

Cytotoxicity of medical materials sterilized with vapour-phase hydrogen peroxide

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A new sterilization system using vapour-phase hydrogen peroxide (VPHP) was recently developed. The cytotoxicity of various medical materials sterilized by the VPHP sterilization system was investigated. After VPHP sterilization, polystyrene, polyurethane (PU8), blend material of silicone and polyurethane (Sil/PU6), poly(methyl methacrylate) (PMMA), fluorosilicone acrylate and poly(2-hydroxyethyl methacrylate) (HEMA) showed strong cytotoxicity, whereas polyethylene and polypropylene did not. Although the cytotoxic potential of most materials is reduced by extension of the aeration time, HEMA and PMMA still retained strong cytotoxicity after 12 h aeration. Addition of catalase to the cell culture eliminated the cytotoxicity of sterilized polystyrene and PU8. Hydrogen peroxide (H_2O_2) residues remaining in the sterilized materials were determined. Large amounts of H_2O_2 (5.1–186 μg) were detected in HEMA, PU8, Sil/PU6 and PMMA. In contrast, silicone and polyethylene contained low levels of H_2O_2 . The amounts of residual H_2O_2 in the materials decreased with increasing aeration time, but the elimination rate of H_2O_2 differed among the test materials. The cytotoxic potential of the VPHP-sterilized materials correlated with the amounts of residual H_2O_2 present. These results indicated that the cytotoxicity of VPHP-sterilized materials was caused by the residual H_2O_2 . To generalize the developed VPHP sterilization system for various medical devices, it is important to validate the aeration of materials for removal of cytotoxic residuals. *Biomaterials* (1995), **16**(3), 177–183

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There are three major sterilization methods for medical devices: autoclaving, γ -ray sterilization and ethylene oxide gas sterilization. Ethylene oxide gas is an effective sterilant for materials that would be destroyed by steam sterilization. However, we have to be aware of the toxicity of residual ethylene oxide in the devices^{1–4}. γ -Ray sterilization often induces deterioration of the mechanical properties of the plastics. Therefore, an alternative sterilization method is required.

Hydrogen peroxide (H_2O_2) solution is a strong oxidizing agent and has been used as a surface disinfectant for surgical implant components, spacecraft hardware, hydrophilic soft contact lenses and packing materials^{5–9}. While aqueous H_2O_2 has a long history of use as a sterilant, the concept of vapour-phase H_2O_2 (VPHP) sterilization was recently developed^{10,11}. The successful application of VPHP technology for surface sterilization of glass, stainless steel and polyethylene has been reported^{12–14}. However, toxicological evaluation of the materials following VPHP sterilization has

not been investigated. H_2O_2 showed strong cytotoxicity^{1,15–18}, so that H_2O_2 residues remaining in materials elicit toxicological responses in skin, eye and mucous membrane.

The present report relates the cytotoxicity of various medical materials exposed to the VPHP sterilization process, as developed by our collaborators. We also determined residual H_2O_2 in the materials, and discuss a cause for the cytotoxicity of the materials.

MATERIALS AND METHODS

Chemicals

Hydrogen peroxide 30% w/v in aqueous solution was obtained from Santoku Chemical Industries Co., Ltd (Tokyo, Japan). Trypsin–ethylenediaminetetraacetic acid solution was purchased from GIBCO Laboratories (Grand Island, NY, USA). Ammonium thiocyanate (NH_4SCN) and iron(II) sulphate heptahydrate (for analysis of H_2O_2 , $FeSO_4 \cdot 7H_2O$) were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Ferrous thiocyanate reagent consisted of 0.5%

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NH₄SCN, 0.5% FeSO₄·7H₂O and 1% sulphuric acid in aqueous solution. The reagent was prepared just before use. Catalase (EC 1.11.1.6) was purchased from Sigma Chemical Co. (St Louis, MO, USA).

