doi:10.1111/j.1365-2591.2007.01364.x

Comparison of operative procedure variables on pulpal viability in an *ex vivo* model

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Abstract

Murray PE, Smith AJ, Garcia-Godoy F, Lumley PJ. Comparison of operative procedure variables on pulpal viability in an *ex vivo* model. *International Endodontic Journal*, **41**, 389–400, 2008.

Aim To measure and compare the responses of pulp tissue to cavity preparation and restoration variables using a novel tooth slice culture model.

Methodology Experimental cavities (265) were continuously cut, under carefully controlled conditions, into the dentine of the labial aspect of 28-day-old Wistar rat incisors, and slices of these teeth maintained in organ culture for up to 2 weeks. The experimental variables examined were: the preparation method, remaining dentine thickness, coolant, drill speed, conditioning with EDTA and filling materials. The reactions of the dentine–pulp complex to the experimental variables were measured using pathohistometric analysis and the correlations between variables were determined using analysis of variance statistical tests.

Results In rank order of surgically induced restorative pulpal injury, from the most to the least injurious were: remaining dentine thickness, absence of coolant during cavity preparation, bur speed, cavity conditioning treatments and the filling material.

Conclusions To reduce pulp injury and to promote pulpal repair activity, the correct use of appropriate materials are important. However, of relatively greater importance is the operative technique adopted, the need to avoid the excess removal of dentine and to minimize trauma during preparation.

Keywords: drill speed, EDTA, necrosis, odontoblasts, teeth.

Received 25 July 2007; accepted 10 October 2007

Introduction

Caries, attrition, abrasion, erosion and trauma are amongst the events which can threaten the survival of the tooth pulp (Bjørndal & Darvan 1999). The operative techniques and materials used to repair and restore the tooth structure following these events present an additional challenge to the survival of cells within the tooth pulp (Cox *et al.* 1992). The preservation of pulp vitality following restorative intervention is dependent on the degree to which the pulpal cell populations can survive, as well as the ability of these cells to detect and respond to pulp injury in order to initiate an appropriate repair response. The most obvious repair response to pulp injury is the deposition of a tertiary dentine matrix (Baume 1980). Unlike primary or secondary dentine, which forms along the entire pulp–dentine border, tertiary dentine is focally secreted by odontoblasts in response to primary and secondary dentine injury. The process of tertiary dentine secretion can be classified as being reactionary or reparative in nature, depending on the severity of the initiating events and the cellular processes through which the newly deposited dentine matrix was formed. In general, reactionary dentine is secreted by pre-existing primary odontoblasts and reparative dentine is

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secreted by newly differentiated odontoblast-like cells (Smith *et al.* 1994). The secretion of reactionary dentine is a key postoperative odontoblast repair response to the presence of a cavity carefully cut into the dentine of a tooth (Smith *et al.* 1994). Reparative dentine, on the other hand, is secreted by a second generation of odontoblast-like cells when irreversible odontoblast injury has destroyed the primary cells (Smith *et al.* 1994). Reparative dentinogenesis is a much more complex process than reactionary dentinogenesis, and is generally observed following injurious cavity preparation or a pulp exposure situation (Murray *et al.* 2000a).

Minimizing pulp injury during cavity preparation is clearly an important clinical goal, as it maintains pulp cell function and viability and reduces the probability of postoperative pulp complications. In order to preserve pulpal viability and function, the potentially injurious individual effects of cavity preparation, cavity conditioning and cavity restoration must be assessed. However, the measurement of pulpal responses to cavity preparation and restoration variables has proved to be extremely difficult, because the pulpal reactions are the end summation of all the variables together.

During restorative procedures, the generation of heat has long been claimed to be the relatively most injurious event to pulp tissue (Zach 1972). Recent investigations have found that the amount of intrapulpal injury generated during cavity preparation and restoration is determined by the drill rotation speed (Hatton *et al.* 1994), size, type and shape of the cutting instrument (Ottl & Lauer 1998), length of time the instrument is in contact with the dentine (Ohmoto *et al.* 1994), the amount of pressure exerted on the hand-piece (Brisman 1996), cavity remaining dentine thickness (Murray *et al.* 2000a), restoration material temperature (Anil & Keyf 1996) and the use of cooling techniques (Lloyd *et al.* 1978). Other factors that have been claimed to be potential sources of pulp injury during cavity restoration include: conditioning of the dentine cavity wall (Tziafas *et al.* 1993), method of placement of the restorative material (Kirk & Meyer 1992) and choice of cavity restoration product (Tobias *et al.* 1982). Potential sources of pulp injury do not end with these restorative procedures; the presence of marginal leakage, causing osmotic pressure to dentinal fluid and, more importantly, bacterial invasion causing pulpal inflammation (Sazak *et al.* 1996) can also influence the fate of the pulp after cavity restoration.

