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Video Article

Development of a Direct Pulp-capping Model for the Evaluation of Pulpal Wound Healing and Reparative Dentin Formation in Mice

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Abstract

Dental pulp is a vital organ of a tooth fully protected by enamel and dentin. When the pulp is exposed due to cariogenic or iatrogenic injuries, it is often capped with biocompatible materials in order to expedite pulpal wound healing. The ultimate goal is to regenerate reparative dentin, a physical barrier that functions as a "biological seal" and protects the underlying pulp tissue. Although this direct pulp-capping procedure has long been used in dentistry, the underlying molecular mechanism of pulpal wound healing and reparative dentin formation is still poorly understood. To induce reparative dentin, pulp capping has been performed experimentally in large animals, but less so in mice, presumably due to their small sizes and the ensuing technical difficulties. Here, we present a detailed, step-by-step method of performing a pulp-capping procedure in mice, including the preparation of a Class-I-like cavity, the placement of pulp-capping materials, and the restoration procedure using dental composite. Our pulp-capping mouse model will be instrumental in investigating the fundamental molecular mechanisms of pulpal wound healing in the context of reparative dentin *in vivo* by enabling the use of transgenic or knockout mice that are widely available in the research community.

Video Link

The video component of this article can be found at https://www.jove.com/video/54973/

Introduction

Dental caries are one of the most prevalent oral diseases and the leading cause of surgical interventions to dentitions in almost all individuals[1,2]. The prognosis of surgical interventions and restorations of a tooth largely depends upon proper pulpal response and successful wound healing. Indeed, dental caries that penetrate deeply through the enamel and dentin frequently lead to the exposure of the underlying pulp tissue that is often "capped" with dental materials, such as calcium hydroxide ($Ca(OH)_2$) or hydraulic calcium-silicate cements (HCSCs), including mineral trioxide aggregates (MTA). The ultimate goal of such a pulp-capping procedure is to expedite pulpal wound healing by regenerating reparative dentin, a physical barrier that functions as a "biological seal" to protect the underlying pulp tissue and to increase the life expectancy of the tooth and the overall oral health. However, the underlying mechanism of pulpal wound healing and reparative dentin formation is not fully understood.

To better understand the mechanisms of pulpal wound healing and reparative dentin formation *in vivo*, several animals were previously used, including monkeys, dogs, and pigs[3-5]. Among them, rats are frequently used because they are relatively smaller in sizes compared to the other animals, but their teeth are large enough to perform direct pulp capping without any technical difficulties[6-10]. These animal models are ideal alternatives to human studies for examining pulpal responses and reparative dentin formation. However, their utilization is limited to observational studies at the cellular level, and they scarcely provide mechanistic insights during reparative dentin formation at the molecular level.

Recent technical advances in genetic engineering provided invaluable and indispensable research tools-mice that harbor a gene that is either overexpressed or deleted-that are instrumental to studying molecular mechanisms of human diseases *in vivo*. The numbers of different strains of transgenic or knockout mice that are strategically inducible in a cell-specific manner are continually growing in the scientific community. Therefore, examining pulpal wound healing and reparative dentin regeneration in these mice would greatly help to expedite our understanding of these processes at the molecular level. However, the use of mice is significantly dampened, as performing a pulp-capping procedure on a mouse tooth is technically challenging due to its miniature size. Here, we present our reproducible method of performing direct pulp capping in mice for the evaluation of pulpal wound healing and reparative dentin formation *in vivo*.

Protocol

Mice were purchased from Jackson Laboratory and kept in a pathogen-free vivarium in the UCLA Division of Laboratory Animal Medicine (DLAM). The experiments were performed according to the approved institutional guidelines from the Chancellor's Animal Research Committee (ARC#2016-037).

1. Mouse Anesthetization

1. Use eight-week-old female C57/BL6 mice (n = 3).
2. Anesthetize the mice using ketamine (80-120 mg/kg of mouse weight)/xylazine (5 mg/kg of mouse weight) solutions and administer intraperitoneally (ip) at a dose of 10 mL/kg.
3. Prepare ketamine (80 - 120 mg/kg)/xylazine (5 mg/kg) solutions and administer them intraperitoneally (ip) at a dose of 10 mL/kg.
4. Confirm that the mice are fully anesthetized by performing a toe pinch.

