ORIGINAL ARTICLE

Cytotoxicity of Strengthened Glass-ionomer Cement by Compounding Short Fibers with $CaO-P_2O_5-SiO_2-Al_2O_3$ Glass

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Synopsis

The purpose of this study was to clarify whether the short glass fibers of $CaO-P_2O_5-SiO_2-Al_2O_3$ (CPSA) glass possessed the ability to reinforce conventional glass-ionomer cement (GIC). Biocompatibility of the set GIC mixed with short CPSA glass fibers was evaluated in a cell culture cytotoxicity test. Moreover, the rate of release fluoride ions from GIC mixed with short glass fibers was measured. The powder of a conventional GIC was mixed with short CPSA glass fibers (diameter, 9.7±2.1 μm; aspect ratio, 5.0±0.9) before mixing with the liquid of the GIC. Set cements of 40 mass% short CPSA glass fibers mixed with GIC powders showed maximum values of 18 MPa in diametral tensile strength (DTS) after aging for 24 hours due to the effects of specific shape of short glass fibers and reactivity between the mixing liquid and short glass fibers. The cytotoxicity of these cements to rat pulpal cells tested by cellular activity showed that the set GIC disks (13 mm in dia. × 1 mm in thickness) with 40 mass% short CPSA glass fibers had cell activity as that of the set GIC or a cell culture coverslip used as control. Moreover, the addition of short glass fibers to GIC did not disturb the release of fluoride from the specimens.

Key words: *glass-ionomer cement, CPSA glass short fiber, mechanical property, cytotoxicity, fluoride release*

1. Introduction

Glass-ionomer cement (glass polyalkenoate cement, GIC) was developed by Wilson and Kent about 40 years ago [1]. Since then, GICs have been widely used clinically in dentistry. GICs possess unique characteristics such as fluoride release and recharge for caries resistance, mild pulp irritation, esthetics, and sufficient adhesion to teeth structures [2, 3]. However, conventional GICs have some disadvantages, including sensitivity to moisture during initial hardening, relative poor mechanical properties and rough surface texture, compared to composite resin, dental amalgam or inlay restoration.

In order to improve the low strength of conventional GICs, resin-modified glass-ionomer cements were developed about 20 years ago and have become widely used. The weak organic-salt matrix of conventional GICs was strengthened by modification of the resin as in methacry-

late-modified systems. Thus, the mechanical properties of resin-modified GICs were superior to those of conventional GICs [4, 5]. As a recent successful study of strengthening conventional GICs, Arita et al. [6] reported that hydroxyapatite-containing glass-ionomer cement had improved flexural and micro-structural properties.

On the other hand, in the year of 2000, FDI recommended the use of conventional GIC for atraumatic restorative treatment (ART) to develop the concept of minimal intervention dentistry [7]. Therefore, research on improving the qualities of conventional GIC is a field of increasing interesting. The addition of short glass fibers to glass-ionomer cement may be more effective for strengthening the cement if the composition of the short fibers is similar to that of the fluoro-alumino-silicate glass [2] of the powder in the cement. Kobayashi et al. [8] reported that such glass fibers composed of $CaO-P_2O_5-SiO_2-Al_2O_3$ (CPSA) possessing a particular aspect ratio (diameter, 9.7±2.1 μm, aspect ratio, 5.0±0.9) could function as a reinforcing agent for conventional GIC. *In vivo* and *in vitro* studies have suggested that CPSA glass fibers have biocompatible potentiality for medical and dental applications [9-12]. Moreover, CPSA glass fibers have a high tensile strength of approximately 2000 MPa [13]. Moreover, Kawano et al. demonstrated that GIC reinforced with short CPSA glass fibers maintained a higher strength than that of conventional cement after aging [14].

The aim of this study was to determine whether CPSA short glass fibers can function as a reinforcing agent in conventional glass-ionomer cement and whether set GIC mixed with short CPSA glass fibers shows biocompatibility and releases sufficient fluoride ion in cell culture.

