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Validation of the Sterilization Procedure of Allogeneic Avital Bone Transplants Using Peracetic Acid–Ethanol

Axel Pruss[1], Bärbel Baumann[2], Michael Seibold[3], Moujahed Kao[1,3], Kathrin Tintelnot[3], Rüdiger von Versen[4], Hartmut Radtke[1], Thomas Dörner[5], Georg Pauli[3]* and Ulf Berthold Göbel[2]

[1]*Institute for Transfusion Medicine, Tissue Bank, University Hospital Charité, Medical Faculty of Humboldt-University, Berlin, Germany;* [2]*Institute for Microbiology and Hygiene, University Hospital Charité, Medical Faculty of Humboldt-University, Berlin, Germany;* [3]*Robert Koch-Institut, Berlin, Germany;* [4]*German Institute for Cell and Tissue Replacement, Berlin, Germany;* [5]*Medical Clinic Centre Rheumatology and Clinical Immunology, University Hospital Charité, Medical Faculty of Humboldt-University, Berlin, Germany*

Abstract. Different procedures are available to inactivate bacteria and fungi, including their spores, as well as viruses in human bone transplants. The most efficient methods are considered to be gamma irradiation and thermal inactivation as well as chemical sterilization methods like the peracetic acid–ethanol treatment (PES). Following national and international standards or draft standards, the antimicrobial effectiveness of this procedure was evaluated. Due to the standardizable size as well as the clinical relevance, defatted human spongiosa cuboids (15×15×15 mm) served as model system. After treatment with PES for 2 and 4 hours, respectively, the titre of living micro-organisms was determined in the supernatant and the cuboid.

A reduction in the titre of viable micro-organisms below the detection level (reduction factor $>5\ \log_{10}$) was already achieved after an incubation time of 2 hours (*Staphylococcus aureus*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Clostridium sporogenes*, *Mycobacterium terrae*, *Candida albicans* as well as spores of *Bacillus subtilis*). No viable micro-organisms could be detected in any of the PES-treated test cuboids. Spores of *Aspergillus niger* were also completely inactivated. The PES procedure proved to be a reliable method for the sterilization of human bone transplants derived from spongiosa.
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Key words: sterilization, bone transplants, spongiosa, peracetic acid.

Introduction

In Germany about 25 000 allogeneic bone graft transplantations are performed annually.[1] Human bone tissue serves as source material for the production of these transplants (among others heads of femur and tibia, body of vertebra, iliac crest). Accordingly, special requirements have to be made on the biological safety of the tissues. Apart from a validated clinical effectiveness of the transplants, the quality assurance measures must focus on the validation of the sterilization process. In accordance with the guidelines for managing a bone tissue bank issued by the Bundesärztekammer (Federal Medical Society),[2] sterilization is particularly indicated if a second testing of the bone tissue donor after an appropriate time cannot be performed, because the tissue was obtained from a cadaveric source. In addition, these validations are required standards in Germany, where all transplants manufactured in national tissue banks are regarded as medicinal products.

So far, no normative specifications exist regarding validation of the disinfection or sterilization of bone tissue intended to be used for transplantation. Table 1 shows a survey of standards or draft standards, on which the design of validation experiments ought to be based.

The aim of the projected validation design was to follow the relevant above-mentioned standards as

*To whom correspondence should be addressed. E-mail: paulig@rki.de

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Table 1. Summary of the relevant standards for validation of disinfection or sterilization procedures for bone tissue transplants

Standard*	Title	Relevant passages
PEI/BfArM [1994-01]	Requirements of validating studies for the proof of the virus safety of medicaments from human blood or plasma	Specification of the model viruses; virus content in the raw material: $\geqslant 10^6$ at least one process step: $R_i \geqslant 10^4$
EN 552 [1994-11]	Sterilization of medicinal products; validation and routine monitoring for the sterilization with irradiation	Specifications to dosimetry, sterilization doses (minimum dose 25 kGy, target: SAL), calibration, validation
EN 1040 [1997-04]	Chemical disinfectants and antiseptics, bactericidal effect (basic test)	Test organisms (*S. aureus*, *P. aeruginosa*); neutralization media; reduction of number of viable micro-organisms: $\geqslant 10^5$
EN ISO 14160 [1998-06]	Sterilization of medicinal products for single use with constituents of animal origin	Test organisms, media, incubation conditions; effects of organic materials; test on inactivation of spores
pr EN 13624† [1999-06]	Quantitative suspension test for investigating the fungicidal effect of the chemical disinfectants for instruments used in human medicine	Test organisms (*C. albicans*, *A. niger*); neutralization media; reduction of the number of viable micro-organisms: $\geqslant 10^5$
pr ISO/DIS 14937† [1999-08]	Sterilization of health care products; general criteria for characterization of a sterilizing agent and development, validation and routine control of a sterilization process	Test organisms (most extensive specification); neutralization possibilities; demand for "worst case" conditions; in-process controls

