Storage media to preserve dentin and their effects on surface properties

Shamim Sultana, BDS,[a] Toru Nikaido, DDS, PhD,[a] Md Asafujjoha, BDS,[b] Junji Tagami, DDS, PhD,[a,c] and Khairul Matin, BDS, PhD[a,c]

[a]Cariology and Operative Dentistry, Department of Restorative Sciences, Tokyo Medical and Dental University, Tokyo, Japan, [b]Department of Conservative Dentistry, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh, and [c]Center of Excellence (COE) Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone at Tokyo Medical and Dental University, Tokyo, Japan

Purpose: This study assessed the probable changes in surface properties of dentin preserved in four different storage media: 1) deionized water (Milli-Q), 2) sodium chloride buffered saline solution (NaCl), 3) Hank's balanced salts solution (HBSS), and 4) phosphate buffered saline solution (PBS).
Materials and Methods: Equal sized dentin beams were prepared from bovine incisors and stored in the above mentioned storage media for different time periods (1, 7, and 30 days) which then subjected to nanoindentation measurements for hardness and elasticity. Surface roughness (R_a) of the samples was analyzed by a confocal laser microscope. Data were statistically analyzed by one-way ANOVA and Dunnet T3 tests.
Results: Nanoindentation showed that storing dentin in Milli-Q or NaCl media resulted in large decreases in hardness and elasticity, 14% and 18%, respectively by 1 day. After 7 days due to demineralization processes in Milli-Q and NaCl media, hardness and elasticity decreased about 27% and 31%, respectively relative to the starting values which were significantly lower than samples stored in the other two media. In HBSS and PBS, there were no significant alterations in mechanical properties during the 30-day long storage. R_a values of all stored samples were more or less the same as control samples except for samples stored in PBS for 30 days, in which the R_a was increased significantly.
Conclusion: PBS is preferred for short term preservation of dentin, as opposed to NaCl or Milli-Q, to avoid alterations in the mechanical properties of dentin surfaces. **(Int Chin J Dent 2006; 6: 123-129.)**
Key Words: dentin, hardness, modulus of elasticity, storage media, surface roughness.

Introduction

Dentin is a hard mineralized tissue whose function is to provide elastic support to the overlying hard enamel, thus enabling the dispersion of mechanical stresses applied during mastication.[1] Dentin consists of an organic matrix made up primarily of collagen and an inorganic phase made up of apatite crystals.[2] The apatite phase contributes to most of the compressive strength that might directly effect hardness, while the collagen phase provides elasticity.[3] Changes in these two phases might affect alterations in the physical properties; hardness and modulus of elasticity of these biological composites. The mechanical properties of dentin are largely determined by the intertubular dentin matrix, which is a complex composite of type I collagen fibers and a carbonate-rich apatite mineral phase.[4] By using a nanoindentation tester, the modulus of elasticity which represents the elastic properties of dentin could be determined.[5,6]

Maintaining the mechanical properties of tooth substrates is important during *in vitro* manipulations as well as being important for clinical tooth preparations. The mechanical property concerns regarding the handling of teeth for research purposes have prompted investigators to evaluate the effects of storage in various storage media on hard tooth specimens as well as extracted teeth during transportation. An aqueous solution is essential for maintaining the hydration of the tooth samples prepared for mechanical testing. The mechanical properties of calcified tissues are also dependent on their mineral content.[7] For *in vitro* studies, primarily nanomechanical testing, bond strength tests, durability studies, caries research and oral biofilm research, storage of samples can become a critical issue since chemical reactions such as etching and dissolution affect the surface layer. The

hardness of enamel and dentin is associated with mineral loss. Obviously, demineralization decreases hardness and remineralization usually restores hardness by deposition of mineral particles.[8-11]

