# Chondroitin sulphate (WF6 epitope) levels in peri-miniscrew implant crevicular fluid during orthodontic loading

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SUMMARY The aim of this study was to monitor changes in chondroitin sulphate (CS; WF6 epitope) levels in peri-miniscrew implant crevicular fluid (PMICF) during orthodontic loading.

Ten patients (seven males and three females; aged  $22.0 \pm 3.4$  years), who required orthodontic treatment with extraction of all four premolar teeth, participated in the study. Twenty miniscrew implants (used as orthodontic anchorage) were placed, two in each patient, buccally and bilaterally in the alveolar bone between the roots of the maxillary posterior teeth. Sentalloy closed-coil springs (50 g) were used to load the miniscrew implants and to move the maxillary canines distally. During the unloaded period, PMICF samples were collected on days 1, 3, 5, and 7 after miniscrew implant placement and on days 14, 21, 28, and 35 during the loaded period. Clinical mobility assessments of the miniscrew implants were recorded at each visit. The competitive enzyme-linked immunosorbent assay with monoclonal antibody WF6 was used to detect CS (WF6 epitope) levels in the PMICF samples. The differences between the CS (WF6 epitope) levels during the unloaded and loaded periods were determined by a Mann–Whitney *U*-test.

During the loaded period, two miniscrew implants were considered to have failed. The CS (WF6 epitope) levels during the unloaded period ranged from 0.00 to 758.03 ng/ml and those during the loaded period from 0.00 to 1025.11 ng/ml. Medians of CS (WF6 epitope) levels, around 'immobile' miniscrew implants, between the unloaded and loaded periods were not significantly different (P=0.07).

CS (WF6 epitope) levels in PMICF can be detected and may be used as biomarkers for assessing alveolar bone remodelling around miniscrew implants during orthodontic loading.

# Introduction

The stability of miniscrew implants has been based on clinical (Ohmae *et al.*, 2001; Miyawaki *et al.*, 2003; Cheng *et al.*, 2004; Liou *et al.*, 2004; Kim *et al.*, 2005; Park *et al.*, 2006), histological (Melsen and Costa, 2000; Ohmae *et al.*, 2001; Kim *et al.*, 2005; Freire *et al.*, 2007), biomechanical (Huja *et al.*, 2005; Motoyoshi *et al.*, 2005, 2006; Chen *et al.*, 2006), and biochemical assessments. Only one study was found in the literature in which biochemical assessments were used to investigate the stability of miniscrew implants (Sarı and Uçar, 2007).

Several biochemical markers have revealed the destruction and remodelling of peri-implant tissue (Last *et al.*, 1995; Johansson *et al.*, 2001; Plagnat *et al.*, 2002; Ma *et al.*, 2003; Liskmann *et al.*, 2004). Numerous studies have monitored glycosaminoglycans, particularly chondroitin sulphate (CS), in periodontal (Shibutani *et al.*, 1993; Waddington *et al.*, 1994; Okazaki *et al.*, 1995; Kagayama *et al.*, 1996; Ababneh *et al.*, 1998; Khongkhunthian *et al.*, 2008) and peri-implant (Last *et al.*, 1995; Okazaki *et al.*, 1996; Johansson *et al.*, 2001) tissue and concluded that glycosaminoglycans in peri-implant crevicular fluid are similar to those in gingival crevicular fluid (GCF; Last *et al.*, 1995; Okazaki *et al.*, 1995, 1996). Increased levels of glycosaminoglycans, particularly CS, in peri-implant crevicular fluid can be a marker for adverse tissue responses, particularly for bone resorption (Smedberg *et al.*, 1993; Waddington *et al.*, 1994; Last *et al.*, 1995).

In the one study in which biochemical assessments were used to investigate the stability of miniscrew implants, interleukin-1 $\beta$  (IL-1 $\beta$ ) levels in peri-miniscrew implant crevicular fluid (PMICF) were used to determine the effects of mechanical stress on the miniscrew implants when used as anchorage for tooth movement. The results demonstrated that IL-1 $\beta$  levels in PMICF of healthy miniscrew implants were not increased during orthodontic loading (Sarı and Uçar, 2007).

