Sterilization of Medical Devices by Ethylene Oxide, Determination of the Dissipation of Residues, and Use of Green Fluorescent Protein as an Indicator of Process Control

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Abstract: Ethylene oxide (EO) is used to sterilize Oxygenator and Tubing applied to heart surgery. Residual levels of EO and its derivatives, ethylene chlorohydrin (ECH) and ethylene glycol (EG), may be hazardous to the patients. Therefore, it must be removed by the aeration process. This study aimed to estimate the minimum aeration time for these devices to attain safe limits for use (avoiding excessive aeration time) and to evaluate the Green Fluorescent Protein (GFP) as a biosensor capable of best indicating the distribution and penetration of EO gas throughout the sterilization chamber. Sterilization cycles of 2, 4, and 8 h were monitored by *Bacillus atrophaeus* ATCC 9372 as a biological indicator (BI) and by the GFP. Residual levels of EO, ECH, and EG were determined by gas chromatography (GC), and the residual dissipation was studied. Safe limits were reached right after the sterilization process for Oxygenator and after 204 h of aeration for Tubing. In the 2 h cycle, the GFP concentration decreased from 4.8 (±3.2)% to 7.5 (±2.5)%. For the 4 h cycle, the GFP concentration decreased from 17.4 (±3.0)% to 21.5 (±6.8)%, and in the 8 h cycle, it decreased from 22.5 (±3.2)% to 23.9 (±3.9)%. This finding showed the potentiality for GFP applications as an EO biosensor. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 91B: 626–630, 2009

Keywords: sterilization; biosensor; membrane oxygenator; measurement/assessment

INTRODUCTION

Ethylene oxide (EO) is the most common sterilizing agent used to sterilize medical devices, which are sensitive to heat or gamma irradiation treatment.[1,2]

However, after the sterilization process (Process), residual concentration of EO and its derivative products, ethylene chlorohydrin (ECH) and ethylene glycol (EG), might remain in the medical devices, depending mainly on the type and size of polymeric plastic material.[3,4]

These residues are potentially toxic, mutagenic, and carcinogenic substances.[3–6] Therefore, it is imperative to remove them from the devices to avoid adverse effects in patients.

Membrane blood oxygenator (Oxygenator) and set of PVC tubing (Tubing) are medical devices used interconnected in cardiopulmonary bypass (CPB) undergoing open-heart surgery providing temporary cardiac and pulmonary support.

After being subjected to EO sterilization, they are commonly maintained in an aeration room to remove the residual excess of EO and its derivatives. After that the devices are subjected to a simulated-use extraction,[7] and the remaining residues are determined by gas chromatography (GC) to check the values against the safety limits.[8]

The Process is monitored by spores of *Bacillus atrophaeus* as biological indicator (BI).[9] However, the BI results do not provide enough information about EO distribution through the Chamber. Still, it is an important factor to know the process and better adjust the sterilization parameters. Besides, it enables economy by reducing costs with EO gas and by reducing the retention time of the medical devices in the aeration room.

To address this need, a biosensor capable of providing information about the distribution and penetration of the EO through the load in the sterilization chamber (Chamber) should be used.

Enzymes and proteins have been used as biological indicators to evaluate the efficacy of industrial procedures, such as blanching, pasteurization, and disinfection treatments[10,11] and EO is a reactive alkylating agent by adding alkyl groups to DNA, RNA, and proteins.[3]

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The Green Fluorescent Protein (GFP) is a compact and globular acidic protein (pI 4.6 to 5.4) with 238 amino acids; it constitutes a monomer with molecular weight between 27 and 29 kDa that exhibits a high thermal stability (It has shown resistance to heat up to 95°C and solutions of pH between 5.5 and 12.0.)[11] and is easily detected using UV light and spectrofluorometry.[12]

The main characteristic of GFP is the presence of one fluorophore (excitation and emission maxima at 394 and 509 nm) that consists of a cyclic tripeptide in the primary protein sequence, which provides a fluorescence emission without using cofactors.[13]

So, it may be applied as a potential biosensor to monitor the Process, to report the extension of the distribution and penetration of EO into the load, by measuring its fluorescent intensity remaining after the Process.[14]

Once the amount of EO gas residuals into the devices and it must be removed after the Process, this work aims to analyze the residual levels of EO, ECH, and EG on the Oxygenator and Tubing, to estimate the aeration time required to a safe use of them in the patients.

