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Enhanced release of bone morphogenetic proteins from demineralized bone matrix by gamma irradiation

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HIGHLIGHTS

- Demineralized bone matrix (DBM) was gamma-irradiated for sterilization.
- Irradiated DBM had higher alkaline phosphatase and osteocalcin production.
- It was reasoned the more released bone morphogenetic proteins by irradiation.
- This result supports the application of radiation sterilization for bone implants.

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ABSTRACT

Gamma irradiation is a useful method for sterilizing demineralized bone matrix (DBM), but its effect on the osteoinductivity of DBM is still controversial. In this study, the osteoinductive activity of gamma-irradiated DBM was examined using a mouse myoblastic cell line (C2C12). DBM was extracted from adult bovine bone and was irradiated at a dose of 25 kGy using a ^{60}cobalt gamma-irradiator. Cell proliferation with DBM was not affected by gamma-irradiation, but alkaline phosphatase and osteocalcin productions were significantly increased in C2C12 cell groups treated with gamma-irradiated DBM. It was reasoned that bone morphogenetic proteins were more efficiently released from gamma-irradiated DBM than from the non-irradiated control. This result suggests the effectiveness of radiation sterilization of bone implants

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1. Introduction

Demineralized bone matrix (DBM) is used extensively for bone implants. Its porous structure is suitable for bone growth, and its matrix proteins like collagen provide an osteoconductive support matrix (Gebhart and Lane, 1991). Many studies have demonstrated the clinical potential of DBM implants in the treatment of bone defects (Piattelli et al., 1996; Trevisiol et al., 2007; Urist, 1965). One of the major challenges with the used of DBM products is ensuring sterility. Although DBM is exposed to concentrated acid and chloroform during the preparation process, subsequent handling or incorporation into a composite can lead to contamination. A safe sterilization method is required to prevent disease transmission and graft contamination.

Gamma irradiation is an effective method for the terminal sterilization of medical devices, as irradiation does not leave any harmful residues and can be applied to the final product with a relatively short processing time (Glowacki, 2005). Therefore, some tissue banks employ gamma irradiation from ^{60}cobalt to kill bacteria, spores, and viruses at a dose ranging from 15 to 25 kGy. However, this method is not utilized by all tissue banks, and some physicians choose not to use bone implants treated with irradiation because of the documented possibility that DBM products become less efficacious following gamma irradiation (Anderson et al., 1992; Currey et al., 1997; Fideler et al., 1995; Gibbons et al., 1991; Noyes et al., 1984; Rasmussen et al., 1994; Salehpour et al., 1995). Several studies have reported the effect of gamma irradiation on osteoinductive activities of DBM, but osteoinductive testing methods varied among studies; therefore, the results were inconsistent.

The purpose of this study was to compare the osteoinductive activity assayed between gamma-irradiated and non-irradiated

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DBMs *in vitro*, and bone morphogenetic proteins (BMPs) of DBM were also isolated to investigate the effect of gamma irradiation.

2. Materials and methods

2.1. DBM extraction

DBM powder was prepared from the spongy portion of bovine bone as per the reported method (Schouten et al., 2005). Separated spongy bones were cut into appropriate sizes. The pieces of bone were then soaked in acetone for 48 h to remove any fatty components. Subsequently, 0.6 M HCl was added to demineralize the spongy bones, after which the bones were washed with double-distilled H_2O. This washing step was repeated 3 times, and the bones were freeze-dried for use in further experiments. The number of donor DBM sources was three.

2.2. Gamma irradiation

The lyophilized DBM powder was irradiated at a dose of 25 kGy by a ^{60}cobalt-irradiator (IR-221; Nordion International Ltd., Ottawa, ON, Canada) equipped with an 11.1 PBq source strength at 22 ± 2 °C and operated at a dose rate of 10 kGy/h. Dosimetry was performed using 5 mm-diameter alanine dosimeters (Bruker Instruments, Rheinstetten, Germany). After irradiation the irradiated samples were stored at 4 °C.

2.3. Cell cultures and cell proliferation

C2C12 cells (5×10^4) purchased from the Korean Cell Line Bank (Seoul, South Korea) were seeded into a 6 trans-well plate (Corning Incorporated, Corning, NY), and incubated in a growth medium (alpha-MEM, 15% FBS, 50 μg/mL ascorbic acid, 10 mmol/L β-glycerol phosphate, 100 units/mL penicillin, and 100 units/mL streptomycin). Cells were treated with non-irradiated (0 kGy) and gamma-irradiated (25 kGy) DBM powder (10, 30, and 50 mg) for 48 h in a 5% CO_2 incubator. The average working volume was about 2 mL. Cell proliferation was detected using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO), as described by Zhanga et al. (2005).