A monoclonal antibody, 3B3, has been used to recognize epitopes of CS in GCF by the enzyme-linked immunosorbent assay (ELISA) method (Shibutani *et al.*, 1993). Some studies suggest that the expression of CS is related to the severity of inflammation, periodontal disease, and hyalinized periodontal ligament (Shibutani *et al.*, 1993; Kagayama *et al.*, 1996; Ababneh *et al.*, 1998). Monoclonal antibody WF6, a novel monoclonal

antibody developed against embryonic shark cartilage proteoglycans, was applied as a biomarker for recognizing an epitope in CS chains. Using the ELISA with monoclonal antibody WF6, trace amounts of glycosaminoglycans present in GCF can be quantified (Khongkhunthian *et al.*, 2008). Two octasaccharides, unsaturated D–C–C–C and C–C–A–D, were recognized by the monoclonal antibody, WF6 (Pothacharoen *et al.*, 2007). This WF6 monoclonal antibody was applied as a serum biomarker for cartilage degradation in an *in vivo* study (Pothacharoen *et al.*, 2006a). Accordingly, the aim of the present study was to apply the competitive ELISA with monoclonal antibody WF6 to detect CS levels in PMICF during orthodontic loading.

# Subjects and methods

The study was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University. Informed consent was obtained from all patients.

#### Subjects

Ten patients (7 males and 3 females; aged  $22.0 \pm 3.4$  years) requiring orthodontic treatment who met the following criteria: good general health; lack of antibiotic therapy during the previous 6 months; absence of anti-inflammatory drug administration in the month preceding the study; healthy periodontal tissue and no radiographic evidence of periodontal bone loss; requirement for four premolar extractions, distal canine movement, and maximum anchorage control, were included in the study.

### Methods

Twenty miniscrew implants (8.0 mm in length, 1.6 mm in diameter; SIN, São Paulo, Brazil) were placed, two in each patient, buccally and bilaterally into the interradicular bone between the maxillary second premolar and first

molar teeth. During the unloaded period, PMICF samples for each miniscrew implant were collected using  $10.0 \times$ 1.0 mm Whatman No. 1 (Whatman International Ltd, Maidstone, Kent, UK) filter paper strips on days 1, 3, 5, and 7. On day 7, after sample collection, a 50 g Sentalloy<sup>®</sup> closed-coil spring (Tomy, Tokyo, Japan) was used to connect the miniscrew implant head and the canine bracket in order to move the maxillary canine distally. During the loaded period, PMICF samples for each miniscrew implant were collected on days 14, 21, 28, and 35 (Figure 1).

# PMICF collection

Before PMICF sample collection, the Sentalloy<sup>®</sup> closedcoil spring was removed. PMICF collection was undertaken following the method of Cliantar and Caruana (1998). Briefly, the miniscrew implant placement site was isolated from saliva and gently air-dried. PMICF samples were collected using Whatman No. 1 filter paper strips. An analytical instrument (Periotron 8000<sup>™</sup>, Oralflow Inc., Plainview, New York, USA) was used to measure the PMICF volume. Care was taken to avoid mechanical injury. Samples containing blood were discarded. The last 2.0 mm of filter paper strip containing the PMICF sample was cut off and individually frozen at −80°C in a microcentrifuge tube for further analysis.

# Clinical mobility assessment of the miniscrew implant

After collecting the PMICF sample, the clinical mobility of each miniscrew implant was assessed using cotton forceps. An extremely light force was laterally applied to the miniscrew implant head. Mobility was scored either as 'yes' (mobile) or 'no' (immobile). If there was any discernible mobility, the miniscrew implant was categorized as mobile. Any miniscrew implants that were loose and thus could not serve as anchorage during the study period were considered as failures. The miniscrew implants that remained stable in the bone until the end of the study period or until intentional



Figure 1 Diagram of the experimental design. \*Unloaded period: sample collection on days 1, 3, 5, and 7 after miniscrew implant placement. \*\*Loaded period: sample collection on days 14, 21, 28, and 35. PMICF, peri-miniscrew implant crevicular fluid.

removal (regardless of mobility) were considered to be successful.

# Competitive immunoassay using monoclonal antibody WF6

PMICF was recovered from the paper strip by the addition of 200  $\mu$ l of phosphate buffer saline, pH 7.4, and the tube was then vigorously shaken for a few minutes. The recovery rate (approximately 98.1 per cent) from each paper strip was determined by a dye-binding assay, using known concentrations of sulphated glycosaminoglycans as standards (Ratcliffe *et al.*, 1988).

