A biocompatible model for evaluation of the responses of rat periapical tissue to a new zinc oxide-eugenol sealer

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We aimed to establish an experimental animal model to evaluate materials for endodontic therapy. We focused on the biocompatibility of new paste-type zinc oxide-eugenol (ZOE) sealer. The results of this sealer were compared with those of conventional powder/liquid ZOE and eugenol-free sealers. The molars of Wistar rats were extracted and repositioned in the original socket after application of the sealers on the root apices. Mild inflammation occurred in the periapical tissue of the replanted teeth with both ZOE sealers on day 7, whereas the eugenol-free sealer induced severe inflammation. On day 14, the lesions induced by all types of sealers were healed and replaced predominantly by fibrous connective tissue. Thus, all endodontic materials showed high biocompatibility, although the extent of inflammatory reactions during the early stages varied depending on the types of materials. We demonstrated that our animal model was useful for the assessment of the biocompatibility of endodontic materials.

Keywords: Biocompatibility, Endodontic sealer, Tooth replantation, Periapical tissue response

INTRODUCTION

Endodontics is the diagnosis and treatment of diseases of the pulp and their sequelae¹. If the pulp is necrotic, the endodontic therapies such as the extraction of pulp tissue, the preparation and cleaning of root canal, and the root canal filling are inevitable. Because the dental pulp and periodontal tissue are intimately related, clinicians have to pay their attention to the anatomic pathways of communication between these tissues. As the tooth matures and the root formation is completed, three main avenues for communication between the dental pulp and periodontal tissue are formed: (1) dentinal tubules, (2) lateral and accessory canals, and (3) the apical foramen¹. Thus, the obturating materials directly contact with the periodontal tissue via these pathways. Two indices are important for obturating materials: (i) biocompatibility; and (ii) the physical and chemical features of the materials selected². Numerous studies using various test methods have evaluated the biocompatibility of endodontic materials. Implantation of endodontic materials with/without polyethylene or silicone tubes or Teflon carriers into subcutaneous connective tissue simulates the conditions whereby stabilization of the materials occurs *in situ* in addition to interaction between the material and connective tissue (although the response of alveolar bone tissue is not observed in these experimental models)^{3–6}. Conversely, intraosseous implantation of endodontic materials with/without polyethylene, silicone or nylon tubes into the tibia^{7,8}, maxilla⁹, mandible^{10,11}, and alveolar bone⁹ provides a suitable model to evaluate the biocompatibility of materials. Infiltration of a few inflammatory cells even in the control samples during the early period may be due to surgical trauma. Test bodies composed of endodontic materials have been implanted in rats^{3–7,11,12}, rabbits^{8,10} and guinea pigs⁹. The experimental tissue

response to root canal-filling materials in dogs has suitably mimicked the clinical tissue response^{13–15}. The use of large animals such as dogs is necessary to obtain information on the response of periapical tissue from the viewpoint of tissue plasticity after endodontic treatment. However, bacterial contamination of the pulp space from the oral cavity is difficult to prevent if the crown is destroyed. Until now, there have been no ideal animal models to evaluate endodontic materials.

Root-canal sealers based on zinc oxide-eugenol (ZOE) are widely used. To date, many new root-canal sealers have been introduced, but ZOE sealers remain the “gold standard” in this category of materials¹⁶. The tissue reaction to these materials is well known, which explains why ZOE-based materials are often used instead of the new materials that have been tested^{3–5,7,8,11,13–15}. Recently, a new paste-type ZOE sealer has become commercially available as a sealer for endodontic applications, extending working time and resulting in easy handling owing to the characteristics of the paste type. However, data on the biological responses to this new sealer are not available. Thus, we aimed to establish the experimental animal model to evaluate the materials for endodontic therapy and assess the biocompatibility of the new paste-type ZOE sealers using the established animal model for tooth replantation.

MATERIALS AND METHODS

All animal procedures conformed to the protocols reviewed and approved by the Ethics in Animal Research Committee of the Kanagawa Dental College (Yokosuka, Japan). These procedures complied with the International Guiding Principles for Biomedical Research Involving Animals (Geneva, 1985).

Eighty female Wistar rats (age, 4 weeks; ~90 g) were used. Sixty rats were used for the experimental group of

the ZOE and eugenol-free sealers, and 20 rats were used as the control group. Under anesthesia (chloral hydrate 350 mg/kg, i.p.), the upper right first molar (M1) was extracted with a pair of Howe's pliers (YDM Company Limited, Tokyo, Japan) or with forceps with hooks. Bleeding from the M1 alveolar socket was inhibited with a sterilized paper point (Pierce Company Limited, Tokyo, Japan). We used two types of ZOE sealers, Canal Sealer™(CS; paste type, Nihon Shika Yakuhin Company Limited, Tokyo, Japan) and Canals™(powder/liquid

type, Showa Yakuhin Kako Company Limited, Tokyo, Japan), and one eugenol-free sealer, Canals N™(Showa Yakuhin Kako), according to the manufacturer's instructions. The composition of the three root-canal sealers is demonstrated in Table 1.

