Enzyme Activity Profiles and Elisa Analysis of Biomarkers from Human Saliva and Gingival Crevicular Fluid during Orthodontic Tooth Movement Using Self-Ligating Brackets

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Abstract

Aim: Profiles of orthodontic tooth movement biomarkers, i.e., Lactate Dehydrogenase (LDH), Aspartate Aminotransferase (AST), Tartrate-resistant Acid Phosphatase (TRAP) and Alkaline Phosphatase (ALP), using Self-ligating Brackets (SLBs) and possible relationships among their activities and total enzymes produced were determined.

Methods: Saliva and Gingival Crevicular Fluid (GCF) were collected from 19 subjects (n=19) before and during orthodontic treatment (5 weeks). The subjects were bonded with SLBs with 100 g or 150 g of orthodontic force. Enzyme assays, ELISA and tooth movement measurements were performed.

Results: A statistical analysis (paired *t*-test) showed that compared to baseline values, significant differences ($p<0.05$) were observed in the saliva levels of AST at week 5, the levels of TRAP at week 2, and the levels of ALP at weeks 1 to 5. In the GCF, LDH showed significant differences ($p<0.05$) at weeks 2, 3 and 4 (100 g) and at weeks 1, 2 and 3 (150 g). AST showed significant differences ($p<0.05$) at weeks 4 and 5 (100 g) and at weeks 3 and 4 (150 g), while TRAP exhibited a significant difference at week 5 (100 g). Pearson's correlation test revealed a weak correlation between enzyme activities and total enzymes. The use of 100 g compared to 150 g of force for tooth movement was not significant ($p>0.05$).

Conclusion: Therefore, 100 g is recommended as a better force for patient comfort. AST, TRAP and ALP in the saliva and LDH, AST and TRAP in the GCF are potential biomarkers in orthodontic tooth movement using SLB systems.

Key Words*: Alkaline Phosphatase, Aspartate Aminotransferase, Lactate Dehydrogenase, Tartrate-resistant Acid Phosphatase*

Introduction

Tooth movement can be divided into physiological tooth movement and Orthodontic Tooth Movement (OTM). Physiological tooth movement is a slow process that occurs mainly in the buccal direction into the cancellous bone or into the cortical bone because of growth. In contrast, OTM can occur rapidly or slowly, depending on the amount and physical characteristics of the applied force and the biological response of the Periodontal Ligament (PDL) [1]. The application of orthodontic force can change the dental and paradental tissues, including the dental pulp, PDL, alveolar bone, and gingiva; it also results in sites of pressure and tension at the tooth [2].

These changes provide information about the process of OTM through the bone remodelling system. According to Perinetti et al. [3], bone remodelling involves four main phases: activation, bone resorption, reversal, and bone formation. Previous studies have shown that several enzymes are expressed during these phases. These enzymes have been described as biomarkers during bone remodelling [4-7]. They include Lactate Dehydrogenase (LDH), Aspartate Aminotransferase (AST), Tartrate-resistant Acid Phosphatase (TRAP) and Alkaline Phosphatase (ALP).

The activation phase is essential for the initiation of tooth movement. During this phase, inflammation and cell necrosis occur and produce the enzymes LDH and AST, respectively [5,7]. OTM can result in areas of pressure and tension around the tooth. The presence of hyalinised zones at the pressured area leads to bone resorption at the PDL, while bone deposition occurs at the opposition site, that is, the tension site. These two processes occur simultaneously and lead to the recruitment of osteoblasts and osteoblast progenitors [8,9]. The presence of TRAP and ALP activity signifies osteoclastic and osteoblastic activity, respectively. Therefore, TRAP and ALP can act as biomarkers for bone resorption and bone formation [4,6].

