The orthodontic bonding properties of human enamel after cryopreservation

Noëmi M. C. DE ROO, Eline DEBOOSERE, Laurent A. M. THIERENS, Chris VERCRUYSSE, Liesbeth TEMMERMAN, Ronald M. H. VERBEECK and Guy A. M. DE PAUW

1 Oral Health Sciences, Department of Orthodontics, Ghent University, C. Heymanslaan 10, 9000 Ghent, Belgium
2 Biomaterials Group, Department of Basic Medical Sciences, Ghent University, C. Heymanslaan 10, 9000 Ghent, Belgium
Corresponding author, Noëmi M. C. DE ROO; E-mail: noemi.deroo@ugent.be

The aim was to investigate the effect of cryopreservation on the enamel bonding properties of orthodontic brackets. Sixty-six human premolars were randomly allocated to a control group or a cryopreserved group. Conventional stainless-steel orthodontic brackets were bonded with a light cure adhesive on the buccal side of the premolars. The shear bond strength (SBS) was determined at a crosshead speed of 1 mm/min. The SBS and adhesive remnant index (ARI) were evaluated respectively by an independent samples t test and Fisher’s exact test ($p<0.05$). The mean failure load was lower in the cryopreserved group. However, this difference in SBS was not significant ($p=0.443$). In both groups, the ARI mostly indicated a failure at the enamel-adhesive interface. The mean ARI scores for both groups were not significantly different ($p=0.099$). Within the limitations of this macro bond strength testing, it can be concluded that cryopreservation does not significantly affect the bonding properties of enamel.

Keywords: Autotransplantation, Cryopreservation, Enamel, Shear bond strength, Adhesive remnant index

INTRODUCTION

Autologous tooth transplantation (autotransplantation) is a well-established treatment modality for tooth replacement. It can be applied in case of trauma, caries, tooth impaction, or agenesis. It is primarily preferred to allogeneic tooth transplantation (allotransplantation), since allotransplants often present a detrimental immunologic stimulus in their periodontal ligament causing inflammatory or replacement resorption. Contrary to a dental implant, an autotransplanted tooth preserves a vital periodontal ligament. Moreover, teeth with an incomplete root formation show revascularization and reinnervation of the pulp and the ability of continuous eruption after autotransplantation. Teeth with complete root formation require an endodontic treatment to prevent or arrest the development of infectious-related root resorption. Pulp healing, periodontal healing, and root development subsequent to autotransplantation were extensively described by Andreasen et al. Their surgical guidelines are still first choice in the contemporary clinical practice. Recently, a systematic review revealed satisfactory rates of 96.3% for survival after 10 years and a weighted estimated success of 96.6% per year after 1 to 24 years. These numbers indicate that the long-term performance of autotransplanted teeth is comparable to other treatment modalities for missing teeth.

Cryopreservation is a reversible process that ceases all biologic functions of living cells or tissues at an ultralow temperature (−196°C). It is routinely performed in diverse branches of medicine, such as reproductive medicine (ovarian tissue and embryos) and ophthalmology (cornea). Bartlett and Reade were among the firsts to apply this process on teeth in the early 70’s. The availability of this technique has extended the scope of autotransplantation of teeth, mainly when the surgical procedures cannot be performed in a one-step procedure. For example when the receptor site needs an orthodontic preparation to (re)gain space to enable transplantation of the donor tooth. In this respect, it is important that the beneficial biological and physical properties of hard and soft tissues of teeth are preserved. In vitro research observed no detrimental effects on the periodontal ligament cells or pulp cells after cryopreservation. Oh et al. observed 96% viability of the periodontal ligament cells after cryopreservation. However, intracellular ice crystallization increased the width of the pulp cells as well as the size of the odontoblastic dendrites in the dentinal tubules. This could result in the formation of cracks in the hard tissues. The literature concerning the influence of cryopreservation on the dental hard tissues (especially on enamel) is scarce. Panighi et al. investigated the influence of cryopreservation on dentin bond strength of resin composite cylinders. They reported that the shear bond strength (SBS) of mid-coriolar and deep dentin was not affected by cryopreservation, contrary to the outer layer of the dentin. Their study emphasized on the bonding properties of dentin only. However, after transplantation of teeth, a resin build-up of the transplant or an orthodontic treatment to close the donor space or to optimize the position of the transplant is often needed. This raises the question if cryopreservation could affect the bonding properties to human enamel. Therefore, the aim of this study was to investigate the effects of cryopreservation on the bonding properties of orthodontic brackets on human enamel.
MATERIALS AND METHODS

Tooth selection
The human premolars selected for the present in vitro study were extracted atraumatically for orthodontic purposes and were excluded in case of dental anomalies (such as amelogenesis imperfecta, dentinogenesis imperfecta, caries, amalgam or composite restorations). This in vitro study was approved by the Ethical Committee of the Ghent University Hospital (ref. B670201524799).

