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# Tissue Processing: Role of Secondary Sterilization Techniques

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## KEYWORDS

• Allograft • Musculoskeletal • Sterilization

The use of allografts in sports medicine surgery has been steadily increasing over the past 10 to 15 years as long-term reports have shown that results with musculoskeletal allografts approach those with autografts.<sup>1-4</sup> The use of musculoskeletal allografts from the American Association of Tissue Banks (AATB) accredited tissue banks increased from 337,338 in 1996 to 1,279,000 in 2003 (**Table 1**).<sup>5</sup> Each year approximately 1.5 million bone and tissue allografts are implanted in the United States, of which approximately 10% are soft-tissue grafts, most commonly bone-patellar tendon-bone (BPTB), Achilles tendon (**Fig. 1**), fascia lata, anterior and posterior tibial tendon (**Fig. 2**), quadriceps and hamstring tendon, and menisci.<sup>6,7</sup> A 2006 member survey by the American Orthopaedic Society for Sports Medicine (AOSSM) indicated that 86% used allografts in knee reconstructive procedures;<sup>8,9</sup> however, despite this widespread use, a substantial number of surgeons expressed concerns about the risk of disease transmission and infection with allografts. A number of advantages of allografts over autografts have been cited, including no donor-site morbidity, shorter operative time, smaller incisions, and greater availability, but all of these have been overshadowed by the most frequently cited disadvantage: risk of disease transmission.<sup>10-12</sup> Recent reports of serious infections associated with allografts have heightened these concerns.<sup>13-15</sup> Of 26 bacterial infections associated with allografts reported to the Centers for Disease Control and Prevention (CDC), 70% were in patients who had anterior cruciate ligament (ACL) reconstructions.<sup>13,14</sup> A 2004 report<sup>16</sup> indicated that of the 875,000 musculoskeletal allografts distributed in 2001, clostridium infections occurred in 0.12% of all sports medicine tissues (tendons, menisci, and femoral condyles).

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Year	No. of Donors	Musculoskeletal Tissue Allografts Distributed
1996	17,010	337,338
2001	20,490	710,064
2003	23,295	1,279,000

Data from Vangness CT Jr. How safe are soft-tissue allografts? AAOS Now, August 2007. Available at: <http://www.aaos.org/news/bulletin/aug07/clinical1.asp>.

### RISK OF DISEASE TRANSMISSION FROM MUSCULOSKELETAL ALLOGRAFTS

Donor screening and testing (**Table 2**) can reduce the possibility of disease transmission, but a “window” period still exists during which a donor with an active viral infection may not have any detectable viral antibodies or antigens.<sup>17</sup> With nucleic acid testing (NAT), this window is approximately 7 days for human immunodeficiency virus (HIV) and hepatitis-C virus (HCV) and about 8 days for hepatitis-B virus (HBV).<sup>18</sup> Currently, the risk of transplanting tissue from an HIV-infected donor is estimated to be 1 in 1.6 million.<sup>11,19–21</sup> Because of the greater prevalence of hepatitis in the general population, estimated to be 1.2 million infected with HBV and 3.9 million with HCV,<sup>17</sup> the risk of the transmission of HBV or HCV is greater than that of HIV. The risk of contracting HCV from unprocessed tissue that is NAT HCV negative is estimated to be 1 in 421,000.<sup>21</sup> McAllister and colleagues<sup>9</sup> noted that the current risk of an allograft-transmitted infection appears to be much less than the overall risk of perioperative nosocomial infection.

More recently, emerging pathogens have become a concern in the use of allograft material. Little information exists about the potential threat from such entities as West Nile virus, severe acute respiratory syndrome (SARS) coronavirus, and prion disease associated with transmissible spongiform encephalopathies such as Creutzfeldt-Jakob disease (CJD) and its variants. Between 1985 and 2002, 97 occurrences of CJD were reported in Japanese patients who had received dura mater allografts; the rate of infection declined after improved processing procedures were introduced in 1987.<sup>22</sup> No prion-disease transmission has been reported in association with musculoskeletal allografts, and the risk of acquiring these diseases as the result of



**Fig. 1.** Commonly used allografts in knee surgery. Top, Achilles tendon graft used for reconstruction of the posterior cruciate ligament. Bottom, Bone-patellar tendon-bone graft used for reconstruction of the anterior cruciate ligament.



Fig. 2. Tibialis allograft.

