Sterilization
of human tissue grafts
<table>
<thead>
<tr>
<th>Standard Code</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO 11137:1995 (EN 552)</td>
<td>Sterilization of Health Care Products - Requirements for Validation and Routine Control - Radiation Sterilization</td>
</tr>
<tr>
<td>ISO 11737-1:1995</td>
<td>Sterilization of Medical Devices - Microbiological Methods ; Part 1</td>
</tr>
<tr>
<td>ISO 11737-2:1998</td>
<td>Sterilization of Medical Devices - Microbiological Methods ; Part 2</td>
</tr>
<tr>
<td>ISO/TR 13409:1996</td>
<td>Sterilization of Health Care Products - Radiation Sterilization - Substantiation of 25 kGy as a sterilization dose for small or in frequent production batches</td>
</tr>
<tr>
<td>ISO 14160:1998</td>
<td>Sterilization of single use devices incorporating materials of animal origin - Validation and routine control of sterilization by liquid sterilants</td>
</tr>
</tbody>
</table>
Sterilization as a concept

sterilization is the process where all types of micro-organisms are either inactivated in terms of their inability to reproduce or they are completely killed. Thus the process of sterilization renders the grafts sterile provided the tissue contains very low level or acceptable level of bioburden.

Word STERILE cannot be used in relative sense like semi-sterile or almost sterile. Grafts are either STERILE or NON-STERILE.
Transmission of infectious diseases with non-sterilized tissue allografts collected under aseptic conditions:

**Bone allografts:**
- bacterial infections \((\text{Tomford et al., 1981, 1990})\)
  including tuberculosis \((\text{James, 1953})\)
- viral infections:
  \[\text{HIV-1} \ (\text{Simonds et al., 1992})\]
  \[\text{HCV} \ (\text{Conrad et al., 1995, Eggen & Norbdo, 1992, Pereira et al., 1993})\]

**Skin allografts:**
- \text{CMV} \ (\text{Abecassis et al., 1994, Aguiar et al., 1994})

**Dura mater, cornea, pericardium allografts:**
- \text{CJD} - prions \((\text{Brown et al., 1992, Lane et al., 1994, Masullo et al., 1989, Thadani et al., 1988, Yamada et al., 1994})\)
Methods of sterilization

- sterilization by steam
- sterilization by glycerol solutions
- sterilization by ethylene oxide
- sterilization by cold plasma
- sterilization by ionizing radiation
Steam sterilization

Steam sterilization or autoclaving is a relatively simple process that exposes the device to saturated steam at 121ºC for a minimum of 20 minutes at a pressure of 121kPa. The process is usually carried out in a pressure vessel designed to withstand the elevated temperature and pressure and kills micro-organisms by destroying metabolic and structural components essential to their replication.

Class B < S < N
Steam sterilization

European Working Group presented the European Draft Standard prEN 13060/1-4 for small sterilizers. This Draft Standard assigned tabletop autoclaves to one of the following classes: Class B (the strictest), Class S, or Class N.

In **Class N** auto-claves, sterilization is allowed only of solid, dense, and unwrapped objects.

In **Class S** auto-claves, sterilization is allowed for solid objects and wrapped or porous (type A, or B) instruments.

In **Class B** auto-claves, sterilization is allowed for all kind of objects: solid, porous elements (fabrics), surgical instruments and wrapped instruments.
Steam sterilization

Although heat (e.g. autoclaving, boiling) is an effective sterilizing agent, it has not been routinely used for tissue grafts, because it impairs the mechanical properties of bone, destroying the osteoinductive capacity of bone and reducing bone graft incorporation.
Pasteurisation (56°C to 80°C for a few hours) inactivates many bacteria as well as a 6 log concentration of HIV-1, a 4.5 log of HCV and a 5 log concentration of non-A, non-B hepatitis viruses. Although pasteurization has no significant negative effect on the mechanical properties of bone nor on the clinical effectiveness of bone allografts, this method is not widely used for sterilization of tissue allografts.

Temperatures over 56°C can denaturate proteins and destroy the osteoinductive properties of bone allograft (BMPs). Diminishes also mechanical properties (reduction of compression strength, and Young modulus) of tissue grafts.
Glycerol sterilization

Glycerol is used for processing non-viable skin allografts, according to Euroskin Bank's protocol of using 85% glycerol for skin sterilization.

This concentration of glycerol will inactivate extracellular viruses, but skin needs to be in 98% glycerol for at least three weeks at room temperature for intracellular viruses to be inactivated.
Glycerol sterilization

Glycerol alone will not sterilise the allograft tissue
EtO sterilization

EtO sterilization is used routinely to sterilise materials that cannot withstand the high temperatures of autoclaving. The procedure involves drawing a vacuum in the sterilization vessel, after which EtO is injected at a concentration of 600–1,200mg/litre. The sterilizer is maintained at the desired conditions of 30–50°C and 40% to 90% humidity for the duration of sterilization, usually between two to eight hours. The critical parameters of the cycle are temperature, pressure, humidity, EtO concentration and gas dwell time. Following the sterilization cycle, the chamber is then evacuated several times to remove residual EtO. Further aeration is usually required after removal from the chamber, with aeration time ranging from two hours to two weeks, depending on the device and packaging.
EtO sterilization

EtO inactivates all types of microorganisms, including bacteria, spores and viruses. It is applied in a gaseous state in a mixture with inert diluents such as CO$_2$ or freons to avoid explosive reactions with oxygen in the presence of moisture.

The sterilization procedure is followed by the removal of EtO. This is essential because of the high chemical reactivity and toxicity of this substance. However, it is practically impossible to remove all EtO from porous tissue allografts and to avoid chemical reactions with their constituents and the production of substances such as chlorhydrin, glycols and other potentially cancerogenic compounds.