A sample size analysis using the software G*power 3.1.9.2 (Heinrich-Heine-Universität, Düsseldorf, Germany) was conducted. The calculation based on the ability to detect a difference in SBS of 20% between the groups with \( \alpha=0.05 \) and \( 1-\beta=0.80 \) revealed a minimum sample size of 56 teeth. The sample size was set at 66 teeth to increase the power. The samples were randomly allocated to two groups (with 33 premolars each): a control group and a cryopreservation group. The extracted teeth were stored in phosphate buffered saline (PBS) until testing (control group) or were transported to the biobank in PBS (cryopreservation group).

Cryopreservation protocol
The teeth were equilibrated with dimethyl sulfoxide (DMSO) using a 4-step procedure, following the established tooth cryopreservation protocol of the Bimetra biobank\(^{17}\). The percentage of DMSO was gradually increased from 2.5 to 10% (Table 1). The basic medium contained 125 mL Dulbecco’s modified Eagle’s medium (DMEM), 15 mL fetal calf serum (FCS), and 1.5 mL antibiotics/antimycotics (AB/AM). The teeth were frozen at a slow freezing rate of \( -1^\circ\text{C/}\text{min} \) from room temperature to \( -80^\circ\text{C} \), plunged in liquid nitrogen and stored in isothermal freezers (\( -175^\circ\text{C} \)). After 5 weeks the teeth were rapidly thawed for 3 min in a \( 37^\circ\text{C} \) warm water bath. DMSO was gradually removed (10–2.5%) and the teeth were transported in PBS to the laboratory, ready for testing.

Sample preparation
All buccal surfaces were cleaned with water and polishing cups at slow speed for 10 s, rinsed with distilled water, and air dried. The adhesion areas (5×5 mm) were demarcated with nail polish and adhesive tape\(^{18}\). After removal of the glue residues with acetone, cleaning and demarcation, the enamel surfaces were conditioned with 37% phosphoric acid gel (Ultra-Etch, Ultradent, South Jordan, UT, USA) for 30 s. Subsequently the teeth were rinsed for another 30 s and air dried until the etched enamel appeared chalky white. A resin-based, light cure bonding agent (Transbond XT primer, 3M Unitek, Monrovia, CA, USA) was applied on the pre-treated enamel surface, gently blown with a stream of air for 5 s and polymerized for 20 s with a power density of 800 mW/cm\(^2\) using a halogen polymerization lamp (Elipar TriLight, 3M-Espe, St. Paul, MN, USA). Stainless steel premolar brackets (Victory Series, 3M Unitek) were bonded with a uniform layer thickness of Transbond XT (3M Unitek). A 300 g weight was applied by means of a tension gauge (Correx, Haag-Streit, Mason, OH, USA) to ensure uniform thickness of the adhesive layer\(^{19}\). The excessive resin was carefully removed with a dental probe. The samples were light-cured for 40 s with a power density of 800 mW/cm\(^2\) at a 5 mm distance (10 s at the occlusal, gingival, distal, and mesial side, respectively).

The bracket base size was 13.45 mm\(^2\). A stainless

| Freezing process | Thawing process |
|------------------|-----------------|
| **Equilibration with cryoprotective agent DMSO** | **De-equilibration of DMSO** |
| Basic medium=675 mL DMEM+75 mL FCS | Basic medium=675 mL DMEM+75 mL FCS |
| • Freezing liquid 1 (2.5%): 175 mL basic medium+4.375 mL DMSO | • Thawing liquid 1 (7.5%): 175 mL basic medium+13.125 mL DMSO |
| • Freezing liquid 2 (5%): 175 mL basic medium+8.750 mL DMSO | • Thawing liquid 2 (5%): 175 mL basic medium+8.750 mL DMSO |
| • Freezing liquid 3 (7.5%): 175 mL basic medium+13.125 mL DMSO | • Thawing liquid 3 (2.5%): 175 mL basic medium+4.375 mL DMSO |
| • Freezing liquid 4 (10%): 175 mL basic medium+17.50 mL DMSO | • Thawing liquid 4: remaining amount of basic medium |

The teeth were incubated for 5 min in each freezing liquid. After freezing liquid 4, the teeth were transferred to the recipient for freezing.