A quantitative ELISA was modified from a previous study (Pothacharoen et al., 2006a) for the epitopes recognized by monoclonal antibody WF6. The standard agent used in the assay was shark cartilage aggrecan (Pothacharoen et al., 2006b). The coating antigen was shark PG-A<sub>1</sub>, and the competitor agent was shark PG-A<sub>1</sub>D<sub>1</sub>. The primary antibody was monoclonal antibody WF6, and the secondary antibody was IgM-specific anti-mouse immunoglobulin with peroxidase. Microtiter plates (Maxisorp<sup>®</sup>, Nunc, Denmark) were coated overnight at room temperature with 10 µg/ml shark PG-A1 fraction (100 µl/well) in the coating buffer (20 mM sodium carbonate buffer, pH 9.6). The following morning, the plates were washed three times with Tris-IB with 0.1 per cent bovine serum albumin (Sigma-Aldrich, St Louis, Missouri, USA; 150 µl/well) and left to air-dry. The uncoated area was then blocked with 150  $\mu l/well$  of 1 per cent (w/v) bovine serum albumin in the Tris-IB for 60 minutes at 37°C. After washing, 100 µl/well of the mixture, which was the PMICF sample or standard competitor (shark PG-A<sub>1</sub>D<sub>1</sub> fraction whose concentrations ranged from 39.06 to 10000 ng/ml) mixed with the monoclonal antibody WF6 (patent number WO 2005/118645 A1; Pothacharoen et al., 2007) at the dilution 1:100, was added. After incubation for 60 minutes at 37°C, the wells were washed and then IgM-specific anti-mouse immunoglobulin with peroxidase (1:2000) was added (100 µl/well; in Tris-IB). The plates were washed again, and the peroxidase substrate (100  $\mu$ l/ well) was then added and incubated at 37°C for 5-20 minutes to allow the colour to develop. The reaction was stopped by the addition of 50 µl/well of 4 M H<sub>2</sub>SO<sub>4</sub>. The absorbance ratio at 492:690 nm was measured using the Titertek Multiskan® MCC/340 multiplate reader (ICN/ Flow Laboratories, Costa Mesa, California, USA).

# Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences version 13 for Windows (SPSS Inc., Chicago, Illinois, USA). The Kolmogorov–Smirnov onesample test was used to determine the distribution of CS (WF6 epitope) levels. The differences between the CS (WF6 epitope) levels during the unloaded and loaded periods were determined by the Mann–Whitney U-test. The results were considered statistically significant at P < 0.05.

# Results

#### Clinical observations

All 10 patients completed the 5-week study period, and PMICF was obtained from 20 miniscrew implants. At placement and during the unloaded period (1 week), all miniscrew implants remained clinically immobile. During the loaded period (4 weeks), two miniscrew implants were mobile. One miniscrew implant was mobile on day 14 and another on day 21. Both were later removed.

### CS (WF6 epitope) levels in PMICF samples

The volume of PMICF collected from the last 2.0 mm of each filter paper strip was 0.1  $\mu$ l (measured by the Periotron 8000<sup>TM</sup>). It has previously been shown that it is possible to measure such a small volume with reliability and also check intra- and interassay variations, including expected recovery values (Pothacharoen *et al.*, 2006b). The CS (WF6 epitope) levels (in nanogram per millilitre) could be detected in almost all PMICF samples collected from peri-miniscrew implant sulci during the unloaded and loaded periods.

During the unloaded period, the CS (WF6 epitope) levels ranged from 0.00 to 758.03 ng/ml and the medians at each visit from 12.63 to 23.18 ng/ml. During the loaded period, the CS (WF6 epitope) levels ranged from 0.00 to 1025.11 ng/ml and the medians at each visit from 19.41 to 28.43 ng/ml (Figure 2).

The medians of CS (WF6 epitope) levels during the unloaded and loaded periods were 17.38 and 23.69 ng/ml, respectively. No significant difference was found between the median of CS (WF6 epitope) level during the unloaded period and that during the loaded period (P=0.07; Figure 3).



**Figure 2** The medians of chondroitin sulphate (CS; WF6 epitope) levels (around 18 immobile miniscrew implants) at each visit during the unloaded (1 week) and loaded (4 weeks) periods.



**Figure 3** The medians of chondroitin sulphate (CS; WF6 epitope) levels (around 18 immobile miniscrew implants) during the unloaded (1 week) and loaded (4 weeks) periods (significant difference: P < 0.05).

The CS levels in the PMICF around one miniscrew implant (MI 5), that was mobile and was later removed on day 21, were high on days 3 and 5, while the CS (WF6 epitope) levels on the opposite side (MI 6, which was immobile) were relatively low (Figure 4).

### Discussion

Since the main component of glycosaminoglycans in alveolar bone is CS, its level in human GCF has been used to investigate alveolar bone remodelling as a result of periodontal disease and orthodontic tooth movement (Waddington *et al.*, 1994). Several studies have monitored CS in peri-implant tissue to evaluate the stability of dental implants and found that the levels of CS in peri-implant crevicular fluid may be an effective method of monitoring changes in bone metabolic activity (Smedberg *et al.*, 1993; Last *et al.*, 1995; Johansson *et al.*, 2001).

