Biological effects of flowable resin composite on erythrocytes and lymphocytes using an *in vitro* model

H. Yamaguchi[1], S. Suzuki[2], K. Kobayashi[1] & T. Arai[1]

[1]Department of Periodontics and Endodontics, Tsurumi University, School of Dental Medicine Yokohama, Japan; and [2]Department of Prosthodontics, University of Alabama at Birmingham, School of Dentistry, AL, USA

Abstract

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Aim The purpose of the study was to evaluate the biological effects of a flowable resin composite on erythrocytes and rat spleen lymphocytes (RSL) using an *in vitro* model.

Methodology Rat spleen lymphocytes were placed into microcentrifuge tubes as a pulp model. An artificial pulp exposure was made through the top of the microcentrifuge tube with a 16G needle. The exposure site was filled with a flowable resin composite and photo-polymerized, and the other specimens were not polymerized. The survival rates were determined by a haemocytometer. Red blood cell (RBC) suspension (0.5%) was reacted with the flowable resin composite. The haemolytic activity was evaluated at room temperature (20 °C) using a supernatant of each sample by measuring 545 nm of absorbency with a spectrophotometer. To evaluate the effect of flowable resin composite on RBCs, supernatant and precipitated samples were analysed by sodium dodecyl sulphate–polyacrylamide gel (SDS–PAGE).

Results The cell survival rate of the polymerized group was significantly lower compared with that of the nonpolymerized group at the same areas ($P < 0.05$). The values of haemolysis (absorbency) were 2.16 for the polymerized group, 2.14 for the nonpolymerized group, and 2.18 for the control group (saline). There were no significant differences among them ($P > 0.05$). The protein component in the supernatant and precipitate exhibited the same reaction with flowable resin composite regardless of the completion of the polymerization, resulting in no change of protein band mobility on SDS–PAGE.

Conclusions The degree of haemolysis was not influenced by the completion of polymerization. The flowable resin composite tested provoked an adverse effect on lymphocytes when it was not polymerized and the degree of haemolysis was not affected by the status of the polymerization.

Keywords: flowable resin composite, haemolysis, lymphocytes, polymerization.

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Introduction

Direct pulp capping is completed when an exposed dental pulp is covered with a dressing or cement that protects the pulp from additional injury and permits healing and repair (Rowe 1967, Brännström & Nyborg 1969). Long-term clinical studies indicate that direct pulp capping can produce success rates of 87.3% (Haskell *et al.* 1978). The direct application of adhesive resin systems onto exposed pulp tissue has been tested clinically (Katoh *et al.* 1997, Kitasako 1997, Katoh *et al.* 1999). The results demonstrated that approximately 11% of pulps became necrotic (Katoh *et al.* 1999), indicating the necessity of further investigation.

It has been suggested that the unfavourable postoperative prognosis of direct pulp capping may be caused

Correspondence: H. Yamaguchi, 2-1-3 Tsurumi Tsurumi-ku Yokohama Kanagawa 230-8501, Japan (fax: +81 45 573 9599; e-mail: yamaguchi-h@tsurumi-u.ac.jp).

by cytotoxicity of the pulp capping material (Mantellini *et al.* 2003). The cytotoxicity of resin composite as a direct pulp capping material by means of a simulated exposed pulp model using lymphocytes has been evaluated (Yamaguchi *et al.* 2003a). Studies have implied that 4-META/MMA-TBB resin affects fibroblast cells to a limited degree but reacts with lymphocytes; which are more easily damaged than fibroblast cells (Yamaguchi *et al.* 2004). The experimental model used (Yamaguchi *et al.* 2004) can estimate the permeation potential of the components of direct pulp capping material from an exposed pulp. The model allows reacted cells to be sampled from the top, middle and bottom of the tube, therefore, morphological changes in cells can be identified immediately. From a clinical prospective, an incomplete seal of the restoration/pulp interface is closely related to the outcome of the pulp capping procedure. Flowable resin composites have been recommended for many clinical uses (Small 1996, Bayne *et al.* 1998, Payne 1999), as they have been formulated with a variety of constituents and viscosities (Moon *et al.* 2002). The resistance to microleakage of flowable resin composite as a restorative material is similar to that of hybrid resin composite (Yazici *et al.* 2003). Cavity lining with flowable resin composite was shown to have the greatest potential to initially maintain the marginal integrity among the materials tested (Haak *et al.* 2003). Taking these features into consideration, it could be speculated that flowable resin composite could be appropriate for direct pulp capping.