Ten microliters of these mixed materials were applied to each of the five root apices of M1 (Figure 1) using a 27-G needle (Terumo Company Limited, Tokyo, Japan) attached to a 1-mL plastic syringe. The extracted tooth with materials was immediately repositioned in

Table 1 Composition of the materials and their manufactures

Materials	Components	
	Base or powder	Catalyst or liquid
Canal Sealer™	Olive oil	Eugenol
	ZnO	Rosine
	Bismuth subcarbonate	Estergum
Canals™	ZnO	Eugenol
	Rosine	Penuts oil
	Bismuth subcarbonate	
	Barium sulfate	
Canals N™	ZnO	Linolic acid
	Rosine	Isostearic acid
	Bismuth subcarbonate	Propylene glycol
	Barium sulfate

Fig. 1 The animal model for tooth replantation to evaluate the endodontic sealers. (a) The alveolar socket of the upper right first molar (M1). (b) M1 possesses five roots. (c) Ten microliters of the mixed material are applied to each of the five root apices of M1. (d) Extracted M1 is repositioned in the original socket after hemostasis has been confirmed.

the original socket after the confirmation of hemostasis, and was classified as the “experimental group”. The replanted tooth did not undergo further treatments according to the method of a previous report¹⁶⁾. Samples were collected from groups of 10 animals at the intervals of 7 days and 14 days after tooth replantation. At each stage, rats were anesthetized and perfused via the transcardial route with physiological (0.9%) saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Maxillae were removed *en block* and immersed in the same fixative for an additional 12 h. After decalcification in a 4% solution of ethylenediamine tetra-acetic acid disodium salt for 4 weeks at 4°C, samples were embedded in paraffin. Specimens for histochemical analyses were cut in the sagittal plane at a thickness of 5 µm, and the sections stained with hematoxylin and eosin (H&E). The histomorphological parameters used in the present study were based on previously described criteria¹⁶⁾. For the determination of scores, sections were evaluated according to four parameters. First, for inflammatory reactions: 0, none or few inflammatory cells and no reaction; 1, <25 inflammatory cells and a mild reaction; 2, 25–125 inflammatory cells and moderate reaction; 3, >125 inflammatory cells and a severe reaction. Quantitative evaluations were done in the micrographs taken from ten areas of sections at ×400 magnification. Second, for cementum resorption: absent or present. Third, for dentin resorption: absent or present. Fourth, for thickness of the periodontal ligament at the apical region: normal, slightly increased, moderately increased, or severely increased. The observer was blinded to

treatment allocation.

Statistical analyses

Statistical analyses of the histopathological parameters described above were carried out using a non-parametric Mann-Whitney U test at the level of 5% significant difference.

RESULTS

Histopathologic findings were demonstrated in Table 2. The control group showed a slight inflammatory response (mild was 20%), no resorption of dentin, little resorption of cementum (10%), and a moderate increase in the periodontal ligament space on day 7, and subsequently no inflammatory response, little resorption of dentin (20%), little resorption of cementum (20%), and slight increase (slightly was 20%; normal was 80%) in the periodontal space on day 14. The CS group showed a moderate inflammatory response (mild was 20%; moderate was 60%; severe was 20%), no resorption of dentin, little resorption of cementum (20%), and a severe increase in the periodontal ligament space on day 7, and subsequently no inflammatory response, little resorption of dentin (20%), little resorption of cementum (20%), and slight increase (slightly was 20%; normal was 80%) in the periodontal space on day 14. The Canals group showed a moderate inflammatory response (moderate was 80%; severe was 20%), little resorption of dentin (20%), little resorption of cementum (20%), and a severe increase in the periodontal ligament space on day 7, and subsequently little inflammatory response (10%), slight