In recent years, nanoindentation has become an excellent tool for the determination of the local mechanical properties of structural features of biological hard tissues.[12-15] This depth-sensing technique allows the performance of nanomechanical testing, including those for nanohardness and elasticity, of dentin to minimize artifacts caused by processing during sample preparation. There are, however, varying reports on the effects of storage media on the mechanical properties of dental hard tissues.[16] In the present laboratory oriented study, four different storage media: 1) deionized water (Milli-Q), 2) sodium chloride buffered saline solution (NaCl), 3) Hank's balanced salts solution (HBSS) and 4) phosphate buffered saline solution (PBS) were assessed using bovine dentin. Previously, HBSS (which is used as a control medium in the present study) has been recommended for short-term preservation in order to maintain the physical properties of human dental tissues.[16] Therefore, this study was designed to investigate the changes in nanomechanical properties of the dentin preserved in the above mentioned storage media using a nanohardness tester and followed with testing for surface roughness.

Materials and Methods

Specimen Preparation

Approximately 1 mm thick and 2 mm wide beam shaped specimens were prepared from the mid-labial portion of twenty sound bovine incisors with a low speed diamond saw (Isomet, Buehler, Lake Bluff, IL, USA). Four equal sized beams were obtained from each tooth as shown in Fig. 1. The specimens were then flattened using a low-speed polisher (Buehler) with silicon carbide paper under running water using grit sizes 1,000, 1,200, 1,500 and polished with water based diamond paste to 0.25 μm and ultrasonically cleaned for 15 s.

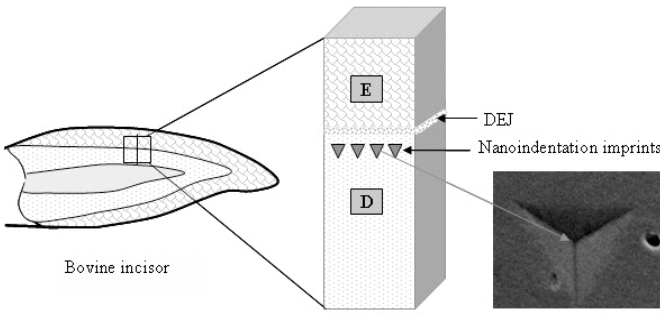


Fig. 1.
Locations of indentation imprints are displayed in the diagrams; E, D and DEJ indicate enamel, dentin and dentino-enamel junction respectively. Four equal sized beams were obtained from mid-labial part of each tooth (two beams from each half). Indentations were applied horizontally and approximately 100 μm inside from DEJ shown in the figure. SEM view of a typical indentation imprint on a control dentin surface is shown on the right. Indentation was programmed and set to place on a dentin cross section where dentinal tubules were perpendicularly cut with the indenter tip aiming at intertubular spaces. Sharp and prominent imprint of the indenter tip, walls and edges are visible.

Preservation in storage media

Before storage all specimens were subjected to nanoindentation tests and the baseline measurement was used as '0' day or control data. The specimens were then stored separately in 4 mL of the following storage media: 1) NaCl, 2) HBSS, 3) Milli-Q and 4) PBS. Chemical components of the storage media are shown in Table 1. The pH of all storage media was measured with a pH-meter (pH/ION Meter F-53, Horiba Ltd., Kyoto, Japan) and the solutions were filtered with Millex-GS 0.22 μm filter units (Millipore Corp., Bedford, MA, USA) before use. One beam from each tooth was stored in each medium at 4ºC for 1, 7, and 30 days time and were then subjected to nanoindentation testing to measure hardness and modulus of elasticity.

Table 1. Chemical components of storage media.

Storage media	pH	Composition (mg/L)	Manufacturer
Normal saline	5.9	900 NaCl	Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan
Hank's balanced salt solution (HBSS)	8.0	400 KCl, 60 KH_2PO_4, 8,000 NaCl, 1,000 glucose, 90 Na_2HPO_4 $7H_2O$, 350 $NaHCO_3$, 1,400 $CaCl_2$, 100 $MgSO_4$ $7H_2O$, 100 $MgCl_2$ $6H_2O$	Sigma Aldrich Co., St. Louis, MO, USA
Milli-Q	6.5	Deionized by water purifier (Millipore)	Japan Millipore Corp., Tokyo, Japan
Phosphate buffered saline solution (PBS)	7.2-7.4	8,000 NaCl, 1,280 Na_2HPO_4, 350 NaH_2PO_4	Wako Pure Chemical Ind., Ltd., Osaka, Japan

