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Evaluation of a new laboratory model for pulp healing: preliminary study

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Abstract

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Aim To assess the feasibility of using the mouse as an *in vivo* model for studying pulpal healing in response to restorative procedures.

Methodology Direct pulp capping on maxillary first molar teeth with mineral trioxide aggregate (MTA), overlaid with light-cured composite resin, was performed on nineteen 3-month-old mice. For control teeth, the composite resin was placed in direct contact with the pulp. Animals were killed at 3 days, 1 week, 2 weeks, 5 weeks and 11 weeks postoperatively. Extracted dental tissues were subsequently analysed by haematoxylin and eosin staining, immunohistochemistry for dentine sialophosphoprotein (DSPP) expression, scanning electronic microscopy and X-ray analysis to determine both pulpal response and dentine bridge formation.

Results Of the 19 mice initially used, 16 were subsequently studied. Histological analyses of pulps directly exposed to MTA for up to 2 weeks demonstrated a distinct structural change in the extracellular matrix. By weeks 5 and 11, a dentine bridge was present in all MTA-treated specimens in which DSPP immunoreactivity was clearly apparent. Scanning electronic microscopy and X-ray analysis enabled confirmation of calcification of the dentine bridge, and demonstrated that it had a globular surface morphology as opposed to the tubular appearance associated with orthodentine.

Conclusions This is the first description of the utilization of a murine model for study of *in vivo* pulpal repair. This approach provides a novel opportunity to enable the use of genetically modified animals to explore cellular and molecular processes during reparative events.

Keywords: dentine bridge, mineral trioxide aggregate, pulp capping, reparative dentine.

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Introduction

Following pulp exposure, direct capping using a suitable material can induce a protective response of reparative dentinogenesis resulting in dentine bridge formation. As pulp exposure results in odontoblast loss, the healing process is consequently more complex requiring the recruitment and subsequent differentiation of new dentine secreting cells. To date, the origin(s) of the recruited cells, the differentiation process and the cellular and molecular reactions involved are not clearly defined. Similarities have been reported between the wound healing response and embryonic developmental events. It is therefore probable that the processes involved in pulp repair recapitulate those of initial odontogenesis, specifically during odontoblast differentiation (Tziafas *et al.* 2000, Smith & Lesot 2001).

Despite extensive study (Smith *et al.* 1990, 2001, Decup *et al.* 2000, Goldberg *et al.* 2001, Six *et al.* 2002), the molecular signalling of cell differentiation

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during reparative dentinogenesis is still not fully characterized. Several studies have characterized the effects of growth factors and/or matrix proteins on reparative dentinogenesis using animal models. Similarities in phenotype of primary odontoblasts and 'odontoblast-like cells' differentiating during the healing process have been clearly demonstrated (D'Souza *et al.* 1995, About & Mitsiadis 2001). However, the markers used have so far not allowed the initial stages of cell recruitment to be followed, nor the presumptive precursors to be precisely defined [pulp fibroblasts, pericytes (Shi & Gronthos 2003), pluripotent cells of bone marrow (Shi *et al.* 2001, Sloan & Smith 2007)]. Study of ^{3}H-thymidine incorporation during reparative dentinogenesis has not allowed elucidation of the origin and the nature of these replacement cells (Fitzgerald *et al.* 1990).

Whilst the presence of a population of stem cells within the dental pulp (Gronthos *et al.* 2000, 2002) or in the apical dental papilla (Sonoyama *et al.* 2008) has been reported, these cells have proven difficult to identify *in vivo*, because of the need for a range of markers to define their phenotype. Nevertheless, some aspects of their differentiation, such as their early expression of transcription factors (Blin-Wakkach *et al.* 2001, Orestes-Cardoso *et al.* 2002, Thesleff 2006, Aioub *et al.* 2007), are known, and this knowledge may allow indirect identification of the stem cells *in situ* within transgenic mice. In addition, although dental pulp stem cells (DPSCs) have been demonstrated to induce the formation of dentine following ectopic transplantation (Gronthos *et al.* 2002), their involvement in the pulp healing process is still to be demonstrated.