Table 2 Histopathologic findings in each study group

Finding	Day 7				Day 14			
	Control	CS	Canals	Canals N	Control	CS	Canals	Canals N
Inflammatory response in periapical tissue (n=10)								
Absent (n)	8	*0	*0	*0	10	10	9	9
Mild (n)	2	2	0	0	0	0	1	1
Moderate (n)	0	*6	*8	0	0	0	0	0
Severe (n)	0	2	2	*10	0	0	0	0
Dentin resorption (n=10)								
Absent (n)	10	10	8	8	8	8	7	8
Present (n)	0	0	2	2	2	2	3	2
Cementum resorption (n=10)								
Absent (n)	9	8	8	8	8	8	8	8
Present (n)	1	2	2	2	2	2	2	2
Apical periodontal ligament space (n=10)								
(apical to bone surface) Normal (n)	0	0	0	0	8	8	*0	*0
Slightly increased (n)	0	0	0	0	2	2	*8	0
Moderately increased (n)	10	*0	*0	*0	0	0	2	*9
Severely increased (n)	0	*10	*10	*10	0	0	0	1

*Statistically significant difference ($p<0.05$) compared with the control group in Day 7 or Day 14.

Fig. 2 Histological images. (a) CS on day 7. Mild infiltration of PMNs, macrophages and lymphocytes ($\times 200$ magnification) (*); inflammatory grade, moderate. (b) CS on day 14. Numerous fibrous tissues and the formation of new blood vessels ($\times 200$ magnification); inflammatory grade, absent. (c) Canals on day 7. Mild infiltration of PMNs, macrophages and lymphocytes ($\times 200$ magnification) (*); inflammatory grade, moderate. (d) Canals on day 14. Numerous fibrous tissues and the formation of new blood vessels ($\times 200$ magnification); inflammatory grade, absent. (e) Canals N on day 7. Severe infiltration of inflammatory cells (PMNs, macrophages and lymphocytes) surrounded by the dotted line. Note the severely enlarged periodontal ligament, many osteoclasts, and bone resorption; inflammatory grade, severe. (f) Canals N on day 14. Numerous fibrous tissues and the formation of new blood vessels ($\times 200$ magnification); inflammatory grade, absent. (g) Control on day 7. Slight infiltration of inflammatory cells (macrophages and lymphocytes) in fibrous tissue (*); inflammatory grade, moderate. (h) Control on day 14. Numerous fibrous tissue and the formation of new blood vessels ($\times 200$ magnification); inflammatory grade, absent.

resorption of dentin (30%), little resorption of cementum (20%), and slight increase (slightly was 80%; moderately was 20%) in the periodontal space on day 14. The Canals N group showed a severe inflammatory response, little resorption of dentin (20%), little resorption of cementum (20%), and a severe increase in the periodontal ligament space on day 7, and subsequently little inflammatory response (10%), little resorption of dentin (20%), little resorption of cementum (20%), and moderate increase (moderately was 90%; severely was 10%) in the periodontal space on day 14.

CS

On day 7, moderate infiltration of inflammatory cells (polymorphonuclear cells (PMNs), macrophages, lymphocytes) was observed in numerous fibrous tissues with new blood vessels (Figure 2a). By day 14, the inflammatory cells disappeared and the periapical tissue recovered, showing normal periodontal tissue (Figure 2b).

Canals

On day 7, moderate infiltration of inflammatory cells (PMNs, macrophages, lymphocytes) was observed in numerous fibrous tissues with new blood vessels (Figure 2c). By day 14, the inflammatory cells disappeared, and the periapical tissue showed normal periodontal tissue (Figure 2d).

Canals N

On day 7, severe infiltration of inflammatory cells (PMNs, macrophages, lymphocytes) was recognized in numerous fibrous tissues with new blood vessels. Periapical tissue showed a severely enlarged periodontal ligament; numerous osteoclasts and bone resorption were observed (Figure 2e). On day 14, the inflammatory cells were reduced in density, and numerous regularly arranged fibroblasts and collagen fibers appeared in the periapical tissue (Figure 2f).

Control

On day 7, an absent or mild infiltration of inflammatory cells (macrophages, lymphocytes) was observed in the fibrous tissue with many blood vessels. Periapical tissue contained a moderately enlarged periodontal ligament and bone resorption was observed (Figure 2g). On day 14, the periapical tissue showed normal periodontal tissue; mild bone resorption was observed (Figure 2h).

DISCUSSION

Tooth replantation is a therapeutic method in which a dropped or dislocated tooth is replaced in its original alveolar socket. Tooth replantation is utilized to treat the complete luxation of teeth, whether intentional or accidental. This procedure interrupts the nerve supply and vascular supply to the dental pulp, leading to the degeneration of pulpal cells¹⁷. In successful cases, pulpal regeneration, re-innervation, and re-vascularization have been shown to occur in humans¹⁸ and in

experimental animals^{19,20}. We aimed to establish a new animal model to evaluate the biocompatibility of endodontic materials by analyzing the periapical tissue response to root-canal sealers using tooth replantation. There are three main advantages in using this animal model. First, the moisture contamination of the apical area could be appropriately controlled. Second, periapical tissue responses could be quantitatively evaluated by histological observation of the periodontal tissue. Third, bacterial contamination of the pulp space could be prevented. The disadvantage of this method was that occasional tissue responses (particularly root resorption and ankylosis) after tooth replantation could be superimposed onto the tissue response to endodontic materials.

