Ethene oxide and bone induction

Controversy remains

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There is controversy as to whether ethene oxide ("ethylene oxide", EO) sterilization destroys the bone-inducing capacity of demineralized bone matrix (DBM) or not. Correctly performed studies seem to support both opinions. Bone conductive properties of fresh frozen, defatted bone grafts are greatly impaired by EO sterilization, whereas purified inductive proteins resist EO. Studies showing destruction of osteoinductive capacity used nonpulverized DBM, whereas the others used powder. This could be the key to resolving the controversy, because if EO treatment reduces the cells' ability to penetrate a cortical graft and to reach inductive proteins inside it, it may appear noninductive after EO sterilization, even though BMP molecules may be intact. On the other hand, cells could easily penetrate the powder implants.

We compared the effect of EO sterilization on the inductive capacity of demineralized cortical bone with that of DBM powder, using allogeneic material in rats. Cortical pieces lost all inductive capacity by EO sterilization, whereas the powder yielded a calcium content which was at best one fourth of the unsterilized. The concentrations of residual EO, ethene chlorohydrin and ethene glucol at implantation were far below approved levels. Another difference between studies is the humidity during EO treatment. In our hands, humidification reduced bone yield by half.

In conclusion, EO sterilization may impair the biological performance of bone inductive implants by reducing cell penetration into bulk material. However, DBM powder, when correctly sterilized, also yielded scanty amounts of bone.

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There is a controversy about the effect of ethene oxide (EO) sterilization upon the bone inductive capacity of demineralized bone matrix (DBM) implants. Some studies have demonstrated a total loss of bone inductive capacity after EO sterilization (Munting et al. 1988, Aspenberg et al. 1990). In many of Urist's papers (e.g., Sato and Urist 1985), protein powders have proven bone inductive properties after EO sterilization. Recently, Solheim et al. (1995) and Zhang et al. (1997) demonstrated retained bone inductive capacity of DBM after EO sterilization. The latter experiment was performed in the same laboratory as that by Munting et al., which reported opposite findings. Other authors have come to the same conclusion (Moore et al. 1990) and even described studies showing inhibition of bone induction by EO as "not reliable" (Sigholm et al. 1992). At first sight, it appears that one of these two positions must be wrong: the most likely explanation being that the studies which report a loss of inductive capacity used implants in which toxic amounts of EO or EO metabolites were still present. In our previous study, in which bone induction was inhibited by EO sterilization, EO residuals were not measured (Aspenberg et al. 1990). However, the procedure was performed, taking all precautions against residual EO, ethene chlorohydrin and ethene glucol. One difference between the two studies which showed destruction of inductive capacity (Munting et al. 1988, Aspenberg et al. 1990) and the others is that the former used intact demineralized segments of diaphyseal bone, whereas the latter in most cases used fragmented material, powders or segments with drilled microperforations. This difference seemed of minor importance, until it was demonstrated that EO sterilization of fresh frozen defatted bone greatly impaired its osteoconductive capacity (Thorén and Aspenberg 1995). In the nonpulverized DBM implants, induced bone is usually formed inside the cortex of the demineralized bone segment. Thus, cells have to penetrate the matrix before they are exposed to sufficient concentrations of inductive proteins. In powders, however, bone forms between the DBM particles. Thus, intact pieces and powders differ by the necessity for penetration. If EO sterilization impairs this penetration capacity, as suggested by its effects on osteoconduction, but leaves inductive pro-

teins intact, this could explain the contradictory results of EO sterilization.

Apart from the type of DBM used, the studies also differ as regards humidity during EO treatment. With low humidity, EO may not penetrate the DBM. If the humidity is below 30%, spores can survive. Humidity may be hard to control in less advanced types of gas sterilizers. An alternative explanation of the conflicting findings on EO sterilization of DBM could be that a low humidity process yields DBM with intact osteoinductive capacity.

This study compares the effect of EO sterilization of demineralized rat femoral segments versus demineralized rat bone matrix powder. Further, we report the effect of humidity and temperature during EO treatment.

Animals and methods

Study 1. Cortical DBM versus powder

Animals and DBM production. Mid-diaphyseal femoral segments were obtained from 25 rats. 8-mm long segments were cut with a cooled saw-blade, cleansed from periosteum and marrow, rinsed in water, defatted in chloroform methanol (1:1 3 h), rinsed in methanol and demineralized in 0.6 M HCl (20 °C, 24 h), thoroughly rinsed in sterile water and freeze-dried. The dried segments weighed 12.4 (SD 1.0) g. Each segment was placed in a separate air-permeable paper and plastic envelope for EO sterilization but, in each of 10 pairs, only 1 segment was sterilized. All segments in the remaining 5 pairs were also sterilized and used to measure EO residuals.