To date, several animal models of reparative dentinogenesis, including the rat, dog, monkey and ferret, have been used; however, to our knowledge, a mouse model has yet to be reported. The mouse represents an interesting and well-characterized laboratory model, specifically with regard to transgenics. These models are predicted to be extremely informative in studies on the molecular signalling involved in pulp healing. The small size of the animal, however, complicates surgical procedures during pulp capping, as traditional instrumentation is not suitable for use on molar teeth whose diameter is approximately 1.4 mm. Miniaturization of these procedures is therefore necessary to exploit the mouse as a laboratory model for pulp-capping research. The aim of this study was to demonstrate the utility of the mouse as a model for reparative dentinogenesis.

Materials and methods

Animal experimentation

All animal experiments were conducted by an authorized person in accordance with French regulations. Nineteen young adult mice (3 months old) were used. Following anaesthesia by intra-peritoneal injection of 2,2,2-tribromoethanol 2-methyl 2-butanol (Avertine®; Sigma Aldrich, Lyon, France) (0.017 mL g^{-1}), a cavity was drilled with a carbide bur (diameter 0.04 mm) (Komet, Paris, France) on the palatal aspect of the maxillary first left molar, in the centre of the tooth according to the mesio-distal plane (Fig. 1) until the pulp was visible through the transparency of the dentine floor of the cavity. A pulp exposure was subsequently created mechanically using an endodontic hand file of 0.15 mm diameter with a 4% taper (C+file®; Dentsply Maillefer, Ballaigues, Switzerland); this approach enabled control of pulp exposure size to approximately 150 μm (size of the tip of the file). Pulp capping was performed using mineral trioxide aggregate (Pro-Root MTA®; Dentsply Maillefer) mixed with sterile water following the manufacturer's recommendations. Mineral trioxide aggregate was placed in contact with the pulp using the tip of a probe, and condensed gently with a sterile paper point (XX-Fine; Henry Schein, Alfortville, France). Subsequently, the cavity was sealed with light-cured composite resin (Point4®; Kerr Hawe, Bioggio, Switzerland) associated with a one-step adhesive system (Prime and Bond® NT™; Denstply Caulk, Paris, France). Animals were placed in individual cages until they recovered from the anaesthetic, and to aid recovery. Paracetamol (0.06 mg g^{-1} day^{-1}) was delivered in their drinking water for 72 h. As a control, the pulp-capping procedure was performed in the absence of MTA on maxillary first molar on two animals, i.e. the composite resin was placed directly in contact with the pulp.

Prior to dental tissue extraction, animals were killed, after deep anaesthesia, by intracardiac perfusion with 4% paraformaldehyde (PFA) (Sigma Aldrich) in phosphate-buffered saline (PBS) 0.1 mol L^{-1} (pH = 7.4) through the left ventricle, using a monostaltic pump for 5 min (Touzart and Matignon, Vitry, France). Treated animals were killed at increasing time periods following the clinical procedure, as follows:

- two treated animals at 3 days postoperatively
- two animals at 1 week postoperatively
- two animals at 2 weeks postoperatively

