Influence of the pulpal components on human dentine permeability *in vitro*

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Abstract

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Aim To examine the influence of the retained pulpal components on permeability of human dentine by monitoring drug diffusion.

Methodology Twelve intact dentine discs were prepared from freshly extracted human third molars. The dentine surface on the enamel side was etched with 10% polyacrylic acid for 30 s. The drug diffusion test was carried out before and after removal of the retained pulpal components. Each dentine disc was inserted between two plastic chambers; enamel- and pulpal-side chambers, which were filled with 0.05 mol L^{-1} naproxen sodium (NA) and phosphate-buffered saline (PBS), respectively. After 10 min, the solution on the pulpal-side chamber was collected to determine the concentration of NA using a spectrophotometer. To remove the retained pulpal components and residual NA, the pulp chamber of each disc was washed out with PBS and placed in an ultrasonic cleaner. After removal of these components and the residual NA, the drug diffusion test was repeated. The inner surface of the pulp chamber was observed using scanning electron microscopy (SEM) before and after the removal of the retained pulpal components.

Results The amount of NA that diffused through dentine into the pulp was significantly higher after the pulp chamber was washed out with PBS (paired *t*-test, $P < 0.05$). SEM observation demonstrated the presence of the retained pulpal components, odontoblastic layer and some parts of subodontoblastic zone, covering the surface of predentine. These components were removed after the pulp chamber was washed out with PBS followed by cleaning in an ultrasonic cleaner.

Conclusions The presence of retained pulpal components had a significant influence on drug diffusion through dentine discs.

Keywords: dentine permeability, naproxen sodium, odontoblastic layer, pulpal components.

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Introduction

Odontoblasts line the outermost surface of the pulp and attach to each other by intercellular junctions (Bishop & Yoshida 1992). Each odontoblast has a process extending into a dentinal tubule, which is the major channel for fluid diffusion across dentine. Therefore, the diffusion of substances applied to dentine occurs through the channels and is influenced by the permeability of dentine (Pashley 1990, Ozawa *et al.* 2002).

Several factors, such as pulp tissue, surface debris and intratubular occlusion, influencing the permeability of dentine, have been examined (Pashley *et al.* 1978). Odontoblastic processes have been considered as a contributing factor to intratubular occlusion to reduce dentine permeability (Pashley *et al.* 1978). The influence of the odontoblastic layer on dentine permeability of rat teeth has demonstrated that perturbation of the odontoblastic layer causes an increase in

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dentine permeability (Turner *et al.* 1989, Izumi *et al.* 2001, Byers & Lin 2003). The damage to odontoblasts by cavity preparation or scrape injury to dentine causes an immediate movement of horseradish peroxidase (HRP) into predentine and dentine (Turner *et al.* 1989, Izumi *et al.* 2001), and an increase in pulpward and outward diffusion of fluoro-gold through dentine in rat molars (Byers & Lin 2003). In addition, odontoblastic processes and outward fluid movement in the dentinal tubules of vital teeth appear to resist bacterial invasion into the human pulp (Nagaoka *et al.* 1995). However, little is known as to what extent the odontoblastic layer would influence drug diffusion through human dentine.

Interestingly, Chadha & Bishop (1996) have demonstrated that the pulpal components, odontoblastic layer and also some parts of the cell-free and the cell-rich zones, are retained around the pulp chamber of human third molars after mechanical separation of the pulp. A simple *in vitro* model utilizing the retained pulpal components might be useful to test their influence on dentine permeability in human teeth.

Naproxen has been reported as a classic nonsteroidal anti-inflammatory drug (NSAID) with established analgesic and anti-inflammatory potencies (McCarthy 1999). Its action is related to cyclooxygenase inhibition and consequent decrease in prostaglandin concentration in various tissues (Papa *et al.* 1999, Vardar *et al.* 2003). As prostaglandin release is involved in pulp inflammation (Okiji *et al.* 1989), topical application of naproxen on dentine might be useful for relieving pulpal inflammation and avoid gastrointestinal side effect. Additionally, naproxen solution has been reported to be tolerable and safe for using in soft tissue (Papa *et al.* 1999). If naproxen could be delivered into the pulp, it may be possible to relieve the inflammatory pulp tissue. Thus, the present *in vitro* study was performed to examine the influence of the retained pulpal components on permeability of human dentine by monitoring drug diffusion.

