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보 문

# Process development of a virally-safe dental xenograft material from porcine bones

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# 바이러스 안전성이 보증된 돼지유래 골 이식재 제조 공정 개발

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**ABSTRACT:** A process for manufacturing virally-safe porcine bone hydroxyapatite (HA) has been developed to serve as advanced xenograft material for dental applications. Porcine bone pieces were defatted with successive treatments of 30% hydrogen peroxide and 80% ethyl alcohol. The defatted porcine bone pieces were heat-treated in an oxygen atmosphere box furnace at 1,300°C to remove collagen and organic compounds. The bone pieces were ground with a grinder and then the bone powder was sterilized by gamma irradiation. Morphological characteristics such as SEM (Scanning Electron Microscopy) and TEM (Transmission Electron Microscopy) images of the resulting porcine bone HA (THE Graft®) were similar to those of a commercial bovine bone HA (Bio-Oss®). In order to evaluate the efficacy of 1,300°C heat treatment and gamma irradiation at a dose of 25 kGy for the inactivation of porcine viruses during the manufacture of porcine bone HA, a variety of experimental porcine viruses including transmissible gastroenteritis virus (TGEV), pseudorabies virus (PRV), porcine rotavirus (PRoV), and porcine parvovirus (PPV) were chosen. TGEV, PRV, PRoV, and PPV were completely inactivated to undetectable levels during the 1,300°C heat treatment. The mean log reduction factors achieved were ≧4.65 for TGEV, ≧5.81 for PRV, ≧6.28 for PRoV, and ≧5.21 for PPV. Gamma irradiation was also very effective at inactivating the viruses. TGEV, PRV, PRoV, and PPV were completely inactivated to undetectable levels during the gamma irradiation. The mean log reduction factors achieved were ≧4.65 for TGEV, ≧5.87 for PRV, ≧6.05 for PRoV, and ≧4.89 for PPV. The cumulative log reduction factors achieved using the two different virus inactivation processes were ≧9.30 for TGEV, ≧11.68 for PRV, ≧12.33 for PRoV, and ≧10.10 for PPV. These results indicate that the manufacturing process for porcine bone HA from porcine-bone material has sufficient virus-reducing capacity to achieve a high margin of virus safety.

**Key words:** dental xenograft material, hydroxyapatite, porcine bone, porcine pathogenic viruses, virus inactivation

Bone grafting is a surgical procedure that replaces missing bone. Autograft, allograft, xenograft, and synthetic bone graft substitute materials play an important role in reconstructive orthopaedic and periodontic surgery (Damien and Parsons, 1991; Bauer and Muschler, 2000; Venkataraman *et al*., 2015). Autogenous bone, with its osteogenic, osteoinductive, and osteoconductive properties, has long been considered the ideal grafting material for bone reconstructive surgery. However, drawbacks with autogenous bone include morbidity, availability and unpredictable graft resorption (Hallman and Thor, 2008). Allografts are graft materials harvested from different human individuals and require processing in order to lessen antigenicity

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and disease transfer. They are osteoconductive and osteoinductive (Khan *et al*., 2005). Xenografts are obtained from the bones of individuals of other species with composition and biomechanical properties that are almost similar to human bone. Two illustrations of xenografts used in dentistry are i) coral-derived bone substitutes with geometry similar to that of human cancellous bone interconnected macropores (200–600 μm) and ii) demineralized animal bone grafts, which are biocompatible and osteoconductive (Jensen *et al*., 2009). Synthetic bone graft substitute materials are osteoconductive alloplastic materials such as calcium phosphate and bioactive glass (Välimäki and Aro, 2006).

The use of xenografts has increased in recent years because adequate amounts can be easily obtained. However, they also have a limitation as the risk of transmission of zoonotic diseases is possible. The best known demineralized xenograft is bovine bone mineral (Bio-Oss®), which has a porous structure similar to human osseous tissue as well as a long resorption time, serving as an ideal scaffold for osteogenesis (Pinholt *et al*., 1991; Haas *et al*., 1998). It is consisted of HA prepared by alkaline treatment and thermal treatment at 300°C to remove organic components of medullar bovine bone. Another bovine xenograft is Gen-Ox® obtained through deproteinization at high temperatures (between 950 and 1,000°C) (Accorsi-Mendonça *et al*., 2008). However, a unique safety issue with using bovine materials, namely the risk of bovine spongiform encephalopathy (BSE) transmission, is rarely addressed in literature and appears to be ignored by practitioners. BSE is a type of transmissible spongiform encephalopathy (TSE) or prion disease, which is a group of fatal neurodegenerative diseases affecting humans and a broad spectrum of animal species (Kim *et al*., 2016). Therefore, porcine bone is considered an alternative to bovine bone.