It has been found that EtO reduces the osteoinductive properties of bone allografts and impairs their incorporation. In our opinion, EtO should not be used for the sterilization of tissue allografts, mainly because of its potential toxicity to the allograft recipient.
Gas plasma sterilization

Two plasma sterilization systems are used as the sterilising agent:

- H₂O₂,
- H₂O₂/peracetic acid (PAA).

H₂O₂ (Sterrad™) procedure comprises a 45-minute period during which vapourised H₂O₂ is diffused through the treatment chamber, following which 300 watts of radio-frequency power are applied at a pressure of 0.5 Torr to create the plasma.

The plasma is maintained for a period sufficient to ensure complete sterilization with a standard phase lasting 15 minutes. The total procedure takes approximately one hour.
Plazlyte™ uses PAA and H$_2$O$_2$ vapour treatment, which is alternated with downstream plasma treatment by microwave excitation of the low-pressure gas mixture comprising oxygen, hydrogen and argon. The equipment operates by vapourising the chemical agents and diffusing the vapour into the chamber, alternating with the plasma. At the end of sterilization, the reactive species combine to form water and oxygen, eliminating the need for aeration.
Gas plasma sterilization

- $\text{H}_2\text{O}_2$ works by the production of destructive hydroxyl free radicals, which can attack membrane lipids, DNA and other essential cell components.

- Inactivation of micro-organisms is dependent on time, temperature and concentration.

- PAA is an oxidising agent that denatures protein, disrupts cell wall permeability and oxidises sulphur bonds in proteins, enzymes and other metabolites.

- It has inferior penetrating ability compared with EtO, but both PAA and $\text{H}_2\text{O}_2$ perform more effectively than EtO in terms of biological kill and sterilant removal.

- It is a fast, low-temperature process with no requirement for aeration.
Gas plasma sterilization

Gas plasma sterilization is suitable for the sterilization of:

- metals, natural rubber, silicone;
- various polymers (polyvinyl chloride, polyethylene, polyurethane).

It is not suitable for the sterilization of:

- liquids, oils, powders, biological tissues, paper, cotton and linen.
To: Users of the AbTox Plazlyte™ Sterilization System

FDA is alerting the health care community not to use the AbTox Plazlyte™ Sterilization System for ophthalmic instruments, and is providing clarification regarding the recall of this device and recommending options for alternative sterilization.

Background

AbTox, Inc., of Mundelein, Illinois, issued a voluntary recall on March 31, 1998, to all owners of the AbTox Plazlyte™ Sterilization System. This recall notice said not to use the system on ophthalmic instruments or on any other instruments made with brass, copper or zinc, or which had been soldered. FDA is warning hospitals and physicians against the use of the AbTox Plazlyte™ Sterilization System because of serious eye injuries, including the need for corneal transplantation in some patients, following use of ophthalmic surgical instruments which had been sterilized with the system. The problem appears to be deposits of copper and zinc salts on devices sterilized with this system. Copper compounds are toxic to human corneal endothelial cells.
Radiation sterilisation is a physical process (as light or UV) involving the exposure of a product to high energy ionising radiation

**Ionising radiation sources**

1) g-rays:
   - \(^{60}\text{Co}\) (03-3.0 MCi)  \hspace{1cm} energy: 1.25 MeV
   - \(^{137}\text{Cs}\)  \hspace{1cm} energy: 0.52 MeV

2) high energy electrons
   - electron beam accelerators  \hspace{1cm} energy: 8-10 MeV
Radiation sterilization

The sterilization efficiency of ionizing radiation lies in its good penetrability inside matter (especially gamma rays) and its high effectiveness in the inactivation of microorganisms without incurring the associated problems of heat exchange, pressure differences or hindrances by diffusion barriers. The radiation causes a moderate rise of temperature which allows heat-sensitive biological materials to be sterilized. It is efficient at room temperature and even at temperatures below zero. It allows the sterilization of materials in previously closed wrappings and this way avoids recontamination during packing.
## Sterilization definitions

<table>
<thead>
<tr>
<th><strong>Absorbed Dose</strong></th>
<th>Quantity of ionising radiation. A measure of the energy imparted to a mass of material. The basic unit is the gray (Gy). 1 Gy = 1 joule per kilogram.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bioburden</strong></td>
<td>Population of viable micro-organisms on a product and/or a package.</td>
</tr>
<tr>
<td><strong>Dosimeter</strong></td>
<td>Device or system having a reproducible, measurable response to sterilization, which can be used to measure the absorbed dose in a graft.</td>
</tr>
<tr>
<td><strong>Sterile</strong></td>
<td>The state of being free from viable micro-organisms.</td>
</tr>
<tr>
<td><strong>Sterility Assurance Level (SAL)</strong></td>
<td>The expected probability of a surviving micro-organism on an individual product unit after exposure to a valid sterilization process</td>
</tr>
<tr>
<td><strong>Sterility Test</strong></td>
<td>Test performed to determine the product or fraction of product which are scored positive when subjected to defined culture conditions.</td>
</tr>
</tbody>
</table>
The **absorbed dose** of any type of ionising radiation has in the past been expressed in units called the rad (radiation absorbed dose) which is equal to 100 ergs per gram of matter (10^{-2} J/kg in SI units). The SI unit of absorbed dose is the gray (Gy), which is defined as 1 J/kg and equals 100 rad.

\[ 1 \text{Gy} = 1 \text{J/kg} = 107 \text{ erg/kg} = 104 \text{ erg/g}; \]

\[ 1 \text{ rad} = 100 \text{ erg/g} \text{ or } 6.24 \times 10^{13} \text{ eV/g}; \text{ hence} \]

\[ 1 \text{ Gy} = 100 \text{ rad} \]
Inactivation of microorganisms by ionizing radiation

Target sites:
DNA, RNA

direct effect

indirect effect

single & double-strand breaks of DNA
DNA intra-strand cross-links
loss or damage to bases, sugars or phosphates

inhibition of DNA synthesis
errors in protein synthesis

cell death
Low doses of ionizing radiation

ability to repair damage to DNA (SSB)
(repair enzymes: e.g. DNA polymerase I; recombination)

radiation-resistant mutants
(e.g. Deinococcus radiodurans)
Penetrability of various types of ionising radiation

