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Effect of ethylene oxide sterilization on enamel and dentin demineralization in vitro

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ABSTRACT

For in situ studies into caries prevention, sterilization of tooth samples is essential. However, sterilization may influence the caries process itself. The aim of this study was to assess the effect of sterilising sound human enamel and dentin with ethylene oxide on lesion depth and mineral loss before and after in vitro demineralization. Lesion depth and mineral loss were measured using transversal microradiography (TMR). The experiment was carried out with 32 enamel and 32 dentin samples. We found a significant reduction of lesion depth due to sterilization in demineralized enamel (−9.8 μm; 95% CI: −15.1 to −4.4 μm). The small effect of sterilization on demineralized enamel is considered to be irrelevant for in situ studies of de- and remineralization.

1. Introduction

To prevent cross-infection via intra-oral appliances in in situ studies the enamel and dentin samples have to be sterilized. Sterilization should result in the complete destruction of all microorganisms. Pashley et al. stated that all extracted teeth for dental education or research should be either sterilized with steam autoclave, ethylene oxide or gamma radiation.[1] All three methods are able to kill vegetative bacteria.[2–4] For intra oral cariogenicity tests, it is of importance that sterilization does not influence the caries process.

Several studies have evaluated the effect of sterilization on tooth tissues, usually enamel. As steam autoclaving is available in dental clinics it is the easiest sterilization method. It is also safe and cheap.[5] However, softening of the enamel[6] and dentin[7] was reported. Autoclaved specimens also behave differently in lesion remineralization.[5] Gamma radiation caused no significant changes in enamel hardness[6] and in dentin permeability.[8] However, it may enhance enamel resistance to artificial caries attack.[9] It also changed the enamel colour when the standard dose of 25 kGy is used, which may be the result of denaturation of the organic component.[10] A dose of 4.09 kGy caused no discoloration, and still resulted in full sterilization.[11] However, Kielbassa et al.[12] reported that dentin is severely affected by irradiation with a dose up to 60 Gy, shown by significant changes in Knoop hardness. A study on the effect of ethylene oxide (EtO) on remineralization of lesioned enamel reported no difference with the control in lesion depth or mineral loss.[5] For ethylene oxide sterilization of dentin, White et al. found a decrease of

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the magnitude of the phosphate peak measured by FTIR spectroscopy, indicating loss of mineral on the dentin surface.[8] The group also found a small colour change in dentin, as measured by UV/VIS/NIR spectroscopy. Sang et al. found that EtO sterilization was more effective than gamma radiation for sterilizing contaminated bone chips used for bone grafts.[13] Thus, ethylene oxide seems to be the method of choice.

Ethylene oxide is a common method for sterilizing medical items. The sterilization method for biomaterials recommended by The Central Sterile Service Department of the University Medical Centre Groningen (UMCG) is ethylene oxide. It is the oldest reported sterilization method for sterilizing extracted teeth; already in 1974 bovine enamel blocks were treated using 1% ethylene oxide vapors.[14] Unknown is the sterilization effect of ethylene oxide on sound enamel and demineralized dentin.

Gold standard for in situ studies on de- and remineralization is transversal microradiography (TMR). The aim of the present study was to assess the effect of sterilising sound human enamel and dentin with ethylene oxide on lesion depth (LD) and mineral loss (ML) before and after in vitro demineralization, as measured by TMR in vitro.

2. Materials and methods

2.1. Sample preparation and experimental set-up

Freshly extracted sound human molars and premolars, provided by a local dentist were stored in a 0.2% thymol solution. Approximately 100 μm was abraded from the top surface of the tooth with grinding paper 220 grit (Siawat Abrasives, Bern, Switzerland). Square enamel samples of 3.2 mm × 3.2 mm and about 1.5 mm thickness, were cut from the crown with a water cooled diamond saw. Dentine samples of the same size were cut from the root. Thirty-two enamel and 32 dentin samples were embedded in blocks of 8 samples; only 1 surface was left free of acrylic for caries development. The experiment involved 8 blocks of 8 dentin or 8 enamel samples. Two blocks of each tissue were sterilized. One sterilized and one non-sterilized block of each tissue was subsequently demineralized for 2 weeks in a 2% CMC gel containing 0.1 M lactic acid (titrated to pH 5 with 10 M KOH) at 37 °C (15). A schematic presentation of the experimental set-up can be seen in Fig. 1. Subsequently, planoparallel slices were cut from the centre of the sample, using a water-cooled diamond-coated saw. The slices were polished (800 grit, Siawat Abrasives, Bern, Switzerland) to the appropriate thickness (enamel 80 μm and dentine 130 μm) and transversal microradiography (TMR)-radiographs were taken.

2.2. Sterilization process

Ethylene oxide (EtO) sterilization was performed at WIMAC (Kliniekdiensten B.V., Rotterdam, The Netherlands) according to ISO 9001:2000 and EN 13485:2003. The gas sterilization process consists of three steps. The first step is 16 h of pre-conditioning under 50–80% relative humidity at 38 °C. The second step is EtO gas cycles with a gas concentration of 625 mg/L in an autoclave at low pressure and temperature (40–55 °C) for at least 3 h. The last step is a degassing period of minimal 72 h (i.e. 6 exchanges/h, 40 °C).

