Microbial Sterilization and Viral Inactivation in Bone and Soft Tissue Allografts Using Novel Applications of High-Dose Gamma Irradiation: *Report on the Preservation of Structural Integrity and Biocompatibility*

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ABSTRACT: The goal of this study was to determine whether conditions could be identified that would allow delivery of a high dose (50 kGy) of gamma irradiation to human allografts while preserving the structural integrity of the grafts. We identified a combination of low temperature and pretreatment of tissue with radioprotectants that resulted in tissue integrity as good or better than existing allografts that had been irradiated to lower doses with conventional gamma irradiation. We demonstrated robust pathogen inactivation (viruses, bacteria, fungi) in spiked tissue products. We conclude that gamma irradiation can be used to improve the safety profile of human allografts.

INTRODUCTION

The transmission of bacterial and viral infections to recipients of human allograft tissue has been well documented by the Centers for Disease Control.[1-4] The tissue industry has very few regulatory constraints. However, the Office of the Inspector General recently concluded that the FDA needs to take a more aggressive role to ensure the safety of human tissues for transplantation.[5] In 2001 the FDA proposed a new rule, 66FR1507, which is commonly referred to as the Current Good Tissue Practices. Under this rule, the tissue industry would be required for the first time to report all adverse reactions and certain product deviations.

The clinical benefits of allograft tissue are well established. However, a recurrent concern shared by both surgeons and patients with regard to the use of allografts is the potential risk of disease transmission from the cadaveric tissue. Tissue banks take preventative measures to minimize the risk of disease transmission and infection by allografts. They evaluate the donor's social and medical history, excluding all donors with high-risk behaviors. In addition, tissues are held in quarantine pending serological testing for infectious agents such as HIV-1, HIV-2, HTLV-I, HTLV-II, hepatitis B, hepatitis C, and syphilis.[6] Tissue banks also rely on aseptic procurement and processing that involves removal of organic matter, soaking in various disinfectant solutions, and monitoring for microorganisms at various stages of processing.[7] A major limitation to donor screening, however, is the "window" period of infection when the levels of infectious markers are below the sensitivity of the existing assays. Hepatitis C was transmitted recently by processed tissue from a "window" period donor.[3] Reliance on screening methods is also compromised in that they provide no safety assurance for emerging pathogens such as West Nile virus, SARS, and Avian Flu virus. In addition, aseptic processing procedures suffer from the fact that for tissue to have a sterility claim, the graft must be sterile prior to processing. Clearly this is a difficult, if not impossible, criterion to meet for cadaveric tissue.

Surgeons and patients would benefit from a method that terminally sterilizes allografts without adversely affecting the quality of the grafts. Currently, there is not a general consensus as to a standard terminal sterilization method for tissues. Gamma irradiation is effective in inactivating all types of microorganisms as well as lipid-enveloped and non-enveloped viruses.[8,9] Low to moderate doses of gamma irradiation are used routinely to sterilize medical devices,[10] animal sera used for tissue culture,[11] and allograft tissues.[7] Some tissue banks currently employ gamma irradiation doses of 15-25 kGy to treat final product. Other banks do not supply tissue treated with these moderate doses of irradiation because of the documented possibility that the structural integrity of the allograft is compromised following such treatment.[7] Much of the damage that occurs to the tissue results from free radicals and reactive oxygen species generated as a secondary effect of the ionizing radiation.

Clearant has developed a robust, terminal method for achieving microbial sterility that meets or exceeds a sterility assurance level (SAL) of 10^{-6}, the current standard for medical devices. In addition, the process has demonstrated the ability to inactivate ≥ 4 logs of enveloped and non-enveloped viruses. The Clearant Process® exposes tissues to 50 kGy (5.0 Mrad) of gamma irradiation under well-defined conditions that minimize the untoward effects to the grafts. The process reduces radiation-induced damage by *(i)* pre-treating grafts with a biocompatible radioprotectant solution that minimizes the effects of any free radicals that are generated, *(ii)* performing irradiation at dry ice temperature, a condition believed to substantially limit the diffusion of free radicals, and *(iii)* packaging the grafts in an optimized configuration that ensures a tight dose distribution and maintenance of low temperature during irradiation.

We report here the biomechanical strength, biocompatibility, and microbial and viral inactivation for hard and soft tissue grafts treated with the Clearant Process®.

