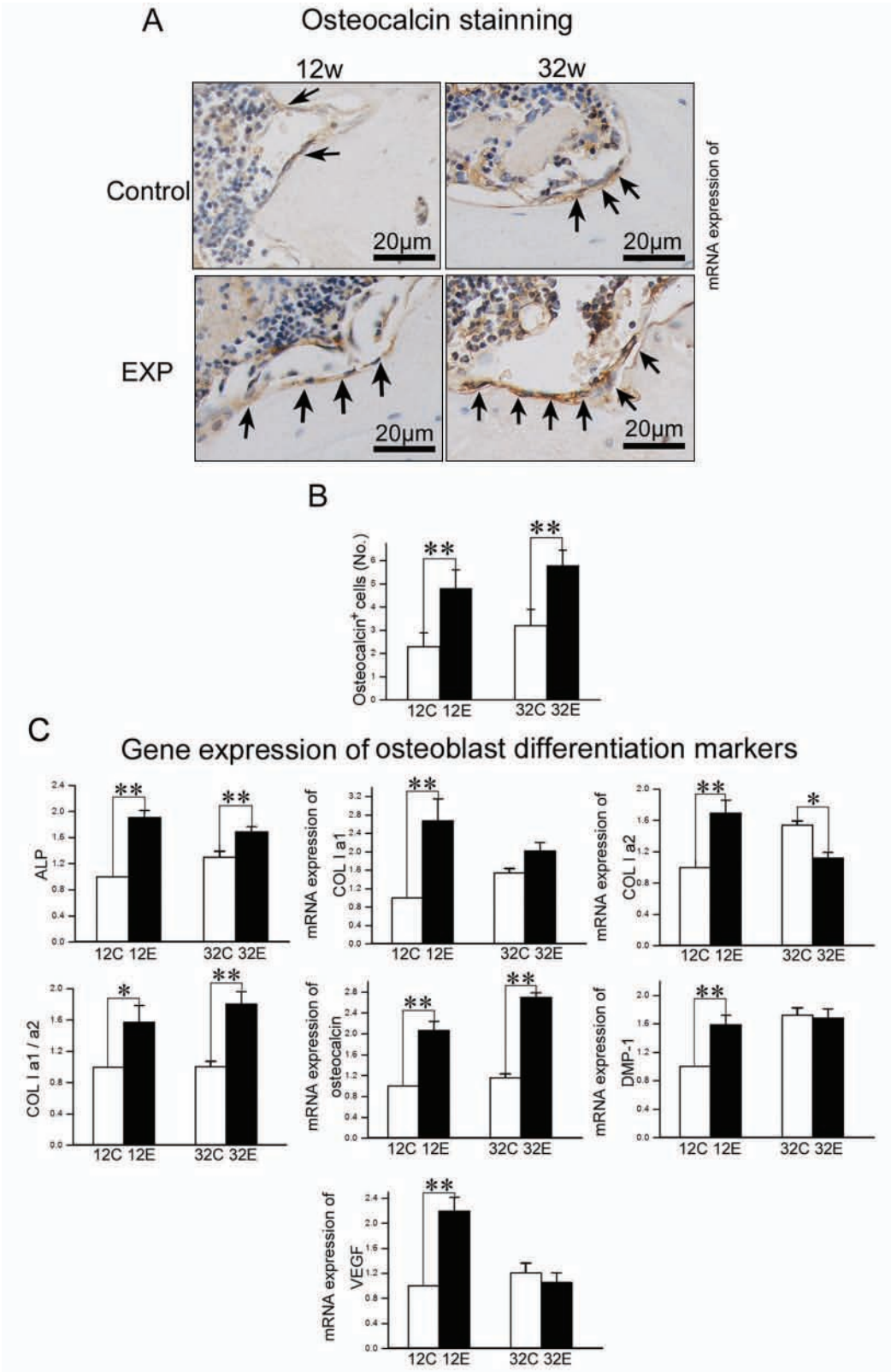


**Figure 4.** Analysis of osteoblast activity in TMJ condylar subchondral bone. **(A)** Osteocalcin immunohistochemical (IHC) staining of TMJ subchondral bone from 12- and 32-week control and experimental groups. **(B)** The number of osteocalcin-positive cells within the selected area was counted. **(C)** Real-time PCR analysis of expression of *ALP, Col1a1, Col1a2*, the ratio of *Col1a1/Col1a2, osteocalcin, DMP-1*, and *VEGF* genes from the subchondral bone of the mandibular condylar head in 12- and 32-week control and experimental groups (*$p < 0.05$, **$p < 0.01$).

and Tb.Sp increased in the experimental groups compared with the control groups ($p < 0.05$) (Fig. 2C). The Tb.N decreased in the 12-week experimental group ($p < 0.05$) but showed no difference in the 32-week experimental group compared with the control groups (Fig. 2C).