In order for direct pulp capping to be biologically successful, the importance of haemorrhage control and prevention of bacterial microleakage should be addressed (Tsuneda *et al.* 1995, Hafez *et al.* 2000). The complete polymerization of adhesive resins might not be achievable during direct pulp-capping procedures. Oxygen has been shown to prevent complete polymerization of adhesive monomers (Rueggeberg & Margeson 1990, Geurtsen *et al.* 1999), and haemorrhagic sites tend to have high oxygen tension. The release of unpolymerized components may also interfere with the function of the local immune system in the pulp tissue, reducing its defence potential (Jontell *et al.* 1995). Neither damage to subjacent pulp lymphocytes nor haemolysis against flowable resin composite systems have ever been discussed.

The purpose of this study was to evaluate the cytotoxicity of unpolymerized flowable resin composite on rat spleen lymphocytes (RSL) using a modified microtube as an exposed pulp model. It was also the purpose of this study to evaluate the *in vitro* haemolysis rates using rat erythrocytes.

Materials and methods

All procedures involving animals were reviewed and approved by the Animal Care and Use Committee of Tsurumi University School of Dental Medicine prior to the experiments. A flow chart of the experimental procedures is presented in Fig. 1. Lymphocytes extracted from rat spleen (Wister male, 5 week old) were used for the evaluation of simulated pulp exposure. Extracted cell suspension was used immediately after the preparation. The medium used for a cell suspension was made with a RPMI 1640 cell culture medium (Meditech, Herndon, VA, USA). The cell suspension was adjusted to a 5×10^6 (cells mL^{-1})

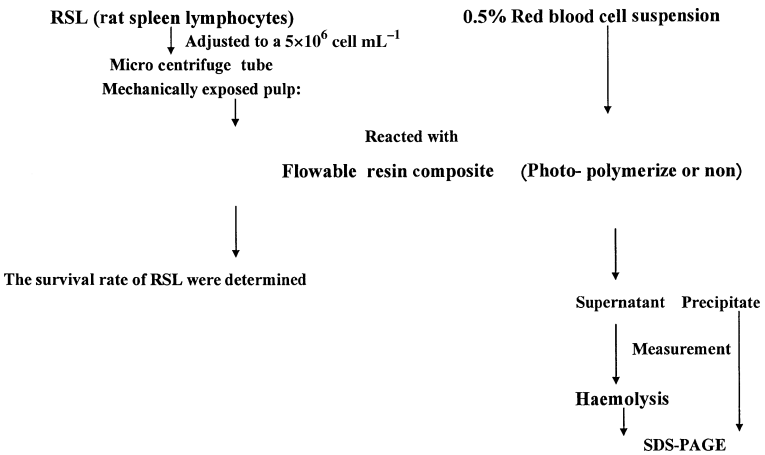

Figure 1 Experimental procedure.