2. Materials and methods

1) Preparation of glass-ionomer cement containing short CPSA glass fibers

Table 1 shows the compositions of CPSA glass fibers and E-glass fibers used in this study, short CPSA glass fibers were prepared in accordance with Kobayashi's methods [8]. The diameter and aspect ratio of short CPSA glass fibers used in the present study were 9.7±2.1 μm diameter and 5.0±0.9 aspect ratio, respectively. Commercially available conventional glass-ionomer cement (HY-BOND GLASIONOMER CX; Shofu Inc., Kyoto, Japan) was used as the base material containing short glass fibers. The powders for cement were prepared by mixing the short glass fibers in GIC powders. The contents of short CPSA glass fibers in the mixed powder were 0, 20, 40 and 60 mass%, respectively. As a control of short E-glass fibers, the mixing ratios of short glass fibers in the cement powder were similar to those for short CPSA glass fibers, because their densities had almost equivalent values (Table 1). The liquid used for mixing the powder was the mixing liquid (polymeric carboxylic acid solution) of the set GIC. To combine the powder and liquid phases, the powder was mixed with the liquid at a powder/liquid ratio (P/L) of 2.0 according to the manufacturer's instructions within 1.0 min by a plastic spatula on a paper plate for dental use. After mixing, the cement paste was placed in a plastic mould for diametral tensile strength and cytotoxicity tests, and the mould was kept in an incubator at 37℃ for 24 hours in relative humidity of approximately 100%.

Table 1 Differences in compositions of short CPSA glass fibers and short E-glass fibers used in this study.

Composition	Glass fiber (mass%)	
	CPSA	E (electric)
SiO_2	31.5	54.7
Al_2O_3	25.4	13.7
B_2O_3		8.0
MgO		0.8
CaO	25.0	22.3
SrO		0.8
R_2O		0.6
TiO_2		0.4
Fe_2O_3		0.2
P_2O_5	18.1	
Density (g cm^{-3})	2.73	2.61

2) Diametral tensile strength (DTS) test

The mechanical properties of set cement were evaluated by a diametral tensile strength (DTS) test. Cement paste was filled in a split mould (6 mm in diameter × 3 mm in height), and the

mould with the paste was stored in an incubator for 24 hours at 37℃. After storage, the DTS of six pieces of cement of each content was estimated using a Universal Testing Machine (AGS-1000B, Autograph, Shimadzu Corp., Kyoto, Japan) with a crosshead speed of 0.5 mm/min. The DTS values were calculated from the maximum failure load, and the mean value and standard deviation were obtained.

3) SEM observation of fractured surface after DTS

Microstructures of the fractured surface after DTS measurement of the set cements with or without short glass fibers were observed by a scanning electron microscope (SEM; S-4000, Hitachi, Tokyo, Japan). Fractured surfaces were sputter-coated with Pt-Pd.

4) Cytotoxicity of GIC mixed with short fibers in cell culture

(1) Preparation of disks

Figure 1 shows the procedures for the cytotoxicity test by cellular activity, pH and fluoride ion measurements. In the cytotoxicity test, 40 mass% CPSA and short E-glass fibers were selected on the basis results of DTS tests because of high strength. Forty mass% content CPSA or E-glass fibers in GIC powder were hand-mixed with liquid according to manufacturer's instructions. A split mould (13 mm in diameter × 1 mm in thickness) was filled with cement paste, and the mould with the paste was stored in an incubator in relative humidity of approximately 100% for 24 hours at 37℃. Then six disk specimens of each material were prepared. After storage for 24 hours, the disks were sterilized under ultraviolet light for 30 minutes and then submitted to the cytotoxicity test. As a control, plastic coverslips of 13 mm in diameter (n=6, Thermanox Nunc, Rochester, NY, USA) were used.

(2) Exposure of cells to test specimens

Cells used in this study were a rat pulpal cell line (RPC-C 2A) established by Kasugai et al. [15]. Stock culture was unfrozen and cultured in Dulbecco's Modified Eagle's Medium (D-MEM, Sigma, Invtrogen, Karlsruhe, Germany) with 10% fetal bovine serum and supplemented with 100 IU/mL penicillin, 10 μg/mL streptomycin and 2 mmol/L glutamine and then incubated in a humidified atomosphere with 5% CO_2 in air at 37℃. The pH of the medium was adjusted to 7.2 with 10% $NaHCO_3$.