Besides, we intend to evaluate the GFP as a biosensor capable of indicating the distribution and penetration of the EO gas into the load, supplying more data to contribute to a better adjustment of the process parameters for the EO sterilization.

MATERIALS AND METHODS

Oxygenators and Tubing

Oxygenators with a total area of 2.0036 m^2 and weight of 1694.45 (±87.10) g, and Tubing with a total area of 0.0885 m^2 and weight of 1714.38 (±14.96) g are made by the same manufacturer (Nipro Medical Ltda., SP, BR) in a clean room ISO class 8,[15] individually packed in polyethylene-Tyvek® bags and placed inside cardboard boxes to be sterilized by EO.

Load

The sterilization cycles were carried out in a Chamber measuring 1.4 × 4.2 × 2.0 (m), with a volume of 11.76 m^3, with capacity to hold three pallets.

For cycles of the Oxygenator, each pallet 1.2 × 1.2 × 1.9 (m) was composed of 48 cardboard boxes, measuring 33 × 56 × 28 (cm), disposed in six layers with eight cardboard boxes per layer, totalizing 144 units with a density equal to 0.07 g/cm^3 for each Oxygenator.

For cycles of Tubing, each pallet 1.2 × 1.2 × 1.7 (m) was loaded with 96 cardboard boxes 35.5 × 39.0 × 13.5 (cm) disposed in 12 layers with eight cardboard boxes per layer, totalizing 288 units with a density equal to 0.16 g/cm^3 for each Tubing.

Sterilization Cycle Parameters and Aeration Conditions

The Process was performed using a mixture of sterilizing gas (10% EO and 90% CO_2) at a concentration of 470 (±30) mg/L, at 50 (±5)°C with exposure times of 2 h (short cycle), 4 h (medium cycle), and 8 h (long cycle).

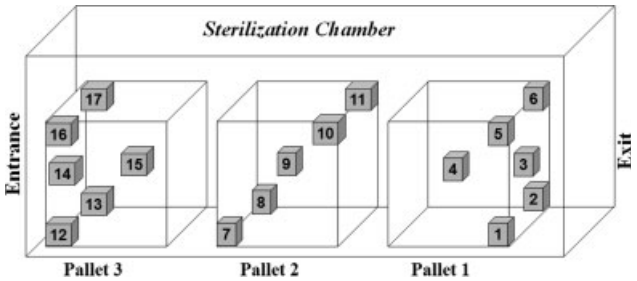

Figure 1. Positions ( ) of temperature and humidity sensors, BIs and GFP distributed in the load of medical devices inside the sterilization chamber.

After each exposure time, the process was finished with five successive cycles of air wash and vacuum 6 (±2) kPa (10-min length each one) to reduce the excess of EO residual content. This facilitates the degassing and also minimizes workers' exposure while unloading.[16]

After being sterilized, the Oxygenators and Tubing were held in an aeration room (30 m^2) at 40 (±5)°C and maximum 30% of relative humidity with 26 changes of air per hour.

BIs (*Bacillus atrophaeus*)

To monitor the sterilization cycles, self-contained BIs of *Bacillus atrophaeus* ATCC 9372 with 1.7 × 10^6 spores inoculated in strips (SGM Biotech, Bozeman, MT) were used distributed geometrically through the load (inside the cardboard boxes) to cover the critical points in the Chamber (Figure 1) determined by previous studies of temperature and humidity. After each sterilization cycle, the BIs were incubated at 36 (±1)°C during 48 h.

Green Fluorescent Protein

The fluorescence intensity of purified recombinant GFP (95% purity, Clontech) was measured in a spectrofluorometer (excitation = 394 nm, emission = 509 nm; RF 5301 PC, Shimadzu Corporation, Kyoto, Japan), which generated a standard curve to determine TPP-extracted GFP concentration: fluorescence intensity (I) = 134.64 + 103.61x (GFP μg/mL); r^2 = 0.98.[14]

An aliquot of 1.0 mL of purified GFP at a concentration of 5.44 (±0.5 μg mL^{-1}), diluted with Tris-EDTA 10 mM buffer pH 8.0 was transferred into glass vials with screw caps and septum of surgical degree paper (100% cellulose, 60 g/m^2), which allows the gas penetration and its contact with the protein.