Tibias and femurs from the remaining donor rats were prepared as described above, but after defatting and rinsing in methanol, they were milled. Particles 125–640 µm were sieved out, demineralized, rinsed and freeze-dried as above. The powder was then divided into three 200 mg portions and packed in air-permeable paper and plastic envelopes. 2 of the portions were sterilized, 1 for implantation and one for EO residual measurement.

Sterilization. The sterilization was done with a Steri-Vac 4 XLP gas-sterilizer (3M Company, St. Paul, Minnesota). The exposure was the standard cycle of 240 minutes at 37 °C, ethene oxide gas concentration 1159 mg/L, and a relative humidity of 63%. After treatment, the packages were air-dried at 37 °C for 20 h. They were then kept dry and ventilated at room temperature until implantation 4 weeks later. The effect of sterilization was controlled by using Bacillus subtilis spores (Attest 3M Company, St. Paul, Minnesota).

The analysis of residual amounts of ethene oxide, ethene chlorohydrin and ethene glycol was performed on the same day as the implantation. The 10 diaphyseal segments were pooled and extracted in acetone during vigorous shaking for 1 hour. The 200 mg powder was extracted separately. The measurement was then performed with gas chromatography using a flame-ionizing detector (Analyslaboratoriet i Lund AB, Lund, Sweden).

Implantation. 10 rats received demineralized femoral segments. Each rat was given 1 sterilized and 1 unsterilized segment from the same donor. The segments were implanted in muscle pouches created by blunt dissection between the 2 oblique muscle layers lateral to the rectus abdominis. 10 rats similarly were given DBM powder, 20 mg per implant site, 1 sterilized and 1 unsterilized specimen per animal.

Evaluation. All rats were killed after 3 weeks. The implants were recovered, fixed in buffered formaldehyde and decalcified in 10 mL Parengy's solution (chrome trioxide 0.15%, nitric acid 4.3% and ethanol 27%), embedded in paraffin and serially sectioned for qualitative histology. Decalcification was done for 3 weeks and the decalcifying solution was sent for analysis of calcium concentration, using atomic absorption spectrophotometry. The Ca content of each EO sterilized implant was divided with its unsterilized control to produce a ratio. The ratios for segments and powders were compared, using Student's t-test.

Study 2. Effect of humidity

DBM production. 600 mg DBM powder was produced and packed in 4 portions of 150, exactly as above.

Sterilization. This time, we used a gas sterilizer specially designed for research on gas sterilization (Vacudyne Altair; normally used in conjunction with development of sterilization cycles for hemodialysis products at Gambro AB). All procedures consisted of the following steps: Prevacuum 50 mbar absolute pressure, conditioning at preset humidity for 20 min, injection of sterilization gas to a predetermined pressure dependent upon temperature and humidity, exposure to an EO gas concentration of 500 mg/L (gas mixture 10% EO and 90% CO_2 by weight), removal of sterilization gas by evacuation to m100 bar and air inlets to 950 mbar 4 times, and finally pressure equilibration to atmospheric pressure.

Temperature, exposure time and humidity were changed to produce 4 different cycles, as follows:

1. 37 °C, 4 h, 90% humidity
2. 37 °C, 4 h, <10% humidity
3. 22 °C, 16 h, 90% humidity
4. 22 °C, 16 h, <10% humidity

Table 1. Calcium yield (µg) from bulk and pulverized DBM after EO sterilization 4h in a Steri-Vac 4 XLP gas-sterilizer, humidity 63%. Mean (SD)

	EO	Control
Bulk segment	6 (3)	920 (290)
Powder	1090 (380)	4090 (970)

Table 2. Calcium yield from DBM powder with different EO treatments in a Vacudyne Altair gas-sterilizer. Mean (SD)

Temp, °C	Time, h	Humidity, %	Calcium, µg
37	4	90	340 (290)
37	4	<10	760 (430)
22	16	90	160 (140)
22	16	<10	450 (340)

During the sterilization, biological indicators from AMSCO were used to monitor the sterility assurance level. The indicator organism was *B. subtilis globigii* (lot GL 106C) with a spore load of 3.2×10^6. The spores were inoculated on a cellulose paper strip, and the strip was packed in a glassine envelope. During each sterilization, 6 indicators were placed close to the packages with the DBM. Directly after the sterilization cycle, the biological indicators were removed aseptically and forwarded to a plus/minus control in tryptone soya broth (Oxoid CM 129). The incubation was done at 32 °C and results were read after 7 days.

Implantation. 8 rats received one 15 mg sample from each of the 4 procedures. Implants were retrieved and evaluated as above. There were no measurements of EO residue or metabolites.