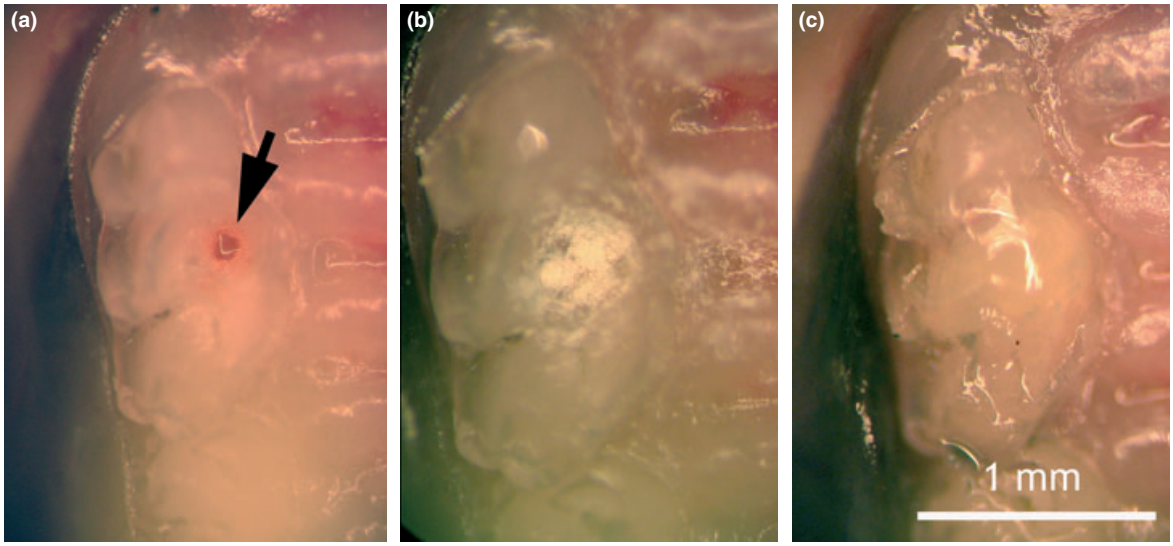

Figure 1 Clinical procedure of pulp capping. (a) After preparing cavity with a small bur, the pulp was exposed using an endodontic hand file. (b) Pulp capping was performed by placing mineral trioxide aggregate (Pro-Root MTA®; Dentsply Maillefer); (c) then the cavity was filled with a light-cured composite.

- eight treated animals and the two 'controls' at 5 weeks postoperatively
- And finally two animals at 11 weeks after treatment.

Following removal of most of the soft tissues, heads of animals were immersed in 4% PFA for 6 h at 4 °C.

Histology

The entire maxillae of 10 experimental animals and the two control animals were dissected, rinsed in PBS for 20 min, and decalcified for 7 weeks in a 4.17% ethylenediamine tetraacetic acid (EDTA solution) with 0.2% PFA (pH = 6.8) and agitation at 4 °C. The solution was renewed every 2 days for the first week, and then every week until the decalcification was complete as confirmed by X-ray analysis. Samples were dehydrated through increasing grades of ethanol and embedded in paraffin or Epon resin block according to standard procedures. The samples embedded in paraffin were cut with a microtome (Leica, Berlin, Germany) in sections of 7 μm, and then stained with haematoxylin and eosin (H&E). Samples embedded in Epon resin were cut in sections of 0.5 μm and stained using a solution of mixed Methyl Blue and Blue Azur II (50% : 50%).

Slides were mounted with Depex (Sigma Aldrich) and observed by light microscopy.

Immunohistochemistry

Immunohistochemistry was performed on sections mounted on SuperFrost® Plus (Manzel Glase, Braumschweig, Germany) slides. Deparaffined and rehydrated sections were incubated for 30 min in 3% H_2O_2/PBS to quench endogenous peroxidase activity, and then rinsed for 10 min in PBS. Nonspecific protein binding was blocked by incubation for 30 min in 5% goat serum in PBS. Specimens were incubated overnight at 4 °C in a humidified chamber with the polyclonal rabbit anti-mouse dentine sialophosphoprotein (DSPP) antibody (kindly provided by Pr Larry Fisher, National Institute of Health, Ref LF 153). Sections were washed three times in PBS at room temperature prior to treatment for 30 min at room temperature with the secondary biotin-labelled goat anti-rabbit antibody (Streptavigen Multilink Kit; Biogenex, Dorset, UK). Subsequently, sections were incubated for 30 min at room temperature with peroxidase linked to avidin (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). After rinsing with three changes of PBS, the immunoreactivity was visualized by development for 2–5 min with 0.1% 3,3-diaminobenzidine and 0.02% hydrogen peroxide (DAB substrate kit; Vector Laboratories). Sections were counterstained with Mayer's haematoxylin, mounted with permanent mounting medium (XAM®; BDH Laboratory, Poole,

UK) and examined by light microscopy. A positive control was performed on untreated mouse teeth and a negative control on mouse oral mucosa. A further negative control involving omission of primary antibody was also performed on mouse teeth.