According to previous studies, the complete cycle of bone remodelling takes 30–40 days to complete [10,11]. However, the total force and the type of bracket used are among the factors that must be considered because they can influence the rate of tooth movement [12-14]. Conventionally, orthodontic forces have been categorised as "light" or "heavy," and it has been assumed that light forces are softer and therefore more natural [2]. Moreover, orthodontic brackets, such as Conventional Ligating Brackets (CLBs) and Self-ligating Brackets (SLBs), have their own advantages and disadvantages in terms of treatment time, friction force and aesthetic value. In particular, these two brackets differ in the frictional force produced between the bracket and arch wire; SLBs produces less friction than CLBs, and SLBs are therefore said to reduce the treatment time [15].

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LDH, AST, TRAP and ALP are secreted during orthodontic tooth movement, and they thus can potentially serve as biomarkers for monitoring tooth movement. Enzymes are complex proteins that are excreted in all parts of the body. The presence of active or inactive enzymes can be detected using specific antibodies, while the activities of active enzymes can be detected by colorimetric enzyme assays [16]. The detection of these enzymes as diagnostic biomarkers is important in establishing basic knowledge for the development of future rapid and specific tools to monitor the progression of tooth movement.

Therefore, this study was performed to determine the profiles of LDH, AST, TRAP and ALP activity and the correlations among the activities of these enzymes with the total amount of enzymes produced during tooth movement using SLBs.

Materials and Methods

Subject selection

A total of 19 healthy orthodontic subjects (5 male, 14 female) aged between 16 and 28 years old were recruited for this study (n=19). These subjects were from the Postgraduate Orthodontic Clinic, Faculty of Dentistry, Universiti Kebangsaan Malaysia, in Kuala Lumpur, Malaysia. The subjects were conveniently selected using the following inclusion criteria: (1) healthy subjects without any systemic diseases, (2) good oral hygiene and healthy periodontium with plaque scores (IMPS) less than 20%, (3) not pregnant, (4) not a smoker, (5) mild to moderate crowding of the maxillary and mandibular arch that required the extraction of the upper first premolar, (6) canine relationship of class II½ or more, (7) class II/1 incisal relationship with overjet greater than 6 mm, (8) overbite not greater than 50%, (9) no previous orthodontic or orthopaedic treatment, and (10) no craniofacial anomalies. During the study period, the subjects were not allowed to take any anti-inflammatory drugs or use any mouthwash that contained chlorhexidine. The subjects received full mouth scaling and polishing 4 weeks prior to the study. Informed consent was obtained from the subjects or their parents or guardians (for those younger than 18 years old) prior to the commencement of the study. This study was approved by the ethics committee of the Universiti Kebangsaan Malaysia (No. 1.5.3.5/244/SPP/DD/030 (1)/2010).

Orthodontic appliances and experimental teeth

The transpalatal arch with Nance appliance (TPANB) was fitted to the maxillary first molars before extraction of the upper first premolar. Pre-adjusted, straight-wire, Self-ligating Orthodontic Brackets (SLBs) with 0.022 × 0.028-inch slots (Forestadent, Quick Bracket; MBT prescription) was bonded to the buccal surfaces of the maxillary incisors, canines and second premolars. The initial levelling and alignment stage utilised 0.014-inch copper nickel titanium (Cu-NiTi) archwire (Biostarter, Forestadent). This stage was completed when a 0.018 × 0.025-inch Cu-NiTi archwire (Biotorque, Forestadent) was obtained. Later, a 0.019 × 0.025-inch stainless steel (Omco Truforce) working archwire was inserted and left *in situ* for 4 weeks to allow passivity of the archwire before proceeding to the canine retraction stage. Canine retraction was then performed with a 0.019 × 0.025-inch stainless steel wire using light nickel titanium (NiTi) eyelet-closing coil springs (American Ortho, 9 mm), which were hooked from the maxillary canine bracket to the maxillary first molar band. In a split-mouth design, the subjects received 100 g or 150 g of force, on either the right or left side of the maxillary arch, which was determined by a random coin toss. The force applied was determined using the Correx gauge (dial-type stress and tension gauge; Dentaurum, Germany).

