Inactivation of enveloped and non-enveloped viruses on seeded human tissues by gamma irradiation

Mark A. Moore

Abstract Human tissue allografts are widely used in a variety of clinical applications with over 1.5 million implants annually in the US alone. Since the 1990s, most clinically available allografts have been disinfected to minimize risk of disease transmission. Additional safety assurance can be provided by terminal sterilization using low dose gamma irradiation. The impact of such irradiation processing at low temperatures on viruses was the subject of this study. In particular, both human tendon and cortical bone samples were seeded with a designed array of viruses and the ability of gamma irradiation to inactivate those viruses was tested. The irradiation exposures for the samples packed in dry ice were 11.6–12.9 kGy for tendon and 11.6–12.3 kGy for bone, respectively. The viruses, virus types, and log reductions on seeded tendon and bone tissue, respectively, were as follows: Human Immunodeficiency Virus (RNA, enveloped), >2.90 and >3.20; Porcine Parvovirus (DNA, non-enveloped), 1.90 and 1.58; Pseudorabies Virus (DNA, enveloped), 3.80 and 3.79; Bovine Viral Diarrhea Virus (RNA, enveloped), 2.57 and 4.56; and Hepatitis A Virus (RNA, non-enveloped), 2.54 and 2.49, respectively. While proper donor screening, aseptic technique, and current disinfection practices all help reduce the risk of viral transmission from human allograft tissues, data presented here indicate that terminal sterilization using a low temperature, low dose gamma irradiation process inactivates both enveloped and non-enveloped viruses containing either DNA or RNA, thus providing additional assurance of safety from viral transmission.

Keywords Viral inactivation · Gamma irradiation · Bone allografts · Soft tissue allografts · Sterilization

Abbreviations

AATB	American association of tissue banks
BVDV	Bovine viral diarrhea virus
CDC	Center for disease control
D_{10}	In this context, the radiation dose required for one log reduction of viral load
DNA	Deoxyribonucleic acid
FrhK-4	Fetal rhesus monkey kidney
HAV	Hepatitis A virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
NAT	Nucleic acid testing
PPV	Porcine parvovirus
PrV	Pseudorabies virus
RNA	Ribonucleic acid
SAL	Sterility assurance level

M. A. Moore (✉)
LifeNet Health, 1864 Concert Drive, Virginia Beach, VA 23453, USA
e-mail: mark_moore@lifenethealth.org

Introduction

Human tissue allografts are widely used in a variety of clinical applications. In particular, the use of bone

and soft tissue allografts for orthopedic repair has gained wide acceptance over the past two decades (Zhang et al. 1994; Peterson et al. 2001; Fanelli et al. 1996; Gasser and Uppal 2006; Mirzayan 2005). The American Association of Orthopedic Surgeons estimates that over 1.5 million musculoskeletal allografts are used annually (Joyce et al. 2008). Since the 1990s, most clinically available allografts have been disinfected to minimize risk of disease transmission. However, disease transmission is still a risk as highlighted by a 2002 Center for Disease Control (CDC) investigation which identified twenty-six cases of bacterial infections that stemmed from allograft tissue, one of which resulted in the death of a 23-year-old man (Jones et al. 2007; Grieb et al. 2006; Vangsness et al. 2006; CDC report 2002; Kainer et al. 2004). The perceived risk of postoperative infection is noted by several authors as the single largest potential disadvantage in choosing an allograft (McGuire and Hendricks 2009; Shino et al. 2002). While risk of infection or disease transmission is thought to be a major disadvantage of allografts, an American Academy of Orthopedic Surgeons tissue work group (Joyce et al. 2008), reports "Disease transmission is rare when comparing reports of infection versus number of allografts distributed/ yr." The tissue banking community has responded to the remaining risk by developing methods to terminally sterilize allograft tissues in their final package (Joyce et al. 2008). The provision of sterile allograft tissue, which is practiced by some tissue banks, can be accomplished by following several steps which include donor screening, microbiological testing, aseptic recovery and processing, disinfection, and, finally, terminal sterilization (McAllister et al. 2007). These processes can be validated to yield a microbial sterility assurance level (SAL) of 10^{-6} at a low dose of irradiation (Baker et al. 2005; Moore et al. 2004; Nguyen et al. 2011). An SAL of 10^{-6} indicates there is a 1 out of 1,000,000 chance that a viable organism exists with any single graft (Vangsness et al. 2003). Achieving this SAL by a validated process allows labeling of terminally sterilized allografts as sterile (ANSI/AAMI 2008). To address clinical effectiveness, this terminal irradiation sterilization process has also been demonstrated to have no significant impact on biomechanical or biological properties (Balsly et al. 2008; McGilvray et al. 2005; Greaves et al. 2008; Goertzen et al. 1995; Rihn et al. 2006).