Scanning electron microscopy

For scanning electron microscopy (SEM) examination, two samples (5 weeks postoperative) were embedded in self-curing epoxy resin (Araldite; Ciba Geigy, Basel, Switzerland). Samples were prepared with a water-cooled low-speed Isomet diamond saw (Buehler Ltd, Evanston, IL, USA) and polished with an automatic polishing device (Pedemax-2; Struers, Copenhagen, Denmark) with waterproof silicon carbide papers of decreasing abrasiveness (600, 1200, 2400 and 4000 grit) and with soft tissue discs with fine diamond suspensions (3, 1 and 0.03 μm) in combination with DL-lubricant cooling solution (Struers). On the two samples, the three roots were removed and samples ground and polished until the opening of the pulp chamber was reached.

After polishing, samples were immersed in 5% sodium hypochlorite solution for 5 min, ultrasonicated in water for 5 min, etched with orthophosphoric acid (Bisico, Lançon de Provence, France) for 15 s and washed with deionized water for 30 s.

Samples were rinsed with a solution of 0.2 mol L^{-1} sodium cacodylate buffer at pH 7.4 for 1 h with three changes, and then dehydrated through increasing grades of ethanol (25% for 1 h, 50% for 1 h, 75% for 1 h, 95% 2×1 h and 100% overnight). Finally, the specimens were sputter-coated with gold palladium using a sputter coater (SC500; Bio Rad, Microscience Division, Elexience, Paris, France) at 20 mA. Samples were observed under a field emission SEM (JSM-6400 F Scanning Microscope; JEOL Ltd, Tokyo, Japan) at an accelerating voltage of 15–20 kV and a working distance from 6 to 10 mm. The SEM was equipped with an energy dispersive X-ray spectrometer system (Tracor 5500; Tracor, Riddletown, WI, USA). Signals in the energy region for phosphorus (P) and calcium (Ca) were recorded and compared between the primary dentine and the reparative dentine of the dentine bridge. The two samples were analysed, and reparative dentine composition was compared to orthodentine (in another area, but on the same sample). The energy scale of the X-ray detector was calibrated using a cobalt standard.

Results

Surgical procedure

Following the surgical procedure, one animal did not recover from the anaesthesia and died 4 h after treatment. The 18 surviving mice recovered within 6–10 h, and were then housed individually, with food and water *ad libitum*. Paracetamol (0.06 mg g^{-1} day^{-1}) was delivered in water for 2 days for analgesia.

Light microscopy observations

All specimens were cut in a frontal plane with control of the positioning of specimens performed using the opposing first molar of the same maxilla as a landmark. Histological examination confirmed the successful completion of the procedure, the localization of the cavity and the diameter of the pulp exposure at around 150 μm (Fig. 2).

In the majority of the treated teeth (16/18), the perforation of the pulp was located in the mesial half in front of the mesial root canal. The cavity was generally centred on the buccal–palatal aspect. On the two other specimens (5 weeks postoperative), the floor of the pulp chamber was perforated during the surgical procedure and these specimens were considered failures.

In the negative control in which no MTA was used for pulp capping, the histology of the pulp appeared normal, but neither dentine bridge nor any signs of healing of the exposure were evident, up to 5 weeks after treatment. No inflammation was observed in both control specimens (Fig. 2).

At 2 weeks postoperatively, a line with a high affinity for histological dyes was observed. This line precisely followed the contours of the material (Fig. 3).

At 5 weeks postoperatively, a dentine bridge was visible in all of the experimental specimens. The dentine bridge was in close contact with the dentine walls and no gap was apparent between the two structures. The pulp cells were in close association with the new hard tissue; however, a polarized cell morphology was not apparent. In three specimens, dentinal tubules were visible in the matrix of the dentine bridge; these tubules were not linear and their course appeared interrupted (Fig. 4).

In most of specimens, several dentine chips were included within the dentine bridge, but did not appear to adversely affect the healing process (Fig. 2).