*Exclusively available via Beuth Verlag GmbH, 10772 Berlin, Germany.
†Draft standard.

closely as possible. This concerns in particular the selection of the test organisms and the consideration of "worst case" conditions. A standardization in the sense of a validation of disinfectants (EN 1040, prEN 13624) is not feasible due to the heterogeneous material of human origin. The test organisms were selected following the specifications of prISO/DIS 14937. Observing the standard EN ISO 14160, the spectrum was extended by *Enterococcus faecium*. Finally, due to the available literature data, which describe resistance of *Aspergillus niger* against 0·008% peracetic acid,[3] this micro-organism was also included in the validation study.

The selected spectrum of micro-organisms covers the clinically relevant pathogens (in-vivo infection, aerosol contaminants) as well as spores which are prescribed as mandatory for validating sterilization procedures. In accordance with EN 1040 and prEN 13624, the aim was a reproducible reduction in the titre of viable micro-organisms by a factor of at least 5 $\log_{10}$.

At present, several procedures are used for inactivating bacteria and fungi, including their spores, as well as viruses in the context of the production of bone tissue transplants. Gamma irradiation,[4–6] thermal treatment[7,8] as well as peracetic acid–ethanol (PES) treatment under negative pressure conditions,[9,10] are applied.

Apart from a broad spectrum of irradiation doses cited in the literature, ranging between 15–40 kGy,[4,6] especially the production of toxic radicals[5] and the negative influence on biomechanical parameters of the bone[4] seems to be problematic during gamma irradiation. Additionally, logistics is complex. So far, no definite statements/reports exist about the efficiency of the procedure of sterilizing contaminated bone tissues. Earlier experiences with the irradiation of medicinal products prove that a radiation dosage of 25 kGy is obviously sufficient for inactivating relevant pathogens.[11] According to the current opinion of the supervisory authorities, 29·5 kGy is required as minimum irradiation dose.

However, the authors' own investigations imply that a dose of at least 34·0 kGy is necessary in order to achieve a titre reduction of clinically relevant viruses by a factor of $>4 \log_{10}$.[12]

Thermal treatment is widely used in Germany, but is presently restricted exclusively to the disinfection of femoral heads obtained from total endoprosthesis operations. However, the validation methods described for this procedure[8] do not completely meet the requirements of the national and international standards or draft standard (Table 1), particularly regarding the spectrum of the test organisms or ''worst case'' conditions (direct contact of the micro-organisms with the bone tissue). Due to their carcinogenic and mutagenic effect, ethylene oxide (A.P. *et al.*, unpublished results) as well as beta-propiolactone[12] and formaldehyde[13] treatment are no longer considered as suitable methods for sterilization in Germany. Additionally, the latter clearly reduces the osteoinductive effect.

Therefore for as long as the last 20 years, ethanol and peracetic acid have been increasingly used for tissue sterilization.[9,10,14] However, the diffusion of these substances into the tissue is limited. Penetration-inhibiting fat barriers must be removed by treating the spongiosa with a chloroform–methanol mixture or by an equivalent validated procedure.[15]

So far, only incomplete data exist for validating the different methods regarding their inactivating capacity against clinically relevant pathogens, and comparative studies are lacking. Considering the demands of clinicians and patients for optimal protection from infections, the method of the PES sterilization was evaluated not only for viruses,[16] but also for selected bacteria and fungi including spores, following the current guidelines.

