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ORIGINAL ARTICLE

Effects of two disinfection/sterilization methods for dentin specimens on dentin permeability

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Abstract

Objectives To investigate the effects of two disinfection/sterilization methods on the permeability of dentin specimens.

Materials and methods Forty intact human third molars were freshly extracted and cut, close to the pulp chamber, into dentin disks with a 500-μm thickness. The disks were randomized ($n = 20$ each) into a 70% ethanol group (acid-etched dentin disks soaked in 70% ethanol for 15 min) and a steam autoclaving group (acid-etched dentin disks autoclaved for 25 min). The permeability (Lp) of each dentin disk was measured before and after either treatment using a hydraulic device, and intra- and inter-group differences in values before and after treatment were analyzed using *t* tests. Field emission scanning electron microscopy (FE-SEM) micrographs of the dentin surface were acquired and examined. FE-SEM samples were prepared using the critical point drying (CPD) method.

Results Immersion in 70% ethanol increased the Lp values of dentin specimens by 17%, which was not statistically significant. Steam autoclaving significantly reduced dentin permeability by 66% because the dentin collagen mesh became compact and collapsed, as detected by FE-SEM.

Conclusions The disinfection of acid-etched dentin disks using 70% ethanol for 15 min does not significantly affect dentin permeability, whereas sterilization of acid-etched dentin disks via autoclaving significantly reduces dentin permeability.

Clinical relevance Considering the influences of dentin permeability by disinfection/sterilization methods, the disinfection of the acid-etched dentin disks using 70% ethanol for 15 min could be used for the study related to dentin permeability, while the sterilization of autoclaving could not.

Keywords Disinfection · Sterilization · Dentin permeability · Dentin collagen · Dentinal tubules · Critical point drying

Introduction

Permeability is an important property of dentin, which has a tubular structure. Dentin permeability is generally measured during evaluations of the sealing ability of dental restorative materials, such as adhesives or resins [1, 2]; effectiveness of desensitizing toothpaste or materials [3–5]; uptake of substances in the dentinal tubules [6]; cytotoxicity of dental restorative materials in a dentin-barrier model [7, 8]; and effects of various clinical procedures. In such in vitro investigations, the measured dentin permeability should be as close to in vivo values as possible in order to yield clinically significant results. For dentin permeability tests, teeth are generally prepared as disks or crown segments for dentin exposure [9, 10]. Once the dentinal tubule orifices are exposed, any treatment could affect the permeability values.

Dentin disinfection/sterilization should be the first procedure performed before any other preparation or treatment for safety and health purposes and to fulfill the aseptic requirements of cell culture tests using dentin specimens [7, 11, 12]. Several methods for storing and disinfecting/sterilizing extracted teeth or dentin specimens have been developed, such as steam autoclaving [11, 13–15]; immersion in ethanol [7, 16], formalin [16, 17], thymol [16, 17], sodium azide [18], or chloramine T [19]; and gamma radiation [20, 21]. Goodis et al. reported that the type of storage solution or disinfection/

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sterilization method can affect dentin permeability [16]. Among these diverse methods, steam autoclaving and ethanol immersion are commonly used, effective, and rapid disinfection/sterilization methods for dentin, and both are recommended by the International Organization for Standardization (ISO) [22]. Steam autoclaving is a high-efficacy sterilization method for extracted teeth that is widely used in preclinical and in vitro studies [11, 13–15]. In cytotoxicity studies conducted using dentin as a barrier, many researchers chose autoclaving for the sterilization of dentin disks [14, 15, 23, 24]. Pashley et al. showed that dentin permeability was not affected after sound teeth were autoclaved [13]. However, our previous study provided evidence that dentin permeability may be decreased when acid-etched dentin disks, not sound teeth, are autoclaved [7]. Immersion in 70% ethanol was previously found to affect dentin permeability during long-term storage [16]. However, when used as a disinfection procedure before testing (rather than as a storage medium), the immersion time is short. A 15-min immersion in 70% ethanol is recommended by ISO [22] and was implemented in our previous study involving the use of dentin [7]. To our knowledge, no study has investigated the effects of autoclaving sectioned dentin disks or crown segments on dentin permeability. In addition, the effects of immersion in 70% ethanol for a short period of time remain unclear. Because both methods are widely used in various in vitro dental studies, their effects on dentin permeability are an important consideration.