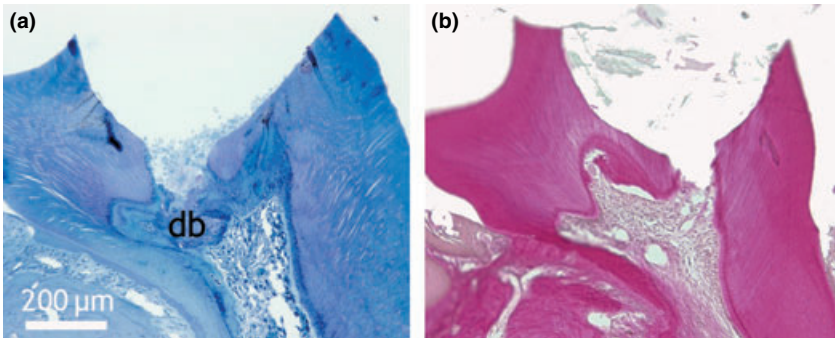

Figure 2 (a) Histology of a mouse first upper molar capped with mineral trioxide aggregate (MTA), 11 weeks postoperatively. 0.5-µm-thickness sections were stained with Methyl Blue/Azur II blue (50% : 50%). The dentine bridge (db) was clearly visible, with inclusion of dentine chips. Dentine chips were probably impacted into the pulp tissue during pulp exposure. (b) Mouse first upper molar treated without MTA, 5 weeks postoperatively. No dentine bridge was visible. Histologically, the pulp tissue appears healthy with no inflammatory process evident. 7-µm-thickness cut stained with haematoxylin and eosin.

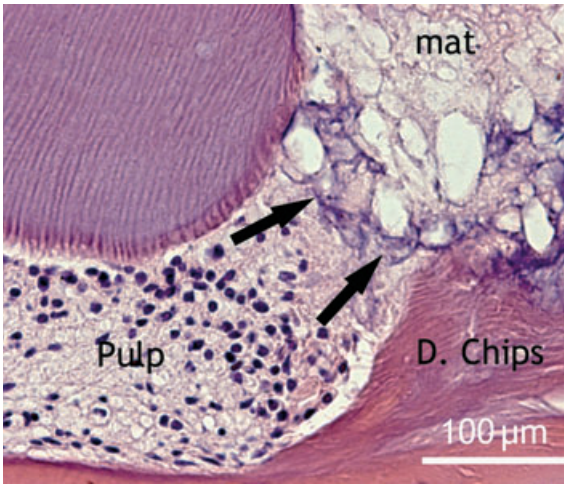

Figure 3 Histology at 2 weeks postoperatively. Haematoxylin and eosin staining. The inflammation process has already decreased, and few cells are present in contact with the material involved in tissue reorganization. A specific line (→), with a high affinity for stain, demarcates the material and the pulp tissue. This line is in very close contact with the material. At this stage, no bacteria or inflammatory cells were visibly present.

Immunohistochemistry

The immunoreactivity for DSPP associated with the dentine bridge was clearly evident (Fig. 4). The cytoplasmic staining is also characteristic of dentine secreting odontoblast-like cells. Orthodentine was also stained as was the intratubular (peritubular) dentine. It was also noticeable that the dentine bridge was stained less intensely than the adjacent orthodentine. The negative controls demonstrated the absence of staining.

Scanning electron microscopy

An inner view of the pulp chamber roof revealed the presence of the dentine bridge. Higher magnification observation revealed a close contact between the dentine bridge and the dentinal wall of the tooth. The structure of the reparative dentine of the bridge had a globular morphology and appeared different from the tubular othodentine structure (Fig. 5).

X-ray spectrophotometeric analysis allowed comparison of phosphorus and calcium levels in different areas of the tooth (Fig. 6a,b). The calcium and phosphorus levels of both types of dentine (reparative and orthodentine) were analysed and the Ca/P ratios of both tissues were similar confirming the calcification of the dentine constituting the bridge. The calcium levels were slightly lower for reparative dentine [62.52% (sample 1) and 65.53% (sample 2)] than for orthodentine [73.64% (sample 1) and 72.53% (sample 2)], suggesting that the reparative dentine samples were less calcified than the orthodentine.

