Effect of gamma irradiation on the osteoinductivity of morphogenetic protein extract from reindeer bone

Tarmo Pekkarinen[1], Oili Hietala1, Timo Jämsä[2] and Pekka Jalovaara[1]

[1]Bone Transplantation Research Group, Department of Orthopaedic and Trauma Surgery, University of Oulu, Oulu, [2]Department of Medical Technology, University of Oulu, Oulu, Finland
Correspondence TP: Tarmo.Pekkarinen@uta.fi
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Background **Bone morphogenetic proteins (BMPs), which are capable of stimulating the production of new bone, must be sterilized before preclinical and clinical use to reduce the risk of infections and associated complications. In this study, we investigated the effects of gamma sterilization on the osteoinductivity of native reindeer BMP extract in the Balb/C mouse thigh muscle pouch model.**

Methods **5 mg of native reindeer BMP extract and 5 mg of bovine serum albumin were administered separately either in gelatine capsules or mixed with gelatine as injections. The dose of gamma irradiation was 4.1 Mrad. Unsterile capsules and injections served as controls. New bone formation was evaluated based on the incorporation of Ca^{45} and also radiographically 3 weeks after implantation.**

Results **Albumin-containing implants and injections did not induce new bone formation, as monitored in radiographs. Gamma sterilization did not reduce the osteoinductivity of native BMP extract in capsules, but a significant decrease in osteoinductivity—measured as area (50%) and Ca^{45} incorporation of new bone (27%)—was seen after injection. Gamma sterilization had no effect on the optical density of new bone induced by native BMP extract administered in capsules or by injection.**

Interpretation **We conclude that, as gamma irradiation did not reduce the osteoinductivity of reindeer BMP extract in gelatine capsules, this method appears to be suitable for sterilization of BMPs to be given in capsule form. Native reindeer BMP extract was more sensitive to irradiation in soluble collagen (gelatine) than BMP in gelatine capsules. This finding must be given serious consideration regarding treatment of patients, but the remaining activity may be sufficient for the induction of bone formation in preclinical and clinical situations.**

Bone morphogenetic proteins (BMPs) have been shown to stimulate the production of bone when combined with an appropriate carrier material (Tuominen et al. 2000, Kujala et al. 2002, 2004, Wozney 2002, Pekkarinen et al. 2003). In clinical situations, it is necessary to sterilize all medical implants and parenteral drug delivery systems prior to surgical placement or injection, to reduce the risk of infections and associated complications. Gamma irradiation and ethylene oxide gas sterilization have been used, but the optimal method for the sterilization of BMPs has been the subject of debate (Munting et al. 1988, Wientroub and Reddi 1988, Wientroub et al. 1990, Aspenberg et al. 1990, Aspenberg and Lindqvist 1998, Sigholm et al. 1992, Ijiri et al. 1994, Zhang et al. 1997, Andriano et al. 2000, Ripamonti et al. 2000, Pekkarinen et al. 2004). The doses of ethylene oxide that are sufficient for sterilization have a detrimental effect on the osteoinductivity of BMPs (Munting et al. 1988, Aspenberg et al. 1990, Aspenberg and Lindqvist 1998, Pekkarinen et al. 2004), whereas gamma irradiation appears to be less harmful in this respect. Most of the reports considering the effects of gamma sterilization on the osteoinductivity of these growth factors have been perfomed on demineralized bone matrix and recombinant

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BMPs (Munting et al. 1988, Wientroub and Reddi 1988, Wientroub et al. 1990, Zhang et al. 1997 Andriano et al. 2000, Ripamonti et al. 2000). There has only been one paper considering the effects of gamma sterilization on native purified BMP (Ijiri et al. 1994).

We investigated the effects of gamma sterilization on reindeer BMP extract administered either as a separate substance in gelatine capsules or mixed with gelatine as injections in the mouse thigh muscle pouch model.