Because of the multi-factorial and interacting effects of operative and postoperative events on the pulp, it is difficult to identify the relative importance of individual factors in a controlled manner *in vivo*. The recent development of a tooth slice organ culture model (Sloan *et al.* 1998) for study of the biological processes in the pulp (Sloan & Smith 1999) and cytotoxicity of dental materials (Murray *et al.* 2000b) offers an experimental approach to study restorative variables in which conditions can be closely controlled. It is also possible to study pulpal responses in the absence of possible complications, such as inflammation and bacterial contamination (Sloan & Smith 1999). Whilst human teeth may be cultured (Magloire *et al.* 1996), individual specimen variability may be problematic for reproducibility of experimental conditions. Few studies have attempted to quantify pulpal injury in response to restorative variables and the first aim of this study was to quantify the effects of cavity remaining dentine thickness, drill speed, use of coolant, dentine conditioning treatments and choice of restoration materials on pulpal cell numbers. As successful resolution of restorative procedures are dependent upon harnessing and utilizing the natural repair responses of the tooth to regenerate dentine, which represents the ideal protection to the pulp (Hilton 1996), the second aim of this study was to quantify the effects of the above restorative variables on reactionary dentine secretion.

Materials and methods

Specimen collection and preparation

Twenty-eight-day-old male Wistar rats (mean weight 91.4 g, SD ± 19.4 g) were sacrificed using isoflurane (Halocarbon, Rivers Edge, NJ, USA) inhalant anaesthesia and a guillotine. The animal protocol (number 07-2006-1) was approved by Miami Children's Hospital Institutional Animal Care and Use Committee according to the NIH Office of Laboratory Animal Welfare certification standards. The mandibular incisor teeth were extracted and washed with a 70% ethanol solution, and maintained in sterile culture medium consisting of Dulbecco's Minimal Essential Medium (DMEM) (Sigma Aldrich Corp., St. Louis, MO, USA) throughout the preparation process.

Cavity preparation

Cavities were prepared immediately following incisor extraction, and a cavity preparation apparatus was

used to ensure the precise positioning and cutting of cavities and cavity depths (Tyas *et al.* 1980). A total of 265 cavities (one per incisor) were cut into enamel and dentine on the bucco-labial aspect, 1 mm above the gingival tissues of rat incisors, using an ISO 001006006 Ash steel drill (Dentsply, York, PA, USA) in an electric handpiece (Dentsply Maillefer, Ballaigues, Switzerland) advanced at a set rate of 50 μm s^{-1} in a fixed apparatus, until an estimated 10 μm cavity remaining dentine thickness remained for each of the drill speed and cavity conditioning test groups. The width of the cavities was measured with a reticule under light microscopy and was found to be between 312 and 345 μm. Exposed cavities ($n = 75$) were prepared by puncturing the base of the cavity preparations with a sterile DG16 explorer probe. The test protocol for examining the cavity cutting and restoration variables is shown in Table 1.

Drill speed during cavity cutting

Cavities ($n = 60$) were cut using each of the following drill speeds; 500 rpm, 4000, 8000 and 20 000 rpm in the presence of coolant, and with 8000 rpm in the absence of coolant (Table 1). Coolant was delivered by a raised water reservoir containing distilled deionized sterile culture water; plastic tubing directed the water flow into the cavity at a constant flow rate of 36 mL min^{-1} at room temperature.

Smear layer removal with EDTA to condition instrumented cavities

A JEOL (Tokyo, Japan) scanning electron microscope (SEM) was used to examine the cavity floor of preparations following dentine preparation. All the prepared cavities ($n = 30$) were placed into three categories of cavity conditioning treatment (Table 1).

Placement of restorative product into prepared cavities

Dycal (Dentsply, York, PA, USA) a self-setting dental liner with a 24% calcium hydroxide content, Hypocal (Elleman, New York, NY, USA) a setting dental restorative compound with a 45% calcium hydroxide content and One-Step (Bisco, Schaumburg, IL, USA) a light-cured adhesive, were freshly prepared strictly according to manufacturer's instructions, and aseptically packed into freshly prepared cavities ($n = 112$) using hand instruments, immediately prior to culture (Table 1). The restoration of cavities with pure calcium hydroxide (BDH Lab Supplies, Poole, UK) was also examined (Table 1). Once packed to the level of the tooth surface, a layer of dental wax was applied to provide a seal, to prevent the loss of material by diffusion whilst in culture. As a control measure, some cavities were sealed with dental wax without the application of any dental material (Table 1).

Table 1 Cavity preparation dependent variables, tooth slice numbers and culture periods

			Number of tooth slices at different culture periods (days)						
Treatment to be investigated	Fixed variables	Test variables	0	1	2	5	10	14	Total
Coolant effects	8000 rpm	No coolant			5		5		10
	60-s EDTA	Water coolant			5		5		10
	Dental wax								
Drill speed effects	60-s EDTA	500 rpm			5		5		10
	Dental wax	4000 rpm			5		5		10
	Water coolant	8000 rpm			5		5		10
		20 000 rpm			5		5		10
Cavity conditioning	Dental wax	0 s EDTA			5		5		10
	8000 rpm	60 s EDTA			5		5		10
	Water coolant	120 s EDTA			5		5		10
Unexposed cavity restoration	8000 rpm	Dental wax	5	5	5	5	5	5	30
	60-s EDTA	Dycal		5	5	5	5	5	25
	Water coolant	Hypocal		5	5	5	5	5	25
		One-step		2	2	2	2	2	10
		Calcium hydroxide		2	2	2	2	2	10
Exposed cavity restoration	8000 rpm	Dental wax		5	5	5	5	5	25
	60-s EDTA	Dycal		5	5	5	5	5	25
	Water coolant	Hypocal		5	5	5	5	5	25

Organ culture of rat incisor tooth slices

Tooth slices were cultured essentially as previously described (Sloan *et al.* 1998, Sloan & Smith 1999, Murray *et al.* 2000b). In brief, the segment of each incisor tooth containing the prepared cavity was sliced into a transverse section of approximately 2-mm thickness, using a segmented diamond-edged rotary saw (Taab, Aldermaston, UK) cooled with sterile culture medium.