Each of the GIC disks was placed in the bottom of 12-well dishes (Figure 1, Falcon, Franklin Lake, NJ, USA). Three mL of 0.5 × 10^5 cells/ml were exposed to the GIC disk surface. After 24 hours of incubation, disks on which cells had adhered were selected and transferred to another fresh 12-well dish, and

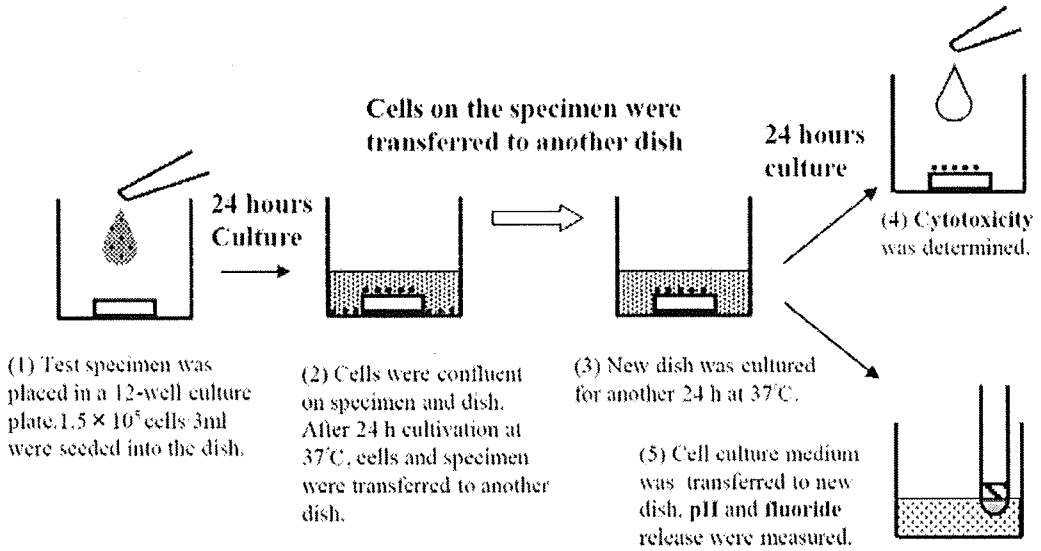

Figure 1 Procedures for the cytotoxicity test and pH and fluoride release measurements in the culture medium.Procedures for cytotoxicity

then 3 ml complete culture medium was added to each dish. Cultures were incubated for another 24 hours for test of cytotoxicity determined by cellular activity. At the same time, pH change and fluoride ion concentration in the culture medium were measured after removal of the disks.

(3) Measurements of pH change and fluoride concentration in the culture medium

The pH change in the culture medium after 24 hours of incubation was measured by a pH meter (Checker, HANNA, Tokyo, Japan). Immediately after pH measurement, the medium was submitted to fluoride ion measurements, and 0.3 mL of total ionic strength adjustment buffer (Tisab III) was added to the dish and stirred by vibration. The amount of fluoride ions released from disks into 3 ml of culture medium was determined by using a fluoride ion-selective electrode (Orion type 9609BN) connected to a pH/ISE meter (Orion 710A, Beverly, MA, USA), which was previously calibrated with standard solution of NaF with morality spanning the concentrations of fluoride ions to be measured.

(4) Cytotoxicity test

Cellular activity of the cells was evaluated after 48 hours of incubation of cultures. The medium was completely removed and 0.5 ml of fresh medium was added to the disks. Then 0.5 mL of Cell-Tilter-Glo Luminescent Cell Viability Assay solution (Progma, Madison, WI, USA) was added to the dish. After 10 minutes of incubation, the plate was vibrated for 15 seconds to destroy the cell membranes. Each 30 µl of test medium was transferred to a 96-well plate. The cellular activity of the cells was determined by using a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA). Cellular activity determined by this method is correlated with cell number. Cytotoxicity is expressed by cell number increase or decrease.

5) Statistical analysis

Results of DTS tests and results of cellular activity and fluoride ion release measurements were statistically analyzed by one-factor analysis of variance (ANOVA, $p<0.05$).