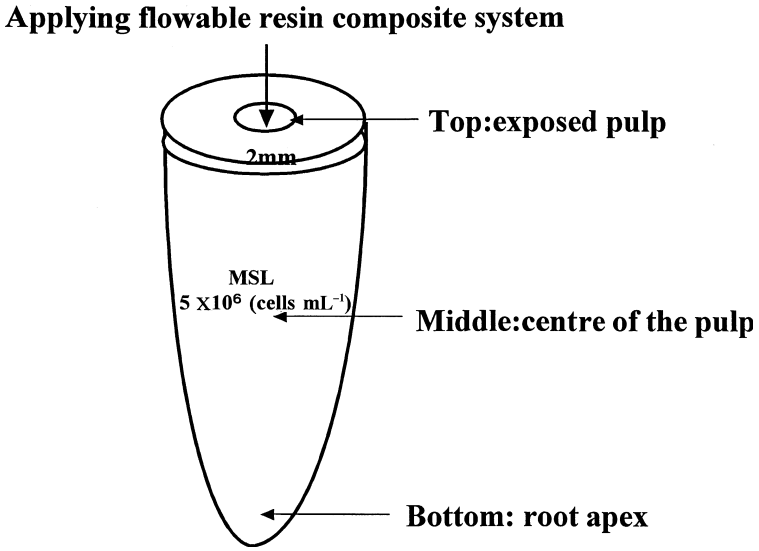

Figure 2 Simulated perforated pulp chamber using modified microcentrifuge tube.

concentration and placed in a modified microcentrifuge tube (model 05-406-15; Fisher Scientific, Pittsburgh, PA, USA), which had a simulated pulp exposure with 2-mm diameter opening on the cap created with a 16G needle (Terumo, Tokyo, Japan). The lymphocyte cells were well suspended and equally distributed in the microtube. The flowable resin composite tested was UniFilLoFlo (GC, Tokyo, Japan). The exposure sites were filled with the flowable resin composite with a CR syringe (Mark 1, Centrix, Shelton, CT, USA) for experimental groups ($n = 10$). Half the specimens were photo-polymerized using a photo-polymerizing unit (Optilux 500; Kerr, Irvine, CA, USA) for 20 s, and the other half were not polymerized. Twenty microlitres of saline was poured into the exposure of the control group. Reacted lymphocytes were then collected from three different levels including the top, middle, and bottom of the tube. The designation of respective levels was; the top as directly below the opening, the middle was the centre of the tube, and the bottom was the bottom of the tube. A pipette (Nichipet EX; Nichiryo, Tokyo, Japan) was inserted in the circumference of the tube, and 20 μL of the suspension was collected at each level independently (Fig. 2). The remaining lymphocytes were stained with trypan blue for 5 min. The number of vital cells was calculated with a haemacytometer (Bright-line; Hausser Scientific, Horsham, PA, USA), and the survival rate was obtained as a percentage. The control model was performed in the same microtube with rat lymphocytes using saline solution, reacted for 5 min, then stained and evaluated in the same manner. The data were statistically analysed by one-way ANOVA and Fisher's protected least significant difference test at a 95% confidence level.

Haemolytic rates were measured according to Fujisawa's method (Fujisawa 1978). Approximately 5 mL of blood was collected from the rat heart at the time of sacrifice. Red blood cells (RBC) were separated by centrifugation (956 *g*). The RBC specimen was adjusted to a 0.5% solution using a physiological saline. Each of 15 test tubes received 5 mL of RBC. A 0.02 g specimen of flowable resin composite was measured by an electric balance with an accuracy of 0.0001 g. The flowable resin composite was photo-polymerized outside of the tube and placed into the tube ($n = 5$). The same amount of unpolymerized flowable resin composite was place into the tube, and designated as a nonpolymerized group ($n = 5$). Two hundred microlitres of saline were placed into the tube for the control group ($n = 5$). All of the tubes were gently agitated for 20 s. Haemoglobin components were produced by haemolytic reaction, and the degree of haemolysis was determined by evaluating the concentration of haemoglobin component in a supernatant of each sample. The value was determined by measuring the 545 nm absorbance level using a spectrophotometer (Hitachi U-1100, Tokyo, Japan). RBC specimens were of mixed with physiological saline and used as a control (Fujisawa 1978). The haemolytic rate was calculated using the following formula:

$$\text{haemolytic rate} = \frac{\text{experimental sample absorbency} - \text{control absorbency}}{\text{complete haemolytic sample absorbency} - \text{control absorbency}}$$