- **γ-rays**
  - $^{60}$Co, energy 1.25 eV

- **fast electrons (e)**
  - energy 10 MeV

**Material density 1g/cm$^3$**

- **One-side irradiation**
  - dose-depth
    - 12 cm of water
    - 3.5 cm of water

- **Double-side irradiation**
  - dose-depth
    - 30 cm of water
    - 8 cm of water

**Material density > 2 g/cm$^3$**

- **Double-side irradiation**
  - dose-depth
    - 10-15 cm of water
    - 3 cm of water
Dose depth distribution of $^{60}$Co gamma rays and beams of 10 MeV electrons in a model matrix of the density 2 g/cm$^3$. One-side irradiation.
Dose depth distribution of $^{60}$Co gamma rays in a model matrix of the density 2 g/cm$^3$. Two-side irradiation.
Dose depth distribution of a beam of 10 MeV electrons in a model matrix of the density 2 g/cm$^3$. Two-side irradiation.

**a** - thickness 3.6 cm: the dose in the center is two times higher than the surface dose;

**b** - thickness 4.2 cm: the dose in the center is by 50% higher then the surface dose;

**c** – thickness 4.7 cm: the dose inside the matrix is by 27% higher then the surface dose;

**d** – thickness 5.2 cm: the dose in the center is by 75% lower then the surface dose.
Radiation sterilization

Radiation resistance of microorganisms

(enzymes, pyrogens, toxins & antigens of microbial origin)

- prions
- viruses
- bacterial spores
- some fungi
- Gram-positive bacteria
- Gram-negative bacteria
Resistance of organisms to irradiation

- Prions
- Viruses, spores, and radiation-resistant bacteria
- Most of bacteria
- Insects
- Men
Factors affecting the sensitivity of microorganisms to ionizing radiation

<table>
<thead>
<tr>
<th>Presence of</th>
<th>Response to irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitizers</strong></td>
<td></td>
</tr>
<tr>
<td>water</td>
<td>increased sensitivity</td>
</tr>
<tr>
<td>oxygen</td>
<td>increased sensitivity</td>
</tr>
<tr>
<td>higher temperature</td>
<td>increased sensitivity</td>
</tr>
<tr>
<td><strong>Protectors</strong></td>
<td></td>
</tr>
<tr>
<td>alcohols</td>
<td>decreased sensitivity</td>
</tr>
<tr>
<td>glycerol</td>
<td>decreased sensitivity</td>
</tr>
<tr>
<td>dimethyl sulphoxide (DMSO)</td>
<td>decreased sensitivity</td>
</tr>
<tr>
<td>proteins</td>
<td>decreased sensitivity</td>
</tr>
<tr>
<td>carbohydrates</td>
<td>decreased sensitivity</td>
</tr>
<tr>
<td>low temperature</td>
<td>decreased sensitivity</td>
</tr>
</tbody>
</table>
Methods

1. The effect of various methods of preservation with subsequent radiation-sterilization on the osteoinductive properties of bone grafts;

2. The effect of preservation procedures with subsequent radiation sterilization on the mechanical properties of bone grafts;

3. The effect of various methods of preservation with subsequent radiation-sterilization on the solubility in vitro of graft collagen;

4. The effect of various methods of preservation with subsequent radiation-sterilization on the susceptibility of in vitro enzyme action;
Material and methods

Femurs for evaluation of osteoinductive and mechanical properties of bone grafts were obtained from 20 week-old WAG male rats.

- Lyophilized irradiated at room temperature with doses 25, 35, 50 or 100 kGy (\(\gamma\)-source)
- Deep-frozen irradiated at -72°C with doses 25, 35, 50 or 100 kGy (10MeV electron beam accelerator)
- Fresh irradiated at room temperature with doses 25, 35, 50 or 100 kGy (\(\gamma\)-source)
- Non-irradiated controls
Scheme of transplantation of bone matrices into muscles of rat abdominal wall

<table>
<thead>
<tr>
<th>Bone matrices</th>
<th>lyophilized</th>
<th>frozen</th>
</tr>
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<tbody>
<tr>
<td>20°C</td>
<td>irradiated</td>
<td>-72°C</td>
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<tr>
<td>doses</td>
<td>35 kGy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 kGy</td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>control</td>
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</table>
X-ray examination at 6 wks after transplantation of bone matrices into rat abdominal wall muscles

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<tr>
<td>20°C</td>
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<td>-72°C</td>
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<tr>
<td>doses</td>
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<td></td>
</tr>
<tr>
<td>35 kGy</td>
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<tr>
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</tr>
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<td></td>
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</tr>
<tr>
<td>50 kGy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
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Computerized morphometric analysis of bone matrices at 6 wks after transplantation into rat abdominal wall muscles

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<th>doses</th>
<th>35 kGy</th>
<th>50 kGy</th>
<th>control</th>
</tr>
</thead>
</table>

- completely resorbed bone matrix
- unresorbed bone matrix
- induced *de novo* cartilage, bone and bone marrow
Mechanical properties (maximal force - N) of rat femurs

- 19 mm span length
- Cross-head speed 0.1 cm/s

<table>
<thead>
<tr>
<th>Condition</th>
<th>Maximal Force (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh nonirradiated controls</td>
<td>138</td>
</tr>
<tr>
<td>Fresh frozen lyoph. 25 kGy</td>
<td>115</td>
</tr>
<tr>
<td>Fresh frozen lyoph. 35 kGy</td>
<td>78</td>
</tr>
<tr>
<td>Fresh frozen lyoph. 50 kGy</td>
<td>58</td>
</tr>
<tr>
<td>Fresh lyoph.</td>
<td>90</td>
</tr>
<tr>
<td>Frozen lyoph. 25 kGy</td>
<td>110</td>
</tr>
<tr>
<td>Frozen lyoph. 35 kGy</td>
<td>110</td>
</tr>
<tr>
<td>Frozen lyoph. 50 kGy</td>
<td>50</td>
</tr>
</tbody>
</table>

