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Original Article

Hylonome

# Gamma irradiation. An effective procedure for bone banks, but does it make sense from an osteobiological perspective?

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**Abstract**

**Background:** Bone grafts promote bone healing by supplying a three-dimensional structure that supports bone ingrowth. Autologous bone therefore still remains the "gold standard" for grafts. Unfortunately, autologous bone grafts are associated with an increased morbidity. In order to avoid such problems, intensive research has been carried out on alternative materials such as allogeneic bone. However, its use is dependent on bone banks and its availability is limited. Gamma irradiation is now becoming established as a procedure for inactivating bacteria, fungal spores and viruses. Its effects on the biomechanical properties of bone have been analyzed in numerous studies. However, the current literature provides little information as to the effects of gamma sterilization on the osteobiology of allogeneic bone grafts. **Purpose:** The purpose of this study was to evaluate the effect of gamma-sterilized bone grafts on immunocompetent cells by an *in vitro* model (a culture of human bone marrow cells). **Methods:** We decided to use the model of human bone marrow cells in culture for the *in vitro* analysis because the physiological conditions in the human body can best be simulated in this model and the observed reactions are applicable to humans. **Results and Interpretation:** In sum, we found a maximum immune response in gamma-irradiated bone grafts, which, interpreted as a sole result, must be seen as a negative biological effect. However, in view of the good clinical results for gamma-sterilized bone grafts other influences would seem to be the determining factors in clinical outcome. Further research is needed to gain a more exact understanding of these factors.

**Keywords:** Bone Graft, Gamma Irradiation, Biological Effects

## Introduction

The goal of modern fracture management is to enable the fractured area of bone to heal reliably and fast and reach complete restoration of the original bone. In cases where there is a critical bone defect or union of the bone is delayed (pseudarthrosis) bone transplantation is frequently necessary.

Bone grafts promote bone healing by supplying a three-dimensional structure that supports bone ingrowth. They also provide a matrix containing live osteoblasts, growth factors and proteins which exert a positive influence on bone metabolism. Autologous bone grafts have these three properties and can thus be termed osteoconductive, osteoinductive and osteogenous materials. Autologous bone therefore still remains the "gold standard" for grafts, since no synthetic bone material has been developed to date that fulfils these requirements[11]. Unfortunately, autologous bone grafts are associated with an increased morbidity. Complications such as infections, wound hematomas, pain and fractures of the ileac crest (the most frequent source) regularly occur[45]. It is also often not possible to obtain sufficient suitable material, particularly in older patients and small children. In order to avoid these problems, intensive research has been carried out on alternative materials such as allogeneic bone, synthetic bone and xenogeneic bone. Allogeneic bone is the most natural alternative. However, its use is dependent on bone banks and its availability is limited. The demand for allogeneic bone grafts has been rising steadily over the past few years, particularly as a result of the increase in long spinal fusions. According to a recent estimate 500,000 bone grafts are expected to be carried out every year in the next decade in the United States alone, and the trend is

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increasing. This was the starting point for the present study. Gamma irradiation is now becoming established as a procedure for inactivating bacteria, fungal spores and viruses[5]. Its effects on the biomechanical properties of bone have been analyzed in numerous studies[28,37,40]. However, the current literature provides little information as to the effects of gamma sterilization on the osteobiology of allogeneic bone grafts. Recently a published Review by Nguyen et al.[33] already mentioned the adversely effects of gamma irradiation on the mechanical and biological properties of bone allografts by degrading the collagen in bone matrix and by activating a inflammatory response due to macrophage activation.

The present study was designed to investigate the effects of gamma-sterilized bone grafts on immunocompetent cells in an *in vitro* model (a culture of human bone marrow cells). At the end of the culture period the cell culture was also examined by raster electron microscopy.