A slow freezing rate of \( 1^\circ\text{C/}\text{min} \) from \( -4^\circ\text{C} \) to \( -80^\circ\text{C} \) for at least 24 h was applied.

Plunging and storage of the samples in liquid nitrogen (\( -196^\circ\text{C} \)).

DMSO: dimethylsulfoxide, DMEM: Dulbecco’s modified Eagle’s medium, FCS: fetal calf serum
steel mountain jig (0.017×0.025 inch) was attached in the bracket slot to minimize bracket deformation during shear testing and to facilitate the alignment of the bracket base to the direction of applied force (Fig. 1)20. The roots of the teeth were then embedded in acrylic resin (Tempron, GC, Tokyo, Japan) up to the level of the cementoenamel junction. Subsequently, the samples were stored in an incubator (Don Whitley Scientific, Bradford, UK) for 20 h at a temperature of 37°C and at 100% relative humidity.

**SBS testing**

SBS is defined as the shear stress needed to break the bond between the tooth and the bracket. It is calculated as the force at failure per unit of area (N/mm² or MPa) by dividing the maximal debonding force (N) by the surface area of the bracket base (mm²)16).

The specimens were tested using a universal testing machine LRXplus (Lloyd Instruments, Bognor Regis, UK) equipped with a 500 N compression load cell. Figure 1 shows the steel rod with one flattened end attached to the crosshead. The adhesive zone between the tooth and the bracket was positioned parallel to the applied force. An occlusal-gingival load was applied to the bracket inducing a shear force at the bracket-tooth interface.

SBS was determined at a crosshead speed of 1 mm/min. The maximum load at failure was recorded with Nexygen software and converted to SBS in megapascals (MPa). After debonding, two independent examiners determined the adhesive remnant index (ARI). The ARI defines the amount of remaining adhesive and the mode of bond failure between the enamel, adhesive, and bracket base (Table 2)21). For the determination of the ARI an optical stereomicroscope was used (10× magnification) and scored as described by Klocke and Nahl-Nieke and Artun and Bergland20,21).

**Evaluation of enamel surface changes**

The possible formation and propagation of enamel cracks was investigated macroscopically. Prior to the SBS testing, the enamel surfaces were stained with a methylene blue dye for 1 min, followed by a 5 s rinse with water. The enamel cracks were evaluated through visual inspection with light microscopy. After cryopreservation and thawing of the samples, the cracks were stained with pink staining fushine. Using this procedure, the existing cracks turned purple, new cracks or the propagation of cracks colored pink. The enamel surface was evaluated with light microscopy with a 10× magnification.

**Statistical analysis**

Statistical analysis was carried out using SPSS Statistics 24 software (SPSS, Chicago, IL, USA). Differences between the groups for SBS were detected using an independent samples t test after checking for normality and homogeneity of variances. The intraclass correlation coefficient (ICC) was calculated to determine the interexaminer agreement for the ARI scores. Differences between the groups for ARI were detected by means of the Fisher's exact test. The significance level was set at 0.05 for all tests.

**RESULTS**

**SBS**

The results of the SBS testing are shown in Fig. 2. Although the mean SBS in the cryopreservation group (15.52 (5.45) MPa) was lower than in the control group (16.48 (4.64) MPa), the mean difference of 0.96 (2.49) MPa was not significant (p=0.443). However, the results of the cryopreservation group were influenced by a positive outlier (Fig. 2). After exclusion of this outlier the independent samples t test was repeated, but the difference between the two groups remained non-significant (p=0.246).

| Score | Description                              |
|-------|------------------------------------------|
| 0     | No adhesive left on the tooth            |
| 1     | Less than 50% of the adhesive left on the tooth |
| 2     | More than 50% of the adhesive left on the tooth |
| 3     | All adhesive left on the tooth, with distinct impression of the bracket mesh |
Fig. 2 Box- and whisker plots visualizing the SBS (MPa) of the control group and cryopreservation group. Remark the positive outlier in the cryopreservation group.

Fig. 3 Distribution of the ARI scores for the control group and cryopreservation group.