In addition to microbial sterility, however, the elimination of viruses is essential in assuring tissue safety. In March of 2005, the American Association of Tissue Banks (AATB) sought to further reduce the risks of Human Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV) transmission by requiring nucleic acid testing (NAT) in donor screening procedures (Rigney 2004). NAT has been shown to markedly reduce the window period of these viruses and was incorporated into blood banking procedures, effectively reducing the risk of HIV and HCV transmission from 1 in 1.5 million blood units and 1 in 276,000 blood units, respectively, to 1 in 2 million blood units for either virus (Stramer et al. 2004). Also, the introduction of NAT has halved the window period for HIV-1 to 12 days from the previous 22 day antibody window (McGuire and Hendricks 2009). Encouragingly, one report indicates that no instances of viral transmission are reported to have ever occurred from tissue processed following proper donor screening procedures that include HIV testing (Peterson et al. 2001). While NAT screening reduces viral risks, these methods only address specific viruses. Previously Chen (2008) described the impact of disinfection processing solutions (US Patents 5,556,379; 5,820,581; 5,977,034; 6,024,735) on a variety of enveloped and non-enveloped viruses. While these processes, in addition to proper screening and NAT, greatly reduce any risk of viral transmission, terminal sterilization has the potential to provide greater assurance of viral safety. Specifically, gamma irradiation has been demonstrated to lead to viral inactivation even at low dosages. (Campbell et al. 1994; Conway et al. 1991; Dugan and Trujillo 1975; Fideler et al. 1995; Grecz et al. 1987; Grieb et al. 2005, 2006; House et al. 1990; Kaupert et al. 1999; Miekka et al. 1998; Pelka et al. 1993; Pruss et al. 2002; Sullivan et al. 1973; Thomas et al. 1982). However indicative, these studies were not comprehensive to the wide array of target viruses, tissues, and desired irradiation conditions.

While these numerous studies all addressed various viruses and conditions, we sought to perform a comprehensive assessment of the impact of gamma irradiation at low temperatures on both seeded human tendon and cortical bone and to examine a designed array of viruses in a single study. Low temperature irradiation exposure was chosen as this has previously been shown to minimize free radical generation

and tissue damage (Anderson et al. 1992; Hamer et al. 1999). Viruses were chosen to represent an array of enveloped, non-enveloped, RNA, and DNA viruses, and included Human Immunodeficiency Virus (HIV), Porcine Parvovirus (PPV), Pseudorabies Virus (PrV), Bovine Viral Diarrhea Virus (BVDV), and Hepatitis A Virus (HAV). Here, the hypothesis that low temperature gamma irradiation of seeded human tendon and bone would result in the reduction of a comprehensive array of RNA, DNA, enveloped, and non-enveloped viruses was tested.