Discussion

The mouse as an experimental model for pulp capping

Several species of animal have been used for pulp-capping studies including the monkey (Pitt Ford *et al.*

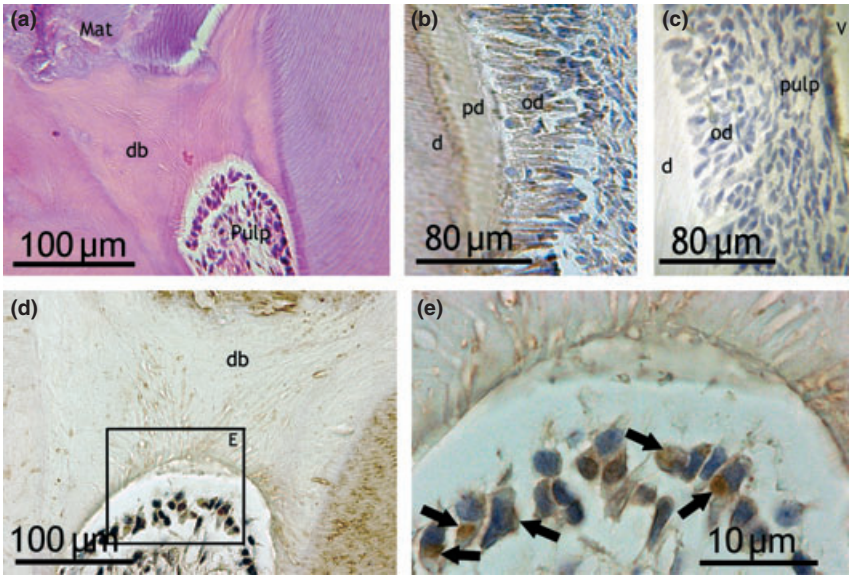

Figure 4 Mouse first upper molar, 5 weeks after pulp capping with mineral trioxide aggregate (MTA). (a) Haematoxylin and eosin staining. The dentine bridge (db) appears very clearly between material (Mat) and the pulp; there is no visible gap between dentine bridge and dentine walls. In the thickness of the db, few tubules are evident; their course is not straight as in orthodentine (×50). (b) Immunohistochemistry with dentine Sialophosphoprotein (DSPP) antibody of untreated mouse molar pulp (positive control). Staining of odontoblasts is evident, and DSPP is apparently also present in the extracellular matrix of the pulp(×50). (c) Negative control staining with omission of primary antibody (DSPP). (d) Immunohistochemistry with DSPP antibody of first upper molar tooth capped with MTA, 5 weeks postoperatively (×50). Dentine bridge staining is very weak compared with orthodentine. (e) At higher magnification, DSPP expression is clearly visible in the cells in close contact with dentine the bridge (×100).

1996), dog (Tziafas *et al.* 1992), ferret (Smith *et al.* 1994) and rat (Decup *et al.* 2000). The monkey and dog are large animals, and are therefore expensive, requiring special care facilities. Moreover, the rules concerning animal experimentation increasingly present difficulties for this kind of work, especially using nonhuman primate species. For these reasons, the number of animals studied is often restricted, potentially influencing the experimental outcomes, reproducibility and subsequent interpretation of data.

A pulp-capping protocol has been described in the rat model, using the mesial surface of the maxillary first molar, after gingivectomy (Decup *et al.* 2000) or on the occlusal surface (Lovschall *et al.* 2005). The molar of the mouse is particularly interesting, because it presents morphological similarities with its human counterpart: it has three roots, three cusps and thus shows analogous conditions of vascularization and innervation. Whilst the rat offers a number of advantages as an experimental model, the availability of genetically- altered models for this species is more restricted than for the mouse.

Use of the mouse as an experimental model provides an opportunity for use of transgenic studies in reparative dentinogenesis; such a model should enable more precise definition of the cellular and molecular mechanisms involved in the process of dentine-pulp repair. Potentially, animals with a modified genome (with reporter gene or gene deletion) could thus be used to:

- characterize the phenotype of the replacement odontoblast-like cells and determine their origin through cell-lineage studies in transgenic mutated mice (Chai *et al.* 2000)
- decipher the cell differentiation process at the molecular level using transgenic null mutant mice for potentially determinant factors [e.g. DSPP null mutant mice (Sreenath *et al.* 2003); Msx2 null mutant mice (Aioub *et al.* 2007)] to better understand the process of differentiation
- evaluate effects of novel bioactive molecules