Materials

Segmented polyurethane 8 (PU8, $M_w = 55\,600$, $M_n = 31\,000$) was obtained from Sanyo Kasei Kogyo Co. Ltd (Kyoto, Japan). The PU8 was synthesized by the reaction of poly(tetramethyleneglycol) 1000, 4,4'-diisocyanatodiphenylmethane and 1,4-butanediol. Silicone rubber film (Sil) was a gift from Dow Corning Ltd. Sil/PU6 was a blend film of Sil and polyurethane 6 (PU6, $M_w = 124\,000$, $M_n = 52\,500$) (1:1). Details of the preparation of PU8, Sil and Sil/PU6 are described in another paper¹⁹. These films were approximately 1 mm thick. Polystyrene film (PS, plastic sheet for tissue culture) was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). The polypropylene film (PP) was a piece of a centrifuge tube manufactured by Sumitomo Bakelite Co. Ltd (Tokyo, Japan). Minisorp[®] tube (Nunc, Inc. Naperville, IL, USA) was used as a sample piece of polyethylene. Poly(methyl methacrylate) (PMMA), fluorosilicone acrylate (FSA) and poly(2-hydroxyethyl methacrylate) (HEMA) films of about 0.5 mm thickness were materials used for contact lenses, and were given by Menicon Co. Ltd (Nagoya, Japan).

Sterilization procedure

Hydrogen peroxide sterilizer (HSB-2000), developed by Sakura Seiki Co. Ltd (Tokyo, Japan), was used. Test materials were cut into 5 × 6 mm² square pieces, placed on a plastic plate and transferred to a sterilization chamber of 210 l volume. The samples were treated according to the manufacturer's instructions. Each sample was preheated at 55°C for 10 min under vacuum. The VPHP generated by heating 30% H₂O₂ solution, was added to the chamber at a rate of 1 ml per 30 s, and samples were exposed to VPHP for 30 min at 55°C. The final concentration of H₂O₂ in the chamber reached approximately 3 mg l⁻¹. After sterilization, the samples were aerated with air introduced by a ventilator at a flow rate of 500 l min⁻¹ in order to remove H₂O₂. As a comparison, samples were separately sterilized by exposure to ultraviolet (UV) light. The conditions of UV exposure did not affect the toxicological and mechanical properties of the samples.

Cell culture

Chinese hamster fibroblast V79 cells were obtained from the Japanese Cancer Research Resources Bank, and grown in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) supplemented with 10% fetal calf serum (Cell Culture Laboratories, Ohio, USA) (FCS-MEM) at 37°C in a humidified atmosphere of 5% CO₂ in air. At the time of the cytotoxicity test, the cells were dissociated with trypsin-ethylenediaminetetraacetic acid solution and resuspended in FCS-MEM.

Cytotoxicity test

The crystal violet (CV) staining method was performed as described by Saotome *et al.*²⁰, with some modification. Cell suspensions (5000 cells/200 μl) were seeded into each well of a 96-well microtitre plate, and incubated at 37°C for 4 h in order to attach them to the bottom of the well. Sterilized materials were added perpendicularly to the bottom of the well, out of contact with the cells. After incubation for 3 d, cells were fixed with 25% glutaraldehyde for 20 min, and were stained with 0.4% CV-methanol solution for 30 min. The plate was then washed in water and air dried. Absorbance of each well at 590 nm was measured by an automatic microplate reader. The absorbance of the control wells, which contained no material, was regarded as 100%, and the percentage absorbance for each well was calculated.

The cytotoxic potential of H₂O₂ was also determined. Cells were cultured with various concentrations of H₂O₂ for 3 d, and the plates were then treated as described above. The H₂O₂ concentration that inhibited the absorbance to 50% of control (IC_{50} (μg ml⁻¹)) was obtained from the dose-response curve.

Determination of residual H₂O₂

The ferrous thiocyanate method²¹ was used. Each sample was immersed in 200 μl distilled water and thoroughly vortexed. Ferrous thiocyanate reagent (67 μl) was added to 200 μl diluted or undiluted extract, or distilled water (blank). After 3 min, 200 μl of the reacted solution was transferred to a 96-well microplate, and the absorbance of each well at 450 nm was measured by an automatic microplate reader. The H₂O₂ concentration was then read off from a calibration curve obtained by standard H₂O₂ solutions.

RESULTS

Cytotoxicity of H₂O₂

Various concentrations of H₂O₂ were exposed to cells for 3 d. The dose-response relationship for H₂O₂ is shown in Figure 1. Its IC_{50} value was 1.03 μg ml⁻¹. The addition of catalase in the cell culture decreased the cytotoxicity of H₂O₂: 10 units ml⁻¹ catalase eliminated the cytotoxic ability of 120 μg ml⁻¹ H₂O₂ (Table 1).