Materials and methods

Spongiosa cuboids

As process-challenge device human spongiosa cubes were used with an edge length of 15 × 15 × 15 mm, originating from donors negative for the following infection markers: anti-HIV1/2-, HBsAg-, anti-HCV and TPHA antibodies. As source material spongiosa tissue was collected under sterile conditions from the columna vertebralis as well as from the epiphyses of femur, tibia and humerus. Adherent fat and connective tissues were carefully removed under aseptic conditions, using scalpel and surgical tweezers. The preparation of the test cuboids was performed at the belt saw in a sterile class B laboratory. The cuboids were rinsed for 30 minutes in sterile water (37°C) to remove the blood from the bone tissue.

In the course of the defatting step, the tissue was always covered with defatting mixture (two volumes of chloroform and one volume of methanol for analysis). The procedure took place under constant agitation (laboratory shaker) over a period of 2 hours (change of defatting medium after 30 minutes). Subsequently, the tissues were flushed with methanol eight times each and were subjected to a 15-minute ultrasonic bath treatment in order to completely remove residual chloroform. Methanol was removed by flushing the tissues twice with sterile deionized water.

Finally, aliquots of the cuboids were tested regarding biological safety in accordance with DAB 97. In all cases, no micro-organisms could be detected.

Test organisms, cultivation and titre determination

The test organisms (in the early stationary phase, see Table 2) were suspended in physiological salt solution and the number of cells was adjusted by densitometry, in the case of *A. niger* and spores of *Bacillus subtilis* by means of a counting chamber. The titre (cfu) of the micro-organisms in the suspensions (number of viable micro-organisms) was determined after appropriate predilution by means of a spiral disk apparatus. Since the inoculum consisted of 100 μl using the spiral disk method, titres <10 cfu/ml were not detected. The titres of the *B. subtilis* spores was determined by mixing 1 ml of appropriately diluted samples with casein soy peptone agar (detection limit $\leqslant$1 cfu/ml). The titre of *A. niger* was determined by plating samples at different dilutions (in each case 100 μl) on Sabouraud glucose agar (detection limit $\leqslant$10 cfu/ml).

Chemicals

Peracetic acid–ethanol mixture (PES): two volumes of peracetic acid 20 g/l; one volume of 96% ethanol; one volume of aqua ad iniectabilia; physiological salt solution (Braun, Melsungen, Germany); sodium thiosulphate (Köhler Chemie, Alsbach, Germany); chloroform for analysis and ethanol for analysis (Merck, Darmstadt, Germany). The sodium thiosulphate was dissolved in physiological salt solution sterilized by filtration (0·22 μm filter) and stored in aliquots at −20°C until use.

Table 2. Test organisms

Micro-organism (ATCC-no.)	Growth properties	Culture medium	Incubation time/ temperature
S. aureus (6538)	Gram-positive cocci in clumps	Casein soy peptone agar (Oxoid)	48 h/37° C
E. faecium (6057)	Gram-positive cocci in chains	Kanamycin-Esculin agar (Oxoid)	48 h/37° C
P. aeruginosa (27 853)	Gram-negative rods	Casein soy peptone agar (Oxoid)	48 h/37° C
B. subtilis (6633)	Gram-positive rods; aerobic spore-forming bacillus	Casein soy peptone agar (Oxoid)	48 h/37° C
B. subtilis (spores) (6633)	Gram-positive rods; aerobic spore-forming bacilli	Casein soy peptone agar (Oxoid)	48 and 72 h/37° C
C. sporogenes (19 404)	Gram-positive anaerobic spore-forming bacilli	Casein soy peptone agar (Oxoid)	48 h/37° C
*M. terrae**	Gram-positive acid-proof rods	Middlebrook 7H10 agar with OADC (Biotest)	3 and 4 weeks/37° C
C. albicans (2091)	Yeast fungi	Sabouraud-glucose agar (SIFIN)	48 h/37° C
Spores of *A. niger* (16 404)†	Mold fungi	Sabouraud-glucose agar (SIFIN)	48 and 72 h/30° C

*Clinical isolate.
†Production of the spore suspension and determination of the titre of *A. niger* followed prEN 13624.

