Ethylene oxide does not extinguish the osteoinductive capacity of demineralized bone

A reappraisal in rats

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We examined the influence of ethylene oxide (EO) and gamma irradiation on the osteoinductive capacity of demineralized bone. Demineralized bone powder prepared from Wistar rats was exposed to EO (55 °C or 40 °C) or gamma irradiation (25 KGy) or was preserved in ethanol. Sterilely-prepared bones served as controls. The powder was packed in a gelatin capsule and implanted for 6 weeks in muscles of 6-week-old female rats. Exposure of demineralized bone particles to EO 55 °C resulted in an almost complete loss of osteoinductivity. Irradiated bones lost about 40% of their osteoinductive capacity, while sterilization with EO at 40 °C resulted in only a slight alteration of the osteoinductivity, as assessed by the recovered weight ratio, calcium content, alkaline phosphatase activity measurements and histomorphometry. Ethanol treatment had no influence on the new bone yield when compared to controls.

As EO exposure at 40 °C is a true sterilization procedure, it can be recommended in a clinical setting for its small effect on osteoinductive capacity as assessed experimentally in rats.

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The availability of several inductive proteins produced by biological engineering will certainly reduce the demand for an osteoinductive material like that prepared from cortical bone by demineralization. Nevertheless, the use of demineralized bone in patients may still remain justified when a large intraosseous defect is to be filled (Kakiuchi et al. 1985, Upton and Glowacki 1992, Whiteman et al. 1993, Wilkins and Stringer 1994). We have previously shown that the modality of HCl demineralization and the type of preservation had no influence on the new bone yield and that freeze-dried demineralized bone had more than a 10-year shelf-life (Delloye 1985, 1986). However, like any implant, demineralized bone must be sterilized for clinical use.

The influence of ethylene oxide (EO) on the osteoinduction capacity of demineralized bone implants remains controversial. The temperature to which the bone is exposed during either the sterilization period or the aeration phase and the presence of toxic by-products generated by ethylene oxide are the main causes of interference with the induction capacity (Arizono et al. 1994, Ijiri et al. 1994),

Because of the conflicting results, we reappraised the influence of ethylene oxide on bone induction and compared it to sterilized and sterilely-prepared implants.

Material and methods

Implant preparation

Femoral and tibial shafts from 8-week-old female Wistar rats were harvested and immediately frozen. After thawing, they were cleaned of marrow and periosteum, then milled to powder that was sieved to retain the fraction containing a particle size of 125–800 μ. The powder was defatted in 1:1 chloroform-methanol for 2.5 hours, sequentially rinsed in methanol and distilled water for 30 minutes at each step and then demineralized in 0.6 N HCl for 48 hours. All the chemical extractions were carried out at 4 °C during constant stirring. The particles were freeze-dried for 2 days and weighed. From the demineralization process to the implantation stage, the bone preparation was carried out sterilely. 30 mg of demineralized bone particles were packed in a number 5 gelatin capsule. All the capsules were sterilized with gamma irradiation at a dose of 25 KGy.

Sterilization

According to the sterilization technique, implants to be sterilized were packed in a double peel-off system and divided into 4 groups.

Group I: Implants were exposed to 10% ethylene oxide in CO_2 at a pressure of 1.1 Kg/cm^2, 50% hu-

midity, at 40 °C for 8 hours. They were aerated for 2 days at room temperature. Every cycle was monitored with 2 samples of *bacillus subtilis* (10^6 organisms) that were cultured for 2 weeks after completion of a cycle.

Group IIa: Implants were processed in the same way as group I, but at 55 °C. This group was discontinued after the first 8 animals and replaced by group IIb.

Group IIb: Implants were exposed to 25 KGy of gamma irradiation for 16 hours at room temperature.

Group III: Implants were immersed in ethanol at 70° for 8 hours at room temperature.

Group IV: Implants in this group served as controls and were prepared sterilely.

Animal implantation

Before implantation, implants were rehydrated with an intracapsular injection of saline. They were recovered after 6 weeks of implantation. 35 6-week-old female Wistar rats received each one capsule from each group in the paravertebral muscles. For each animal, the respective location of the 4 capsules was turned clockwise to avoid any influence of topography. All the implants were recovered and no infection was observed. There was no growth of *bacillus subtilis* after a complete exposure to ethylene oxide at 40 °C or 55 °C.

Analytical procedure

At harvest, all the implants were blot-dried and weighed. A weight index was calculated by relating the weight at recovery to the preoperative weight.

Some of the specimens were further freeze-dried, weighed and assessed for calcium content and enzyme activity. A final dry-weight index was calculated by relating post- and preoperative weights of the freeze-dried implants. Calcium was determined after overnight dissolution of the implant in 12 N HCl by atomic absorption spectrophotometry and expressed as weight of calcium/weight of implanted matrix. For measurement of alkaline phosphatase activity, freeze-dried samples were cut in small pieces and homogenized in ice-cold 0.003 M $NaHCO_3$ buffer at pH 7.4 for 30 seconds and then centrifuged at 4500 rpm at 4 °C for 15 minutes. Supernatants were assayed by the modified method of Lowry et al. (1954), using the p-nitrophenol reaction. Results were expressed as international units (UI) per mg of initial weight.