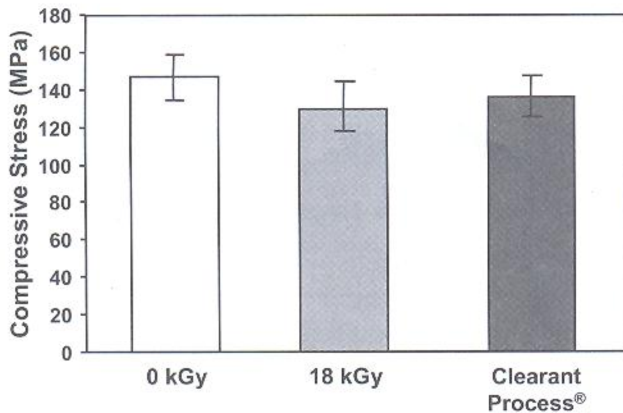

Figure 1. Ultimate compressive stress of cortical bone blocks that were untreated (0 kGy), irradiated to 18 kGy under conventional conditions by the tissue bank, or irradiated to 50 kGy using the Clearant Process®.

METHODS

***Tissue Processing*:** Human femoral cortical bone blocks and bone-tendon-bone allografts that had undergone standard cleaning and processing were provided by Tissue Banks International (TBI, San Rafael, CA). *Bone:* Forty-five cortical bone samples were machined to a size of approximately 5.5 mm X 6.5 mm X 20 mm. Fifteen bone blocks were subjected to the Clearant Process® which includes soaking the tissue in radioprotectant solution (propylene glycol-USP, dimethyl sulfoxide-USP, mannitol-USP, and trehalose), freeze drying bone to an optimized residual moisture, and irradiating to 50 kGy under the conditions described below. Another fifteen bone blocks were freeze dried to <5% moisture and then irradiated to 18 kGy using TBI's former method. The remaining bone samples were fresh frozen and served as the non-irradiated control. *Patellar Tendons:* Two groups of six bone-patellar tendon-bone allografts were gamma irradiated either to 18 kGy by TBI using their former method or to 50 kGy in the presence of the radioprotectant solution.

***Gamma Irradiation*:** The Clearant processed grafts were packaged with dry ice into coolers designed to maintain temperatures below –65°C during the irradiation process. Samples were irradiated to a minimum targeted dose of 50 kGy at Sterigenics. The 18 kGy samples were shipped by TBI to the radiation facility at ambient (bone) or dry ice (bone-tendon-bone) temperatures. The samples were irradiated and shipped under the same conditions.

***Biomechanical Testing*:** *Cortical Bone Blocks:* Compression testing of the bone blocks was performed as described using an MTS Model 858 servohydraulic materials testing machine.[12] *Patellar Tendons:* The bone plugs were potted into PVC pipe rings using steel spring wires drilled into the bone and a polyester resin, making sure that the resin did not contact the insertion site. Cross-sectional areas of the tendons were determined with the use of a specially designed measuring device and a Starrett outside micrometer. Tensile tests were performed using the servohydraulic materials testing machine and custom fixtures to grip the potted bone plugs.

***Biocompatibility*:** In vitro cytotoxicity assays were performed essentially as described using C2C12 murine myoblasts.[13] Cell metabolic activity, an indicator of cell viability, was quantified using an MTT assay per the manufacturer's instructions.

***Pathogen Inactivation*:** Tissue samples that were left untreated or treated with the Clearant Process® were pulverized using a freezer mill and then spiked prior to irradiation with the following pathogens: *E. coli*, *S. aureus*, *B. subtilis*, *B. pumilus*, *A. niger*, *C. albicans*, *C. sordellii*, porcine parvovirus (PPV), and Sindbis virus. Bacterial and fungal cultures were titrated at the time the samples were prepared to determine the spiking titers. Quantification of the microorganisms was performed by plating serial ten-fold dilutions in duplicate on agar plates suitable for each microorganism. Inactivation of PPV and Sindbis virus was quantified by standard $TCID_{50}$ assay.

***Statistical Analysis*:** The effects of treatment were analyzed by one-way ANOVA using Design-Expert 6. A p-value of 0.05 was used to determine statistical significance.

RESULTS

***Biomechanical Testing*:** The strength of bone blocks that were untreated, irradiated to 18 kGy by the tissue bank, or irradiated to 50 kGy using the Clearant Process® was determined using compressive loads. All grafts were tested to failure. The ultimate compressive strength of bone grafts treated with the Clearant Process® was not significantly different from either the untreated, control group or the 18 kGy group (Figure 1).

The patellar tendons were analyzed for structural integrity by tensile strength testing of potted bone-tendon-bone allografts. Because of the high demand for this type of allograft, the study compared only two treatment groups; a conventional 18 kGy group irradiated by the tissue bank, and a group treated with the Clearant Process®. Six samples were tested for each group. The mean tensile strengths were 28.9 MPa and 41.1 MPa for the 18 kGy and Clearant Process® groups, respectively (Figure 2). The difference in strength was not statistically significant ($p = 0.132$). However, there was a striking difference in the types of failures observed between the two groups. A representative failure for each group is also shown in Figure 2. For the 18 kGy group, four failures occurred at the patellar insertion site, one occurred in the tendon midsubstance, and one at the tibial insertion site. In contrast, five of the six failures in the Clearant Process® group occurred in the tendon midsubstance and only one occurred at the patella.