The GFP vials containers were submitted to lyophilization freeze-drying process[11] and then each vial was packed in a Tyvek® envelope and placed together with Tubing and Oxygenators in the cardboard boxes placed in the same place determined for the BIs (critical points) in pallets.

Determination of EO, ECH, and EG Residues

After the Process and forced aeration step, each item was subjected to the simulated-use extraction. One Oxygenator was withdrawn from the load at intervals of 2 h and 4 h of aeration. For the Tubing, one set was withdrawn after 72 h of aeration, and at intervals of 30 h, 50 h, and so on up to 288 h of aeration time. The devices were withdrawn from the critical points of the load (Figure 1). Each cardboard box was replaced with a similar product to keep the load configuration and avoid air flow preference.

Residual Extraction. The simulated-use extraction was performed under conditions which provide the greatest challenge for the intended use as described in ISO 10993-7,[8] which was based on a closed circuit, simulating a CPB, where purified water (<100 CFU/mL and conductivity <1.3 μS/cm at 25°C) in a volume of 4.0 L for the Oxygenator and 2.0 L for the Tubing filled the path from the venous blood inlet in the circuit up to the arterial blood outlet.

The circuits were connected to a peristaltic pump which provided continuous circulation (6 L/min) of purified water kept warm at 37°C (corporeal temperature). After 6 h of CPB simulation, the collected extract was analyzed to measure the EO, ECH, and EG residues by GC.

Gas Chromatography. After desorbing from the medical devices by simulated-use extraction, 1 μL aliquot of the samples of EO, ECH, and EG was automatically injected into the GC model HP6890 (Hewlett Packard Company, Wilmington, DE) operationally controlled by the ChemStation for HP6890 software. A polyethylene glycol capillary column model HP 19091N-133 (30 m × 250 μm × 0.25 μm) for separation using ultrapure nitrogen as carrier gas running at 1.8 mL/min, and a Flame Ionization Detector (FID) at 250°C supplied with a hydrogen gas flow rate at 35.0 mL/min were used. The maximum temperature of the automatic injector was 260°C at 18 psi of pressure. The LOD and LOQ were estimated respectively, as 0.42 μg/mL and 1.38 μg/mL for EO. For ECH, they were 0.37 μg/mL and 1.22 μg/mL, and for EG, they were 16.71 μg/mL and 55.71 μg/mL.

Residual Level Concentration. The residual levels were determined by GC (given in μg/mL) multiplied by the total extract volume used in the simulated-use extraction (2.0 L and 4.0 L for the Tubing and Oxygenator, respectively), and the results of each sample analyzed, expressed as mg/device, and were used to construct the linear dissipation curves, made on the basis of the established limits for Oxygenator and Tubing which determined their aeration time.

Limits

The maximum limits of residues for Oxygenators and Tubing are ≤60 mg for EO and ≤12 mg for ECH. No exposure limits are set for EG, because the risk assessment indicates that when EO residues are controlled as required, it is unlikely that biologically significant residues of EG will be present, according to ISO 10993-7.

RESULTS

Cycle Parameters

Three sterilization cycles were performed according to the parameters of temperature, humidity, and pressure for 2, 4, and 8 h of exposure to EO.

All the 17 points were monitored by humidity and temperature dataloggers with resolution of 0.1°C and 0.1% RH (Testo 175-H1, Lenzkirch, Germany) programmed to record every 5 min.

The evaluation of the three sterilization cycles (Figure 2) indicated that there is a difference of temperature and humidity in the Chamber between the monitored points, mainly those close to the Chamber doors and the ones in the geometric center of the pallets. Despite that there was no statistically significant difference in temperature or humidity either between pallets or between the three sterilization cycles and their parameters were in accordance with the requirements for a successful EO Process,[17] maintaining regularity on the time of the process, qualifying the equipment.

BI and GFP Performance

For each cycle performed, no growth of *Bacillus atrophaeus* spores was detected after incubation.

After the three sterilization processes, lyophilized GFP was rehydrated in sterile water (1 mL), and the remaining concentrations were evaluated by spectrofluorometer and compared with their initial concentration. The results of the decrease in the intensity of fluorescence are shown in Figure 2 together with temperature and humidity data.