Materials and methods

Preparation of native reindeer (Rangifer tarandus tarandus) BMP extract

Native reindeer BMP extract was prepared from reindeer diaphyseal bone. Cortical bones from each animal were chilled immediately after death. The epiphyseal ends, bone marrow and periosteum were removed mechanically, and after freezing in liquid nitrogen, the cleaned cortical bones were ground to a particle size of 1.0 mm^3. The pulverized bone was demineralized in 0.6 M HCl and extracted in 4 M guanidine hydrochloride (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 re-dissolution 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. (Jortikka et al. 1993)

Reconstitution of test materials and sterilization

Implants. 5 mg of BMP extract was introduced into each gelatine capsule (no. 1). The control implants contained 5 mg bovine serum albumin (BSA).

Injection. 75 mg BMP extract and 150 mg gelatine (type A from porcine skin, Bloom 300, Sigma, St. Louis, MO) were mixed with 0.9% saline to obtain 1.5 mL homogenized emulsion. 100 µL of this emulsion was used per injection, and each injection thus contained 5 mg BMP extract. The control injections contained 5 mg BSA. The preparation of implants and injections was done aseptically.

Gamma sterilization of the test materials was performed by a specialized company (Isotron Ltd., Swindon, UK). The dose was 4.10 MRad.

Groups

1. Gelatine capsule + native reindeer BMP (n = 15), non-sterilized BMP group.
2. Gelatine capsule + native reindeer BMP, irradiated (n = 15), sterilized BMP group.
3. Gelatine + native reindeer BMP injection (n = 15), non-sterilized BMP/injection group.
4. Gelatine + native reindeer BMP injection, irradiated (n = 15), sterilized BMP/injection group.
5. Gelatine capsule + albumin (n = 6), non-sterilized BSA group.
6. Gelatine capsule + albumin, irradiated (n = 6), sterilized BSA group.
7. Gelatine + albumin injection (n = 6), non-sterilized BSA/injection group.
8. Gelatine + albumin, irradiated (n = 6), sterilized BSA/injection group.

Implantation and injection techniques

We used male Balb/C mice aged 10–12 weeks, and the administration of capsules and injections was done under neuroleptic analgesia (Hypnorm Janssen, Belgium; Dormicum Roche, Switzerland).

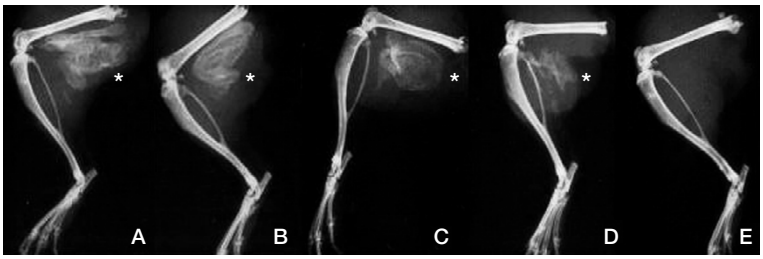
Implantation. Capsules were introduced under sterile conditions into the thigh muscle pouches in the bilateral hind legs. After the implantation, the muscle was closed with 5-0 resorbable sutures, and the skin with 3-0 resorbable sutures.

Injections. 100 µL emulsion was injected under sterile conditions into both thigh muscles using a 1-mL syringe and 20-G needle.

All animals were killed in a chamber with carbon dioxide 21 days later, and the hind legs were harvested (Reddi 1981, Jortikka et al. 1993). The study protocol was accepted by our institutional Ethics Committee.

Radiographic evaluation of area and density of new bone

After harvest, standard lateral position radiographs (100 mA, 20 kV, 0.08 s/exp; Mamex de Maq, Soredex, Orion, Finland) were taken of all hind legs. The radiographic images were transferred into a computer by using an optical scanner (HP Laserjet/Desk Scan). New bone formation was evaluated as



Results of radiography showing the new bone formation induced by reindeer BMP in the muscle pouches of Balb/C mice. (A) non-sterilized BMP group, (B) sterilized BMP group, (C) non-sterilized BMP/injection group, (D) sterilized BMP/injection group, (E) example from BSA group. (*) New bone.

the area (in mm^2) of calcified tissue visible in the radiographs, defined by using Scion Image Beta 4.02 software (Scion Corp., Maryland). The mean optical density (mm Al) 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.