2. Pulp-capping Procedure

1. Place the mouth holder in the mouse's mouth.
2. Secure the mouth holder on the table such that the head is facing upward.
3. Place the microscope (10X) on top of the mouth so that the first maxillary molar is fully visible.
4. Using the ¼-round bur in a high-speed handpiece at 200,000 rpm, remove the enamel part of the tooth in the middle until the pulp is visible through the transparent dentin. Do not expose the pulp with the bur.
5. Using a #15 endodontic K-file (diameter of 150 µm), perforate through the dentin and expose the pulp.
   NOTE: Special care should be taken so that the dentin debris does not get pushed into the pulp. This can be avoided by rotating the K-file quarterly and then pulling the K-file out.
6. Mix MTA with sterile H_2O according to the manufacturer's instructions. Deliver and place the MTA onto the exposed pulp with the tip of the explorer. Use the back side of the paper point (fine) to pack the MTA into the exposed pulp by gentle tapping. The thicker side of the paper point is flat and therefore allows for the proper condensation of the MTA into the exposed pulp.
7. Etch the tooth for 15 s by placing the 35% phosphoric acid etchant where it just covers the tooth. Take special care to limit the placement of the etchant, as it may irritate gingival tissues.
   NOTE: The etchant comes in a syringe and is used to roughen the tooth surfaces so that dental adhesives can flow in to mediate micromechanical bonding onto the tooth. Because they are viscous, it can be self-contained by applying small amounts directly onto the tooth.
8. Use negative-pressured suction to remove the etchant. Use a cotton pellet that is lightly soaked with H_2O to remove the residuals of the etchant. Repeat this step until the etchant is completely removed from the tooth.
9. Using a compressed air duster, gently dry the tooth.
10. Apply the dental adhesives using the backside of the paper point.
11. Make the adhesive layer thin using compressed air for 3 s.
12. Cure the dental adhesives for 20 s using the curing-light unit.
13. Place the flowable composite in small amounts onto the tooth that was capped with MTA. Use the tip of the explorer to flow the composite into the tooth grooves.
14. Cure the composite for 30 s using a light-curing unit to polymerize it. Confirm that the composite is fully cured and hard using the explorer.

3. Post-op Care

1. Administer carprofen (5 mg/kg) subcutaneously (sc) immediately after the pulp-capping procedure.
2. Place the mice on a heating pad at low power to keep the animals warm before they wake up.
3. Return the mice to the vivarium for housing.

4. Tissue Procurement

1. After 5 - 6 weeks, euthanize the mice by cervical dislocation under a complete anesthetic condition with isoflurane.
2. Carefully remove the maxilla out of the base of the skull and put it into a 50-mL tube. Fix the entire maxilla that contains both the pulp-capped tooth and the contralateral uncapped tooth in 4% paraformaldehyde in PBS, pH 7.4, at 4 °C overnight, and then store it in a 70% ethanol solution.
   NOTE: Paraformaldehyde is toxic and carcinogenic. The proper use paraformaldehyde should be monitored as outlined in the standard operating procedures (SOP).
3. Scan the mouse maxillae using the µCT scan. To secure the maxillae during scanning, wrap the samples with gauze soaked with 70% ethanol and place them in the 15-mL cell culture tube.

5. µCT Scanning

1. Prepare the samples for µCT scanning. Briefly, wrap the samples with gauze soaked with 70% ethanol and secure them in a generic 15-mL cell culture conical tube. Mount the tube onto the µCT scanning stage, as outlined in the manufacturer's instructions.
2. Set the X-ray source to a current of 145 µA, a voltage of 55 kVp, and an exposure time of 200 ms.
3. Perform image acquisition with the µCT scanner at a 20-µm resolution and with a 0.5 mm Al filter.

4. Reconstruct the image and visualize it[11].
5. Once the µCT scan is complete, start decalcification with 5% EDTA and 4% sucrose in PBS (pH 7.4) for 2 weeks.

