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ORIGINAL PAPER

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Influence of ethylene oxide sterilization on the activity of native reindeer bone morphogenetic protein

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Abstract We studied the effects of ethylene oxide sterilization (Steri-Vac 4XL, temperature 29°C, exposure time 4 h 10 min, ethylene oxide concentration 860 mg/l) on the osteoinductivity of partially purified native reindeer bone morphogenetic protein (BMP) in a hind leg muscle pouch model of male NMRI mice. BMP was administered in implants containing 3 mg in a collagen carrier. Implants without sterilization and without BMP served as controls. New bone formation was evaluated based on the calcium yield, radiographic and histological examination 3 weeks after implantation. The implants without BMP were not able to induce new bone visible in radiographs. In the sterilized BMP group, the mean area of new bone was 35% (p=0.004) and density 32% (p=0.000) smaller than in the nonsterilized group. Calcium yield was 20% lower in the sterilized group than in the nonsterilized group, but this difference was not significant (p=0.22). It was many times lower in the group without BMP than in the above-mentioned groups (p=0,001). We conclude that ethylene oxide gas sterilization reduces the bone-forming activity of native reindeer BMP by one third.

Résumé Nous avons étudié les effets de la stérilisation à l'oxyde d'éthylène (Steri-Vac 4XL, température 29ºC, temps d'exposition 4 h, concentration de l'oxyde d'éthylène 860 mg/l) sur l'osteoinductivité de la proteine morphogénétique osseuse (BMP) du renne partiellement purifié dans un modèle fait par une bourse du muscle de la patte postérieure de la souris NMRI virile. La BMP a été administré par des implants qui contiennent 3 mg dans un porteur de collagène. Des implants sans stérilisation et sans BMP ont servi de contrôle. La néo- formation osseuse a été évaluée sur la formation de calcium, radiographiquement et histologiquement trois semaines après implantation. Les implants sans BMP n'étaient pas capables d'induire un nouvel os visible sur les radiographies. Avec la BMP stérilisé la surface moyenne d'os nouveau était 35% (p=0.004) et la densité 32% (p=0.000) plus petite que dans le groupe BMP non—stérilisé. La production de calcium était 20% inférieure dans le groupe BMP stérilisé que dans le groupe BMP non—stérilisé, mais cette différence n'était pas significative (p=0.22). Elle était plusieurs fois plus faible dans le groupe sans BMP que dans les groupes susmentionnés (p=0,001). Nous concluons que la stérilisation à l'oxyde d'éthylène réduit d'un tiers l'activité ostéoformatrice de la BMP de renne.

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Introduction

Bone morphogenetic proteins (BMPs), which constitute a large family of bone-inductive proteins, were originally described by Urist in 1965 [23]. The ability of BMPs to induce new bone and to heal bone defects has been well established in many experimental animal and clinical studies [7, 8, 9, 11, 12, 13, 14, 21, 22, 24]. The sterilization of BMP before preclinical and clinical application is mandatory but problematic because BMPs do not tolerate heat management. Different sterilization methods, such as irradiation and chemical agents, have been tested [1, 2, 3, 4, 10, 15, 16, 19, 20, 25]. Ethylene oxide (EO) gas sterilization, which is often used for medical devices [5], has been extensively used [2, 3, 4, 10, 15, 16, 19, 20, 25]. The reports on its effects on BMP are controversial. In general, it has been suggested to decrease partly or totally the osteoinductive activity of BMP [3, 4, 10, 16], but no or only slight alterations have been observed in some other contexts [15, 19, 20, 25].

The aim of this study was to evaluate the effect of EO sterilization on the osteoinductive capacity of native reindeer BMP in a mouse femoral muscle pouch model.

Materials and methods

Preparation of native reindeer (Rangifer tarandus tarandus) BMP

BMP was prepared from reindeer diaphyseal bone. Cortical bones from each animal were chilled immediately after death. The epiphyseal ends, bone marrow, and periosteum were mechanically removed, and after freezing in liquid nitrogen, the cleaned cortical bones were ground into a particle size of 1.0 mm^3. The pulverized bone was demineralized in 0.6 M HCl and extracted in 4 M GuHCl at 4°C. The GuHCl-extracted solution was filtered with a tangential flow system and concentrated. The concentrated solution was dialyzed against deionized water, and the water-insoluble material was collected. After redissolution in 4 M GuHCl solution, the water-insoluble material was dialyzed against 0.25 M citrate buffer pH 3.1. The citrate-buffer-insoluble material was washed with deionized water and lyophilized. [13]

Carriers of BMP and reconstitution of implants

Absorbable collagen sponge (weight 15 mg, size 8×8 mm, Lyostypt, compress from native collagen of bovine origin, mainly consisting of type IV collagen, B. Braun, Tuttlingen, Germany) was used as a carrier of BMP. All implants were made under sterile conditions. According to our preliminary tests, 3 mg of reindeer BMP induced a suitable amount of new bone and was therefore used in all BMP implants (Fig. 1).