Each tooth slice was washed several times in washing medium at 37 °C immediately after cutting and embedded in a culture gel (100 μL) containing DMEM, vitamin C (0.15 mg mL^{-1}), 10% heat inactivated foetal calf serum, L-glutamine (200 mmol L^{-1}), 1% penicillin/streptomycin solution and 1% low melting-point agar maintained at 37 °C (Sloan *et al.* 1998). The culturing gel containing the tooth slice was supported on a sterile 0.8 μm Millipore (Billerica, MA, USA) (mixed esters of cellulose acetate and nitrate) filter. With the aid of a plastic support, the filter was floated on the surface of 4 mL DMEM in 'trowel type' cultures (Bégue-Kirn *et al.* 1992) in 35 × 10 mm petri dishes. The tooth slices were cultured at 37 °C in a 5% CO_2 atmosphere, in a humidified incubator for 1 to 14 days, changing the medium every 2 days. Following culture, the tooth slices were fixed in 10% neutral-buffered formalin for 24 h, de-mineralized in 10% formic acid for 48 h and then processed and embedded in paraffin wax for histological examination. Sections were cut at 6 μm and routinely stained with haematoxylin and eosin.

Histomorphometric analysis

Each tooth slice was analysed blind. Cell numbers within sections of the dental pulp complex of each tooth slice, were counted histomorphometrically (Warfvinge 1987) at ×400 magnification, within a 2101.3 μm^2 unit area, using a microscope eyepiece grid reticule. The intact numbers of odontoblast cells, cells in the subodontoblast layer, and pulpal core fibroblasts were counted. The total number of areas analysed histomorphometrically in each tooth pulp section was six, as the cell numbers of each cell population were counted beneath the site of the cavity preparation and also at an internal control site remote from that of the cavity preparation. The exact RDT (remaining dentine thickness) of the dentine cavities was measured following cavity restoration, between the floor of the prepared tooth cavity and the pulp tissue, and the area of secreted reactionary dentine was also measured using the reticule (Benjamin *et al.* 1985). The wall depth of the restoration, its volume and total wall surface area were estimated as previously described (Murray *et al.* 2000a). The raw data from all the experiments were examined using analysis of variance (ANOVA) tests. The first was Wilk's Lambda statistical test (Wilks 1932) for the multivariate analysis of variance (MANOVA), the second was a repeated measures ANOVA and finally Scheffé's (ANOVA) *post hoc* procedure (Scheffé 1953) claimed to be versatile and the most conservative multiple comparison test (Dawson-Saunders & Trapp 1994). Statistical tests were calculated using STATVIEW software (SAS Institute, Cary, NC, USA).

Results

Maintenance of the dental pulp complex within cultured tooth slices

Transverse slices of Wistar rat incisor teeth were maintained in organ culture for up to 2 weeks in the absence of treatment. Throughout the culture period, little or no morphological tissue alterations were observable. The effect of growing tooth slices in culture for up to 14 days was found to maintain the cell numbers as well as the morphology of the pulpal cell populations (MANOVA, $P = 0.691$). A histomorphological evaluation of the treated tooth slices, revealed the presence of a reactionary (tertiary) dentine matrix in some specimens, showing continuity between the primary dentine matrix and the pre-existing odontoblasts (Fig. 1). This observation was necessary to classify the secreted dentine matrix as being reactionary in origin (Mjör 1983).

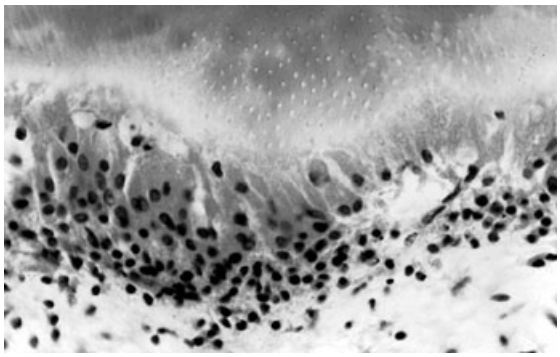

Figure 1 Histology of reactionary dentine.

Cavity remaining dentine thickness controls

The remaining dentine thickness of all the cavity restorations was examined within each of the test groups to remove the possible influence of differences in the remaining dentine thickness of the cavity preparations. The remaining dentine thickness of cavity restorations prepared with the use of 500, 4000, 8000 and 20 000 rpm drill speeds were found to be similar (ANOVA, $P = 0.492$), as was the case with the absence or presence of water coolant (ANOVA, $P = 0.609$) or the EDTA conditioning treatments for 0, 60 and 120 s (ANOVA, $P = 0.781$). The cavity remaining dentine thickness is important, because this is a major initiating factor for the secretion of reactionary dentine (Murray *et al.* 2000a).