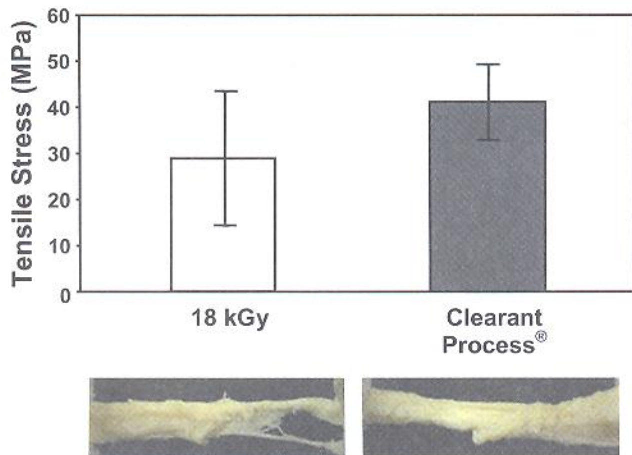

Figure 2. Ultimate tensile strength of bone-tendon-bone grafts that were either irradiated to 18 kGy by the tissue bank or to 50 kGy using the Clearant Process®. A representative failure following mechanical testing from each group is shown.

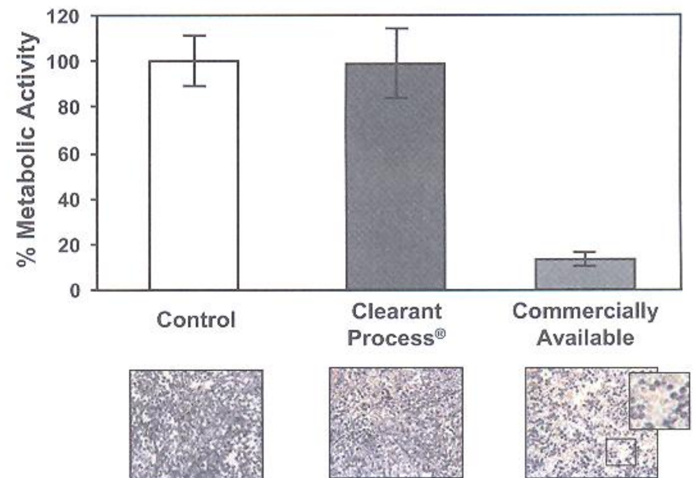

Figure 3. *In-vitro* cytotoxicity of control corticocancellous bone (untreated, 0 kGy) or treated with the Clearant Process® was compared to that of a commercially available product. As an indicator of cell viability, the metabolic activity of each treatment group was measured using an MTT assay. Representative micrographs of C2C12 cells are also shown.

Biocompatibility: *In-vitro* cytotoxicity assays provide a rapid and sensitive method to determine whether significant quantities of biologically harmful extractables are present. The biocompatibility of cortical, corticocancellous, and tendon grafts treated with the Clearant Process® has been tested using a worst-case scenario. Tissue samples were pulverized using a freezer mill in order to maximize the elution of the radioprotectant solution from the tissue, allowing a bolus to be added directly to the cells. A sample similar to a commercially available, bone-based tissue product was also tested. This sample provided a reference for the biocompatibility of a product that is successfully used in patients. The results of one cytotoxicity study for corticocancellous bone treated with the Clearant Process® are shown in Figure 3. There was no significant difference in the metabolic activity of cells treated with extracts from bone subjected to the Clearant Process® and the untreated, non-irradiated, control bone. Extracts from the commercial product resulted in a significant reduction in metabolic activity.

Representative micrographs indicating the viability of the cells prior to quantification are also provided (Figure 3). Cells exposed to the extracts of bone treated with the Clearant Process® had a similar density, morphology, and staining as compared to the untreated control. In contrast, the toxicity observed for the commercial product was evident by a decrease in cell density and cellular staining.

***Pathogen Inactivation*:** The inactivation of nine clinically relevant pathogens (bacteria, fungi, and viruses) was investigated. The pathogens were spiked into bone and tendon grafts in the presence or absence of radioprotectant solution and irradiated to 50 kGy at temperatures below –65°C. Table 1 summarizes the log reduction achieved for each pathogen. All of the bacteria and fungi spiked into the tissues were inactivated, with log reductions ranging from ≥6.0 to ≥9.2 for bone and ≥5.3 to ≥9.0 for tendon, regardless of whether the tissue had been treated with radioprotectant solution. These results indicate that although the radioprotectant solution minimizes the radiation-induced damage to the tissue, it does not protect the microorganisms. The log reductions achieved were limited by the initial titer of the microorganisms spiked into the tissue. No microbial growth was observed when the tissues were placed in culture for 7-10 days, indicating that there were no residual, radiation-resistant microorganisms associated with the tissues.