Graph: F (N) vs (mm)
fibrilar collagen

ionizing radiation

dry state (lyophilized sample)

wet state: $\text{H}_2\text{O} \rightarrow \text{OH}

direct effect: polypeptide chain scission

indirect effect: collagen cross-linking
Material and methods

rat compact bone (♂ 35 year-old)

human compact bone (♂ 14 week-old)

human rib cartilage (♂ 20 year-old)

calf Achilles tendon (♂ 6 week-old)

fresh and lyophilized

irradiated at room temperature with doses 25, 35, 50 or 100 kGy (γ-source)
Material and methods

Evaluation of *in vitro* solubility of collagen of tissue samples

Pulverized tissue samples were extracted with:

(a) 0.5N NaCl (pH 7.0) at 4°C for 48 hrs to determine neutral soluble collagen (*NSC*)

and the residues subsequently with:

(b) citric buffer (pH 3.6) at 4°C for 48 hrs to determine acid soluble collagen (*ASC*)
Material and methods

Evaluation of *in vitro* of collagen solubility of tissue samples

The amount of Pro-OH in extracts was measured and calculated as:

$$\text{mg Pro-OH} / \text{g dry tissue mass}$$

Total soluble collagen (TSC) was calculated as:

$$\text{TSC} = \text{NSC} + \text{ASC}$$
**In vitro** solubility of collagen (TSC) of fresh and lyophilized rat compact bone (♂ 14 week-old) γ-irradiated at room temperature

<table>
<thead>
<tr>
<th>Condition</th>
<th>mg Pr-OH/g dry tissue mass</th>
<th>% of total ProOH content</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.37</td>
<td>(0.39%)</td>
</tr>
<tr>
<td>Fresh</td>
<td>0.88</td>
<td>(0.94%)</td>
</tr>
<tr>
<td>Lyoph. 35 kGy</td>
<td>4.52</td>
<td>(4.81%)</td>
</tr>
<tr>
<td>Lyoph. 50 kGy</td>
<td>1.28</td>
<td>(1.36%)</td>
</tr>
<tr>
<td>Fresh 50 kGy</td>
<td>10.19</td>
<td>(10.84%)</td>
</tr>
</tbody>
</table>

*(total ProOH content of the sample - 94.03 mg ProOH/g dry tissue mass)*
**In vitro** solubility of collagen (TSC) of fresh and lyophilized human compact bone (♂ 35 year-old) γ-irradiated at room temperature

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mg Pr-OH/g dry tissue mass</th>
<th>% ProOH (total ProOH content of the sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-irradiated</td>
<td>0.21 (0.15%)</td>
<td></td>
</tr>
<tr>
<td>Fresh 25 kGy</td>
<td>0.26 (0.19%)</td>
<td></td>
</tr>
<tr>
<td>Fresh 35 kGy</td>
<td>1.29 (0.93%)</td>
<td></td>
</tr>
<tr>
<td>Fresh 50 kGy</td>
<td>0.42 (0.30%)</td>
<td></td>
</tr>
<tr>
<td>Fresh 100 kGy</td>
<td>3.67 (2.64%)</td>
<td></td>
</tr>
<tr>
<td>Lyophilized 25 kGy</td>
<td>0.56 (0.40%)</td>
<td></td>
</tr>
<tr>
<td>Lyophilized 35 kGy</td>
<td>4.62 (3.33%)</td>
<td></td>
</tr>
<tr>
<td>Lyophilized 100 kGy</td>
<td>14.10 (10.15%)</td>
<td></td>
</tr>
</tbody>
</table>

(total ProOH content of the sample - 138.9 mgProOH/g dry tissue mass)
In vitro solubility of collagen (TSC) of fresh and lyophilized human rib cartilage (♂20 year-old) γ-irradiated at room temperature

(total ProOH content of the sample - 79.78 mg ProOH/g dry tissue mass)
In vitro solubility of collagen (TSC) of fresh and lyophilized calf Achilles tendon (♂ 6 week-old) γ-irradiated at room temperature

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mg Pr-OH/g dry tissue mass</th>
<th>% of total ProOH content</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-irradiated</td>
<td>1.37 (1.07%)</td>
<td></td>
</tr>
<tr>
<td>fresh 25 kGy</td>
<td>0.96 (0.75%)</td>
<td></td>
</tr>
<tr>
<td>lyoph. 25 kGy</td>
<td>5.09 (3.98%)</td>
<td></td>
</tr>
<tr>
<td>fresh 35 kGy</td>
<td>2.21 (1.72%)</td>
<td></td>
</tr>
<tr>
<td>lyoph. 35 kGy</td>
<td>6.97 (5.46%)</td>
<td></td>
</tr>
<tr>
<td>fresh 50 kGy</td>
<td>1.03 (0.81%)</td>
<td></td>
</tr>
<tr>
<td>lyoph. 50 kGy</td>
<td>9.57 (7.50%)</td>
<td></td>
</tr>
<tr>
<td>fresh 100 kGy</td>
<td>2.37 (1.86%)</td>
<td></td>
</tr>
<tr>
<td>lyoph. 100 kGy</td>
<td>16.79 (13.14%)</td>
<td></td>
</tr>
</tbody>
</table>

(total ProOH content of the sample - 127.77 mg ProOH/g dry tissue mass)
Evaluation of *in vitro* susceptibility to pepsin digestion of fresh and lyophilized human bone and calf tendon samples irradiated at room temperatures with doses of 25 - 100 kGy