Nanoindentation

The nanoindentation tester (ENT-1100, Elionix Co., Tokyo, Japan) used in this study was a depth sensing computer controlled instrument which has Berkovich indentor, which consists of a three-sided pyramid diamond probe. Just before testing, the specimen was fixed on a flat metallic dais using utility wax to stabilize the specimen and to orient the surface parallel to the stage of the nanoindentation tester with the polished surface facing upwards for indentation. The specimen was kept under dry conditions during measurements since this device can only maintain the temperature in the chamber at 27°C but not the humidity. During indentation, the applied indenter force load and the depth of penetration into the sample are continuously monitored. An indentation load of 0.049 N (5 gf) and 10 indents at 30 μm intervals per specimen were applied 100 μm from the dentino-enamel junction (DEJ) towards the pulp at a speed of 4.9×10^{-6} N/ms (0.5 mgf/ms). Nano-hardness (GPa) and Young's modulus of elasticity (GPa) were calculated and recorded by an attached computer.

Surface roughness

The specimens stored for 30 days were used for the measurement of surface roughness (R_a, μm) by a confocal laser scanning microscope and analyzed by a computerized roughness tester (Keyence Corp., VK-8500, Osaka, Japan). The specimen was fixed on a stage and laser light was applied to visualize the surface. The applied pitch was 0.05 μm and the scan time was 4 minutes. Data were recorded from four corners and the central part of the selected area for each specimen.

Statistical Analysis

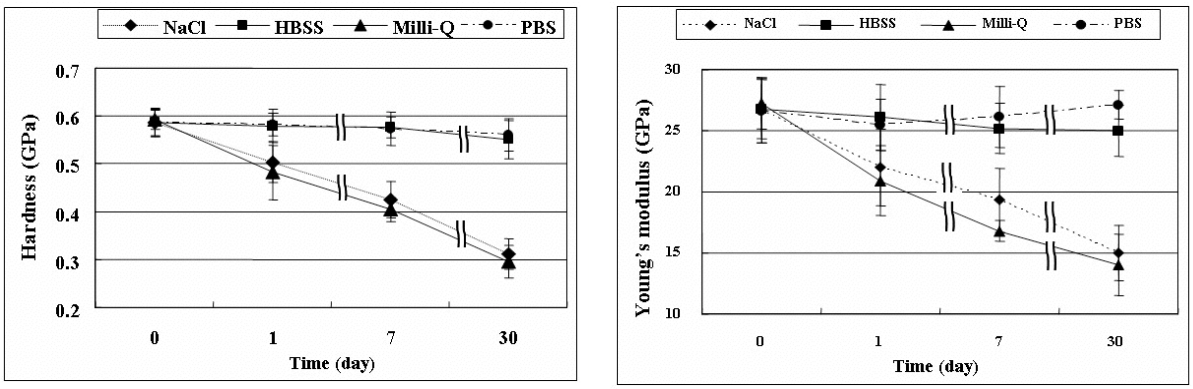
All data were statistically analyzed by one-way ANOVA and Dunnet T3 testing using the Statistical Package for Medical Science (SPSS for Windows release11.0, SPSS Inc., Chicago, IL, USA). The data were analyzed by repeated measurement analysis of variance at the 95% confidence level and the two variables assessed were "storage media" and "time period".

Results

Hardness and modulus of elasticity

The results of this study showed that teeth stored in NaCl or Milli-Q resulted in a large decrease in hardness and modulus of elasticity (Figs. 2a and 2b). At 1 day, a decrease in the mechanical properties values of up to 14% and 18% was observed in case of NaCl and Milli-Q respectively. After 7 days, due to the demineralization process during storage in NaCl and Milli-Q solutions, the mechanical properties dropped to 27% and 31% respectively of their starting values, which were significantly lower than those with HBSS and PBS. In HBSS

and PBS, there were no significant alterations in the mechanical properties for a time interval of 7 days.



a b

Figs. 2a (left) and 2b (right).
a) Reduced nanohardness of bovine dentin versus time stored in NaCl, Milli-Q, HBSS and PBS. Reduction was not significant when stored in HBSS or in PBS (SDs are indicated by error bars, n=10). b) Reduced Young's modulus of bovine dentin versus time stored in NaCl, Milli-Q, HBSS and PBS. Reduction was not significant when stored in HBSS and PBS (SDs are indicated by error bars, n=10).