Therefore, the aims of this study were to investigate the effects of 70% ethanol immersion and steam autoclaving on the permeability of dentin disks and to determine whether both short-term disinfection/sterilization methods are suitable for use in tests involving dentin permeability.

Materials and methods

This study was approved by the Institutional Review Board of the Peking University School and Hospital of Stomatology. The requirement for informed consent was waived by the ethics committee.

Collection of teeth

Forty intact human third molars were extracted from adult patients aged 18–40 years for orthodontic reasons. Following removal of the debris and soft tissues, the teeth were stored in 0.5% chloramine T solution (as recommended by ISO [22]) in deionized water at 4 °C and used within 1 month.

Dentin disk preparation

Dentin disks were prepared by cutting the extracted teeth perpendicular to the long axis, close to the pulp horns, using a low-speed saw (Isomet-Buehler, Lake Bluff, IL, USA). The first cut at the cementoenamel junction was used to remove the root and pulp tissue. Subsequently, cuts at the same angle were used to remove the entire pulp cavity until the highest pulp horn was removed. Then, a 0.5-mm dentin disk was cut. Each crown provided only one disk.

All 40 dentin disks were etched on both sides with 35% phosphoric acid for 30 s and rinsed with deionized water. Then, they were cleaned in an ultrasonic cleanser (Kudos, Shanghai, China) at 53 kHz for 5 min.

Experimental design

The dentin disks were randomly divided into two groups of 20 specimens each according to the disinfection/sterilization method used: a 70% ethanol group, where the etched dentin disks were soaked in 70% ethanol for 15 min and thoroughly rinsed in deionized water, and a steam autoclaving group, where the etched dentin disks were autoclaved at 121 °C and 9.6 MPa for 25 min in 0.9% sodium chloride, cooled to room temperature, and thoroughly rinsed in deionized water.

The dentin hydraulic conductance was measured in both groups before and after the disinfection/sterilization treatment; the permeability values before the treatments was determined as the maximum baseline value.

Measurement of dentin permeability

The equipment for measuring hydraulic conductance was prepared in-house according to the model described by Outhwaite and Pashley [25, 26] (Fig. 1). The water bath was injected with deionized water, which provided a pressure of 32 cm H_2O (3.14 kPa) [9] to the dentin disk. The disk was placed in the middle of the chamber and held in place by the

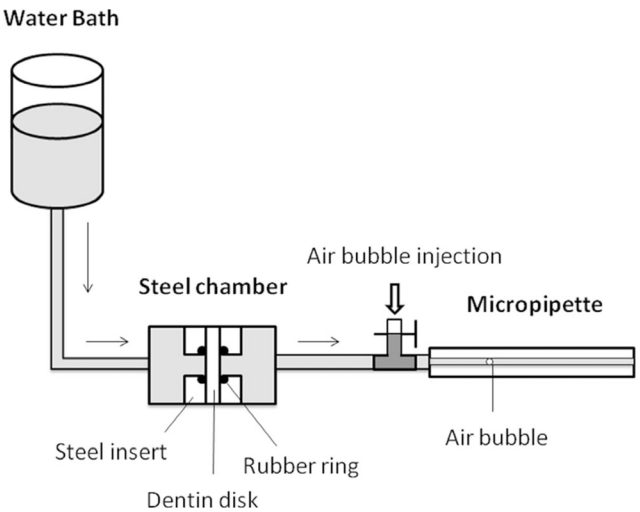

Fig. 1 Schematic diagram of the device (hydraulic conductance device) that was used for evaluating dentin permeability in the present study

steel inserts, with pressure applied from the pulp side to the occlusal side. The measurement area was defined as 0.283 cm^2 at the center of the disk and sealed by a pair of rubber "O" rings with an inner diameter of 6 mm. The chamber filled with deionized water was sealed after the dentin disk was fixed. Then, all parts ware connected via silicon tubes, and the entire system was filled with deionized water. A small air bubble was introduced into the micropipette using a microsyringe from the joint. After the air bubble remained stable for 5 min, the experiment was performed at room temperature. Before every measurement, a glass disk with a size similar to that of the dentin disks was tested to ensure a good seal. The measurement range of the micropipette was 100 μl, and the minimum division value was 1 μl.