Cytotoxicity of VPHP-sterilized materials

Effect of aeration time

Table 2 shows the cytotoxic response of materials aerated for 2, 6 or 12 h following VPHP sterilization. After 2 h aeration, six VPHP-sterilized materials except Sil, PE and PP produced strong cytotoxicity, and no cell growth was observed. The cytotoxicities of most of these materials decreased with increasing aeration time. Aeration for 12 h reduced cytotoxicity to the levels detected in UV-sterilized materials except for PMMA and HEMA. In the case of PMMA, the cytotoxicity was reduced but not eliminated after 12 h aeration. Cells were not grown at all in the culture of VPHP-sterilized HEMA.

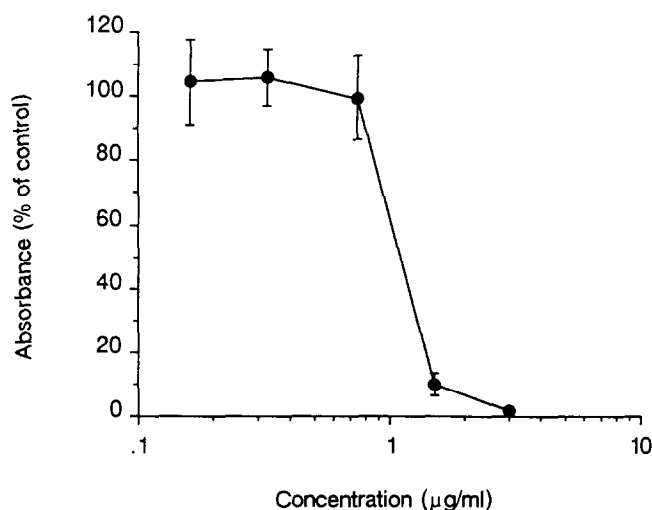


Figure 1 Dose-response relationship for H₂O₂. V79 cells (5×10^3 cells well⁻¹) were incubated with various concentrations of H₂O₂ for 3 d. After incubation, the plate was stained with crystal violet solution and absorbance of each well at 590 nm was measured. Data points are mean \pm standard deviation of quadruplicate wells.

Effect of addition of catalase in cell culture

Three materials (PS, Sil and PU8) were sterilized with VPHP and aerated for 2 h. The cells were cultured with each sample in the presence or absence of 10 units ml⁻¹ catalase. Under the same condition, UV-

Table 1 Effect of catalase on H₂O₂-induced cytotoxicity

Treatment	Absorbance (% of control)
Catalase 10 units ml ⁻¹	99 \pm 16
Catalase 10 units ml ⁻¹ + H ₂ O ₂ 60 µg ml ⁻¹	127 \pm 23
Catalase 10 units ml ⁻¹ + H ₂ O ₂ 120 µg ml ⁻¹	123 \pm 27
Catalase 10 units ml ⁻¹ + H ₂ O ₂ 300 µg ml ⁻¹	59 \pm 16
Catalase 10 units ml ⁻¹ + H ₂ O ₂ 600 µg ml ⁻¹	0

5×10^3 cells were cultured with 10 units ml⁻¹ catalase and various concentrations of H₂O₂ for 3 d. Results are expressed by mean \pm standard deviation of quadruplicate wells.

Table 2 Effect of aeration time on the cytotoxicity of materials

Material	Absorbance (% of control)			
	Vapour-phase hydrogen peroxide sterilization aeration time			Ultraviolet sterilization
	2 h	6 h	12 h	
Polystyrene	0	7 \pm 8	98 \pm 7	93 \pm 10
Polyurethane 8	0	41 \pm 30	77 \pm 23	65 \pm 7
Silicone	41 \pm 16	63 \pm 22	54 \pm 27	31 \pm 21
Silicone and polyurethane 6 blend	0	34 \pm 19	74 \pm 27	57 \pm 15
Polyethylene	96 \pm 6	NT ^a	NT	107 \pm 18
Polypropylene	85 \pm 7	NT	NT	83 \pm 27
Fluorosilicone acrylate	0	98 \pm 11	110 \pm 21	102 \pm 2
Poly(methyl methacrylate)	0	0	31 \pm 34	112 \pm 19
Poly(2-hydroxyethyl methacrylate)	0	0	0	100 \pm 3

^aNT means not tested.