Materials and methods

Dentine disc preparation

Twelve intact third molars that had been extracted for various clinical reasons were used in the experiment. Donor patients (aged 22.33 ± 3.06 years) gave informed consent for their extracted teeth to be used in the present study. Dentine discs were prepared by slicing the teeth 3 mm higher and 0.5 mm lower than cemento-enamel junction (CEJ) (Fig. 1a) using a diamond blade (Isomet®; Buehler Ltd., Lake Bluff, IL, USA) under copious phosphate-buffered saline (PBS) solution irrigation within 1 h after extraction. The enamel-side of dentine disc was etched with 10% polyacrylic acid (Dentine conditioner; GC Co., Tokyo, Japan) for 30 s to remove smear layer and then rinsed with 15 mL of PBS to remove the acid left on the dentine surface. Pulp tissue was pulled out through the pulp chamber with fine tweezers (Ikeda & Matthews 1994).

Drug concentration measurement

Naproxen sodium (NA) (Sigma-Aldrich, St Louis, MO, USA) solution was prepared into a series of concentrations with PBS (pH 7.4) and measured spectrophotometrically at 231 nm (V-550; Jasco, Tokyo, Japan). The measured absorbances and known concentrations were plotted as a standard curve (Fig. 2). The absorbance of each sample, an unknown concentration of NA, was then measured and the concentration was determined from the curve. If the measured absorbance of sample was higher than that presented in the standard curve, sample was diluted with PBS and re-examined.

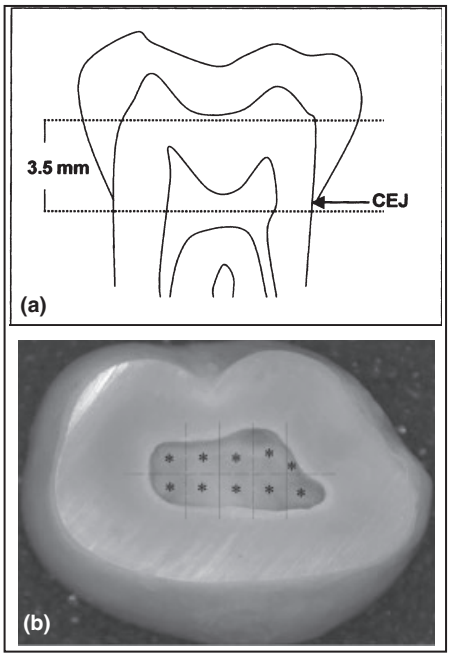

Figure 1 Diagrams showing dentine disc preparation (a) and 10 points for RDT measurement (b).

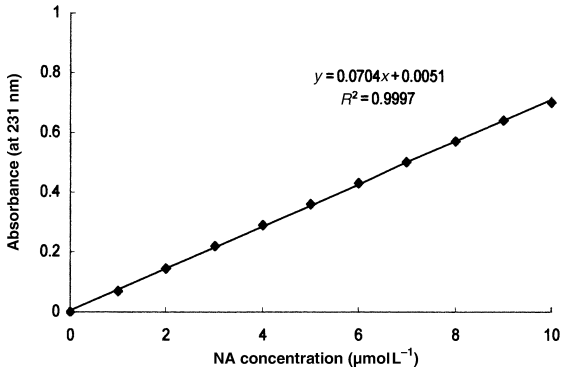

Figure 2 Standard curve used for calculation of NA concentration.

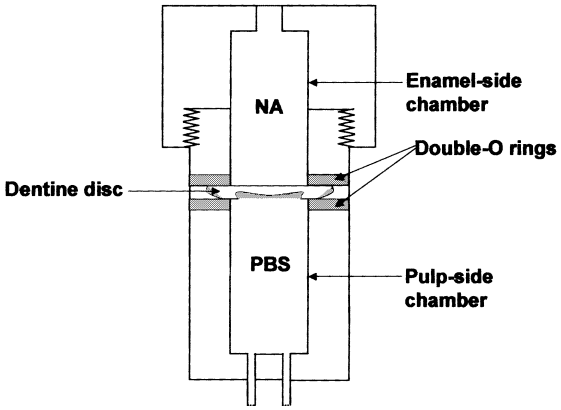

Figure 3 Diagram of the experimental setup for NA diffusion test.