Contamination of the spongiosa cuboids

For each experiment three defatted and dried spongiosa cuboids were placed in sterile 50 ml Falcon plastic tubes (Becton Dickinson, Heidelberg, Germany) with screw-type cap and covered by 15 ml each of the suspensions of micro-organisms. The tubes were sealed with a multiply perforated cover and placed into an exsikkator and incubated under negative pressure (200 mbar; 1 bar $=10^5$ Pa) for 15 minutes at room temperature. Afterwards the suspension was decanted and 15 ml of PES (tubes I and II) or physiological salt solution (tube III, positive control), respectively, added and incubated as described below at room temperature.

The tubes were placed into an exsikkator and incubated under continuous agitation and low pressure. After collecting 0·5 ml (S1) from all tubes, the peracetic acid (one volume) in the remaining supernatant of tubes I and II (S2) was neutralized by adding one volume of sodium thiosulphate solution (0·1 g/ml).[16] A toxic or growth-promoting effect of the sodium thiosulphate solution and of the neutralized medium on the test organisms could be excluded in additional experiments in accordance with the DIN EN 1040. The titres of the micro-organisms in the supernatant S1 and the neutralized supernatant S2 as well as in the homogenate of the cuboid (H) were determined for each organism. The tube incubated with physiological salt solution (tube III) served as positive control.

Homogenization of the treated cuboids and the controls (volume of the cuboid approximately 2 ml) was performed in a sterilized stainless steel beaker of an Omni mixer (type COM, Ivan Sorval Inc., Norwalk, CT, U.S.A.), containing 10 ml of a 1% sodium thiosulphate solution, under cooling in a water-ice bath at 1500 rpm for 2 minutes. The amount of sodium thiosulphate was calculated to neutralize the peracetic acid in the cuboid.

Control of penetration

In order to verify the penetration of the test organisms into the centre of the cuboid, a control experiment was performed using calibration particles (size 1 μm) for flow cytometer (FACS, Becton Dickinson). These particles are approximately the size of clinically relevant bacteria. Since the average diameter of a spongiosa ductule ranges between 500 and 2000 μm, fungi or spores with a diameter of $<10\ \mu$m should also penetrate into the cuboids.

A central cylinder (diameter 5 mm) was punched from a defatted spongiosa cuboid sized $20 \times 20 \times 20$ mm. From both the upper and lower

Table 3. Determination of the titre of viable micro-organisms after treatment with or without PES

Test organisms	Tube no.	Titre S1 (cfu/ml)	Titre S2 (cfu/ml)	Titre H (cfu/ml of cube)
S. aureus	I a/b	Ø/Ø	Ø/Ø	Ø/Ø
	II a/b	Ø/Ø	Ø/Ø	Ø/Ø
	III a/b	$2{\cdot}9 \times 10^8/1{\cdot}6 \times 10^8$	—	$1{\cdot}7 \times 10^8/1{\cdot}3 \times 10^8$
E. faecium	I a/b	Ø/Ø	Ø/Ø	Ø/Ø
	II a/b	Ø/Ø	Ø/Ø	Ø/Ø
	III a/b	$4{\cdot}5 \times 10^7/1{\cdot}2 \times 10^8$	—	$7{\cdot}3 \times 10^6/2{\cdot}1 \times 10^7$
P. aeruginosa	I a/b	Ø/Ø	Ø/Ø	Ø/Ø
	II a/b	Ø/Ø	Ø/Ø	Ø/Ø
	III a/b	$3{\cdot}2 \times 10^7/5{\cdot}0 \times 10^7$	—	$7{\cdot}4 \times 10^7/3{\cdot}1 \times 10^7$
B. subtilis	I a/b	Ø/Ø	Ø/Ø	Ø/Ø
	II a/b	Ø/Ø	Ø/Ø	Ø/Ø
	III a/b	$1{\cdot}8 \times 10^7/6{\cdot}5 \times 10^7$	—	$2{\cdot}9 \times 10^6/1{\cdot}7 \times 10^7$
Spores of *B. subtilis*	I a/b	Ø/Ø	Ø/Ø	Ø/Ø
	II a/b	Ø/Ø	Ø/Ø	Ø/Ø
	III a/b	$2{\cdot}1 \times 10^6/1{\cdot}6 \times 10^6$	—	$6{\cdot}4 \times 10^5/1{\cdot}2 \times 10^6$
C. sporogenes	I a/b	Ø/Ø	Ø/Ø	Ø/Ø
	II a/b	Ø/Ø	Ø/Ø	Ø/Ø
	III a/b	$1{\cdot}7 \times 10^8/5{\cdot}5 \times 10^8$	—	$4{\cdot}3 \times 10^7/1{\cdot}6 \times 10^8$
M. terrae	I a/b	Ø/Ø	Ø/Ø	Ø/Ø
	II a/b	Ø/Ø	Ø/Ø	Ø/Ø
	III a/b	$1{\cdot}5 \times 10^7/9{\cdot}0 \times 10^6$	—	$3{\cdot}0 \times 10^6/5{\cdot}5 \times 10^6$
C. albicans	I a/b	Ø/Ø	Ø/Ø	Ø/Ø
	II a/b	Ø/Ø	Ø/Ø	Ø/Ø
	III a/b	$1{\cdot}0 \times 10^7/1{\cdot}9 \times 10^7$	—	$1{\cdot}9 \times 10^7/3{\cdot}3 \times 10^7$
A. niger	I a/b	Ø/Ø	Ø/Ø	Ø/Ø
	II a/b	Ø/Ø	Ø/Ø	Ø/Ø
	III a/b	$4{\cdot}1 \times 10^5/3{\cdot}2 \times 10^5$	—	$5{\cdot}1 \times 10^4/2{\cdot}5 \times 10^5$