3. Results

1) Diametral tensile strength (DTS)

Figure 2 shows the diametral tensile strength (DTS) values for set glass-ionomer cements containing short CPSA glass or E-glass fibers. The mean DTS of set commercial glass-ionomer cement (CX) was 7.8 MPa. The DTS of set cement mixed with short CPSA glass fibers significantly increased with increase in the content of short glass fibers (20, 40 and 60 mass%), showing significant differences ($p<0.05$) from no fiber content CX. The cement mixed with 40

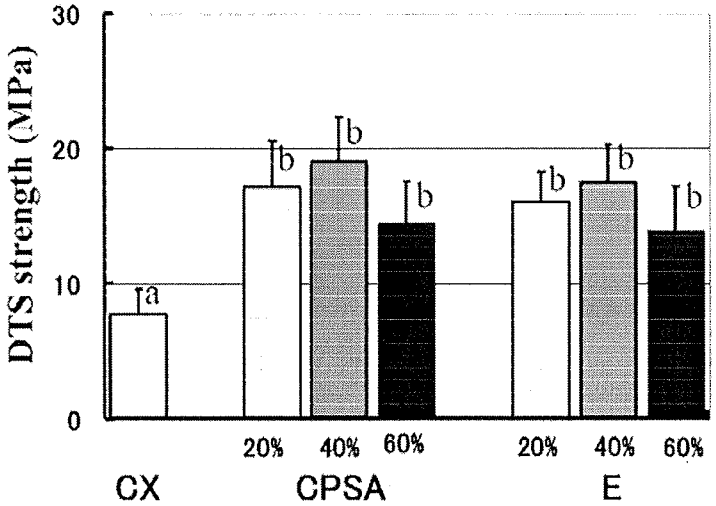

Figure 2 Diametral tensile strengths (DTS) of set CX mixed with short glass fibers. Glass-ionomer cement CX and set glass-ionomer cements containing 20, 40, 60% mass short CPSA glass or E-glass fibers after aging (n=6) for 24 hours. Bars show mean value and 95% confidence intervals. Bars with equal letters are not statistically different.

mass% short CPSA glass fibers showed a maximal DTS value of 18.7 MPa and was stronger than the other set cements tested. The DTS of set cement mixed with short E-glass fibers was also increased by the addition of 20, 40 and 60 mass% glass short fibers. The set cement with 40 mass% short E-glass fibers had the maximal value of DTS. The DTS of cement containing short CPSA glass fibers was higher than that of cement containing short E-glass fibers at each ratio in mean value of strength. However, there were no significant differences ($p<0.05$) between the DTS values of the set cements containing short glass fibers.

2) SEM micro-morphological observation after DTS test

Figure 3 shows SEM microstructures of the set cement of CX (a), cement with 40 mass% short CPSA glass fibers (b) and cement with 40 mass% short E-glass short fibers (c) . These fractures of photographs were obtained after DTS strength test. In the fractured surfaces of set cements containing 40 mass% short glass fibers, the short fibers were clearly observed to be uniformly dispersed into the matrix of the glass-ionomer cement. Shrinkage cracks were clearly apparent in the matrices of both short CPSA glass and E-glass fibers-containing cements. An organic-salt matrix in the glass-ionomer cement was shrunk by water loss under the vacuum condition of SEM. However, it was confirmed that the surfaces of short CPSA glass fibers were partially in contact with the matrix of the glass-ionomer cement (Figure. 3 (b)). The contact area of the cement matrix to short CPSA glass fibers was larger than that to short E-glass fibers (Figure 3 (c)).

3) pH change of medium

The pH changes of the medium after 24 hours of cell culture are shown in Figure 4. The mean pH of the medium in the control plastic disk was 7.2. The pH of the medium of CX was reduced

Figure 3 SEM photographs of fractured microstructures inside the set cements after DTS tests.
(a) Set CX, (b) set cement containing 40 mass% short CPSA glass fibers and (c) set cement containing 40 mass % short E-glass fibers. Arrows shows short glass fibers.