Results

Study 1. Cortical DBM versus powder

1 rat in each group died immediately postoperatively. All other animals completed the study. EO sterilization stopped bone induction in the DBM segments, whereas in the powder the induced amount of calcified tissue was about one third of the unsterilized controls (Table 1). The ratio EO/control was 0.007 for segments and 0.27 for powder ($p < 0.0001$).

Histology confirmed the presence of new bone in all unsterilized implants, except one powder specimen. Apart from the absence of cartilage and bone, sterilized segments appeared similar to the controls. Sterilized powder showed infiltration mostly of spindle-shaped cells with large nuclei, presumably macrophages, between the DBM fragments. Multinucleated giant cells were common, and ossicle formation with marrow was absent. However, it can not be excluded that some areas consisted of new primitive woven bone between DBM fragments. In contrast, controls contained several areas of bone marrow, surrounded by new bone. In areas where no new bone was formed, there was a loose collagenous tissue, markedly different from the cell-rich infiltrate in the EO sterilized samples.

The residual amount of EO was below the detection limit of 10 ppm in segments and powder. Ethene glycol was likewise below the detection limit of 20 ppm in segments and powder. The DBM segments contained 27 ppm ethene chlorohydrine, whereas the powder was below the detection limit of 15 ppm.

Study 2. Effect of humidity

All B subtilis cultures were negative after both high humidity procedures, whereas 5/6 were positive after procedure 2, and 6/6 after procedure 4. Histologically, there was no macrophage infiltration, except partially in one rat. Small amounts of bone were seen in 18 specimens, although only one section from each specimen was studied. The calcium yield was generally lower than before (Table 2). Humidification reduced calcium contents by more than half (2-way Anova; $p < 0.003$) and there was less calcium after the 16-h procedure ($p < 0.04$).

Discussion

EO sterilization eradicated the inductive capacity of demineralized bone segments, whereas the inductive capacity of DBM powder appeared to be partially retained. The amounts of EO, ethene anhydride or ethene glycol in the implants was at least one order of magnitude below the maximum acceptable levels for small bone grafts, according to the US Food and Drug Administration (Gardner 1978). Regarding nonpulverized segments, the present findings confirm those previously reported by us (Aspenberg et al. 1990), this time also with measurements of EO and its main metabolites. The findings by Solheim et al. (1995) and Zhang et al. (1997) also appear to be confirmed, since DBM powder retained parts of its activity, as judged by the Ca content. However, they found no histological differences and no quantitative impairment of mineralization.

As partially purified BMPs (Ijiri et al. 1994) and other members of the TGFß super-family (Puolakkainen et al. 1993) withstand EO sterilization without loss of activity, it seems unlikely that BMPs were des-

troyed in any of the implants in the present study. Therefore, EO sterilization must have effects on other components of the bone induction process, notably on components which are partly dispensable in pulverized implants. These components are probably related to the strong impairment of osteoconductive properties found after EO sterilization of fresh-frozen defatted bone grafts (Thorén and Aspenberg 1995) or to the altered surface ultrastructure of EO sterilized DBM (Doherty et al. 1993). After EO sterilization, the penetration of new cells into DBM segments may be hindered, so that no proper contact with BMPs inside the matrix takes place. Only between DBM particles or small fragments, as well as in drilled microperforations, can osteoinduction still occur as a result of sufficient concentrations of factors that have diffused out from the matrix.

The result that we had hoped for in study 1 was that EO would eradicate bone induction totally in non-pulverized DBM (which it did), and not at all in the DBM powder. In that case, the seemingly contradictory earlier results of various authors might fit together into one single coherent explanation. However, as the calcium yield was diminished to one fourth in the powder, we can only say that EO effects on conduction appears to play a role, besides other factors. The macrophage infiltration and the uncertainty as regards histologic bone in the powder implants caused more concerns.

Therefore, we performed study 2 and found that humidification may also have been partly responsible for our low calcium yield with powder. We had used humidification, which is necessary to ensure the full effect of EO, whereas Sigholm et al. (1992) and Solheim et al. (1995) had not.

According to the results of our studies 1 and 2, the controversy may depend partly on the effects of EO on cell conduction, and partly on humidification. However, neither DBM pulverization nor humidity can explain more than a minor part of the differences between publications. Even when our EO treatment was insufficient to kill B subtilis spores, the powder implants yielded only tiny amounts of bone. It is clear that the effects of EO depend on both the DBM material and the procedure, and the controversy remains unsolved. A larger problem, not addressed in this study, is that there is no evidence for bone induction by DBM in man, and there are reasons to suspect that it does not work, even if not sterilized at all (Aspenberg et al. 1988).

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