Cavity remaining dentine thickness and pulpal activity

Histological analysis of cavity preparation with the fixed hand-piece apparatus, for the assessment of effect of the cavity remaining dentine thickness, showed a range between 2.5 and 100 μm. The intrinsic incisor dentine thickness at the site of cavity cutting was found to average 130 μm. Prepared cavities with a remaining dentine thickness above 50 μm did not influence odontoblast survival or result in a reactionary dentinogenic response (Table 2, ANOVA, $P = 1.000$). Prepared cavities with a remaining dentine thickness between 2.5 and 50 μm, showed a response of reactionary dentinogenesis. There appeared to be a relationship between the area of deposited reactionary dentine and the remaining dentine thickness (Table 2, ANOVA, $P = 0.0054$). As the remaining dentine thickness increased, the area of reactionary dentine secreted decreased. Reactionary dentine formation was absent beneath preparations with a remaining dentine thickness more than 50 μm and also with a thickness less than 2.5 μm (Fig. 2). Only with a remaining dentine thickness between 50 and 2.5 μm were underlying odontoblasts stimulated to secrete reactionary dentine.

Pulp-exposed cavity preparations caused a loss of underlying odontoblast survival (Fig. 3), in comparison with the numbers of odontoblasts at an internal control site remote from the cavity preparation (MANOVA, $P = 0.0001$) shown in Fig. 4. The numbers of odontoblasts beneath the site of pulp exposure reduced as the time period of tooth culture was increased.

Calcium hydroxide materials and pulpal activity

The type of calcium hydroxide product used to restore pulp exposed cavities, appeared to influence pulpal cell survival. Pulp capping with Hypocal had a severe effect on odontoblast density; approximately 60% less odontoblasts were observed in comparison with Dycal pulp capping (Fig. 4), whereas odontoblasts independent of the pulp exposure did not appear to be influenced by the use of different pulp capping materials (Fig. 4). Under the culture conditions used, no reparative dentinogenesis leading to dentine bridge formation was observed.

Table 2 Hierarchy of variables correlated to the odontoblast numbers and reactionary dentine beneath the unexposed cavity

	Measure of pulp injury	Measure of pulp repair
Variable correlations (ANOVA *P* values)	Odontoblast numbers beneath cavity as a measure of pulp injury	Reactionary dentine area as a measure of pulp repair
Cavity remaining dentine thickness	0.0001	0.0054
Pulp exposed restoration material	0.0148	1.000
Absence of coolant during cavity preparation	0.030	0.0001
Bur speed during cavity preparation	0.244	0.0001
Cavity conditioning treatment	0.236	0.0031
Cavity wall depth	0.357	0.0063
Cavity volume	0.872	0.0108
Total cavity wall surface area	0.147	0.0137
Non-pulp exposed restoration material	0.513	0.714

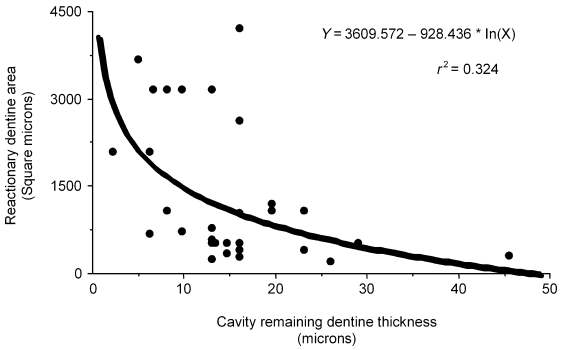

Figure 2 Reactionary dentine secretion beneath unexposed cavity restorations.

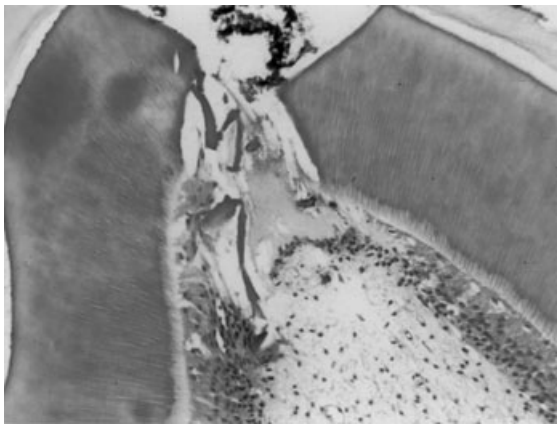

Figure 3 Histology of a pulp-exposed cavity preparation.

When the effect of the calcium hydroxide restoration materials was analysed with respect to the secretion of reactionary dentine, the test materials did not have a positive or negative effect. The Hypocal product appeared to have a slightly positive effect on reactionary dentine secretion, but this was weak (ANOVA, $P = 0.282$). The careful preparation of cavities with a remaining dentine thickness above 2.5 μm did not appear to have appreciable effect on odontoblast cell survival (Fig. 4). Similarly, cavity restoration with the test products did not appear to have significant effect on the odontoblasts (Fig. 4).