Each sample was sterilized with vapour-phase hydrogen peroxide or ultraviolet exposure. The vapour-phase hydrogen peroxide-sterilized sample was then aerated for a period varying from 2 h to 12 h. 5×10^3 cells were cultured with the sample for 3 d, and cytotoxicity was determined. Values are mean \pm standard deviation ($n = 2-6$).

sterilized materials were also tested. Absorbance obtained from UV-treated materials did not vary with addition of catalase. Catalase decreased the cytotoxic responses produced by VPHP-treated PS and PU8 (Table 3).

Determination of residual H₂O₂ in materials

Absorbance gave a linear calibration line at H₂O₂ concentrations in the range from 0.15 to 30 µg ml⁻¹. The detection limit of H₂O₂ was 0.075 µg ml⁻¹. Figure 2 shows the effect of extraction time on the recovery of H₂O₂ from sterilized materials. In the case of PU8 and Sil, extraction for 30 min was sufficient. However, the detectable H₂O₂ in the extract from PS increased with increasing extraction time until 24 h. It was found that the rate of migration of H₂O₂ to water was different among test materials.

We next determined H₂O₂ in the 24 h extracts from PS, PU8 and Sil after aeration for 2, 6 or 12 h. After 2 h aeration, large quantities of H₂O₂ were detected in PS and PU8 (Figure 3). The amount of H₂O₂ decreased with increasing aeration time, and the greater part of H₂O₂ was removed from these materials by 12 h aeration (Figure 3).

Table 4 shows the concentrations of H₂O₂ in the 30 min or 24 h extract from, and the calculated values of H₂O₂ remaining in, 5×6 mm² pieces of sterilized materials. The largest amount of H₂O₂ remained in HEMA of the test materials. In the 24 h extract from HEMA, aerated for 2 h, 931 µg ml⁻¹ H₂O₂ was determined. This means that 186 µg H₂O₂ was retained in a piece of HEMA. Large amounts of H₂O₂ were also detected in PU8, Sil/PU6 and PMMA. In contrast, the residual H₂O₂ levels in Sil and PE were low. In the cases of PS and PMMA, differences in extraction time made a great difference in detectable H₂O₂ concentration. For example, the 30 min extract from PS contained 0.96 µg ml⁻¹ H₂O₂, while the 24 h extract contained 11.1 µg ml⁻¹ H₂O₂. After 12 h aeration the amount of H₂O₂ remaining in the samples became low. However, a piece of HEMA or PMMA still contained 98 µg or 1.5 µg H₂O₂, respectively, even after 12 h aeration.

Table 3 Decrease in cytotoxicity of vapour-phase hydrogen peroxide-sterilized materials by addition of catalase

Material	Catalase 10 units ml ⁻¹	Absorbance (% of control)	
		Vapour-phase peroxide sterilization	Ultraviolet sterilization
PS	- ^a	0	94 ± 7
	+	95 ± 3	116 ± 12
Sil	-	38 ± 3	20 ± 16
	+	47 ± 6	20 ± 11
PU8	-	0	54 ± 14
	+	47 ± 9	68 ± 10

^a-, none added; +, added.

Each sample was sterilized with vapour-phase hydrogen peroxide or ultraviolet exposure. The vapour-phase hydrogen peroxide-sterilized sample was then aerated for 2 h. 5×10^3 cells were cultured with the sample in the presence or absence of catalase for 3 d, and cytotoxicity was determined. Values are mean ± standard deviation of quadruplicate wells.