2.3. Transversal microradiography (TMR)

The radiographic conditions for TMR are employed as earlier described by Inaba et al.[15] The enamel samples were placed on a 35 mm film (Fuji B&W POS/71337) and exposed for 18 s, with a tube charge of 20 kV and 15 mA. The settings for dentin samples were as follows: 25 kV, 25 mA and 10 s. Dentin samples were placed in a Perspex sample holder filled with water to prevent shrinkage.[16]

2.4. Film processing and image handling

After exposure the films were developed with a D-19 developer (Kodak) for 10 min, followed by fixating, rinsing, and drying. A digital image of each microradiograph was recorded with a light microscope (Nikon Eclipse E600) with a magnification 10× and a CCD camera (Teli CS 8310, Tokyo, Japan). The camera was linked to a personal computer equipped with a frame-grabber. The magnified microradiographs were stored as images with a size of 640 × 480 pixels (820 μm × 620 μm), with a resolution of 256 grey values. Further image processing and analysis were performed using the NIH Image program (Scion, Scion Corporation, Frederick, MD, USA) to calculate lesion depth (μm) and mineral loss (vol% μm). Films were only periodically calibrated with the entire stepwedge. Routinely films were evaluated by a software program developed in our lab using two reference areas; an area of the almost radiolucent/black part for reference of no mineral and an area of sound tooth material (almost radiopaque/white) for reference of maximal mineral content. Three scans were made of each enamel and dentin microradiograph at different positions and the average was used for further analysis.

Lesion depth and mineral loss for TMR is defined as the depth/loss difference between points at 4 vol% mineral and

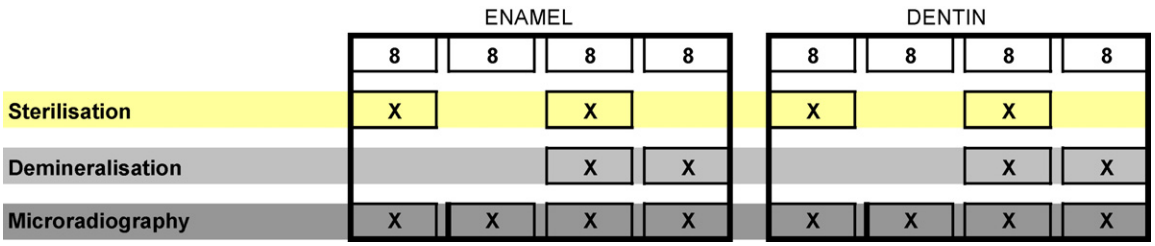

Fig. 1 – Schematic presentation of experimental set-up.

Table 1 – Effect of ethylene oxide sterilization on lesion depth and mineral loss of sound and demineralized enamel and dentin

	Non-sterilized mean (S.D.)	Sterilized mean (S.D.)
Lesion depth (μm)		
Enamel		
Sound	7.2 (3.7)	13.3 (6.5)
Demineralized	114.4 (12.9)	104.5 (10.7)
Dentin		
Sound	10.6 (3.3)	9.1 (1.4)
Demineralized	100.7 (12)	97.8 (15.6)
Mineral loss (vol% μm)		
Enamel		
Sound	671 (158)	478 (292)
Demineralized	5947 (1229)	5386 (932)
Dentin		
Sound	472 (135)	305 (145)
Demineralized	3482 (206)	3379 (676)

5 vol% below the healthy value. These definitions also lead values for 'lesion depth' and 'mineral loss' in sound (non-demineralized) samples.

2.5. *Statistical analysis*

Data were analyzed – separately for dentin and enamel – by two-way ANOVA using SPSS 12.0 software for Windows. In the analysis lesion depth and, separately, mineral loss were included as the response variable and sterilization and demineralization as the fixed effect factors. First, a possible interaction between demineralization and sterilization was studied. The estimated effects are presented together with their corresponding 95% confidence intervals. A p-value of 0.05 or less was considered as statistically significant.

3. Results

The lesion depth and mineral loss values measured in sound samples, which are the result of limitations of the micro-radiographic method, were around 10–30 μm and around 450 vol% μm, respectively. After demineralization for 2 weeks these values were 70–140 μm and 2300–8000 vol% μm, respectively. The results of the study are summarized in Table 1.

Two-way ANOVA showed no interaction between demineralization and sterilization for mineral loss in enamel (0.09) and dentin ($p = 0.7$), nor for lesion depth in dentin ($p = 0.7$). For lesion depth in enamel a significant interaction ($p < 0.001$) was found, therefore lesion depth of sound and demineralized enamel are presented separately in Table 2.