For 2 h of EO exposure, the GFP concentration decrease ranged from 4.8 (±3.2)% to 7.5 (±2.5)% without significant difference between the pallets in the Chamber, although it presented values of 0.17% at point 17 and 10.19% at point 10. The decrease in this cycle was much lower in comparison with the results of 4-h and 8-h cycles.

In the 4-h cycle, the GFP concentration decrease ranged from 17.4 (±3.0)% to 21.5 (±6.8)% and in the 8-h cycle the decrease ranged from 22.5 (±3.2)% to 23.9 (±3.9)%. The 4-h and 8-h cycles showed no significant difference in GFP decrease between either the pallets or the cycles, but presented values of 8.69% at point 14 and 29.62% at point 11 for 4-h cycle and 16.34% at point 2 and 29.41% at point 15 for 8-h cycle.

The 4-h and 8-h cycle allowed better uniformity of internal conditions of the Chamber, and we observed that there is a correlation between the smallest decreasing values of GFP concentration and the coldest points inside the Chamber, which are coincident with the points close to the Chamber doors and its centre. These findings made us believe that there really is a difference in the distribution and penetration of EO in the load inside the Chamber.

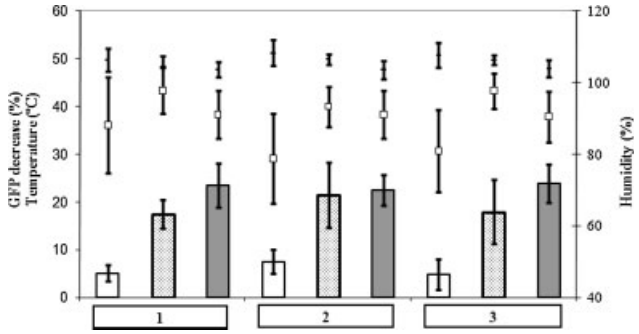

Figure 2. Decrease of the initial concentration of GFP after EO exposure in: ▭ 2-h cycle, ▨ 4-h cycle, and ▬ 8-h cycle. For each cycle, a total of 17 units of GFP biosensors and sensors of (▬) temperature and (▭) humidity were used placed at six points on the pallets 1 and 3 and in five points on the pallet 2.

Gas Dissipation and Residuals Level

For both Oxygenator and Tubing, three sterilization cycles were performed. A total of 18 units of oxygenators were analyzed (six units per cycle) and a total of 24 units of Tubing (nine units in the first and third cycles and six units in the second cycle) were analyzed.

Right after the Process, the oxygenators showed an initial concentration of 35.45 (±3.78) mg/device of EO residual level (Figure 3), what means that these values are lower than the established limits.[8]

After 2 h in an aeration room, 29.21 (±4.32) mg/device of EO residues were remaining in the devices.

Most of the residual concentration dissipates at the beginning of aeration. So, after 16 h under aeration condition, the oxygenators showed a reduction of around 52% of the EO residual comparing with the initial concentration present in this device right after sterilization. This reduction followed the relation: $y = -0.7167x + 29.453$ ($r^2 = 0.94$) for EO residual in mg. Where: y = EO residue in mg/device and x = aeration time.

Regarding these results, Oxygenators did not need to be held in an aeration room after sterilization. As the results of the BI are available only 2 days after sterilization, the Oxygenators are not released before this period. They were held in an aeration room while awaiting these results, when they were released with lower levels of EO around 17.75 (±2.80) mg/device.

The residual levels of ECH (<LOD) and EG (51.70 ± 3.07 mg/device) for the Oxygenator did not exceed the maximum limits established since the first sample was analyzed.

In the Tubing, the first samples of each sterilization cycle were analyzed after 72 h of aeration, when maximum values of EO residual of 283.17 (±81.92) mg/device were found. These values correspond to almost 10 times the EO amount found in the Oxygenator just after its sterilization.

To reach the limits of EO residual established by the current rules, the explicit linear regression (Figure 4) shows that 204 h of remaining in an aeration room are necessary. After this period, the values of 14.97 (±10.63) mg/device of EO residual were reached.