## Material and Methods

*Obtaining the material*

Femoral heads and the bone marrow for the cell culture were obtained during surgery for hip replacement. Bone marrow was removed from the proximal femur and femur heads following femoral head osteotomy (the intervention was approved by the ethics committee of the Faculty of Human Medicine of Philipps University, Marburg, File No. AZ 66/03). Criteria for exclusion were acute or chronic inflammation, a history of malignancy and age over 75 years. In order to be able to obtain statistically meaningful results, two groups of 8 patients (4 men and 4 women) aged between 45 and 70 years were evaluated.

*Preparation of the cells*

A block of spongiosa measuring approx. 7mm x 10mm x 20mm was placed in a sterile vessel containing Liquemine-containing phosphate buffer (0.2% Liquemine, Hoffmann-La Roche; PBS pH 7.4, Seromed). Following mechanical comminution the suspension obtained was passed through a screen with a pore-size of 0.1 mm and filtered to separate off the spongious portion. This cell suspension was layered on to a Ficoll Histopaque density gradient (mixing ratio 2:1; density 1.077). Following centrifugation (20 min., 2200 rpm, 10˚C, without braking, JS-7.5 centrifuge, Beckmann, USA, rmax=142 mm) an interphase was separated from a cell pellet. The interphase thus obtained and the cell pellet were pipetted off and centrifuged for 10 min at 1900 rpm and 10˚C, with braking (JS-7.5 centrifuge, Beckmann, USA, rmax=142 mm) and rinsed three times with PBS. IMDM was used for the final rinse[11,44].

*Processing of the bone grafts*

Femoral heads removed during total hip replacement surgery following femoral head osteotomy were cut into 3-mm thick slices in the horizontal plane under sterile conditions, using a diamond band saw (Exakt 310). Cylinders of spongiosa (further called bone grafts) were cut out of each slice using a specially constructed hollow drill (diameter: 10 mm). Throughout the procedure the bone was rinsed with sterile 0.9% saline (4-6˚C) to prevent its being damaged by the heat that developed during the cutting and drilling and also to prevent its drying out. Each of the cylinders of bone was then rinsed three times for 10 min with Earle's salt solution and once for 20 min with Earle's salt solution with 500IU streptomycin/penicillin, 4 µg amphotericin B and 10 µg gentamycin added.

*Medium*

12.5% FCS (fetal calf serum, Boehringer Mannheim, Germany), 12.5% HS (horse serum, PAA Linz, Austria), 2.4 ng/ml hydrocortisone (Sigma Diagnostic, St. Louis, USA), 50 ng/ml Certomycin (Essex Pharma, Munich), 292.2 ng/ml L-glutamine (PAA Linz, Austria) und 0.3% NaHCO3- (Merck, Darmstadt, Germany) were added to the IMDM (Iscove's Modified Dulbecco's Medium, Life Technologies, Paisley, Scotland). The growth factors interleukin 3 [IL-3] (Aventis, Marburg, Germany) and granulocyte-macrophage colony-stimulating-factor [GM-CSF] (Aventis, Marburg, Germany), each at a concentration of 10.0 ng/ml medium, were added at each change of medium.

*Design of study*

Bone grafts with and without 25kGy gamma irradiation which was done by Beta-Gamma-Service (BGS) (Wiehl, Germany) were inoculated with $3x10^6$ cells. Irradiated bone grafts received cells from a different patient (allogeneous design) while non-irradiated bone grafts received cells from the same patient (autologous design). The bone grafts were covered by 3 ml IMDM and the supernatants were changed every second day. The control groups consisted of cells cultured without bone grafts.