Table 2 Process of allograft procurement, sterilization, and storage	
Donor screening	Precluded by history of autoimmune disease Ingestion or exposure to toxic substances Rheumatoid arthritis Systemic lupus erythematosus Polyarteritis nodosa Sarcoidosis Clinically significant bone disease Blood testing must be negative for antibodies to HIV Nucleic acid test (NAT) for HIV-1 Hepatitis B surface antigen Total antibody to hepatitis B core antigen, Antibodies to hepatitis C virus (HCV) NAT for HCV Antibodies to human T-lymphotropic virus Syphilis
Tissue harvest	Within 24 h of death if body cooled Within 15 h of death if body not cooled Aseptic technique Tissue cultured before processing
Disinfection: removal of contaminants	Antibiotic soaks
Secondary sterilization: destruction of all life forms	Ethyl oxide, other chemical sterilants Gamma/electron-beam irradiation Proprietary protocols (ie, Allowash, BioClense, Clearant)
Storage	Fresh allograft (use within 24 d) Fresh-freezing (3–5 y) Cryopreservation (up to 10 y) Lyophilization (3–5 y at room temperature)

a musculoskeletal allograft is unknown, although it is likely extremely low because of the rarity of these diseases in the general population.<sup>9</sup>

### **OVERSIGHT OF PREPARATION OF MUSCULOSKELETAL ALLOGRAFTS**

In the United States, oversight of tissue banks takes place at 3 levels: the American Association of Tissue Banks (AATB), the Food and Drug Administration (FDA), and state agencies.<sup>6</sup> The AATB has developed standards for tissue banking, and it accredits tissue banks but has no power to shut down a tissue bank, fine or imprison its operators, or order the retention or destruction of tissue that does not comply with minimal requirements.<sup>6</sup> The FDA does have that power, but one of their limitations is that registration of tissue banks has not been required, making it difficult for the FDA to identify and inspect such entities.<sup>23,24</sup> Only a small percentage of tissue banks are AATB-accredited, and few states require tissue banks to be licensed. In 2005, the FDA set up 3 new regulations for entities involved in human tissue products: “registration” rules for tissue banking institutions, “donor eligibility” rules that provide criteria for donor screening and selection, and “current good tissue practices” rules that concern tissue procurement, processing, and distribution.<sup>25</sup> Currently, there is more federal oversight of tissue banks and improved donor screening and testing techniques, including the use of NAT. In the United States, all establishments that collect, process, or handle human cells, tissues, and cellular or tissue-based products must now register with the FDA.

### **DECREASING THE RISK OF DISEASE TRANSMISSION BY MUSCULOSKELETAL ALLOGRAFTS**

The FDA does not require that tissues undergo sterilization nor does it require that recovery and processing of tissues be done in an aseptic manner, both of which are essential to improving allograft safety.<sup>24</sup> Sterilization of musculoskeletal tissues has several inherent problems: the biomechanical integrity of the tissue can be substantially altered by heat and irradiation, not all sterilizing agents have adequate tissue penetration, and musculoskeletal tissues are often contaminated with a large number of organisms.

#### ***Aseptic Procurement***

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Aseptic procurement is a fairly standardized procedure in which standard sterile operating room techniques are used, including using gowns, gloves, and sterile instruments. Aseptically processed tissues, however, should not be considered sterile.<sup>26</sup> Contamination from health care personnel or from the donor (gastrointestinal or respiratory tract) may not be eliminated or even adequately reduced by soaking in antibiotic solution, as is done in most tissue banks to reduce the surface contamination (bioburden) of the allograft tissue. Although culturing of allograft tissue is commonly done to check for the presence of bacteria and fungi after soaking, studies have shown that cultures are, at best, only 78% to 92% sensitive.<sup>27</sup>

#### ***Disinfection and Secondary Sterilization***

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Disinfection—removal of contaminants from the tissue—should not be mistaken for sterilization—destruction of all forms of life, especially microorganisms. Sterility is expressed as a mathematic probability of relative risk. The FDA considers a sterility assurance level (SAL) of  $10^{-3}$  (1 in 1,000 chance that a nonviral viable microbe exists) adequate for implantable biologic medical devices.<sup>24</sup> The AATB requires an SAL of  $10^{-6}$  (less than a 1 in 1,000,000 possibility of a contaminating organism) for tissue bank allografts.<sup>28</sup> Unlike surgical instruments and equipment, it is practically

impossible to absolutely sterilize human tissue without compromising the biomechanical properties or biocompatibility of these tissues. For example, heat and high doses of radiation (>3.0 Mrad) can effectively provide an SAL of  $10^{-6}$ , but both can weaken the collagen structure of the allograft.<sup>29,30</sup>

### **Chemical sterilization**

Chemical sterilization agents have included peracetic acid (PAA), ethylene oxide, hydrogen peroxide, supercritical carbon dioxide, beta-propiolactone, and glutaraldehyde; the last 2 are no longer used because of their toxicity, and the others are generally used in combination with other methods of sterilization.