DISCUSSION

The cytotoxicity test was performed with sterilized materials using the VPHP sterilization system developed by Sakura Seiki Co., Ltd. When the materials were treated according to the manufacturer's instructions, namely, exposure to VPHP for 30 min and aeration for 2 h, six (PS, PU8, Sil/PU6, FSA, PMMA, HEMA) out of nine materials tested showed strong cytotoxicity (see Table 2). CV assay is known to be useful as an *in vitro* alternative method to the Draize eye irritation test²². In our experiment, a sterilized material was added directly into a culture well and incubated with the cells. The cells and material were not tangent to each other, so the cytotoxicity observed was induced by toxic agents leached out from the material, not the results of cell-material interaction due to the physical or chemical property of the material surface. We have already developed a colony assay to evaluate the cytotoxicity of materials using its extract with the culture medium²³⁻²⁵. Colony assay provided a reliable prediction of the inflammatory tissue responses in the implantation test²³⁻²⁵. VPHP-sterilized PS and PU8 showed cytotoxicity by the colony assay (data not shown). Considering the results of the CV and colony assays, when several VPHP-sterilized materials are applied to the eye or tissue, these materials may have a serious influence upon patients. The cytotoxicity of VPHP-sterilized contact lens materials, such as PMMA and HEMA, was not efficiently eliminated by aeration (see Table 2). Therefore, since the eye is a more sensitive organ than muscle or skin to irritants²⁶, it is necessary to be careful in applying VPHP sterilization to contact lens materials.

Meijis *et al.* investigated the degradation of medical-grade polyurethane elastomers by H₂O₂ solution²⁷. Gel permeation chromatographic and infrared spectroscopic studies showed that treatment of PU with 25% H₂O₂ solution for 24 h at 100°C resulted in a significant decrease in mechanical properties accompanied by a reduction in molecular weight, and a decrease in polyether soft segment at the surface²⁷. The degradation is believed to be