The 3 mg of BMP was dissolved in 40 µl of sterile water, pipetted on to the collagen sponge, and lyophilized for 48 h in test tubes.

Sterilization

Sterilization of implants was performed with a 4XL Steri-Vac EO gas sterilizer (3M, USA) at 29°C with 4 h 10 min of exposure and 10 h of aeration. The EO gas concentration was 860 mg/l and relative humidity 60%. The process was carried out according to the manufacturer's instruction. The exposure time was determined by these instructions when the temperature was chosen. The efficacy of sterilization was monitored with samples of *Bacillus subtilis* (3M, Attest 1264).

Study and control groups

The study and control groups were as follows:

1. Collagen + native reindeer BMP, sterilized with EO (n=20). Sterilized BMP group.
2. Collagen + native reindeer BMP, no sterilization (n=20). Nonsterilized BMP group.
3. Collagen, no sterilization (n=10). Collagen group (control group).

Implantation

Male NMRI mice aged 28–35 weeks were used. The implants were introduced in a sterile condition into the thigh muscle pouches in the bilateral hind legs under neuroleptic analgesia (Hypnorm Janssen, Belgium; Dormicum Roche, Switzerland). After the implantation, the muscle was closed with 5–0 and the skin with 3–0 resorbable sutures. The animals were killed in a chamber with carbon dioxide after 21 days of implantation, and the hind legs were harvested. At 3 weeks after implantation, bone formation had proceeded to a stage that allowed quantitative evaluation, and this time point has also been used in many other studies [8, 13, 18]. The study protocol was accepted by the Institutional Ethical Committee.

Area of new bone and its optical density evaluated radiographically

After the harvest, standard lateral position radiographs (100 mA, 20 kV, 0,08 s/exp; Mamex de Maq, Soredex, Orion) were taken from all hind legs of the mice. The radiographic images were transferred into the computer by using an optical scanner (HP Laser Jet/Desk Scan). The new bone formation was evaluated as the area (mm^2) of calcified tissue visible in the radiographs defined by using Scion Image (Scion Corp., USA) software. The mean optical density (mmAl) of the defined area was measured with the same equipment. Calibration of the equipment for the measurement of optical density was performed using an aluminium wedge.