After 30 days, NaCl and Milli-Q showed 47% and 49% loss in hardness and Young's modulus elasticity. In the case of Milli-Q, hardness and elasticity were 0.29 GPa and 14 GPa, respectively. Also for NaCl, these values were 0.31 GPa and 15 GPa. For PBS, it was found that there were no significant changes in hardness and modulus elasticity after 30 days. Also, in HBSS there were no significant changes in hardness and modulus elasticity after 30 days. These results indicated that the effects on sample hardness for each storage media were different. The hardness of dentin stored in PBS and HBSS was not decreased following storage up to 30 days ($p>0.05$). However, with Milli-Q and NaCl hardness was decreased significantly during that same period.

Surface roughness

Surface roughness of the samples after 30 days was mapped by a confocal laser scanning microscope, and the summarized R_a data (mean and SD) are depicted graphically (Fig. 3). No significant differences could be detected between the control dentin surface and dentin surfaces stored in HBSS, Milli-Q or NaCl. Some scattered high and low picks were visible probably because of salt sediments and open dentinal tubules, respectively. Samples stored in PBS significantly increased in surface roughness compared with all other stored and control samples with more high picks and crystalline precipitations detected.

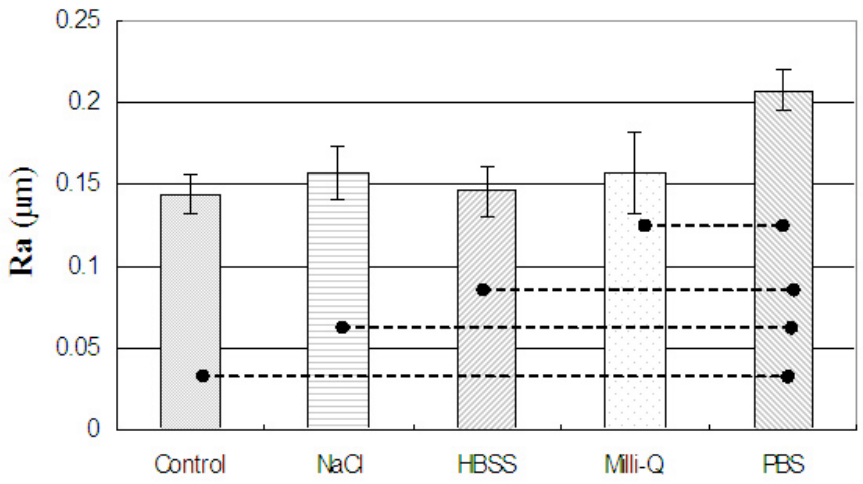


Fig. 3.
Graph represents mean R_a values obtained after preservation in four storage media for 30 days compared with the control (SDs are indicated by error bars, n=15. Broken bars indicate significant differences, $p<0.05$, $F=2.647$).

Discussion

Storage solutions sometimes are used for preservation of tooth substrates in different biological studies. Several available storage media either manufactured or prepared *in situ* with different compositions may affect the expected results of the experiments. Hence, selection of the storage medium for preservation of the samples is a very important factor. To date several methods have been employed to investigate demineralization and remineralization of teeth such as hardness tests,[8] microradiography,[17] contact micrography,[18] electron probe microanalysis (EPMA),[19] QLF,[20] Diagnodent, and many more *in vitro* or *in vivo* assays. The use of nanoindentation is not limited to characterization of the mechanical properties of tooth tissues but is also used for dental materials. The tooth-colored materials are subjected to evaluation using different International Standards Organization (ISO) Testing.[21]