6. Tissue Processing and Staining

1. Embed the decalcified tissues in paraffin. Before embedding, trim the maxilla by making a sagittal cut immediately anterior to the first molar. While embedding, position this surface downward, such that the longitudinal section of the first molar is the cutting surface.
2. Using the microtome, prepare 5 µm-thick slides. The pulp-capping areas usually coincide with the distopalatal (DP) root, which can be used as a landmark. Determine the precise area of interest by examining the histology under the light microscope and comparing the µCT images.
3. For H&E staining, deparaffinize and rehydrate the slides with xylene (2x) and serially diluted ethanol (100% EtOH 2x, 95% EtOH 2x, and 70% EtOH 1x).
4. Rinse the slides with running tap water.
5. Stain with Hematoxylin solution for 2.5 min and rinse with tap water.
6. Dip the slides in 95% ethanol for 1 min.
7. Stain with Eosin solution for 1 min and rinse with tap water.
8. Dehydrate with serially diluted ethanol (70% EtOH 1x, 95% EtOH 2x, and 100% EtOH 3x) and xylene (3x).
9. Mount the slides with mounting solution.

Representative Results

Here, we showed the step-by-step procedures to perform pulp capping on mice teeth. One of the key aspects of pulp capping in mice is to have the appropriate apparatus. In this regard, having the microscope with a 10X power magnification is essential (**Figure 1A**). To create a Class-I-like preparation in the tooth, we used a ¼-round burr in an electric high speed handpiece at 200,000 rpm (**Figure 1B**). Alternatively, any other engines, including those that use compressed air, can be used to prepare a tooth.

In **Figure 2A-2E**, representative steps for performing pulp capping are shown. The Class-I-like preparation was performed (**Figure 2B**). Because a water spray may drown the mice during the procedure, its use is not recommended. For this reason, it is essential to prepare the tooth with gentle and intermittent strokes to prevent overheating. The use of compressed air is also recommended to provide cooling effects. While exposing the pulp with an endodontic file, take caution not to push the dentin debris into the pulp, as this may interfere with the data interpretation in reparative dentin formation (**Figure 2C**). This can be avoided by using the compressed air. Similarly, the MTA should be placed on the exposed pulp without pushing too far into the pulp. MTA placement can be achieved by using the backside of the paper point with gentle tapping motions (**Figure 2D**). Following MTA placement, the tooth should be cleaned using H_2O-soaked cotton pellets to remove any remaining MTA in the grooves, which may interfere with the binding of the composite onto the tooth. Conventional methods of composite restoration are used, including etching the tooth surfaces, priming and bonding with adhesives, and placing and curing the flowable composites (**Figure 2E**).

Six weeks after pulp capping, the mice were harvested, and the top view was photographed to confirm that the composite was still intact (**Figure 3A**). µCT scanning showed significant recession of pulpal space in the pulp-capped group (**Figure 3B**), suggesting that reparative dentin was formed in the pulp. The decalcified tissues samples were subjected to H&E staining to further examine histologically the formation of reparative dentin *in vivo*. In the control group, odontoblastic layers (OB) were prominently evident around the edges of the dentin (**Figure 4A - 4C**). In contrast, the pulp-capping group had significant amounts of reparative dentin (RD) formed in the pulpal space (**Figure 4D - 4F**). Interestingly, a closer examination revealed that reparative dentin (RD) exhibited a typical characteristic of dentin (*e.g.,* striated lines representing dentinal tubules, red arrow), as well as that of bone (*e.g.,* osteocytes representing trapped osteoblasts, black arrowheads). When we stained for Dentin Matrix Protein 1 (DMP1), a marker for odontogenic differentiation[12], we found a significant increase in DMP1 expression in the pulp of the pulp-capped tooth when compared to that of the uncapped tooth (**Figure 5A and 5B**), indicating that reparative dentin was formed within the pulp.