Methods

The study design was to separately seed tendon and bone human tissues with a designed array of viruses and then subject the separate tissues to low temperature, low dose gamma irradiation, and test for surviving viruses. Human tibialis tendons and cortical bone derived from long bones were obtained with generous research authorization. Tendon and cortical bone used in the study came from multiple donor sources. Bone tissue was from two male donors, ages 55 and 77. Tendon was from two male and two female donors, ages 20, 55, 64, and 64, respectively. However, to provide internal control, each individual virus tested per tissue type was tested with a single donor tissue. As an example, all tests for recovery, interference, toxicity, viral reduction, and time = 0 controls to test, e.g., HIV with tendon were perform using individual tendon samples from a single donor. Prior to use, all donors were serologically screened by either antibody or nucleic acid testing and the absence of the following agents was confirmed: Hepatitis B,

Hepatitis C, HIV, and HTLV. The individual tissue portions tested consisted of a single, intact cortical bone piece or tendon segment, respectively. Prior to seeding and irradiation, tissues were processed using the Allowash® disinfection technology (US Patents 5,556,379; 5,820,581; 5,977,034; 6,024,735) to mimic the anticipated tissue condition prior to terminal irradiation. In order to perform a comprehensive study, an array of viruses was tested to include every permutation of DNA, RNA, enveloped, and non-enveloped characteristics. In addition, a specific environmentally relevant virus (HIV) was included. The viruses tested thus included: HIV, HTLV-IIIB strain, an 80–130 nm enveloped RNA containing retrovirus; PPV, NADL-2 strain, an 18–26 nm, non-enveloped, DNA-containing parvovirus; PrV, SHOPE strain, a 150–200 nm, enveloped, DNA-containing virus; BVDV, Singer strain, a 50–70 nm, enveloped RNA-containing pestivirus; and HAV, HM175 strain, 18f, a 28–30 nm, non-enveloped, RNA-containing picornavirus. To qualify the test methodology, toxicity and interference studies were performed at the testing facility (Wuxi Apptec, Philadelphia, PA). To prepare samples for indicator cell toxicity, 1 g tissue portions were mock-spiked with 0.5 mL of virus suspension media and allowed to absorb into the tissue for 15 min at 2–8°C, followed by tissue homogenization (tendon) or suspension (bone) in a total of 9.5 mL of virus resuspension media. Serial dilutions (in serum-free media) of this solution were tested for toxicity against the virus indicator cell lines appropriate for each virus (see Table 1). Serial dilutions (in serum-free media) of this solution were tested for toxicity against the virus indicator cell lines appropriate for each virus (see Table 1). Cell

Table 1 Viral reduction-human tendon (all values in log)

Virus	HIV	PPV	PrV	BVDV	HAV
Indicator cell line	CEM-A	ST	CV-1	BT	FrhK-4
Titer-seeded	6.84	6.72	7.06	7.82	8.40
Gamma-T_o	5.47	5.96	5.77	7.23	7.89
Gamma-treated	<2.57	4.06	1.97	4.66	5.35
Total log reduction	>2.90	1.90	3.80	2.57	2.54
Approximate D_{10} value	<4	6	3	5	5

Viral reduction of seeded human tendon treated with gamma irradiation at low temperatures. Shown are the indicator cell lines, titer seeded onto allograft, titer of recovered virus from seeded grafts (Gamma-T_0), titer of recovered virus from seeded and irradiated grafts (Gamma-T_0), and total log reduction (Gamma-T_0 minus Gamma-treated). Also shown is a rounded approximate D_{10} value calculated by dividing the 11.6–12.9 kGy dose by the total log reduction and assuming a linear dose–response relationship

monolayers were read by visually evaluating the percentage of monolayer remaining per surface area of the well. No toxicity was observed in the negative control wells; therefore 100% of the monolayer was present and intact. For the test sample, if toxicity was observed (i.e., a percentage of that monolayer was missing), that percentage was determined visually by comparison to the negative control. One technician determined the percentage of cytotoxicity (if any) and a second operator was required for verification. Samples which reduced the cell numbers below 80% of controls were considered toxic. Extracts prepared to test interference were likewise prepared, followed by dilution to a non-toxic level, as determined by the toxicity assay. Then, samples were spiked to 5% v/v with virus stock solution. Controls consisted of serum-free media also spiked to 5% v/v with virus stock solution. Samples which altered the virus titer by >0.5 $\log_{10}$ were considered to interfere. The experimental groups (Gamma-treated), were prepared by seeding 0.5 mL of a high titer viral solution (see Tables 1, 2) on 1 g of tissue and allowing absorption for 15 min at 2–8°C. These tissues were placed into a container on dry ice and shipped for irradiation using a ^{60}Co source (Steris, Morton Grove, IL). The irradiation was performed while the samples were still stored on dry ice to maintain low temperatures. The container had previously been mapped for internal irradiation dosage under processing conditions in order to accurately measure tissue exposure. Following irradiation at 11.6–12.3 kGy for cortical bone or 11.6–12.9 kGy for tendon, respectively, the samples were returned to the testing facility and maintained at or below −60°C until viral testing. For testing, the 1 g samples were homogenized (tendon) or suspended (bone) in a total