An electrophoresis was completed to evaluate the effects of flowable resin composite on the RBC. Reacted supernatant and the precipitated protein samples were analysed by sodium dodecyl sulphate–polyacrylamide gel (SDS–PAGE) using 15% gel (PAGEL AE-6000 Atto, Tokyo, Japan). Ten millilitres of both supernatant and precipitated protein samples were dissolved in 100 mL of electrophoresis buffer (1% SDS and 0.01 mol L^{-1} Tris–HCl pH8.0 containing 50% glycerine). Five millilitres of each sample was used for electrophoresis. The electrophoresis was employed to determine the molecular weight (MW) of tested specimens. The MW was determined by comparison with a standard protein specimen. Standard protein: maltose binding protease (MBP)-β-galactosidase (175 kDa), MBP-paramyosin (83 kDa), glutamic dehydrogenate (62 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa), β-lactoglobulin A (25 kDa), lysozyme (16.5 KDa), and aprotinin (6.5 kDa), were used to calculate the MW value. The electrophoresis was carried out using a current of 20 mA for 3 h. The gel was stained with a solution 0.125% Coomasie brilliant blue R-250 (Bio-Rad, Hercules, CA, USA), 7% acetic acid, and 50% methanol. Each gel was destained in 7.5% acetic acid and 5% methanol.

Results

The results of cell survival rates are presented in Table 1. For the specimens being tested without polymerization, the rates at the top, middle, and bottom portion of the microtube were 38%, 49%, and 67% respectively. For the specimens with polymerization, the top portion of the microtube had 59%, followed by 62% for middle portion, and 87% for the bottom portion. The results indicated that cell survival rate of the polymerized group was significantly higher compared with that of nonpolymerized group in the same areas ($P < 0.05$).

The results for the haemolysis values (absorbency) are presented in Table 2. The values were 2.16 ± 0.25 for the polymerized specimens, 2.18 ± 0.22 for the saline control, and 2.14 ± 0.17 for nonpolymerized specimens. There were no significant differences among them ($P > 0.05$). The protein components in the supernatant and precipitate reacted with the flowable resin composite, regardless of the completion of the polymerization, which resulted in no change of protein band mobility on SDS–PAGE (Fig. 3).

Discussion

In vitro biocompatibility tests have been developed to simulate and predict biological reactions to materials when they were placed directly or indirectly onto the pulp tissues. These methods offer a relatively inexpensive way to evaluate newly developed materials. Traditional assays are frequently used to test materials (Nalcaci *et al.* 2004). These traditional assays measure cytotoxicity by means of either terminal-stage events (Hanks *et al.* 1996). In those tests chemical reaction experiments are generally carried out in a test tube to allow cells to react with various solutions. Conventional methods commonly use fibroblasts or epithelial cells, which tend to precipitate at the bottom of the test tube as they have a high specific gravity. In order to obtain sufficient reaction, the test tube is usually well agitated, however, this agitation is artificial because materials are not dispersed into pulp tissue in such a way. The test system used in the present study is unique as it does not require agitation. The RSL are well dispersed in the

Table 1 Results of cell survival rates at each area of the tube

Materials/area	Top	Middle	Bottom
Polymerized	59.10 ± 6.47	62.99 ± 7.64	87.16 ± 9.42
Nonpolymerized	38.24 ± 4.03*	48.96 ± 8.00*	67.16 ± 15.45*
Saline(Control)	61.42 ± 9.11	74.19 ± 11.88	90.58 ± 7.16

Values are in percentage. Mean ± SD ($n = 5$).
*Significantly different with other materials in the same area.