Tube I, contaminated spongiosa cuboids+15 ml PES (2 h incubation time); tube II, contaminated spongiosa cuboids+15 ml PES (4 h incubation time); tube III, contaminated spongiosa cuboids+15 ml physiological salt solution (positive control after 4 h incubation time); S1, supernatant without neutralization; S2, supernatant after neutralization; H, homogenate of the cuboid; a,b, two independent experiments; Ø, below the detection level [⩽10 cfu or 1 cfu (*B. subtilis* spores), respectively]; —, not tested.

part of the cylinder, 7.5 mm was removed. The resulting cylinder (see Fig. 1) was centrally repositioned in the cuboid, and the respective 7.5 mm long openings of the drilling channel were tightly sealed with waterproof bone wax (Ethicon, Somerville, NJ, U.S.A.). The cuboid was submerged in the particle suspension.

The original particle (p) solution was diluted to a final concentration of 19·1 p/μl (determined by FACS analysis). After 15 minutes of incubation under low pressure (see above), a sample of the supernatant was collected and the concentration determined. The same number of p/μl was determined in the supernatant after incubation as in the starting solution. The central bone cylinder was removed from the channel, transferred into an Eppendorf tube containing PBS and centrifuged. After resuspension of the particles in the recovered suspension (267 μl) from the cuboid, 580 particles were counted. From these results a number of 15·2 p/μl in the test cylinder could be calculated, resulting in a recovery rate of 79·6% (19·1 p/μl *vs.* 15·2 p/μl), indicating that the test particles penetrate in 15 minutes into the centre of the test cuboid.

Results

After treatment, no viable cells could be detected in the supernatant with or without neutralization of

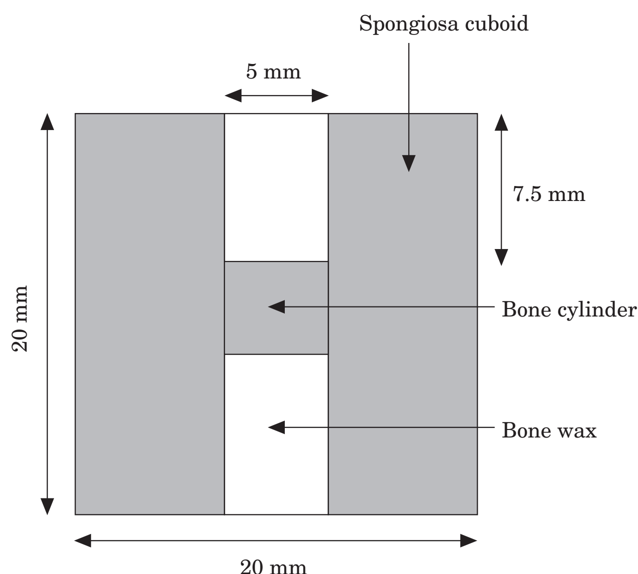

Figure 1. Model of a test cuboid used for the penetration studies.

the PES. Also in the homogenates of the spongiosa cuboids contaminated with the test organisms both after 2-hour and 4-hour treatment with PES under negative pressure conditions, no viable micro-organisms could be detected either (Table 3). Effective penetration of micro-organisms under the experimental conditions was verified in the control experiment (see Material and Methods). PES treatment reduced the titres of all micro-organisms tested by a reduction factor of $\geqslant 5\ \log_{10}$.