*Parameters investigated*

In order to analyze the effects of the sterilization and disinfection procedure on the immunocompetent cell populations *in vitro* the following parameters were determined:

(a) morphological evaluation over the course of the culture procedure
  - raster electron microscopy
(b) determination of vitality with trypan blue stainining
(c) immunoreaction
  - FACS analysis

*FACS Analysis*

The phenotyping of leukocyte subpopulations and their stages of maturity is carried out on the basis of the expression of certain molecules on the cell surface. In FACS analy-

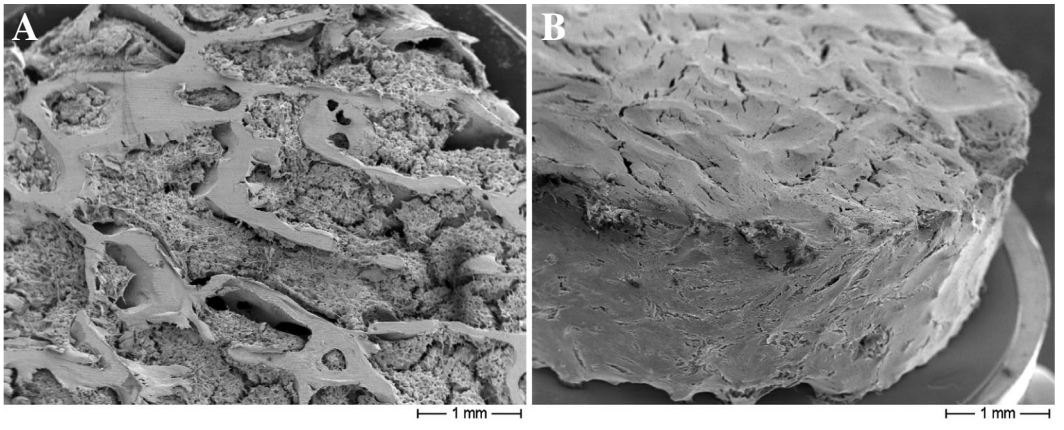


**Figure 1.** (**A**) Bone graft without irradiation before cells were seeded. (**B**) Bone graft without irradiation after 4 weeks *in vitro* culture.

sis these surface molecules are identified with fluorescence-labeled antibodies. The sum of the antibodies typical for a certain surface molecule is termed the "cluster of differentiation (CD)". A FACS analysis was performed on the day on which the cells were prepared and after a culture period of 4 weeks. Accutase® was used to remove the cells from the wells and bone grafts. The cells were then incubated for 2h at room temperature with the following antibodies against specific surface antigens of immunocompetent cells.

- TriTest CD3 FITC/CD19 PE/CD45 PerCP; Becton Dickinson. CD3 positive cells=mature T lymphocytes; CD19 positive cells=B cells
- TriTest CD3 FITC/CD4 PE/CD45 PerCP; Becton Dickinson. CD3 positive/CD4 positive cells=T helper cells
- TriTest CD3 FITC/CD8 PE/CD45 PerCP; Becton Dickinson. CD3 positive/CD8 positive cells=suppressor/cytotoxic T cells
- TriTest CD3 FITC/CD16+56 PE/CD45 PerCP; Becton Dickinson. CD3 negative/CD 16+56 positive cells=natural killer cells (NKCs)

The results were plotted in a system of co-ordinates (dot plot), each cell investigated corresponding to one point. Gates were set to separate individual cell populations and analyze them more exactly. In order to avoid incorrect interpretations for each immunophenotyping procedure a cell sample was stained with a mixture of control cells and antibodies which provided information on non-specific binding and was used to establish an exact setting.

The analysis was performed with the program BD CellquestTM Pro.

## Statistics

For the statistical evaluation the data were analyzed with the aid of the chi-square test. *P*-values<0.05 were considered significant. All values are given as means with the corresponding standard deviations.

## Results

*Raster electron microscopy*

Human bone marrow cells were seeded onto the bone grafts as described above. Figures 1 and 2 show raster electron microscope images that reveal a characteristic spindle-cell-shaped morphology on the bone grafts after 4 weeks' culture.

The bone grafts without irradiation were completely covered after 4 weeks' culture, whereas the gamma-sterilized bone grafts showed a striking reduction in cells and the absence of a fibrous network.