### Osteoclast Activity

In control groups, few TRAP-positive cells were found in the subchondral bone area, while the numbers of TRAP-positive cells were much larger in the 12-week experimental group than in the age-matched control group ($p < 0.01$), but returned to control levels in the 32-week experimental group (Fig. 3B).

The *RANKL* mRNA expression was increased while *OPG* expression was decreased, resulting in an increased *RANKL/OPG* ratio in both 12- and 32-week experimental groups compared with the age-matched control groups ($p < 0.05$) (Fig. 3C). The mRNA expression of *TRAP* and *cathepsin K* was increased in the 12-week experimental group, but returned to control or even lower levels in the 32-week experimental group ($p < 0.05$) (Fig. 3C).

### Osteoblast Activity

Osteocalcin-positive cells were mainly located on the trabecular bone surface, and the numbers of osteocalcin-positive cells in 12- and 32-week experimental groups were larger than those in the age-matched control groups ($p < 0.01$) (Figs. 4A, 4B). Compared with the controls, the mRNA expression of *ALP* and *osteocalcin* and the ratio of *Col1a1/Col1a2* were significantly higher in 12- and 32-week experimental groups ($p < 0.05$), while expression of *Col1a1, DMP-1*, and *VEGF* was significantly increased in the 12-week ($p < 0.01$), but not the 32-week, experimental group ($p > 0.05$) (Fig. 4C).

## DISCUSSION

In this study, we performed a longitudinal observation on condylar subchondral bone changes using an *in vivo* micro-CT method. The current findings demonstrated a predominantly resorptive activity in the subchondral bone of TMJ condyles in the initial phases, followed by increased bone formation in late stages in response to changes in dental occlusion. Consistent with the higher resorptive activity in the 12-week experimental group, expression of *RANKL* and *TRAP* and the *RANKL/OPG* ratio were significantly increased in the experimental group. These changes returned to control levels in the 32-week experimental group. Meanwhile, the osteoblast activity and expression of *ALP* and *osteocalcin* were significantly increased in both the 12- and 32-week experimental groups. The increased ratio of mRNA expression of *Col1a1/Col1a2* suggests a poor quality of newly formed bone (Bailey *et al.*, 2002). These results are consistent with the increased subchondral bone remodeling observed in primary knee osteoarthritis (Ding *et al.*, 2006) and a surgically induced knee OA mouse model (Pelletier *et al.*, 2004) and also agree with our previous reports, in which we demonstrated the OA-like changes in this rat disordered occlusion model (Jiao *et al.*, 2009; Wang *et al.*, 2010).

During normal bone remodeling, osteoclast activity is coupled with osteoblast function. This coupling seems to be interrupted during OA development, characterized by a predominant bone resorption in the early stage and enhanced bone formation with poor quality in the late stage (Petersson *et al*., 1998; Bailey *et al*., 2001; Hunter *et al*., 2003). This enhanced osteoblast activity seems unable to repair the bone loss caused by increased osteoclast activity occurring at the early stage of the present rat model. The newly formed bone, which has a lower BMD value in the experimental group, is more liable to deformation when loading is applied (Day *et al*., 2001). The increased ratio of *Col1a1/Col1a2* also suggests a poor quality of the newly formed bone (Bailey *et al*., 2002). In addition, the increased BS/BV in the experimental group suggests a higher remodeling activity of subchondral bone (Cowin, 2004).

The possible wear of teeth in the orthodontically placed position may be a potential contributing factor for bone formation in the late stage of the experiment. We did not evaluate the occlusal contact because it is beyond our current technical ability. It should be pointed out that human TMJ OA is a complicated disease, caused by different pathological mechanisms. The current animal model may explain 1 of these reasons, so the current findings should be interpreted with caution. The contribution of dental occlusion to TMJ OA development in patients requires further investigation. The current findings may provide significant insight into the role of dental occlusion in TMJ OA development, and thus will help us develop better treatment strategies for patients with TMD problems.

In summary, the present longitudinal *in vivo* micro-CT studies demonstrated a predominantly resorptive activity in the subchondral bone of TMJ condyles in the initial phases and a reparative capability in later stages in response to changes in dental occlusion. The newly formed subchondral bone is of poor quality.

## ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Nos. 30872870, 30901699, and 30801303). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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