Intraosseous models provide an appropriate environment for *in-vivo* testing of endodontic material^{21–23}. However, unset materials such as slow-setting sealers implanted into a freshly prepared bone cavity are likely to be partially displaced by tissue fluids¹¹. With respect to this problem, polyethylene or silicone tubes have been used because they can maintain the unset materials inside their hollow structure. Furthermore, Friend and Browne²³ proposed that containment of a test material within polyethylene tubes allowed for diffusion of its constituents through a more defined area (similar to that of a filling material in the clinical treatment) rather than the larger surface area exposed by direct implantation of the material. Thus, this system could simulate the relationship between the root apices and the periapical tissue, and many researchers have preferred this system to evaluate endodontic materials. However, this system cannot exclude the tissue responses to the polyethylene or silicone tubes themselves. Therefore, evaluating the tissue reaction quantitatively by histological observation of the periodontal tissue using previous animal models is difficult. Therefore, the animal model for tooth replantation described herein could be an alternative system to directly evaluate periapical tissue responses to endodontic materials.

On day 7, the control group showed that the damaged area was filled by well-organized granulation tissue with mild infiltration of inflammatory cells, fibroblasts, a few macrophages, and numerous blood vessels. A moderately increased periodontal ligament space from the root apices to the bone surface was also observed in periapical tissue on day 7. Periapical tissue had almost healed, and the connective tissue was well organized without inflammatory cells by day 14. The mild concentration of inflammatory cells observed in the controls during the early period could have been due to the tissue response to tooth replantation. Thus, this experimental tissue response to endodontic materials using a rat model for tooth replantation suitably mimics the response of clinical tissue.

Several investigators suggested that the irritative features of endodontic materials could be attributed primarily to eugenol and secondarily to zinc ions⁹. Tissue culture studies have shown that ZOE is toxic to pulp

cells and may be more irritative to periodontal ligament cells²⁴. *In-vivo* studies have demonstrated that ZOE-based sealers such as Canals and Canals N do not always cause a dose-dependent increase in genotoxicity²⁵. In one study, the ZOE-based pulp canal sealer initially showed moderate cytotoxic potential, but fibroblast recovery was noticeable 13 days after application²⁶. ZOE-based sealers have good biocompatibility, and the tissue reactions observed at 12 weeks are better than those observed 4 weeks after implantation into the mandibles of rabbits¹⁰. Conversely, eugenol-free sealers (e.g., Canals N) have been developed after taking account of the reports mentioned above on the cytotoxicity or mutagenicity of eugenol-containing sealers. We investigated the effect of two types of ZOE sealers (Canals and CS) and a eugenol-free sealer (Canals N) on periapical tissue using our new animal model to evaluate the tissue response to these materials.

There are no data on the histopathological analyses of periapical tissue responses to endodontic materials except for the use of large animals such as dogs in which the periapical tissue responses to the materials could be assessed after preparation of the root canal. Our experimental animal model precisely showed the effect of root-canal sealers on periapical tissue. The ZOE sealer used in the present study was classified as a “slow-setting material”, so the unset material implanted into periapical tissue would probably be partially dispersed by tissue fluid until 7 days after application. Thus, our animal model simulated the periapical tissue response to the ZOE sealer which overflows from the root apex before root-canal obturation. Regardless of the presence of ZOE components, the inflammatory reaction was recognized during the early stages after the procedure because of the surgical trauma mentioned above. Good biocompatibility was shown in the periapical tissue reaction to the root-canal sealers which set by day 14. These findings support the favorable clinical results observed in ZOE and eugenol-free root-canal sealers. The experimental animal model that we used to evaluate the periapical tissue response to endodontic materials was not totally comparable with the response elicited by human tissue. Under carefully standardized conditions, this model could be used to simulate the inflammatory process induced by iatrogenic means during root-canal obturation in humans, and could therefore be an effective tool to study several aspects of the periapical tissue reaction.

CONCLUSIONS

Our animal model for tooth replantation provided findings of overflowed sealers in periapical tissue. These results showed that the tested ZOE sealers induced an inflammatory response of mild intensity in periapical tissue. The new paste-type ZOE sealer could possess a better tissue compatibility compared with the eugenol-free sealer.

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