The pulpal effect of coolant and speed of the drill during cavity cutting into dentine

The loss of pulpal cell numbers was greatest following cavity cutting in the absence of coolant (Fig. 5) in comparison with cavity cutting with coolant (Fig. 6, Table 2, ANOVA, $P = 0.030$). Cavity cutting at an 8000 rpm drill speed was found to result in the preservation of higher numbers of the odontoblast cell populations, in comparison with cavities cut with a drill speed of 500, 4000 and 20 000 rpm (Fig. 7). However, the difference in odontoblast cell numbers between the use of these drill speeds was not found to be statistically significant (Table 2, ANOVA, $P = 0.244$). There did not appear to be a reparative tissue response during the time-scale of the experiment, as the remaining cells of the pulp did not appear to proliferate or differentiate to replace the injured necrotic cells beneath the site of the

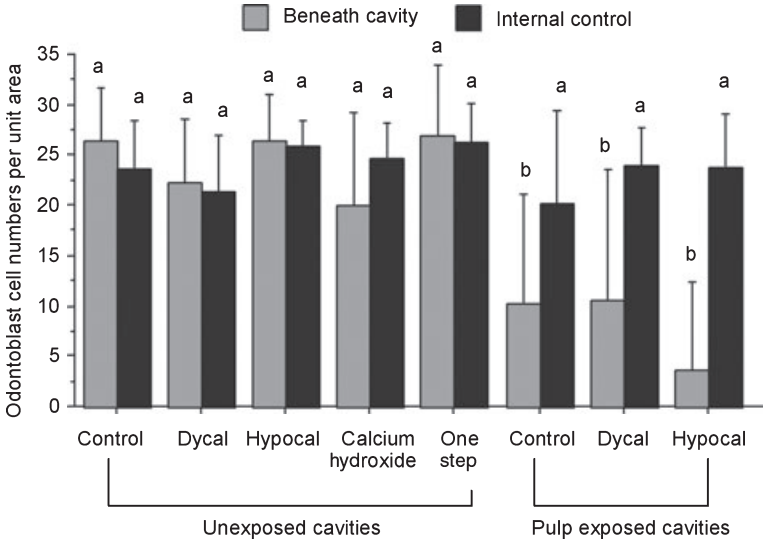

Figure 4 Odontoblast cell numbers following cavity restoration with dental materials. (a) no statistical difference at the $P < 0.05$ significance level. (b) statistical difference at the $P < 0.05$ significance level.

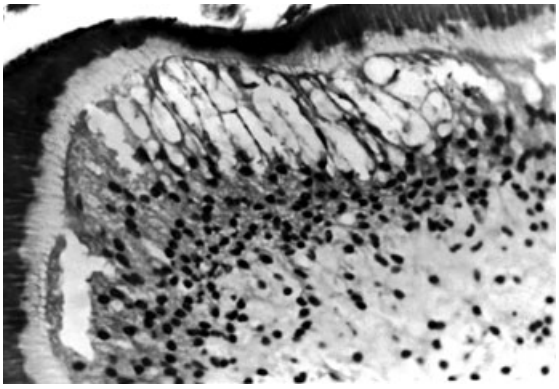

Figure 5 Histology of a cavity cut in the absence of coolant.

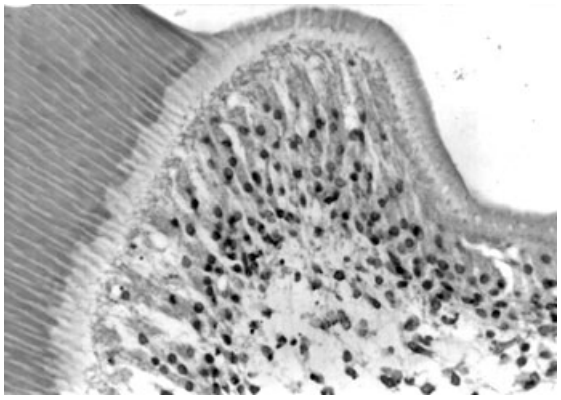

Figure 6 Histology of a cavity cut with coolant.

cavity preparation. The rank order of drill speed induced injury, from the least to the most injurious was: 8000, 4000, 20 000 and 500 rpm with water coolant.

Smear layer

In the absence of cavity conditioning treatment, a smear layer was visible partially covering the dentine tubules in the instrumented cavity floor. When the instrumented cavity surfaces were conditioned for 60 s with static 17% disodium ethylenediaminetetraacetate (EDTA) solution (Roth International, Chicago, IL, USA) pH 8, no smear layer was visible, and an even distribution of dentine tubule orifice sizes was observed. The use of this dentine conditioning treatment was observed to be optimal for removing the smear layer, but did not appear to appreciably remove intra-tubular 'smear layer plugs' from within dentine tubules in the cavity floor. Therefore, a 60-s static EDTA treatment was felt to be reasonably optimal for smear layer removal and was used to examine the pulpal responses (Table 1). The remaining category of cavity conditioning was to condition cavities with 120 s of treatment with static EDTA (Table 1). This conditioning treatment removed the smear layer, as well as causing a loss of some intra-tubular dentine matrix, including smear layer plugs from within dentine tubules. Therefore, 120 s of static EDTA treatment was felt to be an excessive cavity conditioning treatment for smear layer removal and was also used to examine the pulpal responses to such treatment.