Ethylene oxide, commonly used for sterilizing medical devices, was one of the first methods used to sterilize allografts.<sup>31</sup> Chemical residues left by the sterilization process, however, were suggested to cause intra-articular reactions with chronic synovitis, graft failure, and bone dissolution.<sup>32,33</sup> Ethylene oxide has been reported to have some carcinogenic effects in workers exposed to it,<sup>34</sup> but there is no evidence that allografts sterilized with ethylene oxide have induced cancer.<sup>20</sup> In patellar tendon grafts, ethylene oxide can cause a foreign body reaction that results in dissolution of the graft,<sup>32,33,35</sup> termed the “applesauce reaction” by Arnoczky because of the appearance of the dissolved graft.<sup>36</sup> This sterilization method is rarely used today.

PAA has been used since the early 1980s, mainly to sterilize bone allografts. Several preliminary *in vitro* studies suggested that it produced no adverse effects on the structural and mechanical properties of treated bone grafts.<sup>37,38</sup> Analyses of the mechanical function of BPTB grafts *in vitro* revealed no adverse effects of PAA sterilization compared with unsterilized grafts.<sup>39</sup> A more recent study,<sup>40</sup> however, found in a goat model that PAA sterilization delayed or partially inhibited the biological remodeling of PAA grafts, leading to impaired functional knee stability and reduced structural properties of the graft during subsequent healing up to 3 months. The authors recommend caution when considering PAA-sterilized allografts for ACL reconstruction.

### **Radiation sterilization**

Gamma irradiation has been shown to be effective for sterilization of allograft tissues, killing bacteria at doses of 1.5 to 2.6 Mrad;<sup>4</sup> higher doses (>3.5 Mrad) are necessary to kill viruses.<sup>29,30,41</sup> Fideler and colleagues<sup>29</sup> found that some HIV-infected bone-tendon-bone allografts remained positive for the virus after 2.5 Mrad of irradiation and recommended that grafts be exposed to levels as high as 3.6 to 4 Mrad. Heat and high doses of radiation (>3.0 Mrad) can produce an SAL of  $10^{-6}$ , but such high doses substantially affect the biomechanical properties of allografts.<sup>29,30,42,43</sup>

The effects of lower levels of irradiation on allografts remain an area of controversy.<sup>44,45</sup> Schwartz and colleagues<sup>45</sup> confirmed in a goat model that 4.0 Mrad caused 30% and 21% reductions in stiffness and maximal force, respectively, at 6 months after implantation. Even low-dose irradiation (2 Mrad, 20 kGy) has been shown to diminish the strength and increase the cyclic elongation of BPTB allografts.<sup>46</sup> Balsly and colleagues,<sup>47</sup> however, tested bone grafts (dowel and iliac crest wedge grafts) and soft-tissue grafts (patellar, anterior tibial, and semitendinosus tendons and fascia lata) exposed to low-dose (18.3–21.8 kGy) or moderate-dose (24.0–28.5 kGy) gamma irradiation and found no statistically significant differences in mechanical strength or modulus of elasticity for any graft irradiated at low-dose compared with controls. Bone allografts and 2 of the soft-tissue allografts (anterior tibial and semitendinosus tendons) demonstrated strength and modulus of elasticity values similar to those of controls.

Electron-beam radiation has been used for sterilization, primarily of soft-tissue grafts, because of its lower penetrability (8 cm through the density of water) compared with gamma irradiation (30 cm through the density of water), which would be a problem with cortical bone allografts, which have a density of about twice that of water.<sup>48,49</sup> The advantage to electron-beam irradiation is higher processing speed—seconds, compared with hours for gamma irradiation. Although one biomechanical cadaver study of electron-beam radiation combined with tissue-protective measures (low temperature, carbon dioxide) concluded that the process did not impair the mechanical properties of BPTB grafts,<sup>50</sup> another determined that both gamma and electron-beam irradiation caused reductions in tensile strength, elastic modulus, strain, and toughness of rabbit tendons.<sup>49</sup> The decreases in strength and toughness were dose-dependent: the average loss of tensile strength was 36% with 25 kGy and 55% with 50 kGy irradiation compared with controls.