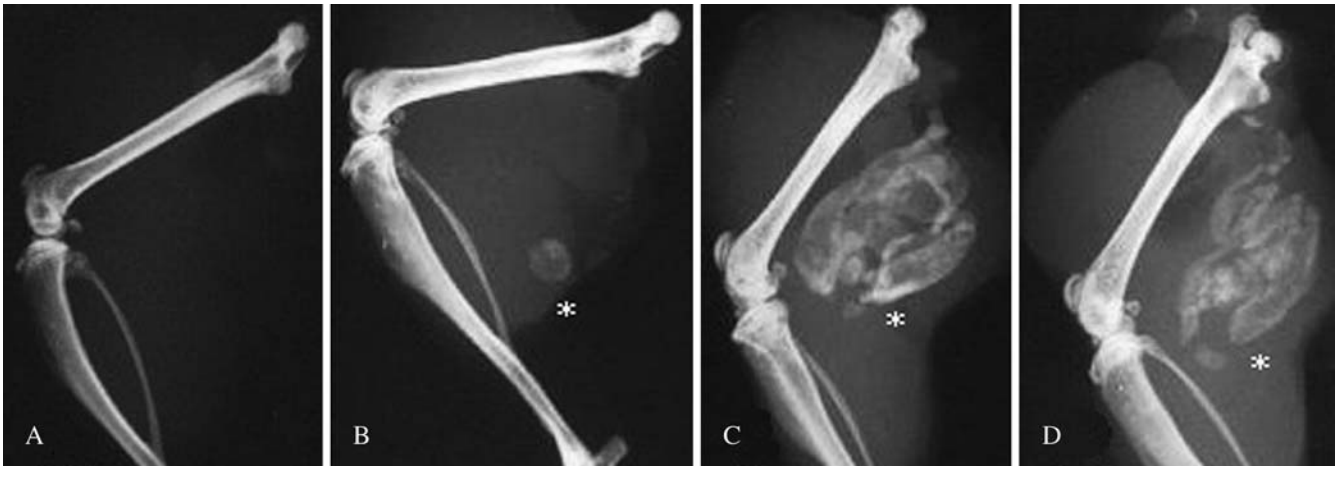

Fig. 1 Radiography shows the new bone formation induced by different amounts of reindeer BMP in the muscle pouches of NMRI mice. **a** Bovine serum albumin, **b** 1 mg of BMP, **c** 3 mg of BMP, **d** 5 mg of BMP, *new bone

Calcium yield

Calcium yield was determined 21 days after implantation from the 14 samples of both groups containing BMP (sterilized and nonsterilized groups). The muscle tissue of the harvested hind leg, including the implant and the newly formed bone, was taken en bloc for a specimen immediately after the radiography. Control samples were taken from the implantation areas of the six hind legs of the collagen group. The specimens were heated in crucibles in an oven at 110°C for 4 h and then ashed in a muffle furnace at 500°C for 2 h and at 800°C for 4 h. The ash was dissolved in 2 ml of 6 N HCl and transferred into a test tube. The calcium content was measured fluoroscopically with a metal complexing dye orthocresolphthalein complexone with calcium C-Test (Wako, Pure Chemical, Osaka, Japan) and expressed as milligrams per milliliter.

Histology

Six implants from the two groups containing BMP (sterilized and control groups) and four implants from the collagen group were used for histologic examination. The muscle tissue of the harvested hind leg, including the implant and the newly formed bone, was taken en bloc for a specimen immediately after the radiography. After dissection, the samples were fixed in 10% neutral formalin solution, decalcified in 0.1 N HCl, cut at 2.0-mm intervals into 5-μm-thick sections, and stained with hematoxylin-eosin.

Statistics

Statistical analysis was performed using the SPSS for Windows statistical package (SPSS Inc., version 9.0). The nonparametric Kruskal-Wallis test was used to evaluate the statistical differences between the groups and the Mann-Whitney test for pairwise comparison. Values of $p<0.05$ were considered statistically significant.

Results

Effect of sterilization

The sterilization method used here proved to be effective, as no growth was observed in *B. subtilis* cultures.

Area of new bone formation evaluated radiographically

There was no radiographically detectable new bone formation in the collagen group, whereas abundant new bone was seen in the groups containing BMP (Table 1). The mean new bone area of the sterilized BMP group was 35% smaller than that of the nonsterilized BMP group (p=0.004) (Table 1).

Table 2 Calcium yield of new bone formation (mean±SD) in different groups

Study groups	No.	Calcium yield (mg/ml)
Nonsterilized group	14	2.74±1.44 *^{x}
Sterilized group	14	2.19±1.30 *^
Collagen group	6	0.02±0.01^{x} ^

* p=0.202 Nonsterilized group versus sterilized group
^{x} p=0.001 Nonsterilized group versus collagen group
^ p=0.001 Sterilized group versus collagen group

Optical density of new bone formation evaluated radiographically

Because the collagen group showed no radiologic new bone formation, its optical density could not be measured. The mean optical density of the new bone in the sterilized BMP group was 22% lower than that in the nonsterilized group (p=0.000) (Table 1).

Calcium yield

Calcium yields of the BMP groups were manifold compared to those in the collagen group (p=0.001) (Table 2). Mean calcium yield was 20% lower in the sterilized BMP group than in the nonsterilized group, but this difference was not significant (p=0.202) (Table 2).

Histological evaluation

There was no new bone formation in the specimens of the groups without BMP. Endochondral new bone formation was seen in the specimens of the groups containing BMP, and this seemed to be most abundant in the nonsterilized group while the decalcified samples did not allow quantitative analysis. There was a substantial amount of hypertrophied and calcified chondrocytes, remodeling ectopic trabecular woven bone, and hematopoietic bone marrow cells. No inflammatory reaction was seen. However, there were no qualitatively histologic features distinctive of the nonsterilized and sterilized groups.