of 9.5 mL virus resuspension media. A portion was diluted to non-toxic and non-interfering levels, as determined by toxicity and interference testing, serially diluted, and infectivity determined in the appropriate indicator cell line (see Table 1). Controls (Gamma-T$_o$) were similarly seeded, extracted as above, and samples maintained at or below −60°C until final testing along with the test groups.

Results

The test methods were qualified using methods described in the "Methods" section and no interference or toxicity was noted towards any of the viruses at the experimentally pertinent dilution levels. In detail, no tissue extracts were found to be toxic to indicator cells at greater than a 10-fold dilution. All test article extracts were diluted 20-fold for final titers, which was beyond any lower level of dilution which may have exhibited toxicity. Likewise, no diluted tissue extracts exhibited interference at greater than a threefold dilution, again, which was a lower level of dilution than the 20-fold dilution used for all test article extracts. In-process dosimetry verified the dose range at 11.6–12.3 kGy for cortical bone samples and 11.6–12.9 kGy for tendon samples. Following seeding and gamma irradiation, the samples were tested as described. Three readings were taken per test sample. The log quantities of virus extracted from controls to gamma irradiated samples, as well as the seeding titer, are shown for tendon (Table 1) and bone (Table 2), respectively. In addition, rounded D_{10} value for the various viruses are also shown in Tables 1 and 2. Total log reduction was

Table 2 Viral reduction human cortical bone (all values in log)

Virus	HIV	PPV	PrV	BVDV	HAV
Indicator cell line	CEM-A	ST	CV-1	BT	FrhK-4
Titer-seeded	6.92	7.30	8.17	7.11	8.12
Gamma-T$_o$	5.77	7.11	8.01	7.01	8.28
Gamma-treated	<2.57	5.53	4.22	2.45	5.79
Total log reduction	>3.20	1.58	3.79	4.56	2.49
Approximate D_{10} value	<4	8	3	3	5

Viral reduction of seeded human cortical bone treated with gamma irradiation at low temperatures. Shown are the indicator cell lines, titer seeded onto allograft, titer of recovered virus from seeded grafts (Gamma-T$_0$), titer of recovered virus from seeded and irradiated grafts (Gamma-T$_0$), and total log reduction (Gamma-T$_0$ minus Gamma-treated). Also shown is a rounded approximate D_{10} value calculated by dividing the 11.6–12.3 kGy dose by the total log reduction and assuming a linear dose–response relationship

Table 3 Virus test groups and log viral inactivation summary

Virus	Nucleic acid	Virus type	Bone	Tendon
Human immunodeficiency virus (HIV)	RNA	Enveloped	>3.20	>2.90
Porcine parvovirus (PPV)	DNA	Non-enveloped	1.58	1.90
Pseudorabies virus (PrV)	DNA	Enveloped	3.79	3.80
Bovine viral diarrhea virus (BVDV)	RNA	Enveloped	4.56	2.57
Hepatitis A virus (HAV)	RNA	Non-enveloped	2.49	2.54