**Materials and methods**

Digestion of samples with 20% pepsin in 0.5N acetic acid at 4°C for 12 hrs

Centrifugation

Evaluation of Pro-OH in extracts (soluble collagen) and in residues (insoluble collagen)
Susceptibility to pepsin digestion of collagen of fresh and lyophilized human bone samples \(\gamma\)-irradiated at room temperature with doses of 25-100 kGy
Susceptibility to pepsin digestion of collagen of fresh and lyophilized calf tendon samples \(\gamma\)-irradiated at room temperature with doses of 25-100 kGy
Effect of ionizing radiation on collagen

- **Direct**
  - (dry state-lyophilized sample)
  - Polypeptide inter- and intra-chain scission

- **Indirect**
  - (wet state: \( \text{H}_2\text{O} \xrightarrow{\text{radiolysis}} \cdot\text{OH} \))
  - Inter- and intra-molecular crosslinking

### In Vitro Effects
- Solubility
- Susceptibility to enzyme action
- Mechanical properties
- Resorption rate

### In Vivo Effects
- Resorption rate

<table>
<thead>
<tr>
<th>Effect</th>
<th>Increase</th>
<th>Decrease</th>
<th>No Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Susceptibility to enzyme action</td>
<td>0</td>
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<tr>
<td>Mechanical properties</td>
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<tr>
<td>Resorption rate</td>
<td>0</td>
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</tr>
</tbody>
</table>

Increase and decrease indicate changes due to radiation exposure.
What is to be understood by validation?

“Validation is a documented procedure for obtaining, recording and interpreting the data required to show that a process will consistently comply with predetermined specifications”.
Sterilization validation

A biological indicator (BI) is an "inoculated carrier contained within its primary pack providing a known resistance to the relevant process."

There are many different types of BIs but the most common include:

- Bacterial spore strips individually packaged in glassine envelopes or pieces of filter paper impregnated with a certified population of a challenge organism that has a known resistance to EtO, such as the spores of Bacillus subtilis var. niger,

- A liquid bacterial spore suspension which is placed on or in the product, which is then referred to as inoculated product.

The BI used for validation and to monitor routine cycles must be more resistant than the bioburden of the product and be placed in a location that is more difficult to sterilize. Comparative resistance testing is an effective means of selecting the BI and its location in the product that presents the greatest challenge to the sterilization process. Such an assessment should be made prior to validation as part of determining the appropriateness of the BI.
Sterilization validation

There are three basic approaches to developing sterilization cycles:

- **The overkill method**, which ensures that the sterilization process will inactivate a specific number of microorganism spores known to be resistant to the particular sterilization process.

- **The combined bioburden and BI method** is used when the two are equally resistant. This method requires routine bioburden and BI testing in addition to a considerable amount of routine sterility testing to develop a cycle that will inactivate the BI challenge population.

- **The absolute bioburden method** requires extensive testing in both the development phase and routine processing. It must be used when the product's bioburden is more resistant than the BI. Such bioburden resistance to the sterilization process can be caused by any number of factors, such as the configuration of the product, the quantity or location of the microorganisms, or the bioburden's intrinsic resistance.
Microbiological performance qualification (MPQ) should be performed using specified products and packaging configured in the same manner in which they will be routinely sterilized. The MPQ can be performed by determining the lethality of the cycle on the basis of the number of D-values applied. The *D-value* is defined as "the time required to reduce a specific microbial population by 90% or one logarithm."
Sterilization validation

The survivor curve construction method involves the direct enumeration of survivors in terms of colony forming units (CFUs) recovered after exposure to graded amounts of the sterilization cycle. A CFU is defined as "a visible outgrowth of a population of organisms arising from a single or multiple cells." A minimum of five cycles should be run.

The fraction-negative method also involves exposing BIs to multiple cycles of graded exposures to sterilization.

At least one sample set that elicits all survivors (growth in all BIs tested).
At least four sample sets that elicit fractional data, i.e., a fraction of the BIs in each set demonstrates growth or survival.
At least two sample sets in which there is neither growth nor survivors.
BIs are immersed directly into the appropriate media and incubated. Results are recorded in terms of the total number of BIs demonstrating growth and the total number eliciting no growth for each set of test samples.
ETO sterilization validation

The half-cycle method determines the minimum time a specific product load must be exposed to an EtO process to guarantee that no survivors exist from the BIs used to monitor the cycle's efficacy.

The data obtained from the survivor curve construction method, the fraction-negative method, or the half-cycle method are used to design the full cycle that will be used to routinely sterilize the product. The full cycle must be capable of reliably demonstrating a required SAL that consists of the minimum time to obtain all negative BIs or CFUs with an additional margin of safety.

Normally this can be expressed as $10^{-n}$ where $n$ is the cumulative probabilities of the log minimum time to sterilize and the log margin of safety. For example, if the half-cycle method was chosen to validate a given sterilization cycle with a required SAL of $10^{-6}$, the half-cycle must demonstrate the ability to ensure that there are no survivors from BIs that have a certified population of $10^6$. Since the BIs have a greater resistance than the product bioburden, it can be concluded that the time is sufficient to achieve product sterility. Adding an equivalent sterilization time period increases the margin of safety. The additional 6 log reduction in the population ($10^{-6}$) is theoretical and is obtained by doubling the half-cycle gas exposure time. If the half-cycle gas exposure time period was 2 hours, the full-cycle gas exposure time would be a minimum of 4 hours.
Validation of the sterilization process

a) qualification of the tissue allografts and their packaging for sterilization,
b) qualification of the sterilization facility,
c) process qualification using a specified tissue allografts or simulated products in qualified equipment,
d) a certification procedure to review and approve documentation of a), b) and c),
e) activities to support maintenance of validation.
Validation of the sterilization process

Design of validation studies

It is not necessary to validate every individual step of a tissue banking process.

Only those steps which are likely to contribute to inactivation/removal of a microorganisms need to be subject to a validation study.
Radiation sterilization validation

Selection of tissue allograft products

Tissue allografts can be prepared from a wide range of tissues such as skin, amnion, bone, cartilage, tendons, and ligaments. If samples can be prepared from these tissues, which are reasonably reproducible in shape, size and composition and also in sufficient numbers for statistical purposes, then the usual sampling procedures apply.