ARI

The distribution of the ARI scores is presented in Fig. 3. Four teeth, two in each group, showed enamel fractures and were excluded. No statistically significant difference in ARI was detected between both groups (p=0.099). In both groups score 1 was obtained for most samples (71.0% and 81.0% for the control group and cryopreservation group, respectively). This indicates that less than 50% of the adhesive remained on the tooth and that failure occurred predominantly at the enamel-adhesive interface.

Enamel surface evaluation

Propagation of existing or initiation of new enamel cracks were not found after cryopreservation of the teeth. All cracks turned purple and were considered pre-existing cracks.

The ICC was 0.878 (0.805–0.925) considering the single measures, indicating an excellent degree of agreement between the two examiners.

DISCUSSION

Insufficient penetration of cryoprotective agents in the pulp can cause intracellular ice crystallization resulting in an increased width of the pulp cells and enlargement of the odontoblastic dendrites in the dentinal tubules. Such expansion can cause damage to the dental hard tissues. Only a few studies investigated the effect of cryopreservation on dentin and enamel. Kühl et al. found no cracks in dentin after cryopreservation and related the enamel cracks to the mechanical forces from the forceps application during extraction22). A recent well-conducted in vitro study observed no changes in elastic modulus or flexural strength of dentin after cryopreservation. However, the fatigue strength of dentin decreased significantly23). Oh et al. concluded that the hardness of enamel did not change after cryopreservation, but longitudinal fractures were found in 25% of the sample15). The aim of this current study was to investigate the bonding characteristics of orthodontic brackets on enamel after cryopreservation in view of the fact that cryopreserved and autotransplanted teeth are subjected to orthodontic tooth movement with bonded brackets in a majority of the cases.

The mean SBS after cryopreservation was lower than the mean SBS of the control group, but the difference was non-significant. Comparison of the results of the present study with other studies is challenging due to the large variation in bracket types, adhesives, demarcation of the adhesion area, storage medium of the specimen, polymerization time, crosshead speed of the tensile tester, and tooth selection (bovine, human)18,24). On the other hand, the values of the SBS of the control group were in accordance with other findings and within the range (5–8 MPa) for a clinically successful result25-27). Owens et al. and Movahhed et al. observed lower values, presumably due to water storage of the bonded specimens, a non-standardized load of application, and/or different storage times before the debonding procedure28,29).

Up to now, only Panighi et al. compared the SBS of resin composite cylinders to dentin of cryopreserved teeth to dentin of non-cryopreserved teeth16). They concluded that cryopreservation significantly affected the SBS on superficial dentin, but not on mid-coronal and deep dentin. The decrease in SBS after cryopreservation might be explained by the short soaking time in DMSO. As a result, cold denatured proteins might undergo an alteration of their tertiary structure and hence affect the SBS30).

For this study, a common assessment method of bond failure was used. The ARI scores of both groups indicate failure primarily at the enamel-adhesive interface and were comparable to those obtained in previous studies for...
non-cryopreserved teeth\textsuperscript{6,20,28,29}. Four teeth were excluded due to an enamel fracture, equally distributed in both groups. Existing damage or damage resulting from the extraction procedure might explain this drop-out. However, the sample size was still sufficient according to the power analysis.

A limitation of this study could be the macro bond shear testing mode, even though no difference between the groups could be detected. Future studies using finite element analysis could give more information on the stress distribution at the bonded interfaces and could help explain the results\textsuperscript{30}. Another limitation is that experimental conditions cannot mimic the actual clinical conditions in the oral cavity. A large number of factors can influence bond testing depending on the materials and the experimental methods, tooth characteristics, storage, or humidity\textsuperscript{15,16}. Therefore, caution is necessary when extrapolating these in vitro results to a clinical setting. Future research using long term storage or aging protocols can match the clinical situation more correctly.

It could be considered a limitation of this study that the enamel surface was only investigated with staining under a light microscope (10× magnification). In this study no macroscopic changes or effect on the SBS of the possible enamel surface changes was found. Future research should focus on the effect of cryopreservation itself on enamel. Interesting methods using SEM were described by Dunbrzyte et al.\textsuperscript{32}. The composition and lay-out of enamel prisms are so specific that the entire crown is needed as a specimen to test enamel characteristics. This is in contrast with studies testing dentin characteristics using standardized dentin blocks. The interindividual morphological differences of the enamel might explain the variability (confidence interval) of the test results.