Saliva and GCF sampling

A total of 5 mL of unstimulated whole saliva was collected from the subjects after 90 minutes of non-oral activity. The subjects were asked to salivate directly into sterile containers for 10 minutes. After collection, the saliva samples were centrifuged at 1000×g for 10 minutes (4°C) to remove insoluble materials. The supernatant was then transferred to a new sterile container and was stored at -20°C until analysis.

Gingival Crevicular Fluid (GCF) was collected using paper strips (Periopaper; Proflow, Amityville, NY, USA) from experimental teeth at week 0 (before the application of tooth movement force) and then every week for 5 weeks after the application of tooth movement force (week 1–week 5). Prior to GCF sample collection, each crevicular sulcus on the test tooth was dried with cotton rolls and a saliva ejector to remove the remaining saliva. Each paper strip was inserted 1-2 mm into the gingival sulcus of the test teeth and was left *in situ* for 60 s [3]. A total of three dipped paper strips were then placed into a 1.5-mL microcentrifuge tube containing 1280 µL of bovine serum albumin (0.01 mg/mL). The tube was then centrifuged for 10 minutes at 400×g (4°C) using a microcentrifuge (Hettich Zentrifugen Mikro 22R, Tuttlingen, Germany) to completely elute the GCF component from the paper strips. The samples were then stored at -20°C for a maximum of 3 days.

Enzyme assays

Lactate Dehydrogenase (LDH) and Aspartate Aminotransferase (AST): Samples were subsequently added to the reaction buffer, which contained 16.2 mM natrium pyruvate, 0.54 mM phosphate buffer (pH 7.4) and 0.2 mM NADH, after the reaction buffer was incubated at 30°C for 5 minutes for the LDH assay. The absorbance was recorded every 30 s for 3 minutes using a microplate reader (Varioskan, Fisher Thermo Scientific, Waltham, MA, USA) at a wavelength of 340 nm.

For the AST assay, the samples were incubated in a mixture containing 0.15 M L-aspartate, 0.2 mM NADH, 0.4 U malate dehydrogenase and 0.1 M sodium phosphate buffer (pH 7.4) for 5 minutes at 30°C. Subsequently, 0.1 mM 2-oxoglutarate was added to the mixture, and after 4 minutes, the absorbance was measured at 340 nm using a microplate reader. Both results were converted to enzyme activity units (1 U=1 mol of NADH consumed per minute at 30°C). The final results were reported as LDH- and AST-specific activities. The specific activities were determined based on units of activity (U) per total protein content in milligrams (mg) and were expressed as U/mg.

Tartrate-resistant Acid Phosphatase (TRAP) and Alkaline Phosphatase (ALP): For the TRAP assay, samples

were incubated for 60 min at 37°C in a mixture containing 0.1 M *p*-nitrophenyl phosphate (*p*NPP), 1 M acetate buffer (pH 5.8), 1.0% (v/v) triton X-100, 0.15 M potassium chloride, 1 mM ascorbic acid, 0.1 mM ferric chloride and 10 mM sodium tartrate. The enzyme activity was then terminated by the addition of 0.9 M NaOH to the mixture. The absorbance was measured with a microplate reader (Varioskan, Fisher Thermo Scientific, Waltham, MA, USA) at a wavelength of 405 nm.

For the ALP assay, samples were incubated for 30 minutes at 37°C in a mixture of 0.1 M carbonate buffer (pH 9.8), 0.3 mM magnesium chloride and 1 mM *p*NPP. After the incubation period, 4 M NaOH was added to stop the reaction. The absorbance then was measured at a wavelength of 405 nm using a microplate reader. Both results were converted into enzyme activity units (1 U=1 µmol of *p*-nitrophenol liberated per minute at 37°C). The TRAP- and ALP-specific activities were determined based on units (U) of activity vs. the total protein content (mg) and were expressed as U/mg.