The other implants were dehydrated, decalcified, paraffin-embedded and longitudinal sections were made for histological assessment of new bone formation. Morphological quantitation was performed with the point-counting technique using an eyepiece graticule at a magnification of 63×. The presence of new bone was evaluated and counted when living osteocytes were observed. The number of counts that intercepted new bone and bone marrow when present was related to the total number of hits occupied by the bone implant (old and new bones). The whole section of the bone implant was scanned and counts were made in triplicate. The surface occupied by the new and old bones was also measured on 3 consecutive sections and expressed in mm^2.

Statistics

All values are reported as means and standard deviation. Comparisons between groups were made by analysis of variance. Analysis of pair-wise differences was performed with Tukey's criterion (Snedecor and Cochran 1980). Differences were considered significant at the 0.05 level.

Results

Sterilization with ethylene oxide and gamma irradiation resulted in a recovery of a lighter implant when compared to alcohol-preserved implants (group III) and sterilely-processed implants (group IV). Using the weight of the blot-dried implants at recovery, exposure to EO at 55 °C showed the strongest impairment in the new bone yield, followed by irradiation and EO at 40 °C (Table 1). Compared to controls, the reduction in weight at harvest of the sterilized implants with EO 55, irradiation and EO 40 represented 66% ($p = 0.00002$), 38% ($p = 0.00005$) and 17% ($p = 0.03$) of the average recovered weight of the sterilely-prepared implants. Alcohol-preserved implants did not differ from controls. As some of the implants had been freeze-dried and weighed, the final freeze-dried weight was also related to the initial weight. Only irradiated implants weighed less than the controls ($p = 0.001$), while implants exposed to 40 °C and those that were preserved in alcohol had an average weight that did not differ from the controls. Since ethylene oxide at 55 °C had a very harmful effect, its further use was discontinued.

There was no difference in the calcium content nor in the enzyme activity of the recovered implants amongst the different groups, apart from trends to less osteogenic activity in the sterilized implants (Table 2).

Histomorphometry showed that there was less new bone and bone marrow in the old matrix that had been sterilized with ethylene oxide at 55 °C ($p = 0.02$), but no difference was found in the other groups (Table 3). Axial section measurement of the area occupied by the old matrix and new bone in the recovered im-

Table 1. Implant data (weight measurements). Mean (SD)

Type of implant	No. of samples	Final weight (final wet/initial weight)	No. of samples	Final dried weight (final dried/initial weight)
EO40 (group I)	35	3.54 (1.05) [a]	18	1.45 (0.40)
EO55 (group IIa)	8	1.45 (0.99) [b]	–	
Co60 (group IIb)	27	2.63 (1.10) [b]	18	1.06 (0.48) [b]
Alcohol (group III)	35	4.63 (0.78)	18	1.95 (0.48)
Control (group IV)	35	4.26 (1.09)	18	1.74 (0.56)

[a] $p < 0.05$ as compared to the control group
[b] $p < 0.01$ as compared to the control group

Table 2. Implant data (chemical analysis). Mean (SD)

Type of implant	No. of samples	Calcium (mg/mg of initial weight)	No. of samples	Alkaline phosphatase (UI/mg of initial weight)
EO40 (group I)	18	0.16 (0.08)	18	24 (7.1)
EO55 (group IIa)	–		–	
Co60 (group IIb)	18	0.13 (0.07)	18	20 (7.4)
Alcohol (group III)	18	0.22 (0.10)	18	26 (9.1)
Control (group IV)	18	0.20 (0.08)	18	23 (6.7)

Table 3. Implant data (morphometry). Mean (SD)

Type of implant	No. of samples	Histomorphometry (ratio of new bone/bone implant)	No. of samples	Longitudinal section (old and new bone area in mm^2)
EO40 (group I)	17	0.20 (0.18)	17	22 (4.1)
EO55 (group IIa)	8	0.05 (0.08) [a]	8	13 (7.5) [b]
Co60 (group IIb)	9	0.25 (0.18)	9	19 (7.2) [b]
Alcohol (group III)	17	0.23 (0.15)	17	29 (6.5)
Control (group IV)	17	0.25 (0.12)	17	27 (5.5)

[a] $p < 0.05$ as compared to the control group
[b] $p < 0.01$ as compared to the control group

plants showed that sterilization resulted in a smaller implant than the non-sterilized implants but only irradiation ($p = 0.005$) and ethylene oxide at 55 °C ($p = 0.0008$) caused an area decrease compared to controls (Figures 1–3). Qualitatively, there was no distinctive histological feature in any groups. New bone was found only in the old matrix.