Drug diffusion test

Each dentine disc was inserted between two plastic chambers (Outhwaite *et al.* 1974) and sealed with double O-rings (6 mm in diameter). The enamel-side chamber was filled with 1 mL of 0.05 mol L^{-1} NA, whereas the pulp-side chamber was filled with sterile PBS (Fig. 3). The pulp-side solution was collected 10 min after NA application. The concentration of the collected solution was determined using the spectrophotometer. The pulp chamber of the dentine disc was washed out with 10 mL of PBS to remove the remaining cells. The dentine disc was immersed in PBS for 15 min, and put in an ultrasonic cleaner for another 5 min three times to remove the residual drug left in dentinal tubules. Then, the disc was inserted in the plastic chambers filled with PBS for 10 min. The spectrophotometer confirmed that PBS in both chambers contained no NA. Then, the second NA diffusion test was performed.

Dentine thickness was measured using a micrometer with a tip fine enough to be inserted into the pulp horn. The mean remaining dentine thickness (RDT) measured from the inner surface of the pulp chamber to the flat occlusal surface at 10 points as shown in Fig. 1(b) was 2.48 ± 0.36 mm and that from the pulp horn to the flat occlusal surface was 1.58 ± 0.47 mm.

Scanning electron microscopic observations

The observations of the inner surface of the pulp chamber were made before removal of the retained pulpal components ($n = 3$), after removal with PBS ($n = 3$) and after ultrasonic cleaning ($n = 3$) by a scanning electron microscope (SEM; S-4500, Hitachi, Tokyo, Japan). Dentine discs were fixed in 2.5% glutaraldehyde in phosphate-buffered solution (pH 7.3) at 4 °C for 3 days. After dehydration through ascending concentrations of alcohol (30, 50, 70 and 90% for 5 min and 100% for 10 min), the specimens were dried by the carbon dioxide critical-point technique (HCP-2; Hitachi, Tokyo, Japan) and coated with platinum using an ion coater (Eiko Engineering, IB-5, Ibaraki, Japan).

Data analyses

The amount of NA diffused through dentine before and after the removal of retained pulpal components was compared by paired *t*-test. A probability value of $P < 0.05$ was considered as significant.

Results

NA diffusion test

The diffusion of NA through dentine before and after the removal of retained pulpal components is shown in Fig. 4. The amount of NA that diffused through dentine into the pulp after removing the retained pulpal components was significantly higher than that before removing them (paired *t*-test, $P < 0.05$).

SEM observations

Prior to removing the retained pulpal components, cells were seen occupying the entire surface of the pulp chamber (Fig. 5a). Missing cells were found in some areas on the roof of the pulp chamber (Fig. 5a). In some

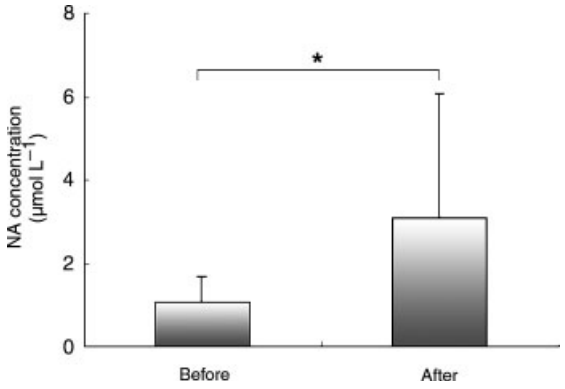

Figure 4 Graph showing the amount of NA concentration diffused through dentine before and after removal of the retained pulpal components. Asterisk denotes significant difference (paired *t*-test, $P < 0.05$).

specimens, dense collagenous structure covering the cells remained (Fig. 5b). Anatomically, this collagenous structure appeared to be that in the subodontoblastic zone lining below the odontoblastic layer. Cells lining predentine were characteristically oval in shape and had an extending process towards dentinal tubules (Fig. 5c), which were morphologically resembled odontoblasts. The processes appeared shrunk and there were spaces between the cell bodies.

