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Ethylene oxide sterilization of bone grafts

Residual gas concentration and fibroblast toxicity

Takeshi Arizono, Yukihide Iwamoto, Kiyotaka Okuyama and Yoichi Sugioka

We examined the concentration of ethylene oxide in bone allografts after gas sterilization. Chips of the human femoral head were investigated. Residual gas concentration was determined by gas chromatography after the bone chips had been subjected to defatting and freeze-drying, followed by ethylene oxide gas sterilization. Bones were prepared in various ways in an attempt to reduce the concentration of residual ethylene oxide. The concentration was higher when gas sterilization was performed before freeze-drying than when it was done afterwards. An experiment performed with fibroblasts showed the high toxicity of residual ethylene oxide in bone chips, even when the concentration was very low. The growth of fibroblast was reduced more in medium which had been shaken with bones sterilized with ethylene oxide before freeze-drying than in medium which had been shaken with bones sterilized after freeze-drying. The higher residual ethylene oxide concentrations resulted in a decrease in fibroblastic culture activity. Our experiment showed the importance of reducing the residual ethylene oxide gas concentration. Defatting and freeze-drying result in lower residual ethylene oxide concentrations.

Department of Orthopedics, Faculty of Medicine, Kyushu University, Fukuoka, 812 Japan
Tel +81 92-641 1151, ext. 2433. Fax -632 1793
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Transmission of disease by bone allografts, especially HIV, cannot totally be avoided even by strict screening tests of the donors (Friedlaender 1982, Kakaiya and Jackson 1990). Sterilization of allograft bones by irradiation is effective but it reduces the biological quality of the bone (Maeda et al. 1988, Loty et al. 1990, Anderson et al. 1992) and the osteoinductive capacity (Skinner 1990). We studied the use of ethylene oxide gas for sterilizing, and evaluated the residual ethylene oxide concentrations in gas-sterilized bones and the toxicity in a fibroblast culture.

Material and methods

*Preparation of bone graft for experiments.*We studied bone chips (5 × 5 × 5 mm) which had been harvested from a human femoral head during surgery and stored at –70 °C. After 1 h in room temperature, defatting, freeze-drying and ethylene oxide gas sterilization were carried out under sterile conditions. To reduce the residual ethylene oxide gas concentration in graft bone, we changed the order of the freeze-drying and gas sterilization steps. Defatting was performed by the following method: the bone chips were placed in a methanol/chloroform mixture (1:1) for 8 h at room temperature, following which they were thoroughly washed with methanol to remove the chloroform (Iwata et al. 1981). Ethylene oxide gas sterilization was performed at room temperature. The gas used was Epone-12, which contains 12% ethylene oxide (Liquefied Carbonic Acid Co. Ltd., Tokyo).

*Measurement of bone ethylene oxide concentration.*The bone ethylene oxide concentration was measured by the head-space method (Romano et al. 1973), using a Shimadzu gas chromatogram (GC-4CM; Shimadzu Co. Ltd., Kyoto, Japan) with a 3-mm diameter, 2-m long column. The column was filled with Flexor-8N8 Chromosorb-W (Shinwa Chemical Industrial Co. Ltd., Kyoto, Japan). The temperature of the injector was 120 °C, and that of the column was 50 °C. The carrier gas, at 25 mL/min was N_2. Propylene oxide was used as the inner standard material to make the standard line representing the ratio of the ethylene oxide concentration to that of propylene oxide. This chromatography was used to evaluate (1) bone chips that were defatted, freeze-dried, and then sterilized with ethylene oxide gas, and (2) bone chips which were defatted, then sterilized with ethylene oxide gas, and freeze-dried. The ethylene oxide concentration was measured 2 months after the treatment. Each procedure was repeated 5 times.

Infiltration of ethylene oxide from a surface of bone. Differences in ethylene oxide concentration at various depths from the surface at which the cancellous bone was measured were investigated. The whole femoral head, which was cut from the base of the neck with an oscillating saw, was defatted and subjected to ethylene oxide gas sterilization. After 24 h, bone chips were removed from various levels below the center of the surface of the osteotomized femoral neck in 3-mm increments to measure gas concentration. Each procedure was repeated 4 times.

*Effects of residual ethylene oxide on fibroblast growth.*The toxicity of the residual ethylene oxide in the 2 groups of bone chips was estimated. Bone chips (0.10 g) from each group were shaken with 10 mL of Dulbecco's Modified Eagles Medium (Life Technologies Co. Inc., Gaithersburg, U.S.A.) supplemented with 10% fetal bovine serum (Life Technologies), for 4 hours at 4 °C. Each medium was then filtered (Dismic-25AS; Advantec Toyo Co. Ltd., Tokyo) and separately poured into 3 dishes, onto each of which 1.00×10^4 human fibroblasts had been attached. The number of live fibroblasts was counted after 72 h. This experiment was repeated 4 times.

Statistical analysis was performed by the Wilcoxon single-rank test.