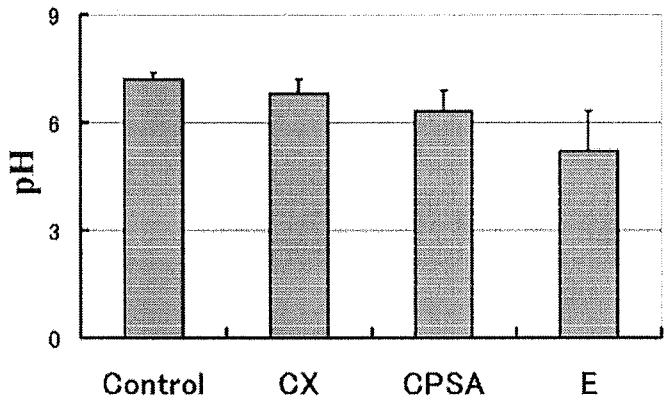

Figure 4 pH change in cell culture medium.
Acid in the specimen diffused into the culture medium and reduced pH value.

to 6.8. The pH medium of CX mixed with short CPSA glass fibers was 6.3, and the pH of the medium of CX mixed with short E-glass fibers was reduced to 5.2. However, there was no significant differences in pH values ($p<0.05$).

4) Fluoride ion release

Figure 5 shows the amount of fluoride released from set GIC into the cell culture medium. The fluoride released from HY-BOND GLASIONOMER CX showed 15 ppm as a base line. The fluoride concentration of 16 ppm released by CX mixed with short CPSA glass fibers is almost the same as that of CX. The addition of short CPSA glass fibers to CX did not disturb the fluoride release from GIC. However, surprisingly, fluoride release from set GIC mixed with short E-glass fibers showed a high value of 62 ppm.

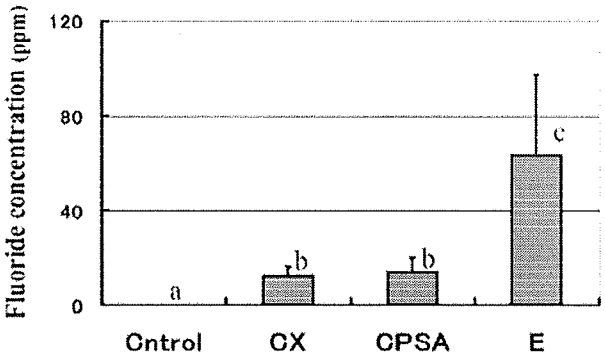

Figure 5 Concentrations of fluoride released from specimens into the culture medium after 24 hours of incubation.
Bars show mean value and 95% confidence intervals. Bars with equal letters are not statistically different.

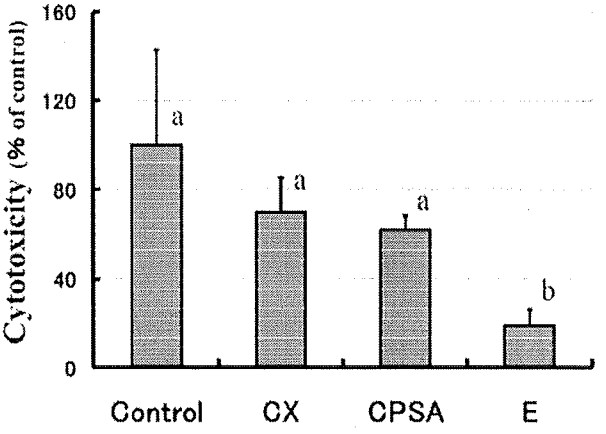

Figure 6 Cytotoxicity measured by cellular activities after culture for 48 hours.
Cell numbers are expressed as percentage of controls (cultures with plastic disk). Bars show mean value and 95% confidence intervals. Bars with equal letters are not statistically different.

5) Cytotoxicity in culture of cells

Figure 6 shows the results of cytotoxicity measured by cellular activities after culture for 48 hours. Cellular activities were expressed as percentage of controls (cultures with plastic disks). Bars show mean value and 95% confidence intervals. Bars with equal letters are not statistically different (one-way ANOVA, $p<0.05$). Cell number of CX was reduced by 24 %, indicating moderate cytotoxicity compared to the control. However, there was no significant difference ($p<0.05$) between the control and CX. The set CX mixed with short CPSA glass fibers also showed moderate cytotoxicity with almost the same cell activity as that of set CX. Interestingly, set CX mixed with short E-glass fibers showed the highest cytotoxicity. Cell activity of set CX mixed with short E-glass fibers was reduced by more than 50% in comparison to that of set CX mixed with short CPSA glass fibers.