Total log reduction of viruses seeded on human tissue by gamma irradiation. Note the variety of viruses used including all permutations of DNA, RNA, enveloped, and non-enveloped viruses. The total log reduction as calculated in Tables 1 and 2 is summarized

considered the difference between the Gamma-T_o and Gamma-treated sample titers. The irradiation dose tested, in the range of 11.6–12.9 kGy, yielded an approximate 2–5 log reduction of the various viruses tested, as summarized in Table 3. In detail, the viruses, virus types, and log reductions on seeded tendon and bone tissue, respectively, were as follows: HIV (RNA, enveloped), >2.90 and >3.20; PPV (DNA, non-enveloped), 1.90 and 1.58; PrV (DNA, enveloped), 3.80 and 3.79; BVDV (RNA, enveloped), 2.57 and 4.56; and HAV (RNA, non-enveloped), 2.54 and 2.49, respectively. Thus, the hypothesis is proven that a low temperature, gamma irradiation process will inactivate DNA, RNA, enveloped, and non-enveloped viruses.

Discussion

The increased use of allograft tissue for musculoskeletal repair has brought more focus to the safety of allogeneic tissue and the efficacy of various sterilization techniques. As previously mentioned, the provision of sterile allograft tissue can be accomplished by following several steps which include donor screening, microbiological testing, aseptic recovery and processing, disinfection, and terminal sterilization (McAllister et al. 2007). One such method of terminal sterilization is gamma irradiation; which can provide both bactericidal and virucidal effects via direct alteration of nucleic acids leading to infectious agent dysfunction and destruction (Vangsness et al. 2003). Of particular interest, numerous reports indicate the inactivation of HIV with gamma irradiation (Campbell et al. 1994; Conway et al. 1991; Conway and Tomford 1992; Hiemstra et al. 1991; Pruss et al. 2002; Salai et al. 1997; Sullivan

et al. 1973). While a few studies have concluded that at least 30 kGy of gamma irradiation is needed to inactivate HIV (Fideler et al. 1994; Hernigou et al. 2000), these reports have assumed HIV is present in high density levels. While 30 kGy may be necessary to inactivate high-density amounts of HIV, it is excessive for lower density levels of the virus that might be found, if at all, in medically screened, NAT tested, and disinfectant processed tissue. In example, Conway et al report a D-10 value for HIV of approximately 4 kGy indicating a potential terminally sterilizing dose of 12 or 16 kGy would yield 3 or 4 log reduction of HIV. Again, the likelihood of any virus, much less large quantities following proper donor eligibility, medical history, and NAT testing is remote to begin with. As demonstrated here, an irradiation dose of 11.6–12.9 kGy administered at low temperature yields an approximate 2–5 log reduction of the various viruses tested including a minimum of 3 log reduction of HIV seeded on both tendon and bone tissues. Please note that this irradiation dosage may be at or below an actual dose used by a manufacturer to achieve terminal sterilization, and thus these values may be considered a worst case situation for log inactivation. To illustrate, if an allograft provider were to sterilize tissue at, e.g., 17.4–19.4 kGy (a 50% higher dose than used in the study reported here) then the anticipated log reduction would rise 50% as well. This is significant as current allograft terminal sterilization methods may range from just over 10 kGy up to as much as 50 kGy (Block 2006). An additional reason for including a terminal irradiation step in allograft processing is to safeguard against those viruses which are currently not tested or are unknown. A study limitation is that individual tests were performed with a single sample of material. While this hampers any conclusion of

statistical significance, note that accompanying controls (recovery, toxicity, and interference tests) were performed with tissue from the same donor to provide internal control. In addition, multiple readings were taken of each test sample increasing confidence in results. Another study limitation is the use of small tissue samples (1 g) and not whole bone or tendon allografts. While whole allografts would have been impractical to test, the relative viral load can be extrapolated from the small samples to a large graft. Enhancing the validity of the study, however, is that the test inactivation agent studied here is gamma irradiation which will fully penetrate tissue and is thus not dependent on tissue size or dimension.

Conclusion

Terminal sterilization of human allograft tissue using a low dose gamma irradiation process inactivates both enveloped and non-enveloped viruses containing either DNA or RNA. This final step in the processing of human allografts complements prior steps in providing additional assurance of viral safety.

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