It is with this new knowledge and an understanding of these processes that new therapeutic strategies can be developed to promote pulp vitality and healing. Within the constraints of the regulations of animal

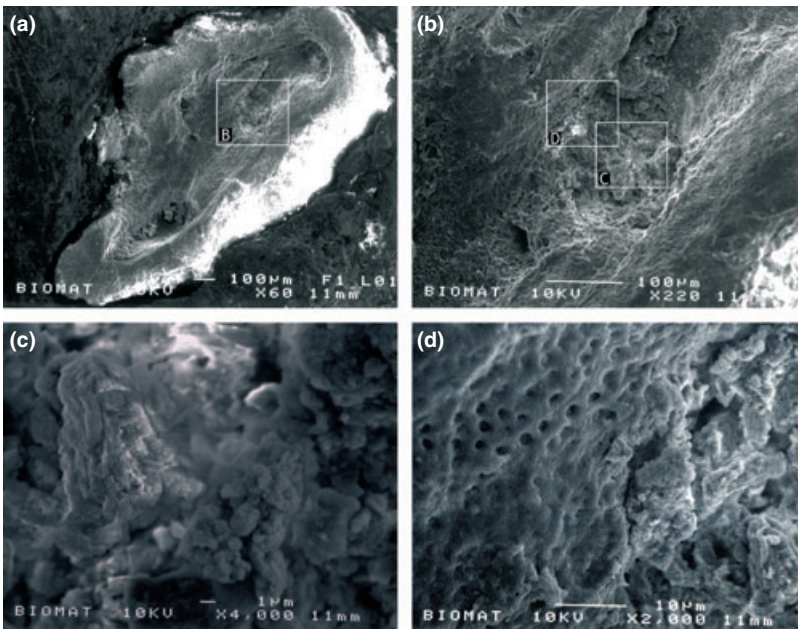

Figure 5 Scanning electron microscopy observation of a mouse molar 5 weeks postoperatively capped with mineral trioxide aggregate (MTA). Roots and pulp chamber floor were removed and the pulp chamber roof was observed at different magnifications. (a) Magnification ×60; dentine bridge appears clearly on the surface of the roof. (b) Observation of the dentine bridge at higher magnification (×200). (c) ×4000 ultrastructure of the dentine bridge is different compared with orthodentine; it is more globular in appareance. (d) Observation at magnification ×2000 of the limit between dentine wall and dentine bridge. No gap is visible.

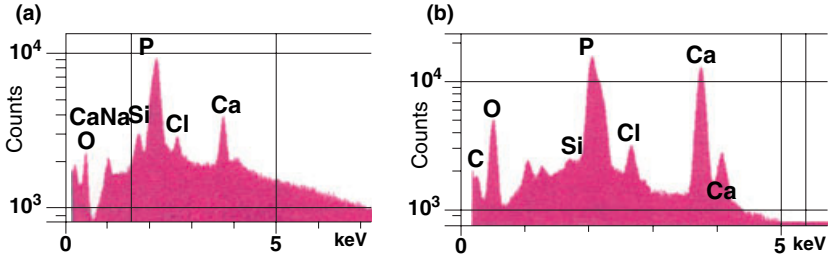

Figure 6 Results of X-ray analyses of orthodentine (a) and reparative dentine of the dentine bridge (b).

experimentation, this model will also facilitate the use of adequate numbers of sample for robust data collection.

A limitation of the model presented is that it currently uses healthy teeth, whilst in the clinical situation pulp inflammation is generally present. However, future experiments could simulate caries-like situations by incorporating bacterial infection models using whole live bacteria or bacterial components. In addition, the presence of dentinal chips or debris arising from the creation of a pulp exposure may contribute to reparative responses in the pulp. Although this may complicate data interpretation, it does reflect the clinical situation where dentine fragments (Tziafas *et al.* 1992) and dissolution products (Smith 2002) contribute to overall pulpal responses. Reproducibility of the pulp-capping procedure was regarded as an important element in the viability of the model especially in view of the small size of the mouse tooth. The histological observations confirmed the reproducibility of our surgical procedure in size and position of the pulp exposure.