In the present study, only the PMICF samples collected from the last 2.0 mm of each filter paper strip were analyzed. The constituent in PMICF that was recognized by the WF6 antibody was CS. It is well known that CS comprises approximately 17 per cent of total glycosaminoglycans in gingival tissue (Bartold, 1987) and only a minor component in the periodontal ligament (Pearson and Gibson, 1982). On the other hand, much higher amounts of CS (94 per cent of total glycosaminoglycans) are present in mineralized tissue, i.e. alveolar bone and cementum (Waddington *et al.*, 1989). Consequently, detectable CS levels in PMICF are due to changes from mineralized tissue remodelling surrounding miniscrew implants, not from the inflammatory status of soft tissue.

In this research, the CS (WF6 epitope) levels in PMICF were investigated in a manner similar to that used for the determination of IL-1 $\beta$  levels of PMICF (Sarı and Uçar, 2007). It was found that the CS (WF6 epitope) in PMICF, both with and without the application of orthodontic force, could be detected. There was no statistical difference between



**Figure 4** Graphs showing the chondroitin sulphate (CS; WF6 epitope) levels around two miniscrew implants (MI 5 and MI 6) at each visit during the unloaded (1 week) and loaded (4 weeks) periods. (a) One miniscrew implant (MI 5) was mobile on day 21 and later removed. The CS (WF6 epitope) levels in peri-miniscrew implant crevicular fluid were high on days 3 and 5, while the CS (WF6 epitope) levels around the miniscrew implant on the opposite side (MI 6, which was immobile) were relatively low. (b) The CS (WF6 epitope) levels around two immobile miniscrew implants (MI 9 and MI 10) in another patient from day 1 to day 35. The CS (WF6 epitope) levels around those miniscrew implants were low.

the median CS (WF6 epitope) level during the unloaded period (1 week) and that during the loaded period (4 weeks). These results indicate that orthodontic force on miniscrew implants might not affect CS (WF6 epitope) levels in PMICF. The findings are in agreement with those of Sari and Uçar (2007), who evaluated IL-1 $\beta$  levels in PMICF during a 3 week loading period. There were no statistical differences in IL-1 $\beta$  levels in PMICF during the 3 week loading period. This indicates that orthodontic force might have a minimal influence on initial bone modelling, subsequent remodelling, and miniscrew implant anchorage stability (Huja *et al.*, 2005; Freire *et al.*, 2007).

In this study, two miniscrew implants were considered failures after the application of orthodontic forces. Miniscrew implant losses after the application of orthodontic forces have been reported (Melsen and Costa 2000). However, several authors have indicated that low-magnitude static force is not detrimental to miniscrew implant stability (Ohmae *et al.*, 2001; Deguchi *et al.*, 2003; Buchter *et al.*, 2005; Freire *et al.*, 2007). Therefore, the 50 g of static force

applied to the miniscrew implants in this study might not affect their stability. A possible explanation for this observation is trauma from the miniscrew placement procedure (Kim *et al.*, 2005). The holes drilled prior to miniscrew implant insertion might decrease the contact area between the miniscrew implants and surrounding bone, which may lead to miniscrew implant failure (Last *et al.*, 1995; Heidemann *et al.*, 2001; Kim *et al.*, 2005; Wilmes *et al.*, 2006).

The levels of CS (WF6 epitope) from the failed miniscrew implants were high 14 days prior to miniscrew implant failure. It is suggested that the elevations of CS (WF6 epitope) level before miniscrew implant failure might be associated with bone resorption around the miniscrew implant. In an earlier investigation, high CS levels were reported to be a potential marker for adverse tissue responses and marked bone resorption (Last *et al.*, 1995). However, the results of the present study should be confirmed by applying various bone resorption biomarkers and increasing the sample size. The increase in sample size may lead to a reasonable conclusion regarding comparisons between successful and failing miniscrew implants, as the number of failing miniscrew implants would be increased.

While the rationale behind this research may be questioned since there is no need for a biochemical test to study, the success or failure of miniscrew implants that are used for a limited period of time, the present study shows the importance of a biochemical test to analyse constituents in PMICF, which was also studied by Sarı and Uçar (2007). Future research should compare the CS levels in GCF around orthodontically moved teeth with those in PMICF or assess the optimal force magnitude required for tooth movement, while a miniscrew implant remains stable, using this novel WF6 antibody.

# Conclusions

CS (WF6 epitope) can be detected in PMICF samples during the unloaded and loaded periods. The CS (WF6 epitope) levels may be used as biomarkers for assessing alveolar bone remodelling around miniscrew implants during orthodontic loading.

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