4. Discussion

The results of DTS measurements showed that the strength of HY-BOND GLASIONOMER CX was 7.8 MPa. The addition of short glass fibers increased the strength to two times that of CX. The results suggested that short glass fibers could be a better reinforcing agent and crack arrestor for strengthening of GIC. However, short CPSA glass and E-glass fibers showed different strengthening effects. Short CPSA glass fibers were more effective than short E-glass fibers. This was due to the reactivity between short glass fibers and glass-ionomer cement because of the different compositions (Table 1) of short CPSA glass and E-glass fibers [8]. The difference in reactivity was also shown by SEM microstructures after DTS tests, because the interfaces between the short CPSA glass fibers and cement matrix were shown to be combined with each other (Figure 3 (b)), though no bonding of the interfaces was confirmed with the use of short E-glass fibers (Figure 3 (c)). In the GICs, cement

powder and liquid were adjusted to react chemically, and then set cement with sufficient mechanical properties was subsequently obtained.

The surfaces of short CPSA glass fibers reacted sufficiently with cement liquid consisting of poly-acrylic acid and chemically hardened in the cement. On the other hand, short E-glass fibers did not react sufficiently and chemically with polyacrilic acid [8]. SEM observation after DTS destruction demonstrated gaps between short E-glass fibers and set cement. This was in conjunction with the results of pH changes indicating non-reacted and residual acid around the short E-glass fibers dissolved into the cell culture medium and reduced pH to 5.2

The results showing that a large amount of fluoride released from the set GIC mixed with short E-glass fibers was also explained by the crack formation in the set cement. In the hardening process of cement, an organic-salt matrix is formed around the fluoro-alumino-silicate glass powders after powders and liquid (acid) are mixed together. Fluoride ions are released into the matrix and are freely released and recharged in the oganic-salt matrix of GIC [16]. Therefore, fluoride concentration depended on the contact area of glass fibers and the set GIC. Many cracks around fibers were observed in the fractures after DTS tests of cement with short E-glass fibers. There were many micro-cracks or spaces around fibers in the cement. The spaces make the area of contact with the medium larger, and a large amount of fluoride is inevitably released from the space in the cement. A large amount of fluoride was released from cracks of GIC mixed with short E-glass fibers. On the other hand, GIC mixed with short CPSA glass fibers was as stable as set CX as suggested by the results of measurements of pH changes and fluoride concentration.

In a cytotoxicity test, it is generally difficult or almost impossible to compare cytotoxicity due to different experimental conditions (such as cell type, contact method between cells and different materials, and time of exposure) [17]. Freshly mixed cement with powder and liquid showed significant cytotoxicity, which diminished after aging [18]. Possible reasons for the cytotoxic effects of GIC are fluoride ion release, release of other substances and residual acid in the set cement [19, 20, 21]. The cytotoxicity could not be explained by fluoride release alone, since cytotoxicity was greater (for one cytotoxic material) than that predicted by its fluoride release. Kan et al. [20] demonstrated a correlation between cytotoxicity and concentration of fluoride released from set GICs. Fluoride release and cytotoxicity were correlated, although the fluoride release did not account for the cytotoxicity observed. Muler et al. [21] reprted that the cytotoxicity of GICs was not due to fluoride but rather that it was attributed to other unidentified toxic components diffusing into the culture medium.

In the present cytotoxicity study, we devised a direct contact cell culture test method between cells and test specimens. Cells were exposed to GIC surfaces. Surprisingly, cells showed good outgrowth on the surfaces of the set CX. Set CX mixed with short CPSA glass fibers showed good cell viability as did cells on the cell culture plastic disk as a control. Set CX mixed with short E-glass fibers showed intensive cytotoxicity due to increased acidity of the culture medium and possibly excessive fluoride release of 62 ppm, which is supported by the result of Kan et al. [20]. However, reduction of pH of the medium of set CX mixed with short E-glass fibers was concluded to be the main reason for the cytotoxicity in this study.

5. Conclusion

Short CPSA glass fibers were shown to be a reinforcing agent for strengthening the GIC and to provide better biocompatibility for clinical use. Release of fluoride from the set GIC mixed with 40% short CPSA glass fibers was stable due to the chemical reaction between short CPSA glass fibers and cement matrix.

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