The effect of EDTA cavity conditioning treatments on pulpal cell numbers following dentine cavity cutting

Pulpal cell numbers in tooth slices containing cavities cut into dentine without EDTA cavity conditioning of instrumented surfaces, in comparison with cavities conditioned with 60 s of static EDTA treatment (Table 2, ANOVA, $P = 0.236$) (Fig. 7), were not found to be statistically significantly different. However, conditioning of instrumented cavity surfaces with EDTA applied statically for 120 s appeared to cause a large decrease in the cell numbers of morphologically intact pulpal cells (*post hoc* ANOVA, $P = 0.0001$) beneath the site of cavity preparation (Fig. 7).

The secretion of reactionary dentine beneath cavities cut and conditioned with EDTA

The use of EDTA solution to remove smear layer and condition the instrumented surfaces of cavity preparations, had an effect on the subsequent secretion of reactionary dentine by the odontoblast cells (Table 2, $P = 0.0031$). Little reactionary dentine was secreted as a result of cavity restoration with an intact smear layer (Fig. 8). Reactionary dentine matrix secretion was maximal following the application of EDTA solution for 60 s and reactionary dentine secretion was slightly reduced following the application of EDTA for 120 s (Fig. 8).

The secretion of reactionary dentine beneath cavities cut with various drill speeds

The use of coolant and the speed of the drill during cavity preparation had an effect on the subsequent secretion of reactionary dentine by the odontoblast cells in unexposed cavities (Table 2, $P = 0.0031$). No reactionary

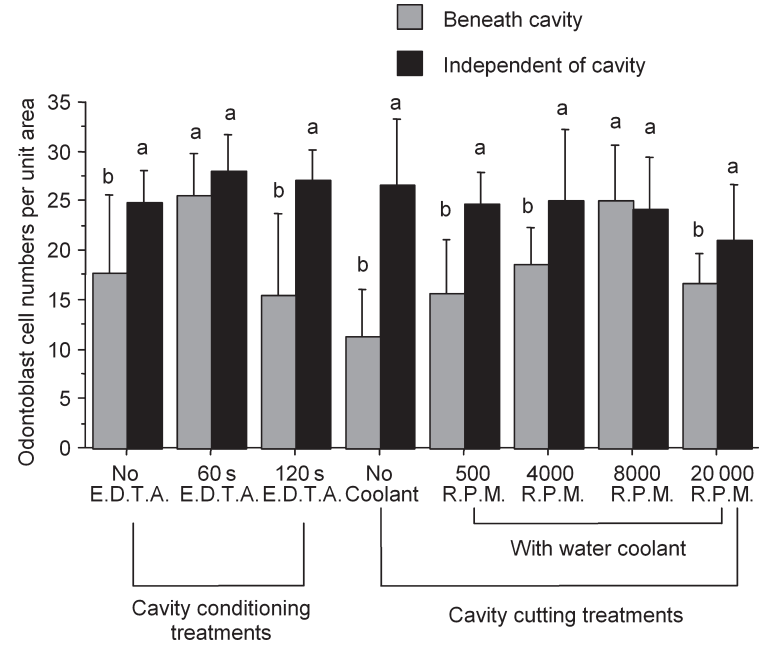

Figure 7 Odontoblast cell numbers following cavity cutting and conditioning with various treatments. (a) no statistical difference at the $P < 0.05$ significance level. (b) statistical difference at the $P < 0.05$ significance level.

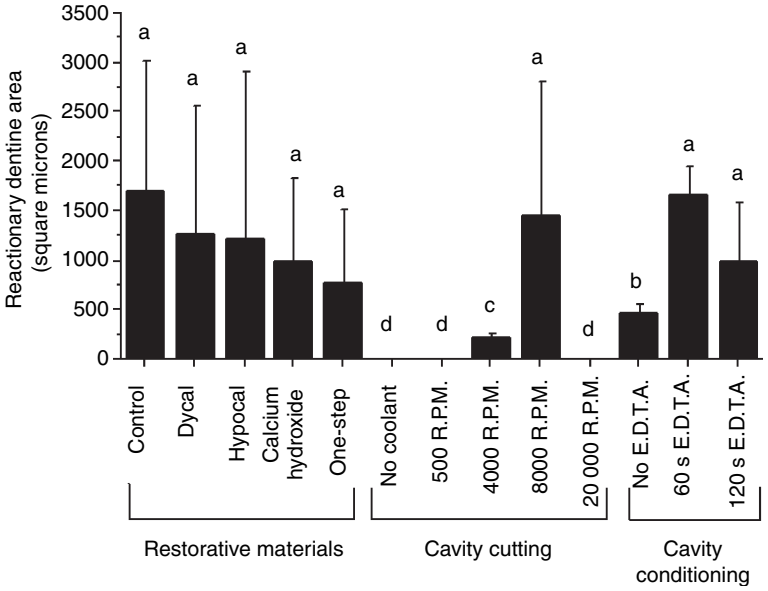

Figure 8 Reactionary dentine secretion beneath cavity preparations. (a–d) no statistical difference at the $P < 0.05$ significance level.

dentine was secreted as a result of cavity preparation without coolant or with 500 and 20 000 rpm drill speeds (Fig. 8), suggesting that the regenerative activity of the odontoblasts had been impaired by these dentine cavity preparation treatments.