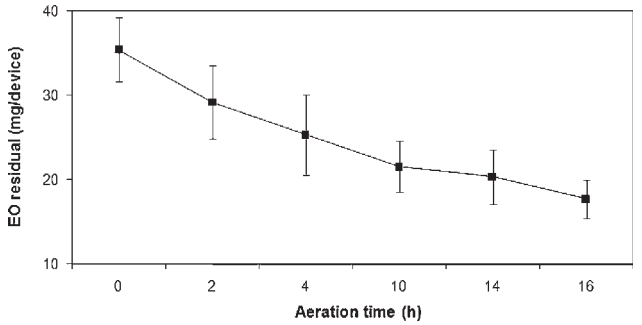

Figure 3. Dissipation of EO residues throughout the Oxygenator. The residual concentrations were below the maximum limit established.

For Tubing, the levels of ECH showed 0.33 (±0.71) mg/device and levels of EG of 66.03 (±18.95) mg/device of residues, which did not exceed the maximum limits established for all samples analyzed.

DISCUSSION

The use of BIs in the Process assures the sterility of the medical devices and the use of GFP allows us to know the extent of the EO distribution throughout the Chamber.

The findings showed that the highest decrease in the GFP concentration was observed at the points located on the surface of load, while the lowest decrease was at the points in the centre of the load.

While the Vital™ oxygenator attained allowed limits of EO residual at the end of the sterilization cycle, its predecessor model (Oxim-II-34 Ultra) required 29 h of aeration to reach safe limits, which may be explained by the difference between the characteristics of each oxygenator.

Vital™ is constructed with polycarbonate housing and uses an area of 2.0 m^2 of polypropylene hollow fiber membrane as a mat format with 180 mL of static priming, whereas Oxim-II-34 Ultra is constructed with polymethyl methacrylate (acrylic) housing and uses an area of 3.4 m^2 of polypropylene hollow fiber membrane as a linear format with 480 mL of static priming.

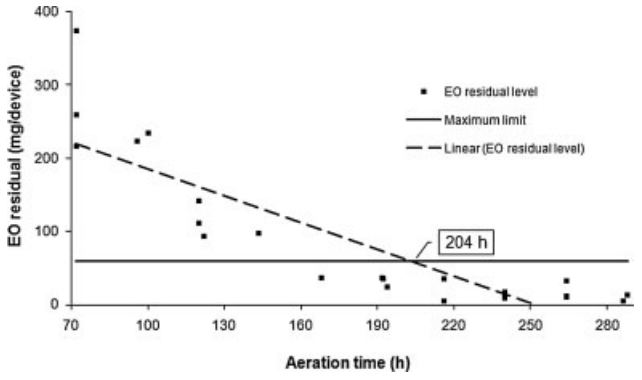

Figure 4. Dissipation of EO residues and determination of aeration time for Tubing. Aeration time (204 h) is needed to reach safe limits.

Vink and Pleijsier studied that although the EO content decreases with the aeration time, it is dependent on the polymer of the device and explained that for PVC the EO concentration is not dependent on the thickness of the material.[18] Even so, PVC is the most widely applied blood-contacting material for the production of blood and blood component storage bags, catheters, and tubing for CPB devices.[19]

PVC is a plastic polymer (molecular weight of 32 g/mol) which is typically added to a plasticizer (softener) to increase the flexibility of the polymer. Di-(2-ethylhexyl)-phthalate (DEHP—molecular weight of 390.56 g/mol) is the plasticizer for most PVC medical devices that increases its chain length[20] and according to Lucas et al.,[3] polymers with increased chain length desorbs EO more slowly, whereas glassy polymers, such as polycarbonate (molecular weight of 134 g/mol) retain the lowest amounts of EO.

Gilding et al.[21] explained that the amount of EO residues is higher in PVC due to the solubility of the EO gas in plasticizers.

The comparison between the results of EO residual in the PVC, polycarbonate, and polymethyl methacrylate run by this study confirms that statement.

CONCLUSIONS

The control of the sterilization process as well as the residues from EO in medical devices is extremely important for the patients' safety.

High amounts of EO adsorbed by PVC articles provide a longer retention time in an aeration room than polycarbonate materials, becoming a great challenge for sterilization companies that can handle it by improvements in sterilization cycle parameters and better aeration conditions.

GFP showed its potential for industrial applications as a biosensor to measure the EO distribution during the sterilization process, reporting the gas action at different points inside the Chamber.

The interaction of GFP with the EO sterilization process is in progress and these preliminary results show interesting perspectives and encourage further studies.

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