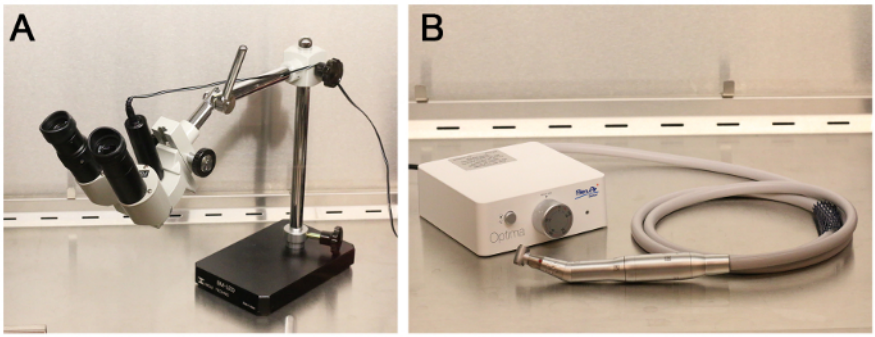

Figure 1: The Equipment Setup for the Pulp-capping Procedure. (A) A microscope (10X) for visualizing the mouse tooth. **(B)** The high-speed handpiece and the electric motor engine for preparing a Class I preparation to expose the pulp. Please click here to view a larger version of this figure.

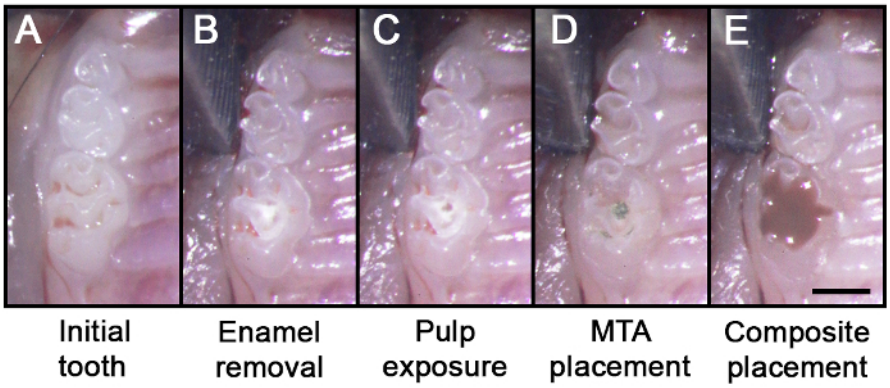

Figure 2: Representative Steps in the Capping Procedure. (A) Unprepared tooth on the maxillary first molar in a mouse. **(B)** Initial enamel removal using the quarter-round bur. **(C)** Pulp exposure using the endodontic file. **(D)** MTA placement in the exposed pulp. **(E)** Composite restoration placement on the tooth. The bar represents 500 µm. Please click here to view a larger version of this figure.

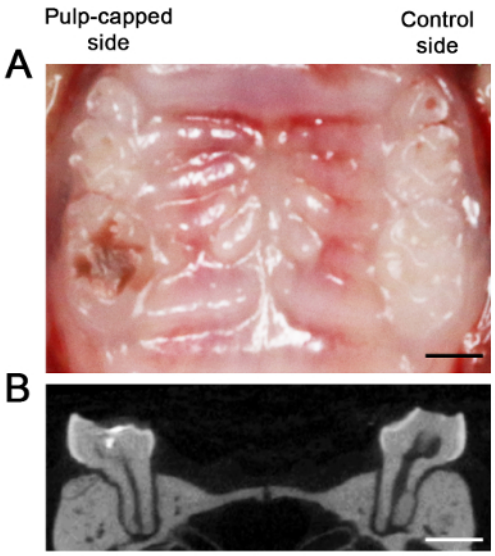

Figure 3: Clinical Presentation and µCT Scanning of the Pulp-capped and Uncapped Tooth in Mice. (A) Occlusal view of the mouse maxillae on the pulp-capped tooth (left) and uncapped tooth (right). **(B)** The cross-sectional µCT images of the maxillae. The bar represents 500 µm. Please click here to view a larger version of this figure.