On the other hand, for restorations dentin is acid conditioned and primed and an adhesive agent applied. Nakabayashi et al.[22] reported that resin-impregnation creates a transitional "hybrid" layer, that is neither resin nor tooth, but a hybrid of the two. The thin layer of resin-reinforced dentin locks the two dissimilar substances together at the molecular level, sealing the surface against leakage and imparting a high degree of acid resistance. The ideal thickness of a well constructed hybrid layer has proven to be less than 10-µm, which involves a submicroscopic undisturbed dentin surface layer. Inappropriate storage could cause damage to this very delicate submicroscopic tooth structure which can influence the data obtained during mechanical testing. Hence, selecting a perfectly matched storage medium with appropriate preservation properties was assessed. In this study bovine teeth were used as an acceptable substitute for human teeth because they are large enough to provide consistent dentin surfaces of sufficient size even in the deep layers and equal sized beams could be prepared in different preservation solutions. Most importantly, controlled base line data in terms of the mechanical properties of tooth substrates could also be obtained which is important for a comparative study.

During indentation, the applied indenter force load and the depth of penetration into the sample are continuously monitored. If the specimen was affected by the solution, loads on the stored specimens were decreased to maintain approximately constant indentation depths. In our study the hardness and modulus of elasticity of dentin were altered within 1 day of storage when preserved in physiological saline (NaCl) and Milli-Q. After 7 days the hardness and modulus of elasticity of dentin were reduced to 27% and 31% respectively of their starting values and further decreased to 47% and 49% after 30 days. However, with PBS or HBSS there were no significant changes of hardness and Young's modulus of elasticity after 30 days. Apparently, there was an exchange of minerals or electrolytes during 30 days storage in all media with PBS and HBSS exhibiting minimum variability.

The pH of the NaCl solution was 5.9 and sufficiently acidic for some demineralization to occur. On the other hand, it lacks calcium and phosphate ions for remineralization. It has been shown that the hardness and Young's modulus of elasticity of dentin decreased when the specimens were stored in physiological saline. This is presumably due to the loss of surface calcium resulting in further exposure of the dentin collagen, which could have an important effect on the hydrolysis of unprotected collagen fibrils. The pH of fresh Milli-Q usually is 6.5, which is not acidic enough for surface demineralization. Habelitz et al.[16] reported that deionized water lacks calcium and phosphate ions and therefore the chemical potential for dissolution of the mineral phase of dentin and enamel is high and is assumed to be the major reason for demineralization and softening of the tissues. Also the hardness was reduced due to the increased permeability of dentin.

Our results showed that there were no significant differences in the hardness and Young's modulus for intertubular dentin when preserved in HBSS. This is consistent with the findings by Habelitz et al.[16] The reason for this is due to the fact that the pH of HBSS is 8.0 which makes it a basic solution. Furthermore, it is highly concentrated in Ca^{2+}, Mg^{2+}, Na^{+}, PO_4^{3-} and Cl^{-}. These molar concentrations are sufficient to maintain ionic and mineral exchange between the liquid phase and dentin. This means that surface demineralization is prevented. On the other hand, there were no significant changes in the mechanical properties of the dentin when preserved in HBSS. It is essential to point out that using HBSS as a preservative solution is also relatively expensive. In addition, it is relatively unstable when exposed to light for short time periods. PBS is a buffered solution with a pH of 7.2- 7.4 containing Na^{+}, PO_4^{3-}, and Cl^{-} ions. This buffer is commonly used in the laboratory especially for bio-medical studies. As a buffer, PBS maintains the electrolytic imbalance in the solution and there was no change in the pH after 1 month storage.

Carvalho et al.[25] reported that long-term storage of EDTA-demineralized human dentin in PBS solution did not cause any significant reduction in its mechanical properties. The present study indicated that storage of solid dentin in PBS solution did not cause any significant changes in its mechanical properties. The R_a value was found to be significantly high due to crystallization of PBS salts on dentin surfaces. Stationary storage in the refrigerator for long periods of time may have allowed the PBS salts to crystallize on the dentin surfaces. Hayakawa et al.[26] have found that there was no precipitation after one day immersion in HBSS in a study onto phosphorylated polymers. After 3 days, the formation of precipitated globules was observed scattered on the phosphorylated polymer surface. After 7 days immersion in HBSS, the phosphorylated polymer surface was completely covered with calcium phosphate globules. In our study, there were very negligible precipitations on dentin while immersed in HBSS. However, comparatively large precipitation globules, rather than salt crystals, were present.