The volume of deionized water filtering through the dentin disk was measured by recording the linear displacement of the air bubble for a consistent time period. The hydraulic conductance of the dentin was calculated using the following equation:

$$\mathrm{Lp} = \mathrm{Jv}/(A \times t \times P),$$

where Lp is the hydraulic conductance of dentin ($\mu l \cdot min^{-1} \cdot cm^{-2} \cdot cm\ H_2O^{-1}$), Jv is the volume of water filtering through the dentin disks during the observation time (μl), A is the measurement area (cm^2), t is the observation time (min), and P is the pressure applied to the dentin disks (cmH_2O).

Statistical analysis

The results are expressed as means and standard deviations of the Lp values for the specimens in the two groups. The relative Lp values after disinfection/sterilization treatment were calculated and are reported as percentages of the maximum baseline-permeability values. Changes in Lp values before and after treatment were compared within groups using a paired-samples t test. The differences in Lp values between the two groups were compared using an independent-samples t test. All statistical analyses were performed using SPSS software, version 20.0 (SPSS, Chicago, IL, USA). The statistical significance was preset at $\alpha = 0.05$.

Field emission scanning electron microscopy

A field emission scanning electron microscope (FES-SEM, HITACHI, SU8010, Japan) was used to observe the surface of the dentin disks before and after disinfection/sterilization. The dentin disks were fixed in 2.5% glutaraldehyde solution for 2 h at room temperature. Then, the disks were dehydrated in a graded series of ethanol (20, 40, 60, 80, and 100%) and dried in a critical point dryer (Leica EM CPD300, Germany). The dried specimens were sputter-coated (HITACHI, E-1045, Japan) with platinum and examined using FE-SEM at 5 kV.

Results

Dentin permeability measurements

Table 1 summarizes the mean dentin permeability values (± SD) obtained before and after the two different disinfection/sterilization treatments and the percent changes in dentin permeability after treatment.

Paired t tests revealed a significant difference in Lp values before and after steam autoclaving ($P < .001$). Specifically, dentin permeability was decreased by 66% after autoclaving at 121 °C for 25 min. On the other hand, dentin permeability in the 70% ethanol group increased by 17%, although the difference before and after treatment was not significant ($P = .057$).

Although independent-samples t tests found no significant differences in dentin permeability between the two test groups before the respective treatments ($P = .911$), there was a significant difference between groups after the treatments ($P < .001$).

FE-SEM evaluation

Representative FE-SEM micrographs of the dentin surface of samples from the two groups are shown in Fig. 2. FE-SEM revealed that most dentinal tubules were completely open after etching with 35% phosphoric acid for 30 s; this indicated that the smear layer and smear plugs had been removed from the dentin surface. The collagen network of the demineralized dentin appeared fluffy, spongy, and well-organized (Fig. 2a, b).

Figure 2c, d shows the FE-SEM images of the dentin surface after treatment with 70% ethanol. The surface morphology was similar to that after treatment with 35% phosphoric acid for 30 s; most dentinal tubules were open and free from debris, and the collagen network still remained fluffy.

Figure 2e, f shows the FE-SEM images of the dentin surface after treatment with steam autoclaving. The originally fluffy collagen fibers appeared condensed, and the collagen mesh became compact. This denatured collagen mesh collapsed in one direction resulting in the reduction of fiber spacing, which induced changes in the surface morphology.

