

Effect of sterilization on osteoinduction

Comparison of five methods in demineralized rat bone

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The aim of this study was to find a safe, effective sterilization method that does not destroy the bone-inductive capacity of demineralized bone implants. Five sterilizing agents were tested in rats. Implants procured and processed under sterile conditions served as controls. New bone formation was evaluated by determining dry weight, calcium content, and Sr-85 incorporation of the induced ossicles.

Glutaraldehyde solution, formaldehyde gas, and ethylene oxide destroyed almost all the bone-inductive capacity. Irradiation by 2.5 Mrads Co-60 resulted in a loss of about half of the inductive capacity. Merthiolate (0.18 per cent) was the only sterilizing agent that did not reduce the bone-inductive capacity of the demineralized implants. Because merthiolate is not sporicidal, gamma irradiation appears to be the most appropriate sterilizing agent for demineralized bone in clinical use.

In bone-banking procedures, tissue may be obtained by nonsterile procedures. Specimens can be trimmed and cut to different sizes and eventually processed chemically. Subsequent sterilization has to rely on an effective agent that does not interfere with osteoconduction and osteoinduction.

Whereas nondemineralized alloimplants have almost no bone-inductive capacity (Dubuc and Urist 1967, Chalmers et al. 1975), demineralized bone has been shown to be bone-inductive orthotopically, as well as heterotopically (Urist 1980, Tuli and Singh 1978).

We have studied the effect of different sterilizing agents on the inductive capacity of allogenic demineralized bone implanted in muscle pouches in rats.

Material and methods

Femurs were excised from 36 inbred, 2-month-old adult female *lou/dec* Wistar rats. Removal and preparation were undertaken under sterile conditions within 3 hours of death. The bones were freed from soft tissues, and periosteum, epiphyses, and marrow were removed. The diaphyses were washed in distilled water and subsequently demineralized in 0.6 N HCl at 4°C for 24 h. The demineralized bones were rinsed in distilled water, cut to 6 mm-long segments, freeze-dried, and stored for 8 months in sterile vials until sterilization and implantation. The dry weight of the implants was determined before implantation.

The implants were divided into five groups, each consisting of 15 specimens that were sterilized as follows:

Gamma irradiation. Implants were exposed to 2.5 Mrads of Co-60 gamma irradiation (Institut des Radio-Éléments, Fleurus, Belgium).

Merthiolate. Each implant was immersed in 15 ml of 0.18 per cent merthiolate in isotonic saline (Thimerosal, Federa Laboratories, Brussels, Belgium) at 37°C for 1 h and then rinsed in distilled water.

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Glutaraldehyde. Each implant was immersed in 15 ml of 2 per cent glutaraldehyde (37°C, pH 7.3) for 45 min and then rinsed in distilled water.

Formaldehyde. Implants were packed in a double-sterile packing system and exposed to a low temperature, steam, formaldehyde cycle for 5.5 h (37°C, 3 mg sterilant/L, humidity 70 per cent). The implants were degassed after sterilization for 48 h in an aeration cupboard to remove residual formaldehyde.

Ethylene oxide. Implants were packed in a double-sterile packing system and subjected to the gas for 6 h (37°C, pressure 10 psi, humidity 33 per cent). Residual ethylene oxide was removed by 48 h degassing.

Exposure times to formaldehyde gas from 2.5-5.5 h were tested. The latter time was found sufficient to ensure destruction of 10^6 *Bacillus subtilis* spores cultured from a AMSCO Spordex biological indicator. The spores were injected as a suspension in the medullary canal of freeze-dried rat tibial shaft and air dried for 24 h under laminar flow. The time to kill 90 per cent of the population of this preparation was 6.8 min when unpacked (Hennebert 1986). The minimum time for sterilization by ethylene oxide was determined in the same way, but on cubes of washed, demineralized, and freeze-dried human cancellous bone. Exposure times of 2, 4, 6, and 8 h were tested. Six hours' exposure time was found to be enough to sterilize samples contaminated with 10^6 *Bacillus subtilis*. Samples of each group were cultured to check the efficiency of the agents. No bacterial growth was observed after 2 days of culture.