Discussion

BMPs are inactivated by a variety of physical and chemical agents [3, 10, 16, 17]. The temperature to which

Table 1 Area and optical density of new bone formation (mean±SD) measured from radiograms

Study groups	No.	Bone area (mm^2)	Optical bone density (mmAl)
Nonsterilized group	20	48.51±20.29 *^{x}	0.59±0.10 ^
Sterilized group	20	31.52±17,89 *$^{\partial}$	0.36±0.10 ^
Collagen group	10	0$^{x\ \partial}$	–

*p=0.004
^ p=0.000 Nonsterilized group versus sterilized group
^{x} p=0.000 Nonsterilized group versus collagen group
$^{\partial}$ p=0.000 Sterilized group versus collagen group

BMPs is exposed during the ethylene oxide sterilization period or the aeration phase and the presence of toxic byproducts generated by EO are the main factors that interfere with the bone inductive capacity of BMPs [2, 10, 25]. Here we used EO at 29°C, which has been demonstrated to be the optimal temperature for gas sterilization, and it may be virtually the lowest temperature at which safe sterilization can be achieved [10]. In the sterilization of clinical materials, the optimal temperature of EO gas is around 60°C, but when the object is heat labile, lower temperatures can be applied. In that case, however, the exposure time must be longer [6].

Most of earlier studies on the effects of EO sterilization on the bone inductive activity of BMPs have been performed by using demineralized bone matrix (DBM), and the results have been controversial [2, 3, 4, 15, 16, 19, 20, 25].

Zhang et al. reported, by using the rat muscle pouch model, that EO sterilization of DBM at 40°C leads to only a slight alteration of osteoinductivity, as assessed by the restored weight ratio, calcium content, and alkaline phosphatase activity of new bone and histomorphometry [25]. Moore et al., Sigholm et al., and Solheim et al. concluded that DBM retained its osteoinductive properties after correctly applied EO gas sterilization [15, 19, 20]. On the other hand, Munting et al. and Aspenberg et al. detected severe impairment of DBM after the time needed for EO sterilization at 37°C by using the rat muscle pouch model [3, 4, 16]. Our results, which showed that EO gas sterilization reduced the bone-forming activity of native reindeer BMP by about 35% compared to nonsterilized native reindeer BMP, are in line with the latter studies [3, 4, 16].

Only one paper was found that reported the effects of EO sterilization on the bone-forming activity of partially purified native BMP [10]. Ijiri et al, reported, by using thoracic subcutaneous rat model, that the majority of osteoinductivity of native bovine BMP was lost after sterilization with EO at 37°C for 4 h and at 55°C for 1 h [10]. Reduction in the activity of native bovine BMP was about half of the control values when the sterilization was performed at 29°C for 5 h [10], which is, by and large, of the same order as in our study.

In addition to decreasing the osteoinductive activity of BMP, EO also exerts other detrimental effects, as it is mutagenous and poisonous due to the formation of reactive free radicals during the sterilization. Despite these facts and the lack of an ideal alternative, EO remains an acceptable method for the sterilization of BMPs.

It is concluded that native reindeer BMP with collagen carrier is sensitive to the effects of EO gas sterilization. Sterilization at 29°C for 4 h 10 min reduced the bone-forming activity of native reindeer BMP by about one third. This does not, however, prevent the use of EO sterilization, as the remaining activity of BMPs is sufficient for the induction of bone formation in preclinical and clinical contexts.