Discussion

Disinfection/sterilization of dentin specimens is a necessary procedure used in diverse preclinical studies and in vitro investigations. Human teeth should be considered as a potential source of blood-borne pathogens, including human immunodeficiency virus, hepatitis B virus, hepatitis C virus, and pathogenic microorganisms [11, 27]. Therefore, freshly extracted teeth are usually disinfected/sterilized and stored in a liquid disinfectant [11, 27]. However, microorganisms can still grow

Table 1 Dentin permeability values before and after disinfection/sterilization treatment using different methods

Sample	Permeability (Lp, μl•min^{-1}•cm^{-2}•cm H_2O^{-1})		Relative Lp value after disinfection/sterilization (percentage of baseline Lp)
	Baseline	After disinfection/sterilization	
70% ethanol group ($n = 20$)	0.372 ± 0.258	0.428 ± 0.314	117 ± 29
Steam-autoclaving group ($n = 20$)	0.364 ± 0.195	0.117 ± 0.0849	34 ± 19

Lp: hydraulic conductance

All values are expressed as means ± standard deviations

from extracted teeth stored in liquid disinfectants [11, 27]. Moreover, in subsequent experiments, once the teeth are sectioned and the dentin is exposed, dentinal fluid originating from the pulp remains on the dentin surface, posing a further potential risk of cross contamination to the environment, personnel, or cell cultures. Therefore, dentin sections and crown segments should be disinfected/sterilized again before subsequent studies. However, the disinfection/sterilization treatment may cause changes in the structure and composition of dentin, which can alter dentin permeability and interfere with the test results [17, 28, 29].

Steam autoclaving and 70% ethanol immersion are commonly used disinfection/sterilization methods for dentin disks before testing [7, 11, 13–15, 22]. Both methods are effective, easy to perform, rapid, and safe to the environment.

Steam autoclaving exhibited 100% efficacy for the sterilization of extracted human teeth in a previous study [11]. Sandhu et al. reported that no visible microorganism growth occurred in the culture medium after teeth were autoclaved at 121 °C at 15 lb for 20 min [11]. However, some researchers did not recommend autoclaving as a suitable sterilization method in studies of the dentin structure and composition because autoclaving produced changes in the organic and inorganic constituents of dentin [28]. These findings are consistent with the results of our study, where steam autoclaving (121 °C, 9.6 MPa, 25 min) significantly affected the permeability of dentin disks. Previously, Pashley et al. reported that autoclaving did not affect dentin permeability [13]. However, they first autoclaved sound teeth and subsequently sectioned them into disks before permeability measurements [13], whereas we first sectioned the teeth into disks and then acid-etched them before disinfection/sterilization treatment. This difference in the sequence of treatment steps may have led to the discrepancy in results. After acid etching, the smear layer was removed and the fluffy collagen network was exposed. Autoclaving led to collagen denaturation and coagulation, and the denatured collagen network became compact that it collapsed in one direction and its fiber spacing decreased (Fig. 2), thereby decreasing permeability. In a study where sound teeth were autoclaved, heat and pressure also caused denaturation of the dentin collagen [30], although this change did not affect dentin permeability [13]. This was probably because the collagen remained embedded in the inorganic phase, even though the teeth were sectioned as disks after autoclaving, and autoclaving showed a small effect on the dentin mineral content [30]. Importantly, in studies where