Because research has supported the hypothesis that gamma radiation-induced allograft damage is caused, in part, by free radical attack on the molecular structure of the collagen,<sup>51,52</sup> a number of radioprotectants have been used to eliminate or decrease the deleterious effects of irradiation. Grieb and colleagues<sup>53</sup> reported that a radioprotective “cocktail” solution, which included propylene glycol, dimethyl sulfoxide (DMSO), mannitol, and trehalose, was successful in protecting mechanical properties of human semitendinosus tendon at 50 kGy under regulated conditions. Akkus and colleagues<sup>51</sup> reported that the use of another free radical scavenger, thiourea, resulted in increased toughness at 36 kGy in bone allografts. Seto and colleagues<sup>49</sup> used crosslinkers, including 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and glucose to add exogenous crosslinks to collagen and compared their effects to those of free radical scavengers (mannitol, ascorbate, and riboflavin) in rabbit tendons. Both treatments protected mechanical properties at 25 kGy, but at 50 kGy crosslinkers were superior. The strength, modulus of elasticity, toughness, and strain of glucose-treated tendon, either gamma or electron-beam irradiated at 25 kGy, were close to those of native tendon. Kattaya and colleagues<sup>54</sup> noted that along with the beneficial effects of radioprotectants there is also the potential of radioprotection of pathogenic organisms and that the ideal radioprotectant should protect graft integrity without compromising sterility.

### ***Combined methods of sterilization***

Combining lower doses of irradiation (1–3.5 Mrad, 10–35 kGy) with other processing techniques, such as antibiotic soaks, is probably the most commonly used method today.

Several companies have proprietary processes for sterilization that each claims will provide a disease-free graft. Cryolife, Inc. (Kennesaw, GA) uses a slow freezing process along with DMSO or glycerol for cryopreservation of grafts. After swab culturing and desiccation, the grafts are treated for an extended period of time with an antimicrobial solution. No secondary sterilization method is used.

BioCleanse (Regeneration Technologies, Inc., Alachua, FL) is a low-temperature chemical sterilization method that is claimed to penetrate the tissue and eliminate endogenous contamination. The process permeates the inner matrix of tissue with liquid sterilants, such as hydrogen peroxide and isopropyl alcohol, followed by pressure variations to drive the sterilants in and out of the tissue. Soft-tissue grafts (bone-tendon-bone, fascia, tendons, and menisci) are treated with this method. Studies have shown that the BioCleanse process does not appear to affect the mechanical properties of BPTB grafts<sup>55</sup> or anterior tibial tendon grafts.<sup>56</sup>

Allowash (Lifenet, Virginia Beach, VA) uses ultrasonics, centrifugation, and negative pressure in combination with reagents, including biologic detergents, alcohols, and hydrogen peroxide. This process claims to increase solubilization and remove lipids, blood, and marrow cells that can act as reservoirs for potential bacterial, fungal, and viral agents. BPTB allografts are terminally sterilized using 13 to 18 kGy of radiation.

The Tutoplast process (RTI Biologics, Alachua, FL) also uses an ultrasonic acetone bath to remove lipids, followed by a series of alternating hyperosmotic saline and deionized water baths to destroy bacteria. An oxidative treatment with hydrogen peroxide is then used to eliminate soluble proteins and destroy nonenveloped viruses and bacterial spores. A final acetone wash is done to ensure that any residual prions are removed and enveloped viruses are inactivated and to dehydrate the tissue; this is followed by vacuum extraction, which allows storage at room temperature. Terminal sterilization is done with low-dose gamma irradiation.

The Clearant Process (Clearant, Inc., Los Angeles, CA) treats tissue with high doses of radiation (50 kGy), which is 2 to 4 times the dose recommended to avoid tissue damage but claims to avoid this by freezing the sample, extracting the water, and adding stabilizers and free radical scavengers. After the tissue is frozen and the water extracted, DMSO and propylene glycol are added as pretreatment radioprotectants.<sup>53</sup>

NovaSterilis (Lansing, NY) developed a technique of sterilization that uses supercritical carbon dioxide at low temperatures and relatively low pressures to induce transient acidification, which is lethal to viruses and bacteria. Although tissue penetration appears to be good with this method, data concerning the effects on the mechanical properties of allografts are limited at this time.