Table 2 Haemolysis value

	Abs (haemolysis value)
Polymerized	2.16 ± 0.25
Nonpolymerized	2.14 ± 0.17
Saline (control)	2.18 ± 0.22

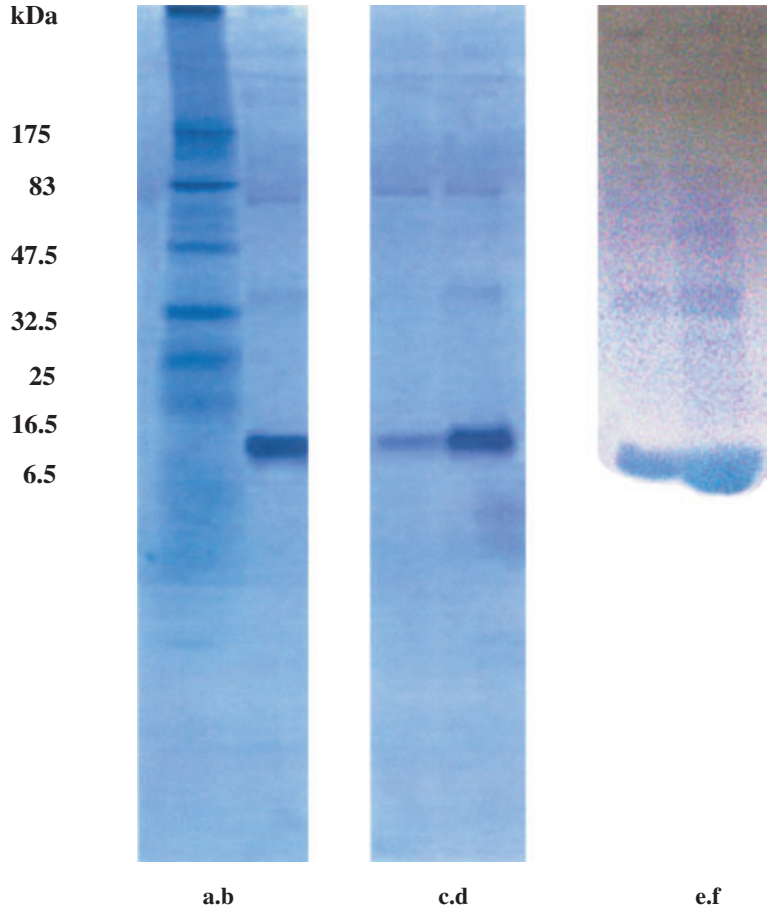

Figure 3 Sodium dodecyl sulphate–polyacrylamide gel. (a) MW, molecular weight protein index. (b) Control: RBC. (c) Supernatant specimen reacted with nonpolymerized flowable resin composite. (d) Supernatant specimen reacted with polymerized flowable resin composite. (e) Precipitate specimen reacted with nonpolymerized flowable resin composite. (f) Precipitate specimen reacted with polymerized flowable resin composite.

microtube as they have a similar specific gravity to the solution. The resin composite paste is applied to an artificial pulp exposure site, and eluted components diffuse into the tube. In such a manner reacted cells in various depths can be collected for evaluation. Although the RSL does not exist in pulp tissues, it works as an immune reactor similar to the lymphocytes in pulp tissues. Another reason for the selection of the RSL was availability as abundant (approximately 2.5×10^7) cells can be collected per spleen.

Direct pulp capping materials get in direct contact with pulp tissue, therefore, exposed pulp tissue was affected by unpolymerized resin components that diffused into the artificially exposed pulp. This study model has the potential to evaluate the distribution of necrotic cells, because the host cells either recover from or succumb to their chemical injury. The shortcoming of this *in vitro* study is inability to superimpose inflammatory and immune reactions resulting from direct pulp capping procedure. Further studies may develop a more realistic method to better utilize the RSL.