Purgo Corp. in Korea is currently producing a dental xenograft material (THE Graft®) from porcine bones. THE Graft® is a resorbable bone substitute material that consists of HA. It is prepared by removing organic compounds from porcine bone pieces using 30% hydrogen peroxide and 80% ethyl alcohol solutions for 12 h each. After rinsing with distilled water, bone pieces are dried at 100°C for 24 h and heat-treated in an oxygen atmosphere box furnace at 1,300°C to remove collagen and organic compounds. The bone pieces are ground with a grinder. Finally, gamma irradiation is performed (Kim *et al*., 2014a, 2014b).

The medical grafting products, developed from porcine tissues, also have a risk of viral contamination (Hodde and Hiles, 2002). Therefore, the ability to remove and/or to inactivate viral contaminants during the manufacturing of animal-derived bone material has become an important parameter for assessing the safety of the products (Forest *et al*., 2007; International Organization for Standardization, 2007; Bae *et al*., 2010, 2012). The manufacturing process for THE Graft® contains an intentional viral inactivation procedure through gamma irradiation. Also it involves a 1,300°C heat treatment procedure which can potentially inactivate viral contaminants.

In this study, we have developed a manufacturing process for a virally-safe dental graft material from porcine bone and evaluated the efficacy of viral inactivation procedures. For this study, the morphological characteristics of the porcine bone HA (THE Graft®) were compared with those of a commercial bovine bone HA (Bio-Oss®). Also, four common-porcine relevant viruses, transmissible gastroenteritis virus (TGEV), pseudorabies virus (PRV), porcine rotavirus (PRoV), and porcine parvovirus (PPV), were chosen as the model viruses for the evaluation of virus safety for porcine-derived medical products (Table 1). The viruses used in this study were selected to represent viruses with a range of biophysical and structural features, which might also present themselves as unknown or unidentified contaminants in the starting material, and display a significant resistance to physical or chemical agents (Inter-

**Table 1. Salient features of viruses used for the evaluation of virus clearance**

| Virus | Family | Host | Shape | Lipid envelope | Genome | Size (nm) | Resistance to physico-chemical reagent |
|---|---|---|---|---|---|---|---|
| Transmissible gastroenteritis virus (TGEV) | *Coronaviridae* | Porcine | *Circular* | Yes | ss-RNA | 100-150 | Low |
| Pseudorabies virus (PRV) | *Herpesviridae* | Porcine | *Icosahedral* | Yes | ds-DNA | 120-200 | Medium |
| Porcine rotavirus (PRoV) | *Reoviridae* | Porcine | *Icosahedral* | No | ds-RNA | 60-80 | Medium |
| Porcine parvovirus (PPV) | *Parvoviridae* | Porcine | *Icosahedral* | No | ss-DNA | 18-26 | High |

national Organization for Standardization, 2007).

## Materials and Methods

### Manufacturing process for porcine bone HA

Porcine bone HA was prepared from swine slaughtered for human consumption in commercial abattoirs (Sunjin). Porcine bone was cut into slices 1 cm thick. Defatting and deproteinization were achieved by chemical and heat treatment. The porcine bone was immersed in distilled water and degreased with 30% hydrogen peroxide solution (Sigma Aldrich) for 12 h and 80% ethyl alcohol solution (Sigma Aldrich) for 12 h. After rinsing with distilled water, bone pieces were dried at 100°C for 24 h and heat-treated in an oxygen atmosphere box furnace at 1,300°C to remove collagen and organic compounds. The bone pieces were ground with a grinder. Finally, glass vials that were packaged with bone powder were sterilized with gamma irradiation at a dose of 25 kGy according to the ISO 11137-2:2006 (International Organization for Standardization, 2006). Gamma irradiation was performed using the continuous type gamma irradiator from Greenpia Tech Inc. using the radio nuclide cobalt 60 ($^{60}$Co) (Fig. 1).