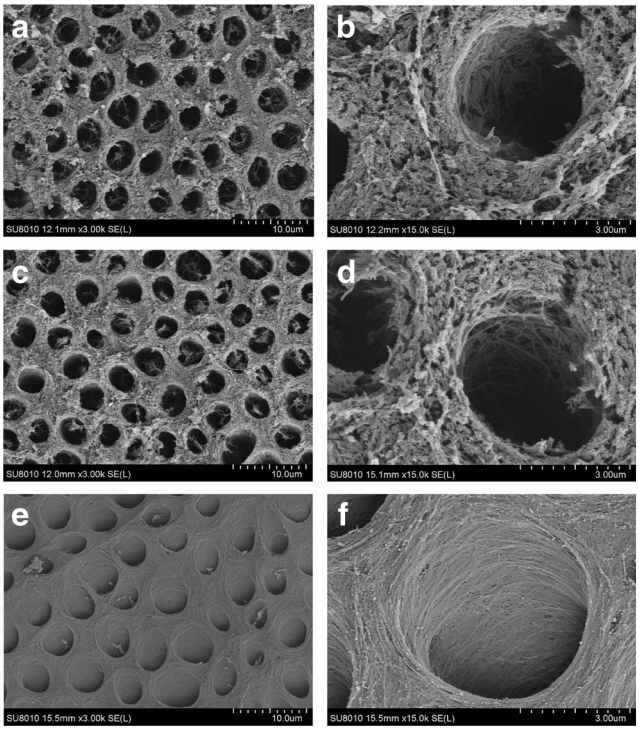

Fig. 2 Representative field emission scanning electron microscopy (FE-SEM) images of dentin surfaces before and after disinfection/sterilization at × 3000 (left) and × 15,000 (right) magnifications **a** and **b** FE-SEM images obtained after acid etching in 35% phosphoric acid for 30 s (before disinfection/sterilization), showing that the dentinal tubules were open, and the dentin collagen mesh was fluffy. **c** and **d** FE-SEM images obtained after acid etching and then disinfection with 70% ethanol for 15 min, showing that there were no significant differences in surface morphology compared with that before disinfection. **e** and **f** FE-SEM images obtained after acid etching and then sterilization via steam autoclaving for 25 min, showing that the dentin collagen mesh became compact and collapsed in one direction

sectioned and acid-etched dentin specimens were autoclaved [14, 15, 23, 24], alterations in dentin permeability should have been considered as a potential adverse effect.

Although immersion in 70% ethanol for 15 min increased dentin permeability by 17%, this increase was not statistically significant. Changes in the organic components of dentin after immersion in 70% ethanol for a short time were not as strong as those after autoclaving. The collagen fibers still retained its fluffy network structure (Fig. 2). In other words, a short (15 min) disinfection treatment with 70% ethanol did not affect the morphology of the dentin surface (Fig. 2) and dentin permeability. Goodis et al. reported that the permeability of crown segments may be affected by storage conditions, including the solution type and storage duration [16]. In their study, they sectioned the teeth into segments and stored the segments in different storage solutions [16], similar to the procedure in our study. They reported that crown segments stored in 70% ethanol for a prolonged time period (up to 6 months) exhibited increased permeability over time [16].

Immersion in 70% ethanol for 15 min is considered less effective in the prevention of microorganism growth compared with steam autoclaving, though ISO has recommended the former as one of the appropriate disinfection treatments for dentin in dentin-barrier cytotoxicity tests [22]. This indicates that short-term 70% ethanol immersion (15 min) can ensure sufficient disinfection of dentin for in vitro experiments involving dentin specimens. Dentin-barrier cytotoxicity tests are in vitro tests performed using dentin disks to separate dental filling materials from cells in order to stimulate the clinical conditions in a tooth cavity. For these tests, dentin permeability is a critical factor that directly affects the cytotoxicity results [7]. The desired permeability of sectioned dentin disks should be as close as possible to the in vivo permeability, which serves as the basis for determining the chemical toxicity of dental materials to pulp tissue in a cavity [7].

Long-term immersion in 70% ethanol was previously shown to cause increased dentin permeability over time as mentioned above [16]; however, the permeability increase found after 15 min of immersion in the present study was not significant. Therefore, immersion in 70% ethanol for 15 min may be an effective disinfection method in tests where permeability is a major factor influencing the results. Dentin disks disinfected in 70% ethanol may be stored in 0.9% sodium chloride solution at 4 °C for up to 3 weeks, though a microbiological examination should be conducted before use [22].

In this study, the critical point drying (CPD) method was used to dry specimens prior to the FE-SEM examination, as this method can prevent drying-related damage to samples and preserve the structural integrity of samples and, in turn, allow for good visualization of the collagen fibers of the demineralized dentin via FE-SEM.

Conclusions

Short-term immersion in 70% ethanol (15 min) was a suitable dentin disinfection method that did not significantly affect the permeability and surface morphology of acid-etched dentin disk specimens. In contrast, steam autoclaving significantly decreased the permeability of dentin disks, indicating that it cannot closely simulate in vivo conditions and may be not an ideal procedure for in vitro tests concerning dentin permeability. Additionally, changes in dentin permeability may be accompanied by changes in dentin composition. Spectroscopy may be used to analyze dentin components in further studies to better understand the effects of these disinfection/sterilization treatments on dentin.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent For this type of study, formal consent is not required.

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