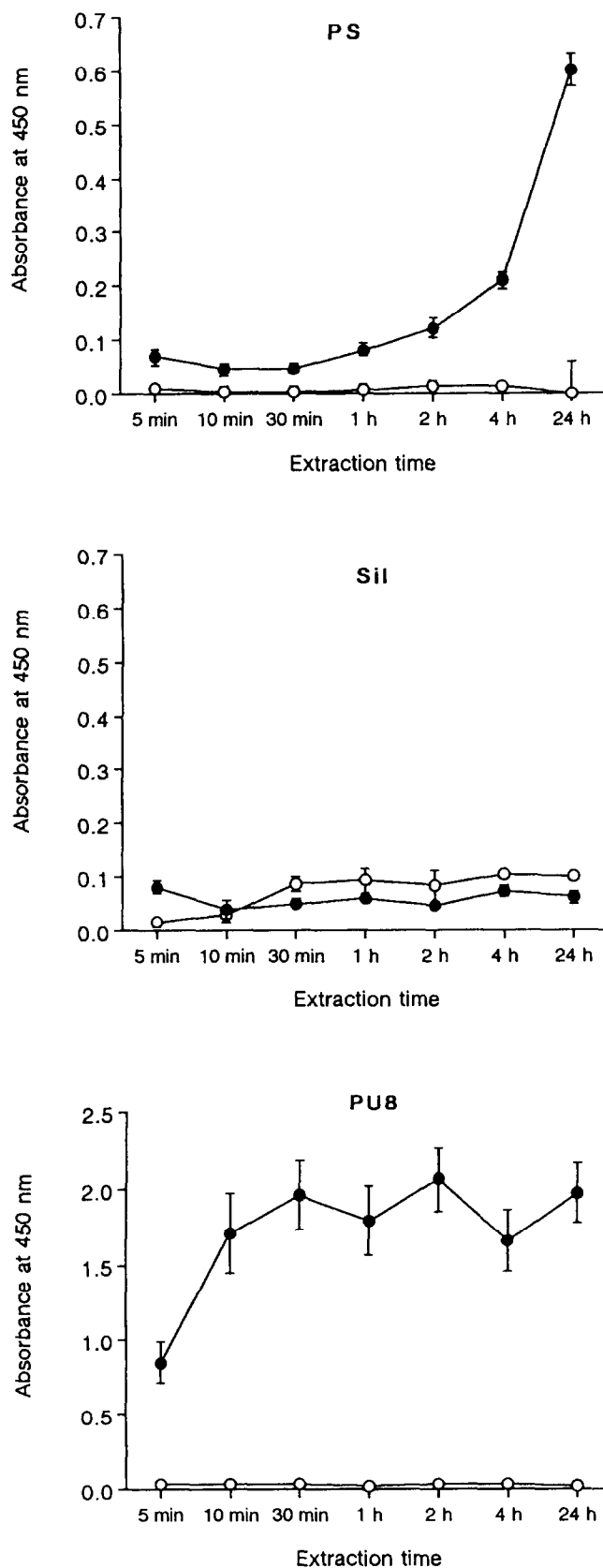


Figure 2 Effect of extraction time on detection of H₂O₂. Each sample was sterilized with vapour-phase hydrogen peroxide and then aerated for 2 h (●), or sterilized with ultraviolet exposure (○). The sample was extracted with distilled water and H₂O₂ concentration in the extract (as an absorbance) was measured. Results are given as mean absorbance ± standard deviation ($n = 3-4$).

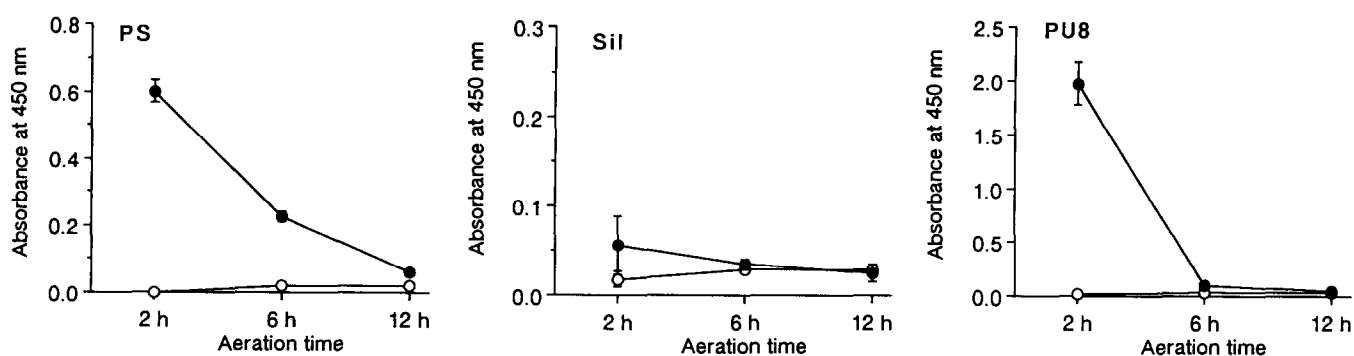


Figure 3 Effect of aeration time on the determination of H₂O₂. Each sample was sterilized with vapour-phase hydrogen peroxide and then aerated for 2 h (●), or sterilized with ultraviolet exposure (○). The sample was extracted with distilled water for 24 h and H₂O₂ concentration in the extract (as an absorbance) was measured. Data points are mean absorbance \pm standard deviation ($n = 3$).

mainly oxidative and catalysed by metal ions^{28–31}. We also investigated the surface change of chemical component of PS, PU8 and Sil/PU6 after VPHP sterilization by attenuated total reflection infrared spectroscopy (ATR-IR). However, there was no difference in the spectra for VPHP-sterilized and non-sterilized materials (data not shown). Our experimental conditions for H₂O₂ treatment were milder than those of Meijis *et al.*²⁷, therefore we think that these materials are not decomposed by VPHP sterilization, and the cytotoxicities observed in the sterilized materials are not due to the suspected degradation products of the materials.

Aeration is effective for the removal of toxic sterilant from the surface of chemical-sterilized materials^{32,33}.

When aeration had been insufficient, the sterilized material was significantly toxic^{2–4}. Although the manufacturer's instructions recommend aeration for 2 h, most samples showed cytotoxicity (Table 2). In the 24 h extract from VPHP-sterilized materials, 0.9–931 $\mu\text{g H}_2\text{O}_2\text{ ml}^{-1}$ were detected (Table 4). The level of residual H₂O₂ in the materials decreased with increasing aeration time (Table 4), and the cytotoxic potential of the materials correlated well with the residual amount of H₂O₂. The residual amount of H₂O₂ left in, and the elimination rate of H₂O₂ from the materials by aeration was different among the materials tested. The reason is unknown. It is considered that the rate of gas permeation may affect the differences in H₂O₂ absorption by the material.