Discussion

Although our investigation indicates that gas and irradiation sterilization impair the formation of new bone, these observations do not confirm our previous findings of a major deleterious effect of EO exposure on the osteoinductive capacity (Munting et al. 1988). Since our initial report of a negative effect of EO, conflicting results have been reported in the literature. Using heterotopic implantation of demineralized bone matrix as a model of osteoinduction, a severe impairment has been observed with EO sterilization by Munting et al. (1988), Aspenberg et al. (1990), Jergesen and Dhillon (1995) whereas Urist (1968), Sato and Urist (1985), Köhler and Kreicbergs (1987), Moore et al. (1990), Sigholm et al. (1992), Solheim et al. (1995) found no difference.

With the use of extracted or recombinant Bone Morphogenetic Proteins (BMP), Yasko et al. (1992) reported good healing of a segmental bone defect in rats after exposure to EO, while Ijiri et al. (1994) found a variable effect according to the sterilization temperature.

As with the residual gas concentration in the implants, the temperature at which EO is delivered is also a critical parameter since initiators of osteoinductivity are proteins. Sterilization of medical equipment with EO is usually performed at 55 °C. The same temperature is used for the degassing phase. However, as

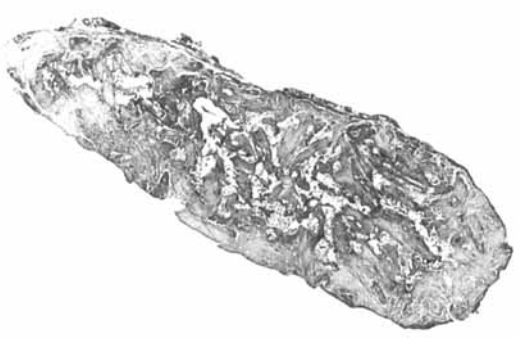

Figure 1. Photomicrograph of a longitudinal section from a retrieved bone implant that has been pulverized, defatted, demineralized and ethanol-preserved. No secondary sterilization was carried out. 30 mg of particles were placed in a gelatin capsule and implanted intramuscularly for 6 weeks. New bone with hematopoietic marrow was found in the old bone matrix (HE, ×18).

Figure 2. Photomicrograph of an axial section from an ethylene oxide (EO, 40 °C)-exposed implant after a 6-week implantation. Qualitatively, new bone formation did not differ from that in the control group (HE, ×18).

Figure 3. Photomicrograph of a longitudinal section from an irradiated bone implant (25KGy) after 6 weeks of implantation. The size of such an implant was reduced but, inside, features of new bone formation were similar to the other groups (HE, ×18).

BMP is heat-sensitive and inactivated at 60 °C, cold cycles of EO sterilization have been developed. Our findings with EO at 55 °C agree with the literature (Ijiri et al. 1994). But even at physiological temperature, conflicting results concerning osteoinduction impairment have been reported. In our laboratory, Munting et al. (1988) observed marked impairment while we did not.

The only major variable that could explain such variation is the more complete defatting performed in this study. Ethylene oxide gas is very soluble in both water and fat. Arizono et al. (1994) showed that defatting and freeze-drying before EO exposure resulted in a lower residual EO concentration than freeze-drying after EO exposure. These authors also found that this difference in the residual gas concentration was large enough to inhibit the growth of cultured fibroblasts. As our implants were thoroughly defatted in chloroform-methanol solution, rinsed in methanol and distilled water, we hypothesize that they contained less EO residuals than in our previously reported experiments (Munting et al. 1988), with less impairment of osteoinductivity. Among other variables that could affect the results are the ages of the animals, which were younger in the present experiments, the use of pulverized bone rather than a bone segment and a 10% rather than 12% concentration of ethylene oxide.

Aspenberg et al. (1990) also reported negative results using EO-sterilized and defatted implants in rats. However, there was already a marked impairment after a short exposure of 5 minutes, so that a systematic negative factor associated with the sterilizing procedure (i.e., temperature and duration of the aeration phase) cannot be ruled out.

On the other hand, our previous findings that gamma irradiation inhibited or only partially destroyed the osteoinductive capacity was confirmed. The negative effect of gamma irradiation was not so strong as previously reported, but this difference can be explained by the variation in the animal's age, the use of particles rather than segments of diaphysis and the time of implantation. As also observed by Schwarz et al. (1988), irradiation differed only from non-irradiated ones by the weight measurements but not by biochemical assays or histomorphometry. In other words, irradiation of an osteoinductive demineralized bone implant results in a smaller residual bone matrix that induces a smaller amount of new normal bone.

From our experiences with EO and osteoinductive implants, we conclude that the chief variable that will interfere with osteoinductivity is the temperature at which implants are exposed to EO. The colder the sterilization cycle, the better preserved the inductive capacity will be. Among other variables that may influence the osteoinductive capacity, the sequence of processing (freeze-drying before sterilization) and the type of processing (demineralization with or without preliminary defatting process) may also interfere with osteoinduction. Since ethylene oxide can produce true sterilization, as its influence on the osteoinductive

property of demineralized bone is low compared to that of other sterilants, when assessed experimentally in rats, its use can be recommended in a clinical setting.

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