The recipients were 65 inbred, 3-month-old female Wistar rats, which were divided into five groups each consisting of 13 animals. Each animal received one sterilized and one control implant. Before implantation the freeze-dried implants were immersed in distilled water at room temperature. Surgery was performed under neuroleptic analgesia (Hypnorm® Janssen, Belgium) under sterile conditions. The implants were placed in the paravertebral muscles at the lumbar level. The animals were fed ad libitum on regular rat chow. Two months after implantation, the animals were killed and the implants recovered. In addition, the iliac bones were harvested to be used as controls of host skeleton Sr-85 activity.

Analytic procedures. Dry weight of all the recovered implants was measured and related to the initial dry weight. The implants and ilia were then hydrolyzed overnight in 10 ml 7N HCl and the calcium content determined fluoroscopically (Marius Instruments, The Netherlands). In 10 samples of nonimplanted demineralized bone, no residual calcium could be detected by this method. Calcium content was found to be 20 parts per million when measured by atomic absorption.

To assess the osteogenic capacity of a given implant, the calcium content was related to initial weight of the implant.

Intraperitoneal injection of 10 mCu of Sr-85 as SrCl₂ per 100 g of body weight, 3.5 days before death, allowed us to estimate new bone formation in the implants and ilia during that period (Elves 1974). Radioactivity was determined in a Packard Model 3385 gamma counter. Background activity was subtracted and data were adjusted to give counts per min per mg of sample. By relating implant activity to ilium activity, an osteogenic index was calculated reflecting the activity exclusively due to bone induction by bone matrix.

The presence of new bone in the different implants 2 months after surgery was assessed by histology. The implants of 2 rats of each group were either demineralized, paraffin embedded, sectioned, and stained in hematoxylin and eosin or dehydrated in methanol, methyl metacrylate embedded, sectioned, and stained in toluidine blue or cyanine solochrome.

Statistical analysis. Mean values and standard deviations were calculated. The results were compared by covariance analysis, taking into account the values of the control implant in order to eliminate interindividual variations. Difference was considered significant at a $P < 0.05$ level (Student's *t*-test).

Results

Calcium content related to the initial weight; merthiolate-treated implants and controls had similar values. Gamma irradiation caused a decrease of about 60 per cent while 83 per cent was lost after exposure to ethylene oxide. Formaldehyde- and glutaraldehyde-treated implants did not contain calcium at recovery (Table 1). A

Table 1. Effect on bone formation by bone matrix subjected to sterilization. Mean values (SD)

	Control n 55	Co-60, Mrads n 11	Merthiolate n 11	Glutaraldehyde n 11	Formaldehyde n 11	Ethylene oxide n 11
Ca content (mg) of recovered implants	1.61 (0.78)	0.63 (0.23)*	1.79 (0.65)	0.02 (0.03)*	0.11 (0.15)*	0.28 (0.40)*
Ca content of recovered implants/weight implanted matrix	0.17 (0.08)	0.07 (0.03)*	0.19 (0.07)	0.00 (0.00)*	0.01 (0.02)*	0.03 (0.05)*
Sr-85 activity	5.9 (10.6)	11.9 (22.5)	4.2 (2.3)	-	-	3.8 (3.5)

Mean values (standard deviation).

* Significant difference as compared with control.

marked difference in the osteogenic index was found among the groups. The Sr-85 uptake was higher in the implants of the irradiated group as compared with the others. The uptake of the implants of the glutaraldehyde- and formaldehyde-treated groups was about the same as the background activity. Therefore, the osteogenic index calculated for these groups was not relevant. In the ethylene oxide group, there was a large

discrepancy in the results; the activity of most of the implants was only slightly above background.

Histology confirmed the absence of bone formation in the glutaraldehyde- and formaldehyde-treated implants. Only scanty bone formation was observed in ethylene oxide-sterilized implants (Figure 1). Control and irradiated implants consistently induced new bone formation (Figure 2).