Porcine parvovirus (PPV) is a model virus for human parvovirus B-19 and hepatitis A. Because it lacks a lipid envelope, PPV is one of the most resistant viruses to inactivate either by gamma irradiation or by other inactivation technologies. Approximately 5 logs of inactivation were detected for PPV in both bone and tendon.

Sindbis virus is a lipid-enveloped virus that is commonly used as a model virus for hepatitis C. Irradiation of tissue containing Sindbis virus resulted in a complete reduction of all of the viral particles that had been spiked into the tissues, ≥4.9 and ≥4.5 logs for bone and tendon, respectively. The inactivation achieved for Sindbis was also limited by the initial viral titer spiked into the tissue.

DISCUSSION

The Clearant Process® is a terminal microbial sterilization and viral inactivation process that includes four distinct parameters important for preserving the structural integrity of allograft tissues. These include: (*i*) control of temperature during irradiation such

Table 1. Log Inactivation of Pathogens Spiked into Bone and Tendon

Pathogen	Type	Bone Log Reduction		Tendon Log Reduction	
		- Radioprotectant	+ Radioprotectant	- Radioprotectant	+ Radioprotectant
E. coli	Aerobic/Gram -	≥ 8.8	≥ 9.2	≥ 8.3	≥ 8.0
S. aureus	Aerobic/Gram +	≥ 8.9	≥ 9.0	≥ 8.8	≥ 8.8
B. subtilis	Aerobic/Gram +/Spore	≥ 7.1	≥ 6.0	≥ 5.5	≥ 5.3
B. pumilus	Aerobic/Gram +/Spore	≥ 8.9	≥ 8.9	≥ 8.4	≥ 9.0
A. niger	Aerobic/Fungi/Spore	≥ 9.2	≥ 9.2	≥ 9.0	≥ 9.0
C. albicans	Aerobic/Fungi	≥ 8.1	≥ 8.0	≥ 7.9	≥ 8.1
C. sordellii	Anaerobic/Gram +/Spore	≥ 6.3	≥ 7.4	≥ 6.8	≥ 6.4
PPV	Non-enveloped/ssDNA	5.3	5.2	5.4	4.9
Sindbis	Enveloped/ssRNA	≥ 5.1	≥ 4.9	≥ 4.6	≥ 4.5

That the temperature of the tissue does not rise above –65°C; *(ii)* pretreatment of the tissue with a biocompatible radioprotectant solution prior to irradiation; *(iii)* attention to the packing configuration and the dose map of the radiation facility to insure a tight min/max exposure of the tissue and *(iv)* for bone, an optimized freeze drying cycle that leaves bone dehydrated rather than freeze dried (<6% moisture). These parameters along with the 50 kGy radiation dose distinguish allografts treated with the Clearant Process® from those currently treated with conventional doses of gamma irradiation.

We were able to provide a safer and efficacious allograft for both the cortical bone and bone-tendon-bone allografts following 50 kGy of gamma irradiation with the Clearant Process®. We and others have demonstrated that when the secondary or free radical effects of gamma irradiation are controlled, the pathogen inactivation kinetics follow a log-linear relationship with increasing dose.[14-16] Thus, if 3 logs of inactivation were achieved with 18 kGy of gamma irradiation, greater than 8 logs inactivation would be achieved with 50 kGy. That is, the risk of transmission of an infectious agent would be reduced by a factor greater than 10,000 over currently irradiated allografts. The Clearant Process® provides a significant enhancement in the safety profile of human tissue. It is particularly relevant to pathogens such as bacterial spores and non-enveloped viruses that are Are relatively resistant to existing pathogen inactivation technologies. This concept is illustrated by the inactivation achieved with 50 kGy of gamma irradiation of porcine parvovirus. Porcine parvovirus is the accepted model virus for human parvovirus B-19 and hepatitis A. Five logs of inactivation are achieved in tissue spiked with this virus (Table 1). Although the clinical consequences of B-19 or hepatitis A are not generally severe, the data highlights the risk of an emerging non-enveloped virus to the patient. Only 2-3 logs of inactivation of this virus are achieved with an 18 kGy dose. Existing chemical inactivation procedures employed in the tissue industry are equally ineffective.[17] The fact that "window period" transmissions of infectious enveloped viruses have been documented emphasizes the need for more effective inactivation treatments for human tissue.[3] In addition, 50 kGy of gamma irradiation provides a greater microbial sterility assurance of tissue products relative to existing gamma irradiation protocols. More importantly, the use of an effective terminal sterilization and viral inactivation protocol could eliminate the need for harsh chemical treatments of tissue such as hydrogen peroxide, which is known to inactivate important factors such as bone morphogenic protein 2.[17] Finally, the data presented here indicate that these increases in the safety margin associated with allografts can be achieved without compromising the functional integrity of the implant.

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