After rinsing the pulp chamber with PBS, the inner surface was cleaner. However, in some areas, a few cells and tissue structures resembled odontoblastic processes retained in the dentinal tubules were left behind (image not shown). After ultrasonic cleaning of the dentine discs, the inner surface was completely clean (Fig. 6a,b).

Discussion

To examine the influence of the retained pulpal components, odontoblastic layer and some parts of

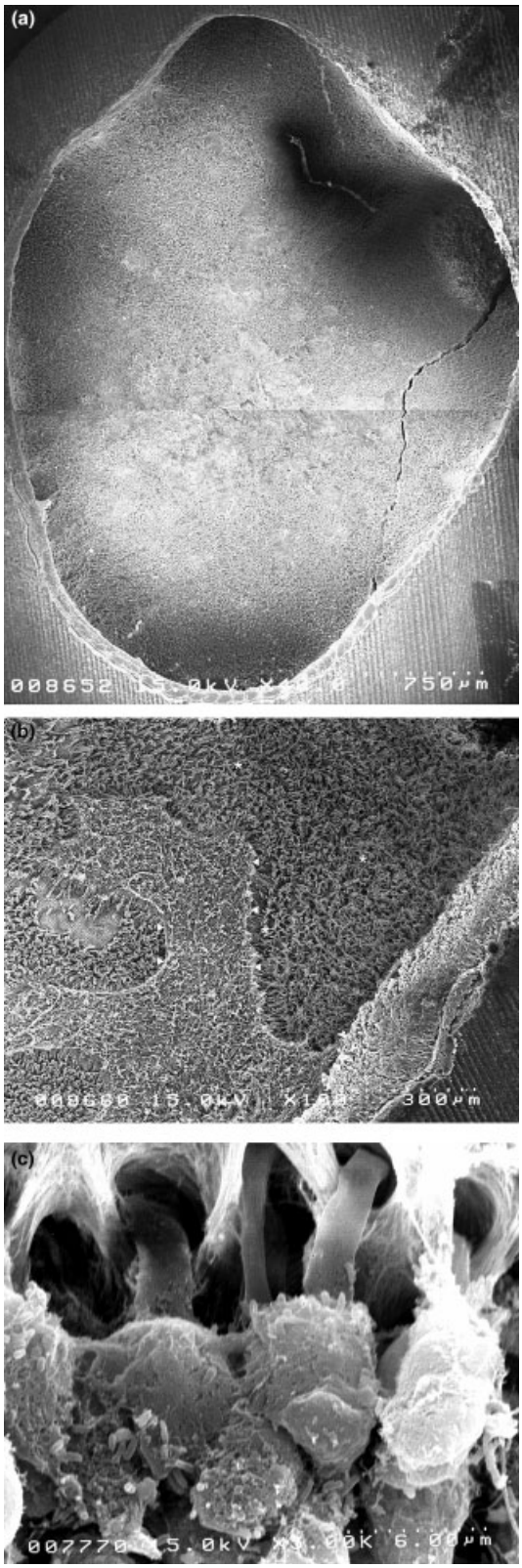

Figure 5 Representative SEM micrographs showing the inner surface of the pulp chamber of human third molars, before removal of the retained pulpal components (Bar = 11 dots on the figure). (a) Overall view of inner surface of pulp chamber showing cells occupied the entire inner surface of the pulp chamber and missing cells on some areas on the roof of pulp chamber. Bar: 750 μm, ×40. (b) Remaining dense collagenous structure (arrowheads) cover on cells (asterisks) lining vertically to predentine. Bar: 300 μm, ×100. (c)Cells having oval bodies and extending processes towards dentinal tubules. Bar: 6 μm, ×5000.