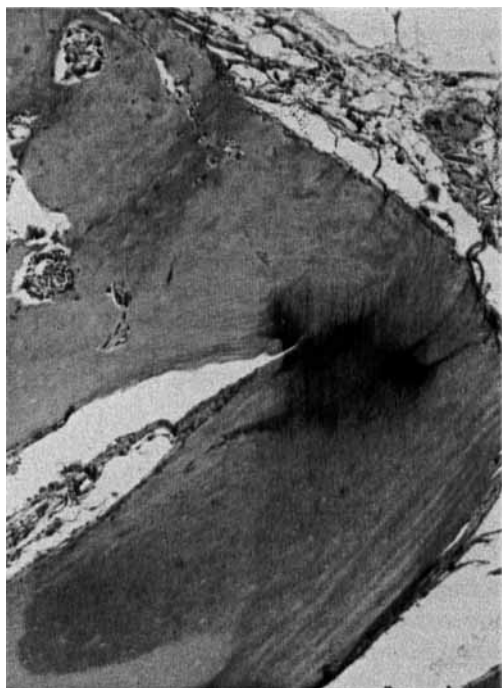


Figure 1. Ethylene oxide-sterilized implant showing mostly old matrix with very scanty bone formation. HE, x16.



Figure 2. Control implant: new bone, osteoblasts, and residual matrix are present undecalcified section, Solochrome cyanide, x16.

Discussion

Over the years, irradiation has been the most widely used method for decontaminating human tissue. Irradiation is lethal for cells and bacteria by direct collision of particles with nuclear proteins. Moreover, the ionized particles split the bonded water molecules, always present in tissue, creating hydrogen and hydroxyl free radicals, which are fatal for bacteria and cells (Turner et al. 1956). Sterilization of freeze-dried tissues requires higher doses to release bound water molecules (Cook et al. 1963). As opposed to the findings of Buring and Urist (1967) and Urist and Mikulski (1975), in our study bone matrix irradiated at 2.5 Mrads retained osteoinductive capacity. Glowacki et al. (1981) have made similar clinical observations. Gamma irradiation beyond 2 Mrads increases the solubility of collagen and glycosaminoglycans and destroys the fibrillar network of the matrix (Buring 1970). This may facilitate resorption and explain the lower bone yield obtained with irradiated implants.

Merthiolate respected the induction principle. Merthiolate (0.01-0.02 per cent) is used as a fungicide and as a preservative of biological products, as, for example, bacterial and viral vaccines. Unfortunately, it has no effect on spores (Russel 1982) and therefore cannot be considered as a true sterilizing agent. Because absolute sterility is not provided by merthiolate, we cannot recommend its use in clinical practice.

Glutaraldehyde and formaldehyde act as alkylants, like ethylene oxide. These agents have been studied extensively by Tulis (1973) and Russel (1982). Obviously, both sterilizing agents destroy all osteoinductive capacity of demineralized bone. The glutaraldehyde-treated implants had the same macroscopic appearance as before implantation, without any trace of resorption, bone induction, or calcium accretion.

The efficiency of ethylene oxide as a sterilizing agent has been confirmed by many investigators

(Philips & Kaye 1949, Robert 1974). Prolo et al. (1980) have studied the effect of ethylene oxide on dense nondemineralized cortical bone. They concluded that ethylene oxide did alter the osteogenic capacity of the implants. It has been used in bone banking of nondemineralized bone (Cloward 1980). Urist and coworkers used ethylene oxide for both demineralized bone and protein extracts containing bone morphogenetic protein (Dubuc and Urist 1967, Sato and Urist 1985). This extremely reactive gas acts by alkylation of nucleophilic N-groups in the purine and pyrimidine bases of RNA and DNA, and can probably also alter proteins involved in osteoinduction. Our results suggest that osteoinductive capacity of demineralized bone is destroyed by an effectively sterilizing ethylene oxide gas cycle. In the ethylene oxide group, the results were highly variable, as indicated by the SD. When present, bone formation was always less than in the controls.

The discrepancy between calcium content and strontium activity may be explained by the fact that the strontium activity reflected bone turnover during the last 3 days, whereas the calcium content reflected total bone formation during 2 months. If bone turnover is high in the final period, strontium activity will be elevated, but this does not necessarily imply high calcium content. By contrast, a high calcium content may be associated with a low strontium activity, as observed in the iliac crest.

Sterile procurement of bone from fresh cadavers - free of bacterial or viral infection, demineralized, freeze-dried, and packed in a sterile environment - remains the best means for preserving the osteoinductive capacity of demineralized bone. This seems to be very difficult to apply in routine bone banking. Therefore, gamma irradiation should be further considered for providing sterile demineralized bone implants, because it preserves, at least partially, the bone-inductive capacity.

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