Ca^{45} activity

24 h before killing, all mice received an intraperitoneal injection of diluted carrier-free Ca^{45} solution (Amersham, UK; 40 µCi/kg of body weight). The muscle tissue of each harvested hind leg, including the implant and the newly formed bone, was taken en bloc for a specimen immediately after the radiography. A piece of intact foreleg muscle was used as reference (10 samples). All specimens were weighed and digested in a mixture of 0.2 mL 70% perchloric acid and 0.4 mL 33% peroxide at 70°C for 3 h. 0.6 mL of the digested solution was pipetted into a diffuse scintillation vial, and 5 mL scintillation cocktail (OptiPhase Hi-safe 3; Wallac, Finland) was added. The samples were counted in a liquid scintillation counter (Wallac 1410, Pharmacia, Finland) with an internal spectrum library. Ca^{45} incorporation into tissue was expressed as DPM/mg tissue.

Statistics

We performed statistical analysis using the SPSS statistical package version 9.0 (SPSS Inc., Illinois). The non-parametric Kruskall-Wallis test was used to evaluate the statistical differences between the groups and the Mann-Whitney test was used for pairwise comparison. Values of $p \leq 0.05$ were considered statistically significant.

Results

The injections and implantations were well tolerated by the mice, and no complications occurred during or after the procedures.

Area of new bone formation evaluated radiographically

There was no new radiographically detectable bone formation in the control groups, whereas there was an abundance of new bone in the groups that received BMP extract (Table 1, Figure).

There was no difference in the new bone area between the non-sterilized BMP group and the sterilized BMP group ($p = 0.4$). A decrease in new bone area could be seen in the sterilized BMP/injection group compared to the non-sterilized BMP/injection group ($p < 0.001$) (Table 1).

Optical density of new bone formation evaluated radiographically

Because the control groups showed no new bone formation radiologically, their optical density could not be measured. There were no differences in optical density between the non-sterilized and sterilized BMP groups ($p = 0.3$) or between the non-sterilized and sterilized BMP/injection groups ($p = 0.2$) (Table 1).

Ca^{45} incorporation

The mean Ca^{45} incorporation was manifold in all groups containing BMP extract compared with the corresponding control groups ($p < 0.001$) (Table 2). There was no difference in Ca^{45} incorporation between the non-sterilized BMP group and

Table 1. Area and optical density of new bone formation measured from radiographs in the different groups. Mean (SD)

Study groups	n	Bone area (mm^2)	Optical density of new bone (mm Al)
BMP group			
Non-sterilized	15	79 (33)	0.39 (0.11)
Sterilized	15	74 (23)	0.41 (0.09)
BMP/injection group			
Non-sterilized	15	55 (13) [a]	0.41 (0.09)
Sterilized	15	27 (14) [a]	0.39 (0.12)

[a] p = 0.000 for non-sterilized BMP/injection group vs. sterilized BMP/injection group.

Table 2. Ca^{45} incorporation in different groups. Mean (SD)

Study groups	n	Ca^{45} incorporation (DPM/mg)
Non-sterilized BMP group	15	313 (256) [b]
Sterilized BMP group	15	281 (120) [c]
Non-sterilized BMP/injection group	15	469 (498) [a, d]
Sterilized BMP/injection group	15	343 (543) [a, e]
Non-sterilized BSA group	6	2.4 (2.0) [b]
Sterilized BSA group	6	5.7 (3.8) [c]
Non-sterilized BSA/injection group	6	3.6 (2.1) [d]
Sterilized BSA/injection group	6	5.0 (3.4) [e]

[a] p = 0.045 non-sterilized BMP/injection vs. sterilized BMP/injection group.
[b] p = 0.000 non-sterilized BMP group vs. non-sterilized BSA group.
[c] p = 0.000 sterilized BMP group vs. sterilized BSA group.
[d] p = 0.000 non-sterilized BMP/injection group vs. non-sterilized BSA/injection group.
[e] p = 0.000 sterilized BMP/injection group vs. sterilized BSA/injection group.

the sterilized BMP group (p = 0.7). A decrease in Ca^{45} incorporation could be seen in the sterilized BMP/injection group compared to the non-sterilized BMP/injection group (p = 0.05).