If allograft products are both few in number (less than 10) and cannot be considered as identical products, then it may be necessary to take multiple Sample Item Portions of a single tissue allograft product for both bioburden analysis prior to sterilization and also for the purpose of establishing a sterilization dose. In such instances, it is important to have confidence in the distribution of microorganisms throughout the sample, obtained, for example, by periodic monitoring of such products.
Validation of the sterilization process

Bioburden estimation

Current methods of sterilization render the grafts sterile, provided the tissue contains a very low or acceptable level of bioburden prior to sterilization. Bioburden estimation is the presterilization count of viable micro-organisms on a product. In ISO document No.11137, bioburden is defined as the total number of viable microorganisms on a health care product unit prior to sterilization processing. We need to test the bioburden for two main reasons:

- Sterilization process validation in order to determine the sterilization dose or verification of its safety margin
- Routine monitoring of the effectiveness of processing controls in terms of the number and nature of micro-organisms present on the grafts and tissue components prior to sterilization
Bioburden determination

- Sample collection
- Transport of the sample to the laboratory
- Removal of micro-organisms from the sample
- Transfer to suitable culture medium and incubation
- Enumeration
- Characterization
Bioburden determination

- For a single large piece of allografts collect the total volume of the eluent solution from the last washing of the tissue allograft processing.
- For large production batches, randomly select units or SIPs of tissue allografts.
- For small production batches take either sample item portions (SIPs) or whole sample from tissues allografts.
- During transportation, tissue samples for bioburden estimation should be kept under the same conditions as for the whole production batch.
Bioburden determination

**Flushing:** The test item is flushed with a known volume of eluent and the resulting solution is collected.

**Stomaching:** This method is particularly suitable for skin, amnion and other soft tissue-like films or in the form of a tube. The test item and a known volume of eluent should be enclosed in a sterile stomacher bag. Reciprocating paddles operate the bag and force the eluent through and around the item. The time of treatment should be recorded.

**Shaking with or without glass beads:** The test item is immersed in a known volume of eluent within a suitable vessel and shaken using a mechanical shaker (reciprocating, orbital, vortex mixing or wrist action). Glass beads of a defined size may be added to increase surface abrasion and thereby recovery efficiency. The time and frequency of shaking should be recorded.

**Ultrasonication:** The test item is immersed in a known volume of eluent within a suitable vessel. The time and ultrasonic intensity of the treatment should be recorded.
Bioburden determination

- Transferring methods:
  - membrane filtration,
  - pour plating,
  - spread plates,

For tissue bioburden determination, the total microbial count should be carried out.

For contaminants that are not commonly found and those suspected to be most radiation resistant, these should be isolated and characterized.
Radiation sterilization validation

Studies should be carried out to establish the types of microorganisms that are normally found on the tissue types to be sterilized as well as their numbers and resistance to radiation. Such studies should take account of the distribution of the microorganisms within the tissue allograft itself since this may not be uniform. This should be determined by taking Sample Item Portions (SIP) of the tissue and demonstrating that there are no significant statistical variations in distribution from SIP to SIP.
Radiation sterilization validation

Bioburden determination

Bioburden determination could include the count of aerobic and anaerobic bacteria, spores, yeasts, molds and anaerobic bacteria. Many factors determine the choice of the tests most appropriate for the tissue allograft. At a minimum, the aerobic bacteria and fungi should be counted.

The objective of the bioburden determination is to:

a) determine the total number of viable microorganisms within or on a tissue allograft and the packaging after completion of all processing steps before sterilization,
b) act as an early warning system for possible production problems,
c) calculate the dose necessary for effective radiation sterilization.
Radiation sterilization validation

Viruses for validation should be chosen firstly to resemble viruses which may contaminate the „product“ as closely as possible and secondly to represent the widest possible range of physico-chemical properties to test the ability of the system to eliminate viruses in general.

The production process must be evaluated for its ability to inactivate/remove infectious HIV.

Examples of viruses representing a range of physico-chemical properties which have been used to evaluate the general ability of a process to remove virus infectivity include:

- SV 40, poliovirus or an animal parvovirus as small non-enveloped viruses,
- a parainfluenza or a murine retrovirus as large enveloped RNA viruses,
- a herpesvirus as a large DNA virus.
The D10-value, value necessary to reduce the initial microbial population by 1 log10, i.e. by 90 %. This value can be read directly from the dose-inactivation curve or calculated using the following equation:

\[
D\text{-value} = \frac{\text{radiation dose/time}}{\log \text{No} - \log \text{N}}
\]

D-value differs greatly among different types of microorganisms, and occasionally considerable variation among different strains of the same organism is observed. The response of microorganisms to radiation also depends on external conditions.
Exponential decrease of bioburden as a function of sterilization
Estimation of unit sterilization dose
\((D_{10} - \text{Value})\) from \(\log_{10}\) bioburden - dose relationship

\(\log_{10}\) bioburden

unit sterilization dose \((D_{10} - \text{Value})\)
“Sterility assurance level - SAL”

is derived from kinetic studies on microbial inactivation, i.e. the probability of viable microorganisms being present on or inside a product unit after sterilization.

SAL $10^{-6}$ would assure that less than one out of million contaminants would survive on or inside the product following sterilization.

Depending on the risk posed by the use of various specimens, different values of SAL ($10^{-3}$, $10^{-6}$) may be recommended.

For tissue allografts a value of SAL $10^{-6}$ or lower is recommended.
Validation of the sterilization process

The log_{10} reduction of the order of 4 logs or more are indicative of a clear effect with the particular test microorganisms under investigation. However, it is emphasised that log number reduction cannot be used as a single, absolute measure of the effectiveness of a step.