Discussion

Animal experimentation has found wide use in the preclinical assessment of dental materials and procedures; however, the International Standards Organization through guidelines ISO 7405 (1995) and ISO 10 993 (1992) has recognized that alternative strategies need to be developed to replace experimentation in live animals.

The usefulness of organ culture of tooth slices for assessment of pulpal cytotoxicity of dental materials (Murray *et al.* 2000b) and dentinogenic activity (Magloire *et al.* 1996, Sloan *et al.* 1998, Sloan & Smith 1999) has already been established. The present study

has demonstrated that tooth slice organ culture provides a useful model for discrimination of both cell injuries after restorative procedures and tissue repair through the formation of reactionary dentine. The ability to closely control experimental conditions has allowed study of the relative importance of individual restorative factors on these tissue responses.

Several factors may affect the ability of odontoblasts to detect dentine injury caused by cavity cutting and restoration and to respond to this injury by secretion of reactionary dentine. These include cavity preparation trauma, restorative dental procedures, operator hand instrumentation, chemicals, dental materials and microleakage (Mjör 1983, Cox *et al.* 1992). A common feature amongst this diverse group of factors may be the extent of injury to the dentine–pulp complex. Detection of injury by the odontoblasts will lead to a response of secretion of reactionary dentine. A clear quantitative relationship has been established in this study between cavity remaining dentine thickness and the stimulation of reactionary dentinogenesis (Fig. 9). An exponential response of reactionary dentinogenesis with decreasing remaining dentine thickness was described once the thickness was less than 50 μm. The lower limit for secretion of reactionary dentine appeared to be 2.5 μm suggesting injury to the odontoblast cell body was extensive at this point (Fig. 9). It could be speculated that the thermal conductivity of dentine in thin sections increases above a threshold level at which point the odontoblast layer becomes susceptible to thermal damage, or that irreversible injurious amputation of the odontoblastic process occurs. With a remaining dentine thickness of less than 2.5 μm, pulpal damage became generally more extensive with the exposed odontoblasts varying in their sensitivity to the pulp capping material (Fig. 9). In the present study, the precise measurements of pulpal responses apply to rat teeth *ex vivo*. This tooth slice model appears to permit more control over all the experimental variables, compared with *in situ* human teeth where bacterial activity and patient variables such as oral hygiene and physiological variables such as age may influence the dental pulp responses. The relationships between remaining dentine thickness, odontoblast survival and reactionary dentine have been shown to exist in human teeth (Murray *et al.* 2000a), but have not yet been so precisely defined to a remaining dentine thickness precision of between 2.5 and 50 μm. Most likely because the combined effects of bacteria, patient and physiological variables have the potential to modify the dental pulp responses to the restorative variables.

The relationship between reactionary dentinogenesis and remaining dentine thickness shown in Fig. 1 suggests that diffusion of stimulatory molecules through the residual dentine beneath the cavity may be an important determinant of this response. This would concur with the concept that endogeneous growth factors sequestered within the dentine matrix may provide the molecular stimulus for reactionary dentinogenesis (Finkelman *et al.* 1990, Cassidy *et al.* 1997, Smith 2003). The greatest secretion of reactionary dentine was observed with remaining dentine thickness of less than 20 μm (ANOVA $P = 0.0295$) concurring with other studies (Weider *et al.* 1956, Cox *et al.* 1992) that suggest cavities must be placed in the inner third of dentine to stimulate formation of tertiary dentine.

The choice of Hypocal or Dycal products to restore unexposed cavities did not appear to significantly influence the effect of cavity remaining dentine thickness on reactionary dentine secretion. Lack of correlation between cavity restoration material and the thickness of reactionary dentine has been previously suggested (Kronfeld 1933). However, there was a difference between these materials in the amount of pulpal necrosis following the capping of a pulp exposure. Hypocal with its greater concentration of calcium hydroxide gave rise to greater pulpal injury. These observations reflect the ability of calcium hydroxide to dissolve necrotic tissue (Hasselgren *et al.* 1988) and the effects of compositional differences in pulpal wound dressings on the repair process (Cox *et al.* 1992).

The present study has highlighted how smear layer removal by EDTA under suitable conditions may contribute to repair through reactionary dentine secretion. Use of a 60-s static EDTA treatment led to optimal secretion of reactionary dentine and also pulpal survival. Absence of cavity conditioning (0-s EDTA) led to minimal reactionary dentine secretion and decreased pulpal cell survival. The presence of a smear layer will not only lead to inadequate pulpal protection from reactionary dentinogenesis, but may also trap bacteria (Bergenholtz *et al.* 1982). Clinically, presence of a smear layer can lead to inadequate marginal seal of the restoration resulting in an increased probability of microleakage and eventual pulpal inflammation (Eick *et al.* 1997). EDTA is primarily used in endodontics, although used in some adhesive systems, it is not very popular; phosphoric acid is the most common conditioner. There is a need to expand research into the