Results of the two-way ANOVA regarding the effect of sterilization are summarized in Table 2. Lesion depth decreased in demineralized enamel (−9.8 μm; 95% CI: −15.1 to −4.4 μm), but increased in sound enamel (6 μm; 95% CI: −0.5 to 11.6 μm). The relative effect of sterilization is reported for the demineralized samples only. Apart from the effects presented in Table 2, a large effect of demineralization was found, which is a logical result of the demineralization procedure.

4. Discussion

The aim of this study was to assess the sterilization effect of ethylene oxide on dentin and enamel on lesion depth and mineral loss before and after in vitro demineralization.

It should be noted that the lesion depth and mineral loss values reported for the sound samples actually represent a radiolucency caused by limitations of radiological techniques such as misalignment and blur, and a variation in mineral concentration. Although the tooth material is sound, the edge of the sample blurred due to the thickness of the sample. This blur contributes to lesion depth as defined and determined by the software. The blur on sound dentin and interpreted as lesion depth is around 12 μm for TMR.[16] Although the values 'lesion depth' and 'mineral loss' are thus actually inaccurately used when reporting measurements on sound (non-demineralized) samples, in the past it has been a common use for TMR measurements on sound samples.[5,17] Therefore the terms lesion depth and mineral loss are used in the present study for sound samples as well.

It is very well possible that the sterilization effects on sound samples differ from the demineralized samples. After all, on sound samples there can only be an effect on the tooth material. On the demineralized samples there can also be an effect on the demineralization process itself. Therefore we

Table 2 – Results from two-way ANOVA, fitted separately for enamel and dentin

	Effect of sterilization (95% CI)	Relative effect demineralization[a]	p-Value
Enamel			
Lesion depth			
Sound	6.0 μm (−0.5 to 11.6)		0.34
Demineralised	−9.8 μm (−15.1 to −4.4)	−9.8/109.6 = −9%	0.001
Mineral loss	−276 vol% μm (−606 to 55)	−276/5666 = −5%	0.1
Dentin			
Lesion depth	−2.2 μm (−6.4 to 2.0)	−2.2/99.2 = −2%	0.30
Mineral loss	−135 vol% μm (−289 to 18)	−135/3430 = −4%	0.08

[a] Percentage of effect/average demineralization value.

have studied the interaction between the parameters sterilization and demineralization. Only for lesion depth in enamel there was an interaction ($p < 0.001$): opposite effects were measured.

Whether the measured effect of EtO sterilization represents a true effect is open for discussion. Since the effects on lesion depth are small and not-consistent, we assume that they may represent a measurement error, presumably caused by limitations of microradiography or by variation in mineral concentration.

If we compare our results with the existing literature, the picture of inconsistency is even enhanced. For lesioned enamel, Toro et al.[5] sometimes found an increase, sometimes a decrease, but never a significant difference in remineralization after gas sterilization, expressed in lesion depth or mineral loss, using transversal microradiography. In the present study we found mineral loss reduction for demineralized enamel ($p = 0.001$). White et al. studied the effect of EtO on sound dentin, they found an indication for loss of mineral on the dentin surface.[8] In the present study a trend ($p = 0.08$) of less mineral loss in both sound and demineralized dentin treated with EtO compared to the unsterilized group was observed. Speculating on the cause of this variability in observations; there may be a small effect of ethylene oxide sterilization, but this effect is not predictable. In all cases ethylene oxide sterilization effects were small.

The relative effect of ethylene oxide sterilization in the present study on demineralized tissue was −5% (ML) to −9% (LD) for enamel, and −2% (LD) to −4% (ML) for dentin calculations (Table 2). Although evaluation revealed statistically significant differences on enamel measuring lesion depth, it is known from former studies of our research group that the coefficient of variation of TMR measurements for lesion depth is around 15% for both dentin and enamel. For mineral loss of enamel it is around 22% and for dentin 15%. Moreover the effect seems to be an absolute effect, because there are no interactions between demineralization and sterilization, except for enamel LD. This would imply that as lesions grow deeper the effect size will not increase, and therefore the relative impact will decrease. In the light of all the inconsistencies mentioned above and the small size of the effects when measured, we assume that the sterilization effects will not influence the outcome of in situ caries studies in a relevant way.

Our study did not evaluate the efficacy of EtO from a microbiological perspective; Inefficacy against Bacillis subtilis-spores (commonly used as a biologic indicator of sterilization), which were placed into the pulp chamber of extracted human molars, was reported for ethylene oxide.[4] Efficacy against this spore was reported for gamma radiation, but White et al.[8] concluded also that their test conditions did not completely demonstrate that gamma irradiation would sterilize through dentin and residual pulp. In in situ studies it is very rare that whole teeth will be used. Instead, small tissue slabs with no pulp chamber or large internal spaces are used, which should improve sterilization efficacy. Additional studies will be required to confirm the effectiveness of EtO for rendering the enamel and dentin samples for in situ studies of extracted teeth free of bacteria and spores.

In conclusion, results of this study indicate that ethylene oxide has a small effect on tooth tissues and their demineralization. However, for in situ de- and remineralization studies, this small sterilization effect is considered to be irrelevant.

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