Discussion

In the present study the efficiency of the peracetic acid–ethanol mixture (PES) under negative pressure was evaluated using human spongiosa cuboids as a model system. The experiments were planned and performed following national and international standards or drafted standard (see Table 1), especially considering the standards DIN EN 1040, prISO/DIS 14937 and prEN 13624.

The bactericidal,[17,18,19,20] fungicidal,[21,22] virucidal[23] and sporicidal[22,24,25] effect of the peracetic acid has long been well-known. By addition of ethanol to the sterilizing medium the surface tension is reduced. By negative pressure air vesicles, which could prevent penetration into the cuboid, are removed and the penetration of the sterilizing medium into the defatted, air-dried bone tissue is promoted by constant agitation of the sterilization containers.

In the present investigation, as early as after a 2-hour incubation the absence of viable micro-organisms in the PES-treated test cuboids contaminated with a variety of micro-organisms could be shown. The calculated dilution factor between original titre and titre in the positive control (tubes III, S1) of approximately $1\ \log_{10}$, which is due to the volume of the contaminating solution in the spongiosa cuboid and the volume (15 ml) of PBS in the incubation tube, could be reproduced for all micro-organisms. The difference between S1/III and H/III, showing a lower titre in the homogenate, can essentially be explained by mechanical destruction of the cuboid in the context of homogenization and the accompanying damage to the respective micro-organisms. An exception here are the *Candida albicans*-contaminated test cuboids, where the titre in the homogenate (H/III) is higher than in the supernatant (S1/III). This could be caused by an adhesion of fungi to the wall of the plastic tube, so that no uniform distribution of the cells was achieved in the supernatant.

Treatment with PES resulted in an inactivation of all micro-organisms below the level of detection. A reduction in the number of viable micro-organisms by $\geqslant 5\ \log_{10}$ could be shown for *Staphylococcus aureus*, *E. faecium*, *Pseudomonas aeruginosa*, *B. subtilis*, *Clostridium sporogenes*, *Mycobacterium terrae*, *C. albicans* as well as for spores of *B. subtilis*. With *A. niger* only a reduction of $>4\ \log_{10}$ could be experimentally shown, due to the low starting titre of $2{\cdot}5 \times 10^6$ spores/ml. However, a reduction by $\geqslant 5\ \log_{10}$ is computationally also granted for *A. niger* considering all negative test results (t=2 hours, t=4 hours, two independent experiments).

It was described that spores of *A. niger* could not be inactivated by 0·008% peracetic acid.[3] When PES is used in higher concentration we could demonstrate an effective inactivation of the spores.

In a previous investigation using the same experimental design, we showed an effective inactivation of viruses in accordance with prISO/DIS 14937. An inactivation factor of $>4\ \log_{10}$ was shown for Pseudorabies virus, bovine virus diarrhoea virus, human immunodeficiency virus type I, porcine parvovirus and poliovirus.[16] Our investigations complement well-known findings on the virucidal effect of peracetic acid on Coxsackie viruses and hepatitis B virus.[17,26] Hepatitis A virus (HAV) showed a relatively high resistance against PES treatment. However, the virus safety of the

spongiosa cuboids regarding HAV could be achieved in the defatting step using chloroform/methanol which resulted in a reduction of HAV by approximately 7 $\log_{10}$.[17] A combined defatting procedure and PES sterilization leads to an effective removal of relevant bacteria, fungi and viruses.

Considering the results reported in this investigation as well as the results of the virus-inactivating study in accordance with DIN EN 1040 and prISO/DIS 13624, we were able to prove the sterilizing effect of PES on contaminated bone tissue transplants. The production process can be recommended for application in bone banks.

However, the sterilization procedure was validated only for bone cuboids sized $\leqslant 15$ mm. For larger bone transplants like femoral heads, epiphyses or complete extremity bones, the inactivation/sterilization procedure will have to be evaluated.

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