Enzyme-linked Immunosorbent Assay (ELISA) analysis: ELISA analysis was performed to evaluate the total levels of LDH, AST, TRAP and ALP present in the saliva and GCF during orthodontic tooth movement. The analysis was performed in duplicate using a sandwich ELISA kit specific for each enzyme (Cusabio Biotech Co. Ltd., China). The total amount of each enzyme was determined using its standard curve, which was plotted using CurveExpert Professional software, version 1.6.5. Total levels of LDH and AST were reported as mIU/mL, while the levels of TRAP and ALP were expressed as ng/mL (mIU/mL=milli-International Units/millilitre).

Canine movement

Canine movement was measured starting at week 0 (after the alignment stage but before force application) and then every week for 5 weeks after force application (week 1 – week 5). The measurements of canine movement were obtained from the margin of the distal bracket of the canine to the margin of the distal tube of the first molar using digital callipers (KERN, Germany). The results were reported as the means (mm) ± standard deviations (s.d.).

Statistical analysis

Paired *t*-tests were used to compare the means between the control (week 0) and test samples, and $p<0.05$ was considered significant. The correlation between an enzyme's activity and the total amount of the enzyme was evaluated using Pearson's correlation test. The r value from the correlation test was divided into three ranges of correlation: 0.0 – 0.3 (weak); 0.4 – 0.7 (intermediate); and 0.8 – 1.0 (strong). These analyses were conducted using SPSS software, version 20 (SPSS Inc., Chicago, IL, USA).

Results

Specific enzyme activities and total enzymes in saliva

The specific activity of LDH in saliva showed no significant differences ($p>0.05$) relative to that at week 0 (control) throughout the course of treatment (*Figure 1a*). Relative to the control, significant differences ($p<0.05$) were observed for AST-specific activity at week 1 (*Figure 1b*), for TRAP-specific activity at week 2 (*Figure 1c*), and for ALP-specific activity at week 1 to week 5 (*Figure 1d*). Moreover, the total amount of the LDH enzyme showed an intermediate negative correlation (r=-0.70) with LDH-specific activity, and the total amount of AST enzyme showed a weak positive correlation (r=0.20). The total amount of TRAP enzyme had a weak positive correlation (r=0.10) with TRAP-specific activity, but it was not significantly correlated ($p>0.05$), and the total amount of ALP enzyme had a weak negative correlation (r=-0.29) with its respective specific activity; however, neither of them was significantly correlated ($p>0.05$).

Specific enzyme activities and total enzymes in Gingival Crevicular Fluid (GCF)

The LDH-specific activity for 100 g of force exhibited significant differences ($p<0.05$) at weeks 2 and 3 (*Figure 2a*), while the activities of AST (*Figure 2b*) and TRAP (*Figure 2c*) were significantly different from control values at week 5. For 150 g of force, there were significant differences ($p<0.05$) in the LDH-specific activities at weeks 1, 2 and 3 (*Figure 1a*) and in the AST-specific activities at weeks 3, 4 and 5 (*Figure 2b*), but no significant differences ($p>0.05$) in the TRAP-specific activities (*Figure 2c*) were observed compared to the controls. Moreover, there were no significant differences ($p>0.05$) in

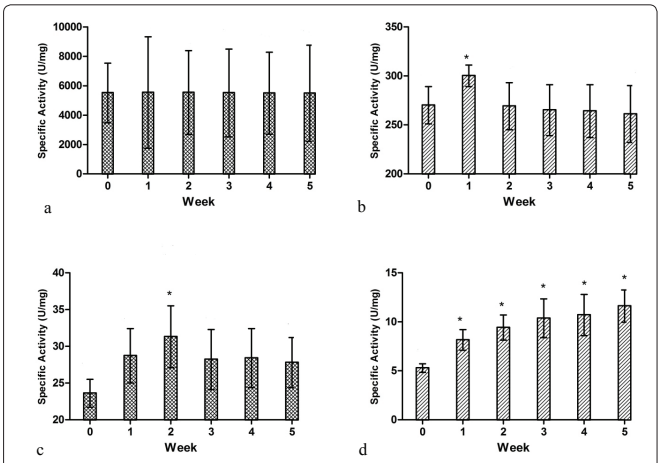

Figure 1: *Specific enzyme activities:*
*a) Lactate dehydrogenase, b) Aspartate aminotransferase, c) Tartrate-resistant acid phosphatase and d) Alkaline phosphatase in the saliva over the five tested weeks (n=19); *= significant ($p < 0.05$).*