Discussion

An important problem in the clinical application of BMPs and their carriers is sterilization. Ethylene oxide gas is used in many protocols, but it reduces the osteoinductive activity of BMPs, and the formation of free radicals during ethylene oxide sterilization is a further cause for concern (Munting 1988, Aspenberg et al. 1990, Aspenberg and Lindqvist 1998, Ijiri et al. 1994, Zhang et al. 1997, Pekkarinen et al. 2004). Many reports have suggested that gamma sterilization is less harmful in these respects, and it would thus be a better alternative for the sterilization of BMPs (Wientroub and Reddi 1988, Wientroub et al. 1990, Andriano et al. 2000, Ripamonti et al. 2000).

Wientroub et al. (1988, 1990) used allogenic demineralized bone matrix with endogenous native BMPs and reported that samples could tolerate up to 5–7 MRad of gamma irradiation as long as appropriate temperatures (4ºC or less) were maintained. Andriano et al. (2000) showed that gamma irradiation at doses of 1.5–2.5 MRad did not reduce the activity of a combination of polymeric and bovine-derived BMPs, and thus appeared to be a promising method for sterilization of BMPs. These authors even reported that irradiation of the polymer matrix actually improved the extent of new bone formation, but this trend was not supported by statistical analysis (Andriano et al. 2000). Ripamonti et al. (2000) investigated the effects of gamma irradiaton at doses of 2.5–3.0 MRad on the osteoinductivity of human OP-1, and observed that gamma-irradiated human OP-1 combined with irradiated xenogeneic bovine collagenous matrix carrier is effective in regenerating and maintaining the architecture of induced bone. Our results are, by and large, in accordance with these studies. We have shown that gamma irradiation at a dose of 4.1 MRad does not reduce the osteoinductivity of BMP extract in gelatine capsules.

There have also been reports with results that conflict with ours. Zhang et al. (1997) and Munting et al. (1988) reported that gamma irradiation at a dose of 2.5 Mrad reduced the osteoinductive capacity of demineralized bone matrix (DBM) by about 40–50%. Ijiri et al. (1994) reported that exposure of bovine-derived BMP with type-I collagen carrier to 2.5 MRad of gamma irradiation reduced the activity of BMP to 4.4% of that of the controls. Moreover, these authors suggested that irradiation of bovine type-I collagen carrier alone with 2.5 MRads dramatically reduced the osteoinductivity of non-irradiated bovine-derived BMP released

from this carrier. Here, we observed a reduction in the osteoinductivity of BMP extract when it was exposed to 4.1 MRad of gamma irradiation in an injectable mixture with soluble gelatine.

It has been shown that irradiation changes the consistency of collagen. Buring (1970) reported that gamma irradiation in excess of 2.0 MRad increased the solubility of collagen and destroyed the fibrillar network of the bone matrix. It is possible that these changes have harmful effects on BMPs, or that collagen potentiates these deterious effects of irradiation on BMPs by some unknown mechanism.

Currently, the standard dose recommended by the Food and Drug Administration (Rockville, MD) is 2.5 MRad. However, even when we used a larger dose (4.1 MRad) here, BMP maintained its osteoinductivity well. Because the safety requirements allow the use of even lower doses than the one used here, gamma irradiation can be recommended for the sterilization of BMP material for clinical purposes.

We conclude that gamma irradiation does not reduce the osteoinductivity of native reindeer BMP extract in gelatine capsules and is a suitable sterilization method for BMPs administered in this way. Reindeer BMP extract in soluble collagen (gelatine) for injections seemed to be more sensitive to irradiation than BMP in gelatine capsules, and this difference must be given serious consideration. The remaining activity may, however, be sufficient for the induction of bone formation in preclinical and clinical situations.

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No competing interests declared.

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