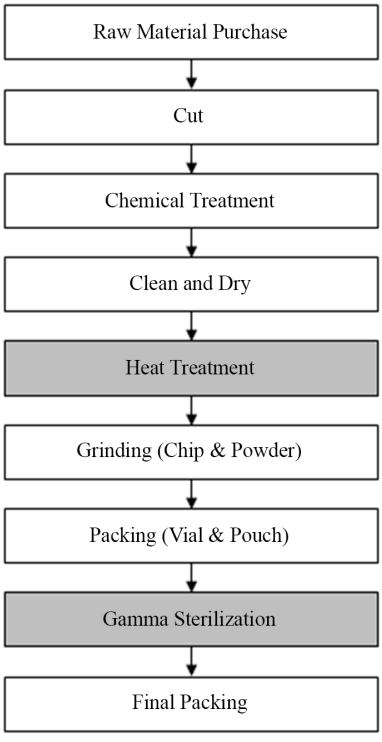


**Fig. 1. Flow diagram of manufacturing process for porcine bone hydroxyapatite (THE Graft®).** The grey boxes indicate the validation steps employed for virus inactivation.

### Morphological characterization of porcine bone HA

The morphological characteristics of the porcine bone HA prepared from this study were compared with those of a grafting material of bovine origin, Bio-Oss®. Morphological characterization of the materials was carried out through scanning electron microscopy (SEM, LEO SUPRA55, Carl Zeiss) at 20 kV of electron acceleration and transmission electron microscopy (TEM, JEM-3010, JEOL) at 300 kV of electron acceleration. Pore size analysis was performed on SEM images taken at various magnifications. Pore size was defined as the longest distance across a single pore.

### Preparation and titration of viruses

For the propagation and titration of TGEV (ATCC VR-763), PRV (YS-400 strain), PRoV (KVCC-VR0000176), and PPV (ATCC VR-742), ST cells (ATCC CRL-1746), Vero cells (ATCC CCL-81), MA-104 cells (ATCC CRL-2378), and MPK cells (ATCC CCL-166) were used, respectively. All the viruses and cells were obtained from American Type Culture Collection. Prior to viral propagation, all the host cells were grown in high glucose Dulbecco's modified Eagle's medium with L-glutamine (HG DMEM, HyClone) containing 10% fetal bovine serum. ST cells, Vero cells, and MPK cells were subcultured in DMEM containing 2% fetal bovine serum for the propagation and titration of TGEV, PRV, and PPV, respectively. Meanwhile, for the propagation and titration of PRoV, MA-104 cells were grown in DMEM plus 0.1% trypsin (Gibco).

An aliquot from each sample used in the virus inactivation studies and an appropriate control were titrated immediately after being collected in 7-fold serial dilutions to the end point using a quantal 50% tissue culture infectious dose ($TCID_{50}$) assay (Kärber, 1931). For titration of the viruses, indicator cell monolayers in 24-well culture plates were infected using at least eight replicates of 0.25 ml of the appropriate dilution of each sample or the positive control. Negative control wells were mock-infected using at least eight replicates of 0.25 ml of the culture medium. The plates were then incubated at 35°C for

approximately 1 h, and the wells were fed with 1 ml of the tissue culture medium. After 7–14 days incubation, the wells were examined for cytopathic effect (CPE).

As a part of the virus validation protocol, cytotoxicity, interference and load titer tests were performed. The cytotoxicity tests were performed on those samples generated for virus titration in virus spiking experiments to control for any possible cytotoxic effects on the indicator cells that might interfere with the virus titration. The interference tests were performed to determine whether the starting materials for virus spiking studies exerted an inhibitory effect on the ability of the cell lines to permit the detection of the virus. The load titer assays were performed to determine precisely the point at which spiking level leads to a loss in the virus titer.

### Virus inactivation studies

Virus inactivation studies were conducted using the validated scale-down processes. To evaluate the effectiveness and robustness of the 1,300°C heat treatment in inactivating viruses, 1.5 ml of virus stock solution was spiked with 1 g of porcine bone material before heat treatment. The virus-spiked samples were incubated at room temperature for 10 minutes to allow the adsorption of the virus solution. The virus-spiked samples were treated at 1,300°C for different durations (0, 0.5, 1, and 2 h). As a hold control, one of the virus-spiked samples was kept at 4°C during heat treatment in order to determine precisely the amount of virus titer lost during the virus inactivation study. Samples were collected at different times. Each sample was mixed with 5 ml of virus culture media, vigorously shaken with a vortex mixer in order to withdraw viruses from the virus-spiked porcine bone material, and then centrifuged. The supernatant was collected. The above procedure was