References

1. Andriano KP, Chandrashekar B, McEnery K, Dunn RL, Moyer K, Balliu CM, Holland KM, Garrett S, Huffer WE (2000) Preliminary in vivo studies on the osteogenic potential of bone morphogenetic proteins delivered from an absorbable puttylike polymer matrix. J Biomed Mater Res 53:36–43
2. Arizono T, Iwamoto Y, Okuyama K, Sugioka Y (1994) Ethylene oxide sterilization of bone grafts. Acta Orthop Scand 65:640–642
3. Aspenberg P, Johnsson E, Thorgren KG (1990) Dose-dependent reduction of bone inductive properties by ethylene oxide. J Bone Joint Surg [Br] 72:1036–1037
4. Aspenberg P, Lindqvist SB (1998) Ethene oxide and bone induction. Controversy remains. Acta Orthop Scand 69:173–176
5. Babb JR, Phelps M, Downes J, Ayliffe GA (1982) Evaluation of an ethylene oxide sterilizer. J Hosp Infect 3:385–394
6. Ernst RR (1974) Ethylene oxide sterilization kinetics. Biotechnol Bioeng Symp 4:865–878
7. Gao TJ, Lindholm TS, Marttinen A, Puolakka T (1993) Bone inductive potential and dose-dependent response of bovine bone morphogenetic protein combined with type IV collagen carrier. Ann Chir Gyn 82:77–84
8. Gao TJ, Lindholm TS, Marttinen A, Urist M.R (1996) Composite of bone morphogenetic protein (BMP) and type IV collagen, coral-derived coral hydroxyapatite and tricalcium phosphate ceramics. Int Orthop 10:321–325
9. Gao TJ, Lindholm TS, Kommonen B, Ragni P, Paronzini A, Lindholm TC, Jalovaara P, Urist MR (1997) A coral composite implant containing bone morphogenetic protein repairs a segmental tibial defect in sheep: mechanics and immune assay. Int Orthop 21:194–200
10. Ijiri S, Yamamuro T, Nakamura T, Kotani S, Notoya K (1994) Effect of sterilization on bone morphogenetic protein. J Orthop Res 12:628–636
11. Johnson EE, Urist MR (2000) Human bone morphogenetic protein allografting for reconstruction of femoral nonunion. Clin Orthop 371:61–74
12. Jortikka L, Marttinen A, Lindholm TS (1993) Partially purified reindeer (Rangifer Tarandus) bone morphogenetic protein has a high bone-forming activity compared with some other artiodactylous. Clinic Orthop 297:33–37
13. Jortikka L, Marttinen A, Lindholm TS (1993) Purification of monocomponent bovine morphogenetic protein in a water-soluble form. Ann Chir Gyn 82:25–30
14. Kujala S, Raatikainen T, Ryhänen J, Kaarela O, Jalovaara P (2002) Composite implant of native bovine bone morphogenetic protein (BMP) and biocoral in the treatment of scaphoid nonunions—a preliminary study. Scand J Surg 9:186–190
15. Moore TM, Artal R, Arenas M, Gendler E (1990) Influence of postmortem time and temperature on osteoinductive activity of demineralized microperforated ethylene oxide-sterilized syngeneic bone implant in the rat. Clin Orthop 259:239–244
16. Munting E, Wilmart J-F, Wijne A, Hennebert P, Delloye C (1998) Effect of sterilization on osteoinduction. Comparison of five methods in demineralized rat bone. Acta Orthop Scand 59:34–38
17. Prolo DJ, Oklund SA (1983) Sterilization of bone by chemicals. In: Friedlaender G, Mankin H J, Sell KW (eds), Osteochondral Allografts. Little, Brown, Boston. pp 233
18. Reddi AH (1981) Cell biology and biochemistry of endochondral bone development. Coll Relat Res 1:209–226
19. Sigholm G, Gendler E, McKellop H, Marshall GJ, Moore TM, Sarmiento A (1992) Graft perforations favor osteoinduction—studies of rabbit cortical grafts sterilized with ethylene oxide. Acta Orthop Scand 63:177–182
20. Solheim E, Pinholt E-M, Bang G, Sudmann E (1995) Ethylene oxide gas sterilization does not reduce the osteoinductive potential of demineralized bone in rats. J Craniofacial Surg 6:195–197

21. Tuominen T, Jämsä T, Tuukkanen J, Nieminen P, Lindholm TC, Lindholm TS, Jalovaara P (2000) Native bovine bone morphogenetic protein improves the potential of biocoral to heal segmental canine ulnar defects. Int Orthop 24:289–294
22. Tuominen T, Jämsä T, Tuukkanen J, Marttinen A, Lindholm TS, Jalovaara P (2001) Bovine bone implant with bovine bone morphogenetic protein in healing a canine ulnar defect. Int Orthop 25:5–8
23. Urist M.R (1965) Bone formation by autoinduction. Science 150:893–899
24. Wozney JM (2002) Overview of bone morphogenetic proteins. Spine 15(S):2–8
25. Zhang Q, Cornu O, Delloye C (1997) Ethylene oxide does not extinguish the osteoinductive capacity of demineralized bone. A reappraisal in rats. Acta Orthop Scand 68:104–108