*Vitality tests with trypan blue staining after 4 weeks and separation of the cells*

The vitality of the cells after 4 weeks' culture with the bone grafts showed significantly fewer ($p<0.05$) cells in, on and around the irradiated bone grafts than in the control cultures, which were defined as 100%. It was not possible to perform a satisfactory vitality test of the bone grafts without irradiation as all the cells could be removed from the bone grafts owing to the newly formed extracellular matrix.

*FACS analysis*

The figures show the percentages of the subpopulations of all CD45+ cells (lymphocytes) whose phenotypes were determined. A total of 10,000 events within the CD45+ cell population were analyzed in each case.

- Control cultures

At the start of the culture period ~31.7% of the CD 3+ cells were found. This percentage fell to 9% over the course of the 4 weeks. The percentage of CD $^3/_4$+ cells fell from

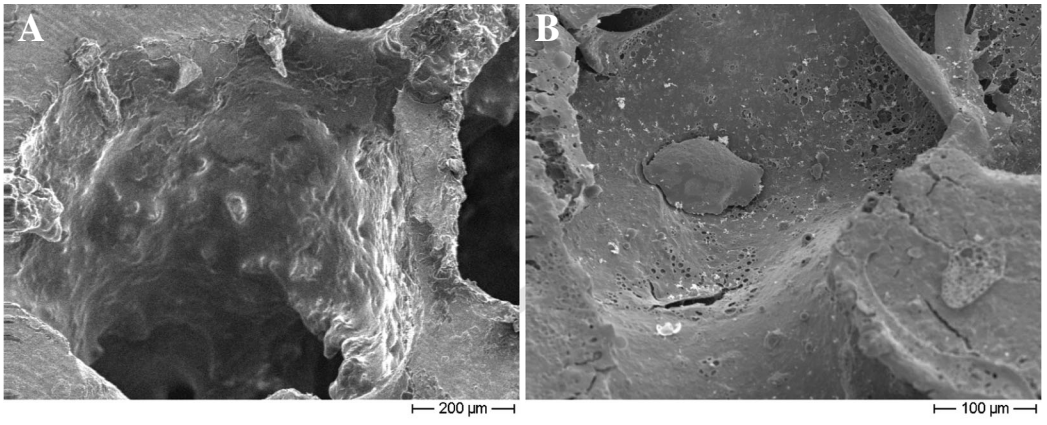


**Figure 2.** (**A**) Bone graft sterilised using 25kGy gamma irradiation before the cells were seeded. (**B**) Bone graft sterilised using 25kGy gamma irradiation after 4 weeks *in vitro* culture.

~33.9% to ~23.2% and that of the CD3/8+ cells, which was initially ~32%, also decreased to ~14.6%. In contrast, the percentage of CD3-/16+/56+ cells rose from ~8.5% to ~12.6% over the 4-week period, while that of the CD19+ cells fell from ~23.1% to ~14.9%.

- Bone grafts without irradiation

After 4 weeks the percentage of CD3+ cells (mature T cells) had increased markedly to ~28% ($p<0.05$), that of the CD3/4+ cells (T helper cells) to ~61% ($p<0.05$), that of the CD3/8+ cells (suppressor/cytotoxic T cells) to ~54% ($p<0.05$) and that of the CD3-/16+/56+ cells (natural killer cells) to ~38% ($p<0.05$).The percentage of CD19+ cells (B cells) remained almost unchanged (~12%).

- Irradiated Bone grafts

During irradiation with 25kGy there was a distinct shift with excessive cell proliferation of CD3/8+ cells (suppressor and cytotoxic T cells) to ~92% ($p<0.05$), CD3/4+ cells (T helper cells) to ~99% ($p<0,05$) and CD3-/16+/56+ cells (natural killer cells) to ~96% ($p<0.05$). There was also a drastic reduction in CD3 and CD4+ (mature T and B cells) to ~3 and 0.1% ($p<0.05$), respectively (Figure 3).