Contrary to many of the studies reported with calcium hydroxide (Schröder & Granath 1972, Horsted *et al.* 1981, Fitzgerald *et al.* 1990), neither a necrotic layer nor a persistent inflammatory zone was noticed.

The completion of the pulp capping in a very short time and adaptation of a clean procedure (sterilization of the material) limited the duration of the pulp exposure to the oral cavity and thus bacterial contamination. Moreover, the use of a light-cured composite bonded with a one-step bonding system avoided any bacterial leakage post-restoration (Pradelle-Plasse *et al.* 2003). Even at 11 weeks, the coronal filling was still intact, and did not show any evidence of deterioration at the tooth–restoration interface.

In the present study, continuous bridge formation was observed, in continuity with the dentine of the walls of the cavity and the discontinuity between the bridge and the dentinal wall, often described with the use of calcium hydroxide, was not apparent. This result confirms several published reports with other animal models after capping with MTA and also clinical reports (Holland *et al.* 2001, Aeinehchi *et al.* 2003, Dominguez *et al.* 2003, Asgary *et al.* 2006, Nair *et al.* 2008).

Two weeks postoperatively, the first signs of the healing process were observed as the presence of a line in direct contact with the capping material. This line followed the contours of the material precisely and was visible on all stained sections. This junctional extracellular matrix (JEM) appears as a structural reorganization of the matrix in this area, and not a necrotic layer as described in many publications about pulp capping with calcium hydroxide. The structural modification of the matrix at this level may play an important role in the process of differentiation of the second generation of odontoblast-like cells, as suggested by experiments using growth factors (Rutherford *et al.* 1993). This could reflect recapitulation of developmental events in which signals from the inner dental epithelium mediated by the dental basement membrane are responsible for the induction of odontoblast differentiation. In the mature tooth, epithelial cells and a basement membrane are absent; however, in the healing process of the pulp, similar signalling processes for odontoblast differentiation may exist but of different derivation (Smith & Lesot 2001). The structural modification of the matrix in the area adjacent to the material at the exposure site might play a key role in the process of differentiation of the second generation of odontoblasts. It could be speculated that the matrix in this area may substitute for the role of the basement membrane during odontoblast differentiation in tooth development.

The modification of the matrix at this level could be obtained by:

- adsorption of dentinal matrix proteins (and predentine), produced by odontoblasts prior to cavity preparation
- serum components leaked into the pulpal parenchyma, at the time of surgery resulting from inflammation
- local cellular secretion by either various pulpal cell populations or newly differentiated odontoblast-like cells.

More investigations are needed to characterize this junctional area, which may reflect changes in cell secretory behaviour during dentine bridge formation at the material interface.

This study has allowed the chronological monitoring of the healing process. The initial inflammatory phase was very short (the first 2 days) (data not shown), which concurs with the results published by various authors in preceding studies of other species (Abedi *et al.* 1996, Torabinejad & Chivian 1999). The subsequent dentine bridge formation showed evidence of a dentinogenic response.

The staining of the new odontoblast-like cells with DSPP antibody, confirmed the secretion of a matrix with at least some dentine characteristics, namely the secretion of dentine sialoprotein. However, further characterization of the matrix with other markers (such as Matrix Extracellular Phosphoglycoprotein, nestin, Alkaline Phosphatase Alkaline for example) may also be beneficial.

The SEM and X-ray spectrometric analysis confirmed that the dentine bridge was calcified, although indicating that reparative dentine is probably less calcified than orthodentine. These observations are of interest and are potentially novel as most of our knowledge on reparative dentine is based on histological evidence that requires decalcification of the hard tissues and therefore may generate processing artefacts. The Ca/P ratios of the reparative dentine may be indicative of the presence of other forms of calcium phosphate rather than just hydroxyapatite, emphasizing the pathological nature of the process of reparative dentinogenesis.

Conclusion

The present study has demonstrated the feasibility of using the mouse as a laboratory model for analysis of the healing process following pulp capping. This opens exciting opportunities for use of new tools such as genetically modified animals to explore cellular and molecular processes during reparative events.

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