Considering storage and the post-extraction time, the use of formaldehyde should be avoided because of its potential to affect the pH of the storage medium\textsuperscript{33}. According to Shaffer et al. storage of teeth in 2% glutaraldehyde, 1% sodium hypochlorite for 24 h, steam autoclaved for 20 min at 127°C or left untreated before bonding gave no significant differences in bond strength to enamel\textsuperscript{34}. Finnema et al. reported that water storage of the bonded specimens decreased the bond strength\textsuperscript{23}. For these reasons, post-extraction time was kept to a minimum and the extracted premolars were transported in PBS at room temperature. After bonding, the teeth were stored in an incubator for 20 h at a temperature of 37°C and 100% relative humidity.

The bonding procedure was performed as standardized as possible. According to Van Noort et al., the geometry of the adhesive interface can have a significant effect on the results\textsuperscript{19}. As the SBS is based on the force at failure per unit of contact area, the surface area is of great importance\textsuperscript{19}. Therefore, great care was taken to keep the experimental parameters reproducible: a 300 g weight was applied by means of a tension gauge when placing the brackets to ensure uniform thickness of the adhesive layer and the excessive resin was carefully removed with a dental probe. However, it can be remarked that this excess removal still might induce small defects around the bracket edges.

**CONCLUSION**

Generally, it can be concluded that there was no significant influence of cryopreservation on the bonding characteristics to human enamel. Both study groups showed comparable SBS and ARI values. Therefore, it can be supposed that the bonding of orthodontic brackets on cryopreserved and autotransplanted teeth can be performed successfully.

**ACKNOWLEDGEMENTS**

This research has been made possible through a collaboration with the Bitema biobank, a high quality biorepository for the Ghent University Hospital and Ghent University.

**REFERENCES**

1) Temmerman L, De Pauw GA, Beele H, Dermert LR. Tooth transplantation and cryopreservation: state of the art. Am J Orthod Dentofacial Orthop 2006; 129: 691-695.
2) Yan Q, Li B, Long X. Immediate autotransplantation of mandibular third molar in China. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010; 110: 436-440.
3) Gonnissen H, Politis C, Schepers S, Lambrichts I, Vrielinck L, Sun Y, et al. Long-term survival and survival rates of autogenously transplanted canines. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010; 110: 570-578.
4) Schwartz O, Andreassen JO. Allo- and autotransplantation of mature teeth in monkeys: a sequential time-related histoquantitative study of periodontal and pulpal healing. Dent Traumatol 2002; 18: 246-261.
5) Andreassen JO, Paulsen HU, Yu Z, Bayer T, Schwartz O. A long-term study of 370 autotransplanted premolars. Part II. Tooth survival and pulp healing subsequent to transplantation. Eur J Orthod 1990; 12: 14-24.
6) Andreassen JO, Paulsen HU, Yu Z, Schwartz O. A long-term study of 370 autotransplanted premolars. Part III. Periodontal healing subsequent to transplantation. Eur J Orthod 1990; 12: 25-37.
7) Andreassen JO, Paulsen HU, Yu Z, Bayer T. A long-term study of 370 autotransplanted premolars. Part IV. Root development subsequent to transplantation. Eur J Orthod 1990; 12: 38-50.
8) Andreassen JO, Paulsen HU, Yu Z, Ahlquist R, Bayer T, Schwartz O. A long-term study of 370 autotransplanted premolars. Part I. Surgical procedures and standardized techniques for monitoring healing. Eur J Orthod 1990; 12: 3-13.
9) Rohof ECM, Kerdijk W, Jansma J, Livas C, Ren Y. Autotransplantation of teeth with incomplete root formation: a systematic review and meta-analysis. Clin Oral Investig 2018; 22: 1613-1624.
10) Konc J, Kanyo K, Kriston R, Somoskoi B, Cseh S. Cryopreservation of embryos and oocytes in human assisted reproduction. Biomed Res Int 2014; 2014: 307268.
11) Armitage WJ. Cryopreservation for corneal storage. Dev Ophthalmol 2009; 43: 63-69.
12) Bartlett PF, Reade PC. Cryopreservation of developing teeth. Cryobiology 1972; 9: 205-211.
13) Temmerman L, Beele H, Dermert LR, Van Maele G, De...
Pauw GA. Influence of cryopreservation on the pulpal tissue of immature third molars in vitro. Cell Tissue Bank 2010; 11: 281-289.