Calculation of reduction factors
The microorganism reduction factor, R for an individual inactivation or removal step is given by the expression:

\[ R = \log \left( \frac{V_1 \times T_1}{V_2 \times T_2} \right) \]

where \( V_1 = \text{volume of starting material} \), \( T_1 = \text{concentration of microorganisms in starting material} \), \( V_2 = \text{volume of material after the step} \), and \( T_2 = \text{concentration of microorganisms after the step} \).
Validation of the radiation sterilization

Selection of required sterilization dose

The choice of 25 kGy (2.5 Mrad) for sterilization of medical products was first suggested in 1959. The choice was not arbitrary, but was determined on the basis of available data. However, the addition of a 40% safety factor was obviously excessive. Even though the exponential relationship between dose and numbers of surviving cells had been discovered and documented some 30 years ago, the decision on 25 kGy was still based on belief in a philosophy of absolute sterility.
Validation of the sterilization process

In recent years, new methods of selecting the sterilizing dose have been proposed. These are based upon an appreciation of the probabilistic nature of microbial inactivation which, in turn, allows sterility, as applied to a population of product items, to be defined quantitatively. Two methods of dose selection, based on the new concept, are described in the ISO document. The methods were first put forward by the Association for the Advancement of Medical Instrumentation (AAMI) Guidelines as B1 and B2 methods in 1984. Basically, the choice of dose is made on the basis of a knowledge of the microbiological contamination on product items before sterilization which is called bioburden and a prediction that the selected SAL will be achieved. For products like tissue grafts where the usage is intended to come into contact with compromised tissue i.e. tissue that has lost the protection of the natural body barriers, the SAL of $10^{-6}$ is widely accepted.
**Radiation sterilization validation**

**Method A1** For establishing specific sterilization doses for Standard Distribution of Resistance and other microbial distribution for samples sizes between 10 and 100, an adaptation of Method 1 of ISO 11137:1995 may be used. Method 1 of ISO 11137 is normally used for multiple batches containing a large number of samples per batch. For batches of 100 samples for example, verification dose experiments are carried out for a SAL of $10^{-2}$. A successful experiment (up to 2 positive tests of sterility) will then enable the dose required to achieve a SAL value of $10^{-6}$ to be calculated from the survival curve of a Standard Distribution of Resistances (SDR). The verification doses for SAL values between $10^{-2}$ and $10^{-1}$ may be found for bioburden levels up to 1000 cfu per allograft product. These SAL values correspond to relative low sample sizes of 10 – 100.
Radiation sterilization validation

**Method A2** A similar approach can also be undertaken when the distribution of microbial radiation resistances is known and different to the SDR. This method is applied to a microbial population which has a different distribution of radiation resistances than the SDR.

However, it should be noted that, for both Methods A1 and A2 above, low bioburden levels combined with low sample numbers, will give rise to an increased probability of failure of the verification dose experiment.
Radiation sterilization validation

Method B For substantiation of a 25 kGy sterilization dose, the Method in ISO/TR 13409:1996 may be used to calculate the verification dose. This is an accredited method and is essentially a modification of Method 1 of ISO 11137-1 and applies only to a Standard Distribution of Resistances. In this method, the verification dose for a given SAL is approximated to the initial bioburden by a series of linear relationships. Each linear equation is valid for a particular ten-fold domain of bioburden level e.g., 1-10 cfu. The method in ISO/TR 13409:1996 can only be used to substantiate a dose of 25 kGy. It should be noted that the statistical approach allowing up to 1 positive test for sample sizes up to 30 and up to 2 positive tests for sample sizes above 30 does not offer the same level of protection as for the 100 samples in ISO 11137-1 until the sample size reaches 100.
Radiation sterilization validation

Method C For substantiation of a 25 kGy sterilization dose, an alternative and more recent method in AAMI TIR 27 may be used. This method may be adopted as a formal replacement of Method B (ISO/TR 13409) for the use of 25 kGy as a sterilization dose. The modification takes into account how the verification dose varies with bioburden level for a given SAL (and sample size) on the assumption that an SAL of 10^{-6} is to be achieved at 25kGy. Depending on the actual bioburden levels to be used (1-50 or 51-1000 cfu per allograft product), a linear extrapolation of the appropriate SDR survival curve is made from either (log N 0,0 kGy) or (log 10^{-2}) to (log 10^{-6}, 25 kGy) for 1-50 cfu and 51-1000 cfu respectively. For bioburden levels less than 1000 cfu per allograft unit, these constructed survival curves represent a more radiation resistant bioburden than would otherwise be the case. The validity of this approach arises from the purpose of the method which is to validate a sterilisation dose of 25 kGy for all bioburden levels below 1000 cfu per allograft product.
Example 1 of sterilization procedures

Limited number of amnion samples with low bioburden and low bacterial resistance using Method A2 to calculate the verification dose applied to sample sizes of less than 100 in a single production batch.

The single batch of 20 amnion membranes (5 x 5 cm) from which 10 are used for the bioburden determination and 10 are used for the verification dose experiment.
Example 1 of sterilization procedures

- Typical microbial levels of procured tissue before processing - *in the range of 5000 - 10000 cfu per tissue*

- Typical bioburden levels of processed and preserved tissues - *57 cfu per allograft product*

- The most radiation resistant microbes were assumed to have a $D_{10}$ value of 1,8 kGy.
Example 1 of sterilization procedures

Calculation of the sterilization dose

Stage 1
Production batch size 40 - 5 x 5 cm amnion samples
Test sample size for bioburden determination - 10
Test sample size for the verification dose experiment - 10
Verification dose required for SAL $10^{-1}$ (=1/10)

Stage 2
Obtain samples - 20
10 for bioburden; 10 for verification dose

Stage 3
SIP 1
The whole allograft product is used average bioburden - 57
Bioburden results: 28, 91, 90, 30, 30, 86, 28, 64, 91, 32

Stage 4
Verification dose calculation - 4.96 kGy
Example 1 of sterilization procedures

Stage 5
Irradiation dose 5.0 kGy

Result: 1 positive / 10 samples

The sterility test yielded one positive test out of ten and therefore the verification dose experiment was successful and the sterilisation dose for \( \text{SAL} = 10^{-6} \) can be calculated 13.96 kGy.
Example 2 of sterilization procedures

Limited number of bone samples with very low bioburden and SDR using ISO/TR 13409:1996 to calculate the verification dose (SIP-1)

Introduction.