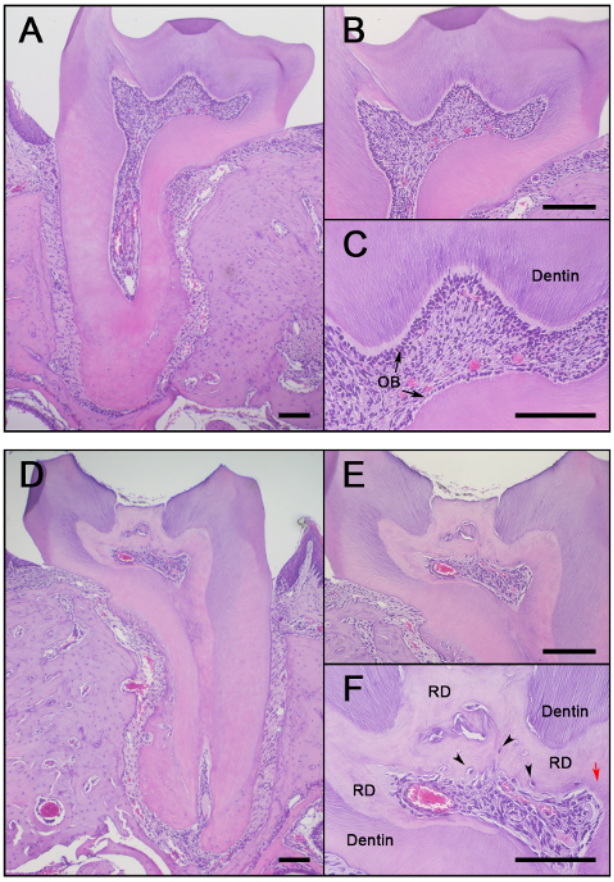

Figure 4: Histological Evidence of Reparative Dentin Formation *In Vivo*. (A-C) H&E staining of the uncapped maxillary first molar in a mouse at 100X, 200X, and 400X. **(D-E)** H&E staining of the pulp-capped maxillary first molar in a mouse at 100X, 200X, and 400X. The bar represents 100 µm (OB = odontoblast layers; RD = reparative dentin; black arrowheads = osteocytes; red arrow = dentinal tubules). Please click here to view a larger version of this figure.

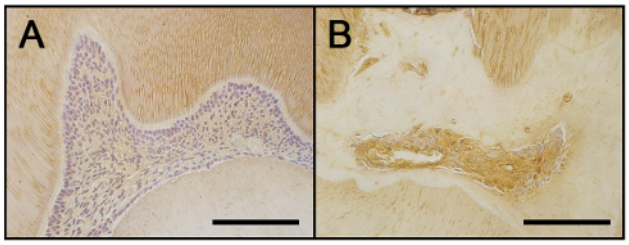

Figure 5: Immunohistochemical Staining of DMP1. (A) DMP1 staining of the uncapped maxillary first molar in a mouse at 400X. **(B)** DMP1 staining of the pulp-capped maxillary first molar in a mouse at 400X. The bar represents 100 µm. Please click here to view a larger version of this figure.

Discussion

Currently, there are several different experimental models available to validate the *in vivo* effects of dental materials, scaffolds, or growth factors on odontogenic differentiation of dental pulp stem cells (DPSCs)[13]. These models include ectopic autologous transplantation of DPSCs into an organ, such as the renal capsule, or subcutaneous transplantation of DPSCs into immunocompromised mice with scaffolds[14,15]. However, these methods are limited in that their odontogenic effect on DPSCs is not performed in the orthotopic pulp environment. On the other hand, orthotopic transplantation into the pulp or pulp-capping procedures on a tooth are used in larger animals[16,17]. Although these models are valuable in evaluating odontogenic potential in the orthotopic environment, the use of those models is largely observational in nature, providing limited mechanistic insights on pulp wound healing and reparative dentin formation.

In this paper, we present a detailed method to perform pulp-capping in mice. This step-by-step procedure includes anesthetizing the mice, preparing the Class-I-like cavity, placing the pulp-capping materials, harvesting the maxillae, analyzing with the µCT scan, and assessing tissue samples for reparative dentin formation. Our pulp-capping mouse model will be instrumental in investigating the fundamental molecular

mechanisms of pulpal wound healing *in vivo* in the context of reparative dentin by enabling the use of transgenic or knockout mice, which are widely available in the research community.