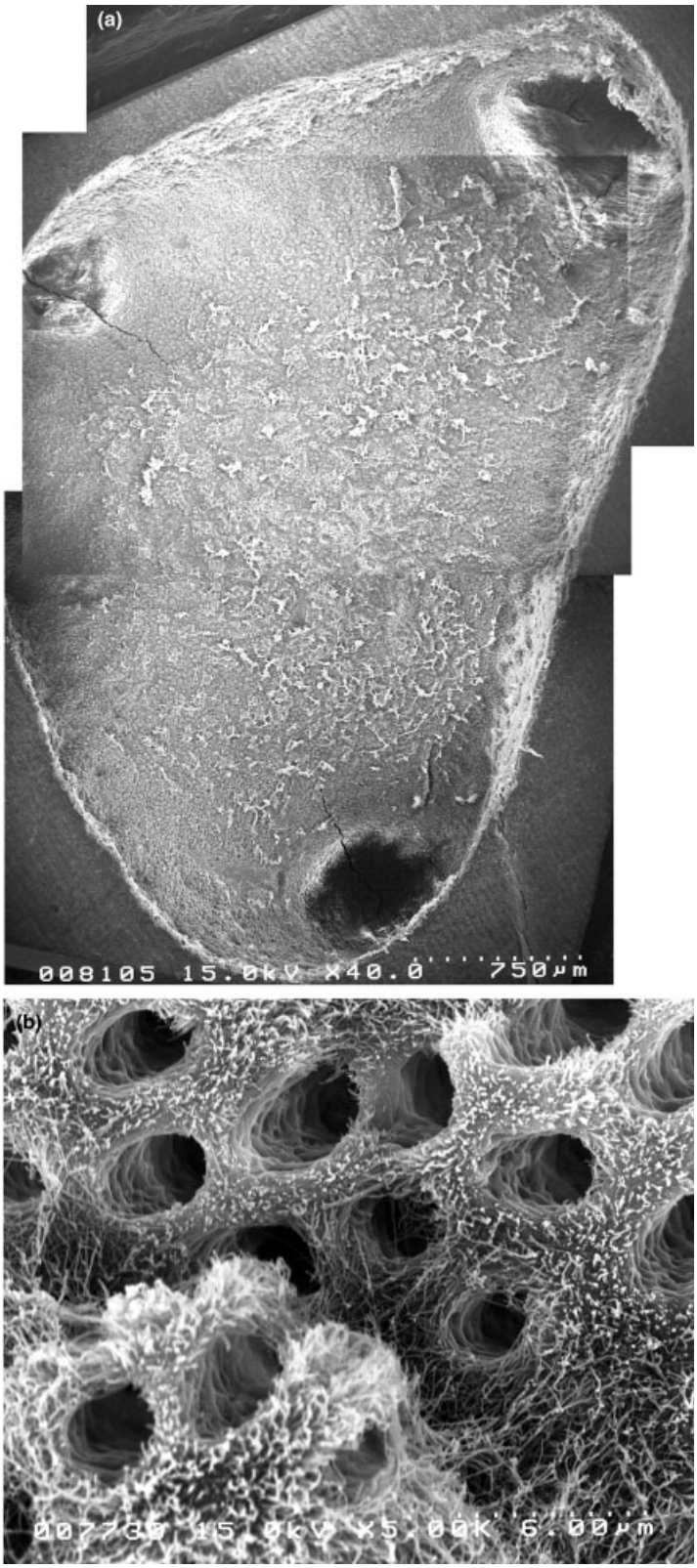

Figure 6 Representative SEM micrographs showing the inner surface of the pulp chamber of human third molars, after ultrasonic cleaning. (a) Overall view of inner surface of pulp chamber showing no cells. Bar: 750 µm, ×40. (b) Magnified view of (a) showing unmineralized collagenous structure of predentine and empty dentinal tubules. Bar: 6 µm, ×5000.

subodontoblastic zone, on dentine permeability in human teeth, a simple *in vitro* model was used that is capable of measuring drug diffusion. NA diffusions through dentine were compared before and after removal of these components by washing out the pulp chamber with PBS followed by cleaning in an ultrasonic cleaner. NA diffusion significantly increased after the pulp chamber was washed out. This result indicates that the presence of the pulpal components that line closely to predentine may resist NA diffusion through dentine. It is speculated that the odontoblastic processes, which occupy considerable space in the dentinal tubules and are assumed to serve as an effective physical barrier against bacterial invasion into the vital tooth (Nagaoka *et al.* 1995), and collagen within the dentinal tubules contribute to reducing dentine permeability. The disappearance of these tubular contents after removing cells might be reasons for the increase in the diffusion of NA.