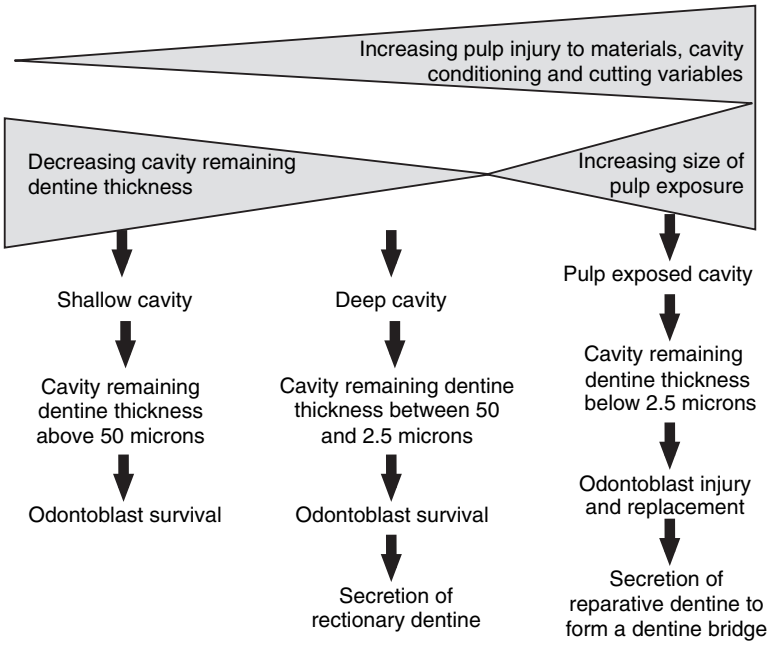

Figure 9 Diagram of odontoblast activity in response to the remaining dentine thickness of cavity restorations.

tissue reactions of teeth to common conditioning, chelating or etching agents, including phosphoric and citric acid, and self-etching primers, because the current study has demonstrated the effects of EDTA are not limited to smear layer removal.

The direct application of bonding agents (Pameijer & Stanley 1995), EDTA and other dentine conditioning agents has long been an issue for pulpal viability. The cytotoxicity of EDTA has led to the recommendation for it not to be used in pulp exposures or very deep cavity preparations (Lindemann *et al.* 1985). Prolonged EDTA treatment (120 s) in the present study confirmed this potential cytotoxic effect. Although it has been suggested that smear layer retention may inhibit or delay bacterial colonization by reducing dentine permeability, there is no clear scientific consensus on this issue (Sen *et al.* 1995). During, the 1990's, the consensus was moving toward smear layer removal to reduce the microflora and associated endotoxins, enhance the sealing ability of restoring materials and to decrease the potential of bacteria to survive and multiply (Pashley 1992). The recent consensus was moving towards *all-in-one* self-etching adhesive systems that did not remove smear layer in order to be less time-consuming (Perdigão *et al.* 2000). Although the development of early adhesive products which did not condition the cavity walls, were not very successful, and led to low bond strengths (Tao *et al.* 1991). The current consensus appears to favour the removal of smear layer using a separate acid etch step because some *all-in-one* self-etching adhesive systems, are demonstrating a faster loss of marginal integrity during clinical service (Peumans *et al.* 2007).

The stimulatory effects of EDTA on reactionary dentinogenesis may be a result of solubilization of endogeneous growth factors from the dentine matrix (Finkelman *et al.* 1990). Growth factors, such as transforming growth factor-beta and bone morphogenic proteins, may play a critical role in dentinogenic events (Sloan *et al.* 1998). EDTA can solubilize a number of dentine matrix components including growth factors and its chelating action at neutral pH 7 may contribute to this action (Smith & Leaver 1981). Thus, smear layer removal with EDTA in accordance with the manufacturer's recommended treatment, may be beneficial to the patient both in terms of pulpal repair response and to achieve greater bond strength of restorations (Blomlöf & Lindskog 1995), but it is unlikely to be effective if the remaining dentine thickness is above 50 μm.

Recommendations for the selection of drills and drill speeds have been extensively reviewed (Summitt 2006). Whilst choice of drill speed has been suggested to be a matter of personal preference, this study has shown the consequences of inadequate coolant during cavity cutting and the possible effects of different drill speeds on pulpal activity. Absence of water coolant caused the greatest loss of pulpal cell survival, which concurs with

previous views (Tobias *et al.* 1982) and the resulting injury prevented a reactionary dentinogenic response. Cavity preparation with drill speeds of 500 or 20 000 rpm in the presence of water coolant also reduced pulpal cell survival and prevented reactionary dentinogenesis. Cavity cutting with 8000 rpm drill speed and to a lesser extent 4000 rpm in the presence of coolant were found to be less injurious to the pulpal cells and led to reactionary dentinogenesis. These observations concur with those previously reported in human teeth (Swerdlow & Stanley 1958, Croft & Stanley 1966, Diamond *et al.* 1966).

Conclusions

The present study has allowed quantitative investigation of a number of individual restorative factors on both pulpal cell injury and also repair through secretion of reactionary dentine. Importantly, it has demonstrated the value of tooth slice organ culture in the investigation of these factors in a reproducible and carefully controlled manner. It provides greater clinical relevance than many cell culture approaches and also allows pulpal responses to be studied in the absence of processes such as inflammation. Whilst this allows dissection of the biological processes taking place during pulpal injury and repair, *in vivo* experimentation is required to ultimately bring together the various systemic and local tissue events.

Acknowledgements

This publication was made possible in part by Grant Number DE015573 from the National Institutes of Dental and Craniofacial Research (NIDCR/NIH). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NIH.

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