This method uses Method B (from ISO/TR 13409:1996) and applies it to a sample of 40 small pieces of amnion.

Typically, very low bioburden levels are found after processing. In this example, very low SIP values are used so that most of the allograft product can be retained for use.
Example 2 of sterilization procedures

Calculation of the sterilization dose

Stage 1
Production batch size 40 - 5 x 5 cm amnion samples
Test sample size for bioburden determination - 10
Test sample size for the verification dose experiment - 10

Stage 2
Obtain samples - 20
10 for bioburden; 10 for verification dose

Stage 3
SIP 1, bioburden - 57
Bioburden results 28, 91, 90, 30, 30, 86, 28, 64, 91, 32
Example 2 of sterilization procedures

Stage 4

Verification dose 4.6 kGy
Verification dose formula: I + (S x log (average SIP bioburden)) kGy
According ISO/TR 13409:1996, I and S values are 0.67 and 2.23 respectively.
= 0.67 + (2.23 x log 57) = 4.6 kGy

Stage 5

Verification dose experiment 4.5 kGy (delivered dose)
2 positive / 10 samples

The test sterility yielded 2 positive from the 10 SIPS tested. Therefore, the verification experiment was not successful.
Example 3 of sterilization procedures

Limited number of bone samples with very low bioburden and SDR using ISO/TR 13409:1996 to calculate the verification dose (SIP<1)

Introduction.

This method uses Method B (from ISO/TR 13409:1996) and applies it to a sample of 40 small pieces of bone.

Typically, very low bioburden levels are found after processing. In this example, very low SIP values are used so that most of the allograft product can be retained for use.
Example 3 of sterilization procedures

Calculation of the sterilization dose

Stage 1
Production batch size 5 - bone cut into 40 small pieces (1 cc each)
Test sample size for bioburden determination - 10
Test sample size for the verification dose experiment - 10

Stage 2
Obtain samples - 20
10 for bioburden; 10 for verification dose

Stage 3
SIP 0.025 calculated from 1/40 SIP, bioburden - 1
Bioburden results of 1, 0, 2, 0, 1, 2, 1, 1, 1, 1
The average bioburden for the product tested was calculated as follow:
1/0.025=40.
Example 3 of sterilization procedures

Stage 4

Verification dose calculation 1.3
Veriﬁcation dose formula: I + (S x log (average SIP bioburden)) kGy
According ISO/TR 13409:1996, I and S values are 1.25 and 1.65 respectively.
= 1.25 + (1.65 x log 1) = 1.25 kGy = 1.3 kGy

Stage 5

Verification dose experiment 1.3 kGy (delivered dose)
0 positive / 10 samples

The test sterility yielded 0 positive from the 10 SIPs tested. Therefore, the verification experiment was successful and no further action was necessary.
Validation of the sterilization process

Inactivation of HIV by radiation

The dose of irradiation needed to reduce the viral load by $1 \log_{10}$ - the $D_{10}$ value is 4 kGy or even 5.6 kGy. Taking into consideration the required SAL $10^{-6}$, assuming the average HIV bioburden to be about $10^3$ virions/ml for the state of acute infection and a D10 value of 4 kGy, a reduction of 9 (6+3) units or a dose of 36 kGy (9x4 kGy) would be required. On the other hand, if the $D_{10}$ value is 5.6 kGy, then a dose of >50 kGy would be needed to inactivate HIV.
Validation of the sterilization process

Sterility test

Sterility test is a test designed to reveal whether viable forms of microorganisms are present in or on health care products which have gone through the process of sterilization.

As sterility is generally understood to represent an absolute condition in which no life forms are present, it is assumed that there are only two possible conditions, a graft may be sterile (no life-forms present) or it may be non-sterile (the presence of one or more viable microorganisms).
Before sterilization

Irradiation indicator
Sterilization protocol
After sterilization

Irradiation indicator
Radiation sterilization validation

Dosimetry
Several dosimetric systems are being used for dose measurements in industrial irradiators:

- **the Fricke dosimeter** - commonly used for the calibration of all other high dose dosimetric systems. The dosimeter can measure doses in the range of 40-400 Gray (Gy).

- **the ceric-cerous dosimeter** based on reduction of ceric ions in acidic aerated aqueous solutions both in the presence and absence of cerous ions - used widely for industrial irradiators.

- **plastic dosimeters** such as red perspex, clear perspex, radiochromic dye film, cellulose triacetate film and cellulose diacetate film are also available for use but show a response which is dependent on environmental factors i.e. temperature, humidity and light.

- **free radical dosimetry** systems using alanine and glutamine.
Measurements of absorbed dose of ionizing radiation

a/ water calorimeter  [0.5 - 50 kGy]
b/ foil PVC dosimeter  [5 – 50 kGy]
c/ L-alanine  [0.005 – 100 kGy]  EPR
d/ bone powder  [0.05 – 40 kGy]  Spectrometry
Relation between the dose of ionizing radiation and the intensities of the epr signals recorded with bone dosimeter.
Relationship between the heights of the epr signals (recorded with l-alanine and bone dosimeters) and the dose of $^{60}$Co radiation.
## Comparison of sterilization methods

<table>
<thead>
<tr>
<th>CONSIDERATIONS</th>
<th>AUTOCLAVE</th>
<th>ETHYLENE OXIDE</th>
<th>RADIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue materials</td>
<td>Mainly damaged</td>
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<td>Only at high doses</td>
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<td>Packaging</td>
<td>Special</td>
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Sterilization of Medical Materials

- thermic methods
- radiation sterilization
- EtO

(%)
thank you