The present findings suggest that the retained pulpal components significantly influence the permeability of dentine. However, the pathways through which drug diffuses in the odontoblastic layer could not be identified. It was difficult to evaluate the conditions of intercellular spaces and cell-to-cell junctions during drug diffusion test. Drugs might penetrate through the inter-odontoblastic space (Torres-Quintana *et al.* 1999) and empty dentinal tubules. Thus, the present study could not evaluate the permeability of the odontoblastic layer, a topic that has been extensively examined in the extracellular movement of tracers between the pulp and dentine in animal studies. Researchers have reported the discrepant results in the existence of a physiological barrier comprised of odontoblastic layer (Turner *et al.* 1989, Bishop & Yoshida 1992, Torres-Quintana *et al.* 1999, Izumi *et al.* 2001). Further study is necessary to examine the permeability of human odontoblastic layer. Additionally, it is of interest to determine the influence of this layer on fluid movement across dentine. However, a technique that is more effective in maintaining the integrity of the odontoblastic layer is required. Transmission electron microscopic technique may also be useful to confirm the conditions of cells and their connections instead of SEM. Supposedly, the present model may provide smaller drug diffusion resistance than in intact teeth that contain many kinds of cells in good condition, supporting pulp tissue, and an outward dentinal fluid flow (Vongsavan *et al.* 2000). All these factors might provide greater pulp protection from external stimuli.

In the present study, the retention of cells was observed under SEM. Almost all cells appeared to attach to superficial pulpal predentine before the pulp chamber was washed out. These cells resembled closely the morphology of odontoblasts; however, it was not possible to differentiate these cells from other pulpal cells under SEM. There are several possible factors that affect the retention of odontoblasts to predentine, such as the odontoblastic process extending into the dentinal tubule, fibronectin attaching cell to extracellular matrices (Yoshiba *et al.* 1995), von Korff fibres extending from the pulp into the mineralized dentine (Bishop *et al.* 1991), and the cell-to-cell junctions between odontoblastic cell bodies (Holland 1987, Ikeda & Suda 2002). Disruption and loss of cells were found in some areas on the roof of pulp chamber. Cell disruption might have occurred during pulp tissue separation or preparation of the specimens for SEM, by breaking the intercellular connections and enlarging the intercellular spaces (Jean *et al.* 1986). Cell loss might have been caused by uncontrolled pressure application to dentine by the tips of tweezers, which has been reported previously (Chadha & Bishop 1996).

The technique used in the present study for removing cells from the pulp chamber has been applied to isolate a large number of odontoblast-like cells from cat and human teeth (Ikeda & Matthews 1994, Ikeda & Suda 2002). Using this technique, the present SEM images showed that almost all cells came off the dentine surface, but some odontoblast- and process-like structures were retained in the dentinal tubules. However, after rinsing the dentine disc in an ultrasonic cleaner, the inner surface was completely clean. A possible explanation for the detachment of cells was that the water pressure and ultrasonic force were strong enough to disrupt the intercellular junction or cell-to-extracellular matrix adhesion. In addition, the pulling force applied to separate the pulp tissue might have helped the odontoblasts to detach easily on washing out or sonication.

NA may be suitable as a tracer because of its small molecular size (8.26 Å) (Sugano *et al.* 2002) compared with HRP (40–50 Å) (Chen *et al.* 1997), which can pass through the odontoblastic layer to predentine and adjacent dentine in rat teeth (Izumi *et al.* 2001). In addition, it has an anti-inflammatory effect (Papa *et al.* 1999), which may be useful for reducing pulpal inflammation when used as a topical agent. Additionally, we found that NA application to the dentine disc did not significantly influence the dentine permeability even after washing the disc in an ultrasonic cleaner (Puapichartdumrong *et al.* 2003).

As the presence of the retained pulpal components appeared to resist the diffusion of small molecular drug across dentine, dentine permeability would significantly increase without these components. The present study provides a simple *ex vivo* method to examine the influence of the retained pulpal components on drug diffusion through human dentine. The present method may be useful for experiments using other tracers. Additionally, when freshly extracted teeth are used, researchers should be aware of the influence of these components on dentine permeability.

Conclusion

Presence of retained pulpal components significantly influences drug diffusion (NA) through dentine. However, the further studies are needed to elucidate the efficacy and toxicity of NA for relieving the inflammation of pulp tissues.

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