The Musculoskeletal Transplant Foundation (MTF, Edison, NJ), a non-profit organization, also uses a series of chemicals, including nonionic detergents, hydrogen peroxide, and alcohol, to treat most cortical and cancellous grafts, without terminal sterilization with irradiation. This process has been demonstrated to maintain osteoconductivity for up to 1 hour; compressive strength, impact strength, and shear strength are reported to be unaffected by the cleaning procedure.<sup>57</sup> BPTB and other soft-tissue allografts are treated with an antibiotic "cocktail" of gentamicin, amphotericin B, and Primaxin (imipenem and cilastatin). The antibiotics are washed out at the completion of processing to nondetectable levels. Low-dose gamma irradiation (12–18 kGy) is used for tissue that is found to have a bioburden (the number of contaminating organisms on a given amount of material before sterilization) greater than what could be sterilized by the antibiotic cocktail.

## STORAGE OF PROCESSED ALLOGRAFT TISSUE

Once the allograft tissue has been processed, it must be preserved and stored until needed. Articular cartilage allografts may be used as "fresh" grafts, within 24 days of donor death, but most other allograft tissue is fresh-frozen, freeze-dried, or cryopreserved.

Fresh-freezing or deep-freezing is the simplest and most widely used storage method for ligament and meniscal tissue. After sterile tissue harvest, the tissue is cultured and then frozen while serologic tests are done; the tissue is then soaked in an antibiotic solution, packaged, and frozen. The AATB requires storage at a temperature of at least  $-40^{\circ}\text{C}$ , but most tissue banks keep allografts at  $-70^{\circ}\text{C}$  to  $-80^{\circ}\text{C}$ , which allows storage for 3 to 5 years,<sup>4,20</sup> at a temperature near  $-196^{\circ}\text{C}$ , grafts can be preserved for as long as 10 years.<sup>4</sup>

Freeze-drying or lyophilization (residual moisture content of less than 5%) destroys all cells within the tissue but has the advantage of allowing vacuum-packed storage at



room temperature for 3 to 5 years. This method is not often used for sports medicine procedures in the United States because the process can degrade the mechanical properties of soft-tissue allografts.<sup>3</sup> A disadvantage is the need for a minimum of 30 minutes of rehydration of the graft before use, especially if a bone block is attached to the soft tissue. Freeze-drying alters the material properties of collagen but has not been shown to have a clinical effect.<sup>20</sup> One study<sup>58</sup> noted a significant association between the failure of freeze-dried allografts used for ACL reconstruction and the time from procurement to implantation, suggesting that the shelf life of freeze-dried tissues is limited. Another study<sup>59</sup> found that the ultimate strength of cancellous bone was reduced by 19% and stiffness by 20% in rehydrated lyophilized grafts, suggesting that the mechanical properties of lyophilized BPTB grafts may be inferior to those of fresh-frozen allografts.

Cryopreservation is a process by which the tissue undergoes controlled-rate freezing to  $-135^{\circ}\text{C}$  while cellular water is extracted by glycerol and DMSO. Packed in a cryoprotectant solution, the graft has a shelf life of 10 years, and up to 80% of cells can remain viable.<sup>4,9,20</sup>

## SUMMARY

No sterilization techniques have been definitively proven to be more effective than others, and their biomechanical and biological effects on allograft tissue remain largely unknown. Despite recent highly publicized occurrences of infection from allografts, however, the current risk of an allograft infection appears to be much less than the risk of infection surrounding the surgical procedure itself.<sup>5,9</sup> Most of these incidents involved questionable practices, violations of FDA regulations, and even alleged illegal activities by recovery agents.<sup>13,15,24</sup> According to a report from the AATB covering data from 2003 and 2004, of 192 reports of suspected allograft-related infections, 42% involved soft-tissue grafts and 37% involved bone grafts, with an overall incidence of 0.014%; 59% involved orthopedic sports medicine procedures.<sup>9</sup> The American Academy of Orthopaedic Surgeons (AAOS) recommends that surgeons choose tissue provided by an AATB-member tissue bank and that they be familiar with the different sterilization processes used for allografts.<sup>60,61</sup> With appropriate donor screening, improved donor testing, including NAT, and adherence to AATB standards, the risk of disease transmission or infections can be eliminated or substantially decreased.

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