Recent studies demonstrated several mouse models in which dentin formation was observed[18,19]. Saito *et al.* created a Class-I-like preparation without pulp exposure, which stimulates reactionary, not reparative, dentin formation. Both reactionary dentin and reparative dentin are classified as tertiary dentin, which forms following external stimulation to the tooth. However, unlike reactionary dentin, which is formed by existing odontoblasts, reparative dentin is formed by odontoblast-like cells, such as DPSCs, when the pulp becomes exposed and the odontoblastic layers are breached[20]. Therefore, it does not represent an actual pulp-capping procedure in the clinic. In another study, glass ionomer was used to cap the pulp exposure[19]. However, a clinical study showed that the glass ionomer induced chronic inflammation, but not reparative dentin[21]. In this regard, our pulp-capping mouse model better represents the actual pulp-capping procedure in patients.

It is noteworthy that when we harvested mice after more than 6 weeks, reparative dentin formation occurred throughout the pulp chamber and the root canals (**Figure 4**). Such an observation is rather unexpected, as we anticipated reparative dentin formation at the junction between the pulp-capping material and the pulp. However, potent mineralization of the pulp is also observed in clinical settings, especially in relatively young patients[22]. Because the 8-week-old mice used in this study are considered to be "young adults"[23], a possibility exists whereby these mice still harbor significant odontogenic potential. Therefore, it would be worthwhile to examine the ageing effects of reparative dentin formation in mice.

Our histological examinations revealed that, although reparative dentin was clearly formed in the pulp-capped tooth, there were characteristics of both dentin and bone formation, as evidenced by the presence of dentinal tubules (red arrow) and osteocytes (black arrowheads) in the reparative dentin (**Figure 5**). Such observations suggest that reparative dentin formation may be induced by locally residing odontoblast-like dental pulp stem cells, as well as infiltrating mesenchymal stem cells from the surrounding bone.

Compared to the dentin-forming cells, we found no dentin-resorbing cells within the pulp, as determined by tartrate-resistant acid phosphatase (TRAP) staining (data not shown). Indeed, pulpal or periapical inflammation induces osteoclast formation on the bone surfaces around the tooth, but not on the dentin surfaces due to as-yet-unknown mechanisms[24]. Of note, there was a clear demarcation between the existing dentin and the newly formed reparative dentin (**Figure 4**). A previous study demonstrated a similar phenomenon; when a tooth is extracted in the presence of bisphosphonate or anti-RANKL antibody, both of which inhibit the functions of osteoclasts, there were clear demarcations between existing lamellar bone and newly formed woven bone[25]. This notion further supports the absence of dentin-resorbing cells in the pulp. Collectively, our established mouse model would provide unique opportunities to examine the mechanisms of pulp wound healing and reparative dentin formation *in vivo*.

There is a limitation to the pulp-capping mouse model. Genetic makeups between humans and mice are clearly different. The complete genome has been sequenced in humans and mice, and there is about 85% similarity in the protein-coding regions between mice and humans[26,27]. In line with this notion, it was suggested that findings related to pulp capping in animals do not necessarily reflect those in humans[28]. Nonetheless, animal models are extensively used in the research community to recapitulate human diseases *in vivo*, such as collagen-induced arthritis for rheumatoid arthritis[29], ovariactomy-induced bone loss for osteoporosis[30], lipopolysaccharide (LPS) administration for systemic shock[31], and ligature placement for periodontitis[32]. As such, the pulp-capping mouse model will be essential to examine the molecular mechanisms of pulp wound healing and reparative dentin formation *in vivo.* Nonetheless, just like other animal models, interpretation and validation of the findings from the pulp-capping mouse model should be carefully evaluated.

In summary, the current study demonstrates successful pulp capping in mice. Unlike other known models, this pulp-capping mouse model will provide an invaluable research tool in the field of pulp regeneration and reparative dentin formation because it provides: 1) an opportunity to utilize widely available genetically engineered mouse strains to elucidate the underlying mechanisms at the molecular level and 2) an economically efficient way to obtain statistically significant results by increasing sample sizes. Further studies await, including objective quantification of reparative dentin formation *in vivo*, age-dependent effects of reparative dentin formation, evaluation of clinically available pulp-capping materials, and validation of molecular determinants that are required for proper pulpal wound healing and reparative dentin regeneration.

Disclosures

The authors have nothing to disclose.

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