## Discussion

Gamma sterilization has been employed as a safe and effective procedure for sterilizing medicinal products, including bone grafts, for many years[18]. Since the introduction of tissue banks, increased emphasis has been laid on adherence to standards and quality requirements. The goal is to set or find a standard dose that guarantees a high enough level of sterility while at the same time reducing the negative biological effects of the gamma irradiation to a minimum. The dose most widely used for sterilization by tissue banks is 25kGy, although opinions are still divided as to the optimal dose. In their evaluation of the protocols of 36 American tissue banks Vangsness et al. showed that the dose employed varied between 10 and 35kGy[40]. Most of the tissue banks investigated[3,15,22,29,32] follow the recommendations of the IAEA[18-20].

Other groups postulate that a dose of 25kGy is not sufficient to eliminate microorganisms and recommend higher doses of 30kGy and more[2,10].

However, biomechanical studies have demonstrated that at doses >20kGy bone matrix proteins are destroyed and their mechanical properties is compromised[6,24].

A dose of between 15 and 20kGy is therefore recommended. The IAEA and AATB officially recommend a minimum dose of 25kGy for sterilizing bone allografts[1,20].

Biomechanical changes in bone grafts resulting from gamma irradiation have been well investigated[4,9,14,16,42], whereas only few studies on the biological effects of gamma sterilization have been carried out and published.

The remodeling of grafts takes place in two steps. First there is an activation of osteoclasts, followed by bone resorption. During this time the unresorbed portions are available for the bone of the recipient. Later the allograft is completely replaced by new ingrowth of viable bone tissue[12,37].

This process requires osteoconduction and osteoinduction[25,26].

Although the main focus of attention, of studies dealing with bone grafts, is the formation of new bone, the activation of the osteoclasts is the decisive first step in the remodeling process[27]. As soon as a bone graft is implanted a cellular immunoreaction is triggered[33]. This results in the activation of the osteoclasts and resorption of the graft, which is considered essential[30].

It was this situation that prompted the design of the present study. The aim of the study was to investigate the cellular immunoreactions in an *in vitro* model. The main objective

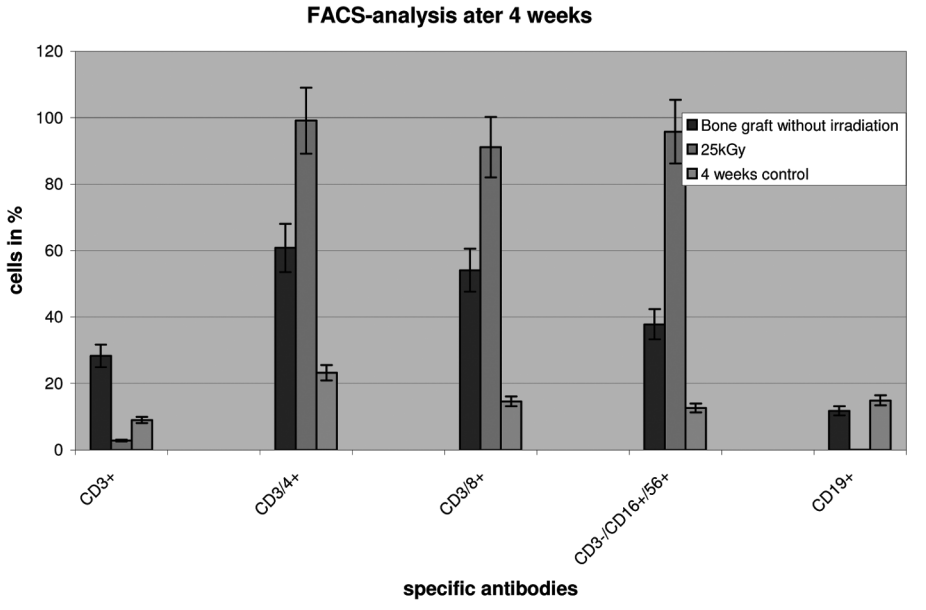


**Figure 3.** FACS analysis after 4 weeks *in vitro* culture.

was to recognize and interpret any changes in the immunocompetent cell populations in a culture of human bone marrow cells.