Table 4 Amount of H₂O₂ in vapour-phase hydrogen peroxide-sterilized materials (a) 30 min extraction

Material	2 h aeration		12 h aeration	
	H ₂ O ₂ ($\mu\text{g ml}^{-1}$) ^a	H ₂ O ₂ ($\mu\text{g piece}^{-1}$) ^b	H ₂ O ₂ ($\mu\text{g ml}^{-1}$)	H ₂ O ₂ ($\mu\text{g piece}^{-1}$)
Polystyrene	0.96 \pm 0.21	0.19 \pm 0.04	0.09 \pm 0.12	0.02 \pm 0.02
Polyurethane 8	34.2 \pm 13.9	6.8 \pm 2.8	0.57 \pm 0.06	0.11 \pm 0.01
Silicone	0.90 \pm 0.27	0.18 \pm 0.05	0.21 \pm 0.30	0.02 \pm 0.02
Silicone and polyurethane 6 blend	30.9 \pm 6.21	6.2 \pm 0.6	0.26 \pm 0.05	0.05 \pm 0.01
Polyethylene	0.51 \pm 0.12	0.10 \pm 0.02	NT ^c	NT
Fluorosilicone acrylate	3.1 \pm 0.7	0.62 \pm 0.14	0.18 \pm 0.03	0.04 \pm 0.01
Poly(methyl methacrylate)	5.4 \pm 2.8	1.1 \pm 0.6	0.44 \pm 0.10	0.09 \pm 0.02
Poly(2-hydroxyethyl methacrylate)	976 \pm 68	195 \pm 14	216 \pm 147	43 \pm 29

(b) 24 h extraction

Material	2 h aeration		12 h aeration	
	H ₂ O ₂ ($\mu\text{g ml}^{-1}$)	H ₂ O ₂ ($\mu\text{g piece}^{-1}$)	H ₂ O ₂ ($\mu\text{g ml}^{-1}$)	H ₂ O ₂ ($\mu\text{g piece}^{-1}$)
Polystyrene	11.1 \pm 0.1	2.2 \pm 0.0	1.17 \pm 0.13	0.23 \pm 0.03
Polyurethane 8	41.5 \pm 2.1	8.3 \pm 0.4	0.93 \pm 0.80	0.19 \pm 0.16
Silicone	1.2 \pm 0.3	0.24 \pm 0.06	0.43 \pm 0.19	0.09 \pm 0.04
Silicone and polyurethane 6 blend	90.0 \pm 2.0	18.0 \pm 0.4	0.45 \pm 0.12	0.09 \pm 0.02
Polyethylene	0.90 \pm 0.10	0.18 \pm 0.00	NT	NT
Fluorosilicone acrylate	7.1 \pm 0.8	1.4 \pm 0.2	0.38 \pm 0.05	0.08 \pm 0.01
Poly(methyl methacrylate)	25.3 \pm 1.5	5.1 \pm 0.3	7.4 \pm 0.0	1.5 \pm 0.0
Poly(2-hydroxyethyl methacrylate)	931 \pm 34	186 \pm 7	490 \pm 77	98 \pm 15

^aH₂O₂ concentration in the extract. ^b5 \times 6 mm² square piece. ^cNT means not tested.

Each sample was sterilized with vapour-phase hydrogen peroxide and aerated for 2 or 12 h. The sample was then extracted with 200 μl distilled water at room temperature for 30 min (a) or 24 h (b). The H₂O₂ concentration in the extract was measured, and the residual H₂O₂ amount in a sample piece was calculated. The results are given as mean \pm standard deviation ($n = 3–4$).

In conclusion, strong cytotoxicities were observed in sterilized PS, PU8, Sil/PU6, FSA, PMMA and HEMA using the VPHP-sterilization system developed. The cytotoxic potential of the sterilized materials correlated with the amount the H₂O₂ remaining in the materials. A long aeration time decreased cytotoxicity as well as residual H₂O₂ in all these materials except PMMA and HEMA. Therefore, the cytotoxicity of VPHP-sterilized materials was caused by residual H₂O₂ left in the materials. In order to apply this VPHP sterilization system to various medical devices, it is important to validate the aeration of materials for the removal of cytotoxic H₂O₂.

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