Recent studies reported that a successful outcome required absolute haemorrhage control, regardless of the material used for direct pulp capping (Gerzina & Hume 1996, Matsuo *et al.* 1996, Akimoto *et al.* 1998). The effects of bonding systems, including a self-etching primer and a bonding monomer, have been evaluated on mice spleen lymphocytes (Yamaguchi *et al.* 2001). A previous study suggested that the self-etching primer may have an adverse effect on lymphocytes (Yamaguchi *et al.* 2003b). The present study proved that the flowable resin composite tested provoked an adverse effect on lymphocytes when it was not polymerized, however, it showed little effect on haemolysis. An adverse effect on RBCs caused by the use of a self-etching primer has also been reported (Yamaguchi *et al.* 2001). In that study it was speculated that high haemolytic activity was probably attributed to the breakage of haemoglobin components because of an oxidation–reduction reaction caused by an organic solvent included in the primer. The incorporation of unpolymerized monomers with RBCs probably makes

haemorrhage control difficult. It is suggested that the effects of haemolysis is of fundamental importance for direct pulp capping procedures. This present study proves that flowable resin composite had little effect on the RBC protein bands, regardless of the completion of photo-polymerization, as the results demonstrated negligible changes in the protein bands with SDS–PAGE.

Unpolymerized monomers diffuse directly into the pulp at the exposure site besides the dentinal tubules, and the monomers act as a cytotoxin to pulp cells (Pashley 1988, Hanks *et al.* 1994, Pashley *et al.* 2000). A recent report demonstrated that the cytotoxic effects on pulp cells were increased when the adhesive resin was not completely polymerized as a consequence of low light intensity (Chen *et al.* 2001). Cytotoxic responses were observed when adhesive resins were applied directly to human pulps *in vivo* (Hebling *et al.* 1999) which might be attributable in part to the incompleteness of adhesive resin polymerization and subsequent release of cytotoxic component at the site of pulp exposures. Unpolymerized and partially polymerized adhesive resin induced apoptosis very rapidly (Mantellini *et al.* 2003). An evaluation of cytotoxicity requires immunological analysis, and immuno-histological studies have shown the presence of immune components in the pulp (Honjo *et al.* 1970, Pulver *et al.* 1978). Although T-lymphocytes account for only 1–2% of total pulp cells (Mangkornkarn *et al.* 1991), they play an important role in the defence against bacteria (Jontell *et al.* 1987). If pulp tissue was infected, bacteria would be eliminated by phagocytosis, and then macrophages work as antigenic cells to lymphocytes. If lymphocytes in the pulp tissue are damaged, the pulp cells cannot recognize the antigen, therefore, it is believed that defence system will be affected by the damage of lymphocytes.

The present study showed that flowable resin composite provoked an adverse effect on lymphocytes when it was not polymerized. Further analysis for the immune reaction of unpolymerized flowable resin composite on pulp lymphocyte is required.

The haemolytic test had an adequate correlation with tissue culture and subcutaneous implant tests (Klotzer *et al.* 1970, Sela *et al.* 1973). Haemolysis values provoked by contact with flowable resin composite tested were similar to the value of saline control, regardless of the polymerization condition. When the application of flowable resin composite is considered for direct pulp capping, cytotoxic effect to pulp cells is dependent upon the degree of polymerization. Further investigations are required to determine the potential of direct pulp capping using flowable resin composites. This study model is possible to evaluate an immediate effect of materials on lymphocytes when direct pulp capping procedures are employed with resin composites. The cell collection from each level is designed to simulate the reaction of pulp cells at respective areas including immediately below the exposure to near the root apex. As the evaluation is limited to an immediate effect, further investigations including analysis of immune reaction are suggested. The evaluation of inflammatory responses of lymphocytes using the cell culture model is also promising.

Conclusions

1. Flowable resin composite provoked an adverse effect on lymphocytes when it was not polymerized.
2. Haemolysis was not substantially affected by the completion of polymerization.

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