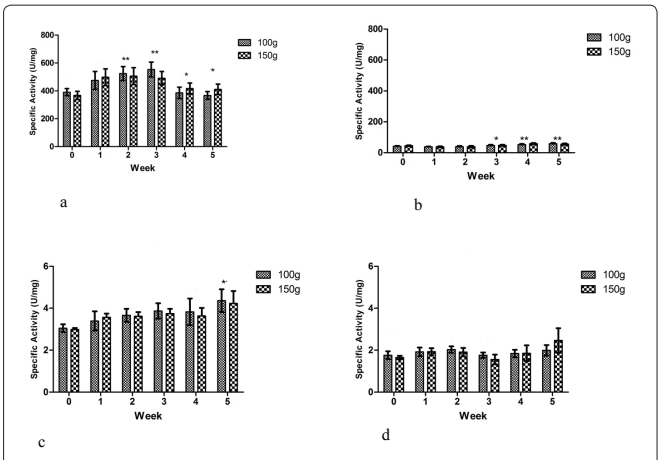

Figure 2: *Specific enzymes activities*
*a) Lactate dehydrogenase, b) Aspartate aminotransferase, c) Tartrate-resistant acid phosphatase and d) Alkaline phosphatase in the Gingival Crevicular Fluid (GCF) with 100 g or 150 g of force during the five tested weeks (n=19), *= significant ($p < 0.05$)*

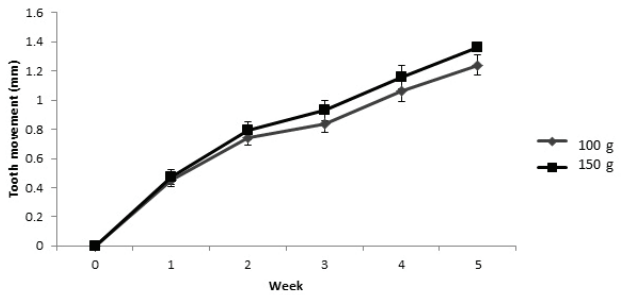

Figure 3: *Mean cumulative canine movement (in mm) ± SDM after the application of 100 g or 150 g of orthodontic forces over 5 consecutive weeks (n=19).*

the ALP-specific activities at both forces (*Figure 2d*).

At 100 g of force, the total amount of LDH enzyme showed a weak positive correlation (r=0.25), whereas the total amount of AST enzyme showed an intermediate negative correlation (r=-0.72). The total amount of TRAP enzyme, meanwhile, showed a weak negative correlation (r=-0.001) with its respective specific activity, as opposed to the total amount of ALP enzyme, which showed an intermediate positive correlation (r=0.35). A statistical analysis using Pearson's correlation for all four enzymes showed no significant correlations ($p>0.05$). At 150 g of force, the total amounts of LDH (r=-0.19), TRAP (r=-0.15) and ALP (r=-0.26) showed weak negative correlations with their respective specific activities, whereas the total amount of AST enzyme showed an intermediate negative correlation (r=-0.38) with AST-specific activities. However, no statistical correlations for any of four enzymes studied were observed ($p>0.05$).

Specific activities and tooth movement: 100 g vs. 150 g Throughout the course of the treatment, there were no significant differences ($p>0.05$) in the specific enzyme activities between 100 g and 150 g of force for any of the enzymes involved in this study. The movements of the canines also showed no significant differences ($p>0.05$) between 100 g and 150 g of force throughout the 5 weeks of treatment (*Figure 3*).