From the results of surface roughness measurements it can be concluded here that PBS contains sufficient salts so that during long term preservation the salts were deposited on to the dentin surface. On the other hand, deposition of the salts onto the surfaces may interfere with some results of the experiments. Therefore, the surface can become rough compared to samples in the other three preservative solutions. On the other hand, the samples preserved in NaCl and Milli-Q showed smooth surfaces like the control. This may have resulted from the sedimentation of the dissolved mineral particles back onto the demineralized dentin surfaces. Opening of the dentinal tubules of these samples resulted in a widened but partially occluded aperture resulting in low R_a values after laser detection. Therefore, our study suggests that both hardness and Young's modulus of elasticity were reduced as a result of several media properties including the pH, buffering capacity and the ion content of the storage media.

Previous investigators have found that formalin storage was effective for infection control purposes.[27] It cannot, however, be recommended as a storage medium for dentin bonding studies due to the alterations in dentin bond strength resulting from its use.[28] Other possible alterations can be due to variations in specimen wetness, mineralization, microtubule density and orientation.[29] The significant influence of various parameters including the origin of the dentin, types of teeth, tooth storage temperature, maximum storage time of teeth, dentin depth, as well as the storage of bonded specimens (medium, temperature, and time) need to be further evaluated.

Acknowledgment

This work was supported by the Center of Excellence (COE) Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone at Tokyo Medical and Dental University, Tokyo, Japan, and a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science (No. 16390544). Also, the authors would like to express their gratitude to Prof. Howard K. Kuramitsu, State University of New York at Buffalo for his kind contributions in editing the manuscript.