2.4. Alkaline phosphatase (ALP) assay

C2C12 cells were plated into a 6 trans-well plate (5×10^4 cells per well, 2 mL of working volume), and alkaline phosphatase (ALP) was measured in cell lysates from cells treated with non-irradiated and gamma-irradiated DBM powder (10, 30, and 50 mg) after 48 h. To estimate the ALP concentration, cell layers were washed with ice-cold 50 mM Tris-buffered saline (pH 7.4), scraped into 0.5 mL ice-cold Tris-buffered saline (50 mM) containing 0.2% NP-40, and sonicated on ice (20 s). An ALP assay was performed using the supernatants of the sonicated cells according to the manufacture's instructions (Sigma Co.). Enzyme activity was detected using spectrophotometry at 405 nm after 30 min of incubation. Standard curves were plotted using p-nitrophenol standard solution in 0.08 mmol/L in NaOH. The number of ALP assay replicates in a experiment was three, and the assay was carried out 3 times.

2.5. Osteocalcin production

The amount of osteocalcin secreted into the culture medium was determined using a radioimmunoassay kit (Biomedical Technology, Inc., Stoughton, MA), and the experiment was carried out 3 times.

2.6. Bone morphogenetic proteins (BMP) extraction

BMP was extracted from 100 g of non-irradiated and gamma-irradiated DBM powder using the modified Urist method (Hu et al., 2004). In brief, 100 g of DBM powder was mixed with 1 L of distilled water containing 6 M urea and 0.5 M $CaCl_2$ for 1 h and dialyzed (molecular weight cut-off, 1,000 Da; Spectrum Laboratories Inc., Rancho Dominguez, CA) against distributed water for 72 h. The precipitate was collected and reconstructed in 1 L distilled water containing 6 M urea and 0.5 M $CaCl_2$, and then dialyzed (molecular weight cut-off, 1,000 Da; Spectrum Laboratories Inc.) against 11 volumes of 0.25 mol/L citric acid buffer (pH 3.1). The resulting precipitate was collected and defatted in chloroform–methanol (1:1) for 4 h and lyophilized as purified BMP.

2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein concentration of the BMP extracts was determined using the bicinchoninic acid (BCA) method (Krieg and Yan, 2005), and the contents of the loaded protein per lane adjusted to 28.3 μg. Electrophoresis was performed using precasted 4–20% Nu-PAGE Bis–Tris gels (Invitrogen, San Diego, CA) at 100 V for 1 h in a Nu-Page MES SDS running buffer system (Invitrogen) according to the manufacturer's instructions. A SeeBlue Plus2 prestained standard protein marker (Invitrogen) was used to determine the molecular masses of the protein bands. The gel was then stained with Coomassie Brilliant Blue R-250 for visualization.

2.8. Statistical analysis

The assays were carried out 3 times and the average values were presented. The data were analyzed using the Statistical Package for the Social Sciences (SPSS Inc. Chicago, IL) program. Differences among the mean values were obtained using Duncan's multiple comparison tests at a significance level of $p < 0.05$.

3. Results and discussion

3.1. Effect of gamma irradiation of DBM on cell proliferation and ALP contents

Many studies have described *in vivo* and *in vitro* test systems that can be used to measure the osteoinductive performance of DBM. The most desirable method would be an *in vitro* assay that is inexpensive, highly reproducible, and simple to perform. C2C12, a mouse myoblast cell line, can convert its differentiation pathway from myoblasts to osteoblasts in the presence of DBM (Jortikka et al., 1998). Fig. 1 shows cell proliferation and ALP contents of C2C12 cells treated with gamma-irradiated and non-irradiated DBM. No cytotoxic effects were observed between groups treated with non-irradiated and gamma-irradiated DBM under the indicated weight of DBM (12.5, 25, and 50 mg) at 48 h. In this study, a dose of 25 kGy of gamma irradiation was applied as this has been the recommended dose for terminal sterilization of medical products including bone implants for several decades. In a previous study, proliferation of rat bone marrow cells was found to be inhibited with non-irradiated and low-dose gamma-irradiated DBM (Han et al., 2008). However, we found no difference in cell numbers among all groups treated with DBM. The differences in the results between the previous study and this experiment may be due to the use of different cell types and DBM extraction processes.

When the weight of DBM powder was fixed at 50 mg, ALP content was measured in lysates from cells treated with

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