Discussion

Orthodontic tooth movement was regulated through the bone remodelling process. Activation, bone resorption, reversal and bone formation constitute a sequence of active events that occur during bone remodelling. Inflammation and cell necrosis are two biological processes that occur during the activation phase [17]. When orthodontic force is applied to the teeth, periodontal tissues will respond to mechanical stress with inflammatory reactions and cell necrosis, thus promoting the release of various enzymes, including LDH and AST, into the Gingival Crevicular Fluid (GCF) and saliva [7].

LDH-specific activity in the GCF implies the presence of gingival inflammation and tissue destruction [18,19]. Furthermore, LDH in the GCF has the potential to serve as a biomarker for detecting inflammation during orthodontic treatment [20,21]. The presence of LDH causes periodontal vasodilatation and leukocyte migration from the periodontal ligament capillaries [22]. In this study, when compared to the controls, the specific activity of LDH in the GCF increased significantly ($p<0.05$) at weeks 2 and 3 for 100 g of force and at weeks 1, 2 and 3 for 150 g of force. These findings showed that inflammation occurred earlier when 150 g of force was applied, which might induce a painful sensation that starts earlier and lasts longer. Therefore, 100 g of force was preferable for patient comfort during treatment.

LDH-specific activity in the saliva during this study showed no significant differences ($p>0.05$) compared to the baseline. This result indicated that the treatment had minimal effects on the activity of LDH. This minimal response of LDH may be a result of the different type of ligation bracket system used in this study. This study used SLBs instead of CLBs, which was used in previous studies by Rohaya et al. and Shahrul Hisham et al. [4-7]. LDH-specific activity in the saliva using the CLB system showed significant differences ($p<0.05$) at days 3, 7 and 10 [5]. The minimal response of LDH in the saliva might have been caused by SLB, which has the potential to reduce the frictional resistance produced by the archwire and the bracket [23]. Moreover, saliva itself is one of the factors that can affect frictional resistance [24].

AST is an intracellular enzyme that is normally confined to the cell cytoplasm but is released into the extracellular environment upon cell death [25]. According to Böhl et al. [26], necrotic tissue was formed during the second phase of tooth movement (after approximately 2 days of force application). However, the level gradually decreased over the next 3 weeks of treatment. We observed a significant increase ($p<0.05$) in AST-specific activity in the saliva as early as the first week of force application, which later decreased in the subsequent weeks. This finding is in agreement with the findings of Perrson et al. [27] and Rohaya et al. [7].

AST-specific activity in the GCF increased significantly ($p<0.05$) only at week 5 when 100 g of force was applied; in contrast, when 150 g of force was applied, the AST activity increased significantly at weeks 3, 4 and 5. The presence of necrosis might be due to the process of tooth movement, or it may also be due to deleterious effects, such as root resorption and bone destruction, which usually result from heavy force [2]. The 150 g force is a heavy force, and it produced significant AST-specific activity or necrosis earlier than the application of 100 g of force during the orthodontic treatment. However, its deleterious effects must be considered during orthodontic treatment.

AST in the GCF is considered to be important in regulating alveolar bone resorption during orthodontic tooth movement [25]. Bone resorption occurs at the pressure site after the removal of the hyalinised area of necrotic tissue, which develops after the application of force. Osteoclastic activity is present during bone resorption, as demonstrated by TRAP-specific activity [28]. In contrast, bone deposition occurs at the tension site, as evidenced by ALP-positive osteoblastic cells [2].

In saliva, TRAP-specific activity increased significantly at week 2 ($p<0.05$) compared to the control values. These results show that bone resorption occurs at day 14, or week 2, and is followed by the process of bone formation from week 3 until week 5, in agreement with a previous study [5]. However, TRAP-specific activity in the GCF showed a significant difference ($p<0.05$) compared to baseline values only at week 5 for 100 g of force, while no significant differences ($p>0.05$) were observed for 150 g of force throughout the course of

treatment. This finding indicates that light force has the ability to evoke frontal resorption of the bone, while heavy force results in undermining of bone resorption [29]. Frontal resorption is the removal of alveolar bone by multinucleated cells, whereas undermining resorption is the removal of alveolar bone by osteoclasts, in which the pressure applied to a tooth results in a loss of vitality in localised areas of the periodontal ligament [2,25].