Sterilization of S. aureus *in bone.*The effects of ethylene oxide gas sterilization on bacteria were assessed in bones at various stages of the procedure. *Staphylococcus aureus* Smith diffuse strain was incubated in brain-heart infusion broth (Difco Laboratories, Detroit, MI) for 6 h. The bone chips were placed in the bacterial suspension, and this was then incubated for 6 h. The bone chips were then taken out and washed with phosphate-buffered saline. They were examined for the presence of viable bacteria at three stages of preparation. Bone chips in the first stage were subjected to bacterial examination after defatting; those in the second stage were examined after defatting and freeze-drying. In the third stage, the bone chips were defatted, freeze-dried, and sterilized with ethylene oxide gas before being examined. To determine the presence of viable bacteria, the bone chips in each stage were homogenized with 0.5 mL of phosphate-buffered saline and cultured on normal agar plates 1 week after the preparation of each stage.

Results

*Sterilization of graft bone.*In the bones obtained at the first and second stages, more than 100 colony formations by bacteria were confirmed. However, all the agar plates of the third stage bone chips, which had been sterilized with ethylene oxide gas, were sterile.

Ethylene oxide concentration in bone chips treated with various methods. The concentrations of ethylene oxide in the graft bones differed according to the bone chip preparation. The mean concentration in the group 1 bone chips, which were defatted, freeze-dried, and then sterilized with ethylene oxide, was 2.61 ± 1.3 μg/g, while the group 2 bone chips, which were defatted, sterilized with ethylene oxide gas and finally freeze-dried, had a mean concentration of 18.1 ± 3.8 μg/g ($P < 0.05$).

*Ethylene oxide concentration in bone at different depths from the surface.*The concentration was highest at 6–9 mm from the bone surface ($1.85 \times 10^3 \pm 77$ μg/g), while the concentration at 0–3 mm from the surface was lowest ($1.08 \times 10^3 \pm 1.0 \times 10^2$ μg/g). This indicates that ethylene oxide had already begun to diminish in the surface bone 24 h after gas sterilization and that this gas had infiltrated to a depth of at least 9 mm.

Inhibition of fibroblast growth. Fibroblast growth was inhibited in medium that had been shaken with the gas sterilized bone chips ($P < 0.01$). The rate of inhibition differed in the various preparations. The number of fibroblasts in group 1 (in which the medium was shaken with bone chips, defatted, freeze-dried, and finally sterilized with ethylene oxide gas) was 19100 ± 2900 and that in group 2 (in which the medium was shaken with bones that were sterilized before freeze-drying) was 14000 ± 1300. The corresponding number in the control group (in which the medium was shaken with bones that were only freeze-dried) was 24600 ± 2100 ($P < 0.01$).

Discussion

Gas is frequently used to sterilize clinical materials, including bone grafts. However, the residual gas concentration after sterilization with ethylene oxide is a problem since the gas is toxic (Chichester 1968).

Residual ethylene oxide gas concentration in bone grafts have not been studied. The preparation of bone before gas sterilization varies; no optimal method has yet been found. Moore et al. (1990) reported that bone morphogenetic protein is resistant to degradation by ethylene oxide treatment and that demineralized and ethylene oxide-sterilized bone showed osteoinductive capacity. We believe that a minimal residual ethylene oxide concentration might enhance the osteoinductive capacity.

Herron and Newman (1989) reported on the failure of gas-sterilized bone grafts in spinal fusion; in his study, the graft bones were ethylene oxide-sterilized before they were freeze-dried. At first we believed that freeze-drying after gas sterilization might reduce the residual concentration, since freeze-drying is performed in a vacuum, which may promote aeration in the ethylene oxide gas-sterilized bone. We found a higher gas concentration in group 2 bones (i.e., those which were sterilized before being freeze-dried). This result pointed out the importance of dehydration before gas sterilization. Ethylene oxide gas is very soluble in both water and fat (Chichester 1968). When water remains in the bone, large amounts of ethylene oxide are dissolved, and a high concentration of ethylene oxide infiltrates into the bone chips. After dehydration by freeze-drying, the high concentration of ethylene oxide which has infiltrated may remain in the tissue. Ethylene oxide gas reacts with H_2O and is converted into ethylene glycol (Chichester 1968) which is cytotoxic, and gas sterilization is not effective if this conversion to ethylene glycol occurs. Because of this, graft bones should be dehydrated before ethylene oxide gas sterilization is carried out. Our results indicate that the best method of reducing the residual gas concentration when carrying out gas sterilization of graft bone is first defatting, then freeze-drying, and, finally, sterilizing with ethylene oxide gas.

In our preliminary experiment the number of alive bacteria on graft bones which had been incubated with a bacterial suspension decreased remarkably after defatting. Both chloroform and methanol have bactericidal effects. However, in the procedure of defatting they do not sterilize graft bones totally. Bacteria on graft bone may be different from free bacteria.

The inhibition of fibroblast growth was consistently dependent on the residual ethylene oxide concentration. According to the criteria of the Food and Drug Administration, the maximum permitted concentration of ethylene oxide in bulk materials which weigh more than 100 g and are inserted into the human body, is 25 µg/g (Gardner 1978, Roe et al. 1988). However, we found that even a low concentration of ethylene oxide affected fibroblast growth. This suggests a high toxicity of ethylene oxide and the necessity of reducing its residual concentration.

When ethylene oxide gas is used for graft bone sterilization, defatting and freeze-drying before such sterilization seem to be essential for reducing the residual concentration of ethylene oxide.

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