References

1. Nakabayashi N, Pashley DH. Hybridization of dental tissues. Quintessence Publishing Co. Ltd.; Chicago: 1998. p.22.
2. Katz JL. Hard tissue as a composite materials, Bounds on the elastic behavior. J Biomech 1971; 4: 455-73.
3. Martin RB, Boardman DL. The effects of collagen fiber orientation, porosity, density, and mineralization on bovine cortical bone bending properties. J Biomech 1993; 26: 1047-54.
4. Kinney JH, Pople JA, Marshall GW, Marshall SJ. Collagen orientation and crystallite size in human dentin: a small angle X-ray scattering study. Calcif Tissue Int 2001; 69: 31-7.
5. Urabe I, Nakajima M, Sano H, Tagami J. Physical properties of the dentin-enamel junction region. Am J Dent 2000; 13: 129-35.
6. Poolthong S, Mori T, Swain MV. Determination of elastic modulus of dentin by small spherical diamond indenters. Dent Mater J 2001; 20: 227-36.
7. Gustafson MB, Martin RB, Gibson V, et al. Calcium buffering is required to maintain bone stiffness in saline solution. J Biomech 1996; 29: 1191-4.
8. Pereira PNR, Inokoshi S, Yamada T, Tagami J. Microhardness of in vitro caries inhibition zone adjacent to conventional and resin-modified glass ionomer cements. Dent Mater 1998; 14: 179-85.
9. Akimoto N, Yokoyama G, Ohmori K, Suzuki S, Kohno A, Cox CF. Remineralization across the resin-dentin interface: In vivo evaluation with nanoindentation measurements, EDS, and SEM. Quintessence Int 2001; 32: 561-70.
10. Marshall GW, Habelitz S, Gallagher R, Balooch M, Balooch G, Marshall SJ. Nanomechanical properties of hydrated carious human dentin. J Dent Res 2001; 80: 1768-71.
11. Zheng L, Nakajima M, Higashi T, Foxton RM, Tagami J. Hardness and Young's modulus of transparent dentin associated with aging and carious disease. Dent Mater J 2005; 24: 648-53.
12. Kinney JH, Balooch M, Marshall SJ, Marshall GW Jr, Weihs TP. Hardness and Young's modulus of human peritubular and intertubular dentine. Arch Oral Biol 1996; 41: 9-13.
13. Kinney JH, Balooch M, Marshall SJ, Marshall GW Jr, Weihs TP. Atomic force microscope measurements of the hardness and elasticity of peritubular and intertubular human dentin. J Biomech Eng 1996; 118: 133-5.
14. Marshall GW Jr, Balooch M, Gallagher RR, Gansky SA, Marshall SJ. Mechanical properties of the dentinoenamel junction: AFM studies of nanohardness, elastic modulus, and fracture. J Biomed Mater Res 2001; 54: 87-95.
15. Chaiwat M, Nikaido T, Foxton RM, Tagami J. Effect of glass ionomer cements on nanohardness of caries-affected dentin. Int Chin J Dent 2003; 3: 122-130.
16. Habelitz S, Marshall GW Jr, Balooch M, Marshall SJ. Nanoindentation and storage of teeth. J Biomech 2002; 35: 995-8.
17. ten Cate JM. Remineralization of caries lesions extending into dentin. J Dent Res 2001; 80: 1407-11.
18. Creanor SL, Awawdeh LA, Saunders WP, Foye RH, Gilmour WH. The effect of a resin-modified glass ionomer restorative material on artificially demineralised dentin caries in vitro. J Dent 1998; 26: 527-31.
19. Kitasako Y, Nakajima M, Foxton RM, Aoki K, Pereira PN, Tagami J. Physiological remineralization of artificially demineralized dentin beneath glass inomer cements with or without bacteria contamination in vivo. Oper Dent 2003; 28: 276-82.
20. Pretty IA, Ingram GS, Agalamanyi EA, Edgar WM, Higham SM. The use of fluorescein-enhanced quantitative light-induced fluorescence to monitor de-and re-mineralization of in vitro root caries. J Oral Rehabil 2003; 30: 1151-6.
21. International Standards Organization. Dentistry -resin-based filling, restorative and luting materials 2000; ISO 4049.
22. Nakabayashi N, Nakamura M, Yasuda N. Hybrid layer as a dentin-bonding mechanism. J Esthet Dent 1991; 3: 133-8.
23. Kinney JH, Balooch M, Marshall GW, Marshall SJ. Measurement of the microhardness and young's modulus of human enamel and dentine using an indentation technique. Arch Oral Biol 1996; 41: 539-45.
24. Kinney JH, Balooch M, Marshall GW, Marshall SJ. A micromechanics model of the elastic properties of human dentine. Arch Oral Biol 1999; 44: 813-22.
25. Carvalho RM, Tay F, Sano H, Yoshiyama M, Pashley DH. Long-term mechanical properties of EDTA-demineralized dentin matrix. J Adhes Dent 2000; 2: 193-9.
26. Hayakawa T, Yoshinari M, Sakae T, Nemoto K. Calcium phosphate formation on the phosphorylated dental bonding agent in electrolyte solution. J Oral Rehabil 2004; 31: 67-73.
27. DeWald JP. The use of extracted teeth for in vitro bonding studies: a review of infection control considerations. Dent Mater 1997; 13: 74-81.
28. Zheng TL, Huang C, Zhang ZX, Wang S, Zhang G. Influence of storage methods on microtensile bond strength of dentin adhesive system. Shanghai Kou Qiang Yi Xue 2005; 14: 147-50.
29. Kinney JH, Balooch M, Marshall GW, Marshall SJ. A micromechanics model of the elastic properties of human dentine. Arch Oral Biol 1999; 44: 813-22.

Correspondence to:

Dr. Shamim Sultana
Cariology and Operative Dentistry, Department of Restorative Sciences
Graduate School, Tokyo Medical and Dental University
1-5-45, Yushima, Bunkyo-ku, Tokyo, 113-8549, Japan
Fax: +81-3-5803-0195 E-mail: rumasultana2000@yahoo.com

Received November 8, 2006. Accepted November 30, 2006.