Bone resorption triggers the activity of osteoblasts for bone formation. Increased activity of osteoblasts is characterised by an increase in ALP-specific activity. ALP-specific activity in saliva increased significantly ($p<0.05$) at week 1 until week 5 of treatment. Conversely, ALP-specific activity in the GCF showed no significant differences ($p>0.05$) compared to baseline for either 100 g or 150 g of force. This finding may be due to the low activities of TRAP and ALP in the GCF during tooth movement, which could not be detected by the assay system. The use of SLB tends to induce less bone resorption and bone formation, although tooth movement still occurs (*Figure 1*) [30].

Tooth movement and the specific activities of LDH, AST, TRAP and ALP were compared between 100 g and 150 g of force for each week of treatment. There were no significant differences ($p>0.05$) in tooth movement or enzyme-specific activity between 100 g and 150 g of force, which may have been due to the difference in the bracket system used (SLB vs. CLB). According to Proffit (2000), approximately 50% of the force applied to move a tooth is used to overcome friction between the archwires [31]. SLB has the ability to engage itself to the archwire and is assumed to reduce friction by eliminating the ligation force [32]. Thus, only minimal force is needed to start the motion of the tooth. A minimal amount of force is preferable in orthodontic treatment because it lowers the risk of unwanted developmental effects and is less painful [33].

Our findings regarding enzymatic biomarker profiles in the saliva reflect the process that occurs during tooth movement, as described by Perinetti et al. [3]. AST-specific activity increased significantly ($p<0.05$) at week 1, as represented by the activation process, and was followed by the bone resorption process, as demonstrated by a significant increase ($p<0.05$) in TRAP-specific activity at week 2. Along with the increase in TRAP-specific activity, ALP-specific activity also started to increase significantly ($p<0.05$) at week 1 through week 5. These findings showed that there were overlaps between the bone resorption and bone formation processes during the reversal phase at week 2. This finding further shows that bone formation can occur as early as week 2, which contradicts the bone remodelling process described by Perinetti et al. [3], who suggested that bone formation occurs after the bone resorption process. These enzyme biomarker profiles showed that the remodelling cycle in SLB might be completed earlier, thus shortening the treatment time. However, the enzyme-specific activities in the GCF were very low and thus did not present a clear picture of the remodelling process. Therefore, GCF samples would require more sensitive detection methods to obtain a clearer picture of the remodelling cycle.

There were no strong correlations ($r<0.8$) between the LDH, AST, TRAP and ALP proteins and their respective enzyme activities. The protein expression, as analysed by ELISA, did not yield a profile similar to the enzyme-specific activity as analysed by enzyme assays. This finding showed that there is a combination of active and inactive enzymes in the saliva and GCF [34]. Hence, an enzyme assay is preferred to ELISA for the observation of active biomarker profiles during orthodontic tooth movement because the enzyme assay only presents the activity of active enzymes.

Conclusion

ALP, TRAP and AST from the saliva and LDH, AST and TRAP from the GCF may potentially be used as biomarkers for monitoring orthodontic tooth movement, and 100 g of force was sufficient for orthodontic treatment using the SLB system. There was a weak correlation between enzyme activities and the total amounts of the enzymes in both the saliva and the GCF (100 g and 150 g of force). Therefore, the enzymatic assay approach is preferable to ELISA.

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Contributions of each author

NAK conducted the lab work and generated the data; MAS, IZZA and SS analysed the data; AAJ performed the statistical analysis; RMAW and SHZA designed, analysed, and generated the data and were also involved in writing the manuscript. All of the authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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