We decided to use the model of human bone marrow cells in culture for the *in vitro* analysis because the physiological conditions in the human body can best be simulated in this model and the observed reactions are applicable to humans.

Current reports on the effects of gamma sterilization on the osteoinductive potential of bone grafts in the literature are inconsistent[8,21,31] Some study groups have demonstrated that the capacity for osteoinduction is reduced or partially destroyed by gamma sterilization[8,21,31]. However, other groups have failed to find any effects of gamma sterilization on the osteoinductive properties of bone grafts at all[34,35,43]. In our study design we did not attempt to investigate the effect on osteoinductive proteins or osteoinductive properties, e.g. BMPs, but to identify any changes in the immunocompetent cells induced by gamma sterilized bone grafts. The images produced by the raster electron microscope show the typical patterns of cell colonization and matrix formation, despite differences in magnification. The non-irradiated bone grafts were completely covered by homogenously distributed, spindle-shaped cells, while the gamma sterilized bone grafts exhibited a glaze-like surface and the trabecular spaces were colonized by only a few cells (Figures 1, 2). In the bone grafts without irradiation the cellular immunoreaction was most clearly evident in the proliferation of mature T cells, T helper cells, suppressor/cytotoxic T cells and natural killer cells. In contrast, following irradiation with a dose of 25kGy a distinct proliferation of both suppressor and cytotoxic T cells, T helper cells and natural killer cells, while the proportion of mature T and B cells was substantially reduced. Cells brought into contact with foreign material react with defense mechanisms. T helper cells mediate cellular immunoreactions and are involved in the differentiation of B cells into plasma cells. Mature T cells lead to the activation of macrophages, thus triggering a reaction that reaches the level of an inflammatory reaction. As their name suggests, cytotoxic T cells, and also NKCs, lead to unmediated destruction. Osteoimmunologically, T cells are associated with the activation of osteoclastogenesis via the production of mediators. Various studies have revealed indications that T cells play a major role in the development and functioning of osteoclasts. Whether cytokines and chemokines released by activated T cells or other immune cells affect bone metabolism *in vivo*, and if so, how, remains unclear[39]. From the viewpoint described above, activated T-cells and proliferation would be an undesirable rather than a desirable reaction. However, when autologous bone received from donors is employed, rapid and complete osseointegration occurs and thus this immune response seems to be a necessary prerequisite for and the expression of bone remodeling processes. In a study in T cell-deficient nude mice we also found that the absence of T cells led to a reduction in the amount of type III collagen, with an associated impairment of osteoconduction[11]. However, most of the few clinical reports on studies in which gamma sterilized spongiosa has been employed in both femoral[36] and acetabular[7,17] revision surgery are inconsistent with our *in vitro* results. Only Robinson et al. found, for example, no bone integration of the gamma sterilized spongiosa[36]. In contrast, Holt's group found radiological evidence of complete incorporation of gamma-sterilized bone at follow-up after 6 and 13 months[17]. Buckley et al. found an 88% survival rate of gamma-sterilized grafts used

in acetabular revision surgery[7]. This is roughly the same as the survival rate for unirradiated grafts. These good clinical results were also confirmed by Hamer et al., who found histological evidence of complete bone incorporation of the gamma-sterilized grafts employed in 5 cases (re-revision surgery)[16]. A possible explanation for the discrepancies between the clinical findings and the experimental results we have presented could be that the osteoinductive properties are less important that the osteoconductive properties, so long as the grafts were grafted on to healthy bone. This is also corroborated by the observations of Enneking and Campanacci, who investigated allografts removed for histopathological assessment and concluded that their findings suggest that large structural allografts are osteoconductive rather than osteoinductive[12].

In sum, we found a maximum immune response in gamma-irradiated bone grafts, which, interpreted as a sole result, must be seen as a negative biological effect. However, in view of the good clinical results for gamma-sterilized bone grafts other influences would seem to be the determining factors in clinical outcome. Further research is needed to gain a more exact understanding of these factors.

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