14) Temmerman L, Dermaut LR, De Mil M, Van Maele G, Beele H, De Pauw GA. Influence of cryopreservation on human periodontal ligament cells in vitro. Cell Tissue Bank 2008; 9: 11-18.

15) Oh YH, Che ZM, Hong JC, Lee EJ, Lee SJ, Kim J. Cryopreservation of human teeth for future organization of a tooth bank: a preliminary study. Cryobiology 2005; 51: 322-329.

16) Panighi MM, Allart D, Jacquot BM, Camps J, G’Sell C. Influence of human tooth cryopreservation on dentin bond strength. Dent Mater 1997; 13: 56-61.

17) Temmerman L, Vral A, Meire M, Verbeeck RM, Deschepper E, Dermaut LR, et al. Pulpal regeneration and root development after subcutaneous transplantation of cryopreserved immature teeth in rats. Cryobiology 2012; 64: 81-90.

18) Van Noort R, Cardew GE, Howard IC, Noroozi S. The effect of local interfacial geometry on the measurement of the tensile bond strength to dentin. J Dent Res 1991; 70: 889-893.

19) Eliades T, Brantley WA. The inappropriateness of conventional orthodontic bond strength assessment protocols. Eur J Orthod 2000; 22: 13-23.

20) Klocke A, Kahl-Nieke B. Effect of debonding force direction on orthodontic shear bond strength. Am J Orthod Dentofacial Orthop 2006; 129: 261-265.

21) Artun J, Bergland S. Clinical trials with crystal growth conditioning as an alternative to acid-etch enamel pretreatment. Am J Orthod 1984; 85: 333-340.

22) Kührl S, Deyhle H, Zimmerli M, Spagnoli G, Beckmann F, Muller B, et al. Cracks in dentin and enamel after cryopreservation. Oral Surg Oral Med Oral Pathol Oral Radiol 2012; 113: e5-e10.

23) Yan W, Tenwalde M, Oilo M, Zhang H, Arola D. Effect of cryopreservation of teeth on the structural integrity of dentin. Dent Mater 2018; 34: 1828-1835.

24) Finnema KJ, Ozan M, Post WJ, Ren Y, Dijkstra PU. In-vitro orthodontic bond strength testing: a systematic review and meta-analysis. Am J Orthod Dentofacial Orthop 2010; 137: 615-622.

25) Mews L, Kern M, Ciesielski R, Fischer-Brandies H, Koos B. Shear bond strength of orthodontic brackets to enamel after application of a caries infiltrant. Angle Orthod 2015; 85: 645-650.

26) Eslamian L, Borzabadi-Farahani A, Tavakol P, Tavakol A, Amini N, Lynch E. Effect of multiple debonding sequences on shear bond strength of new stainless steel brackets. J Orthod Sci 2015; 4: 37-41.

27) Pseiner BC, Freudenthaler J, Jonke E, Bantleon HP. Shear bond strength of fluoride-releasing orthodontic bonding and composite materials. Eur J Orthod 2010; 32: 268-273.

28) Movahhed HZ, Ogaard B, Syverud M. An in vitro comparison of the shear bond strength of a resin-reinforced glass ionomer cement and a composite adhesive for bonding orthodontic brackets. Eur J Orthod 2005; 27: 477-483.

29) Owens SE Jr, Miller BH. A comparison of shear bond strengths of three visible light-cured orthodontic adhesives. Angle Orthod 2000; 70: 352-356.

30) Privalov PL. Cold denaturation of proteins. Crit Rev Biochem Mol Biol 1990; 25: 281-305.

31) Braga RR, Meira JB, Boaro LC, Xavier TA. Adhesion to tooth structure: a critical review of “macro” test methods. Dent Mater 2010; 26: e38-e49.

32) Dumbrzyte I, Linkeviciene L, Linkevicius T, Malinauskas M. Enamel microcracks in terms of orthodontic treatment: A novel method for their detection and evaluation. Dent Mater J 2017; 36: 438-446.

33) Silverstone LM. The histopathology of enamel lesions produced in vitro in teeth previously exposed to calcifying fluids. Caries Res 1970; 4: 31-48.

34) Shaffer SE, Barkmeier WW, Gwinnett AJ. Effect of disinfection/sterilization on in-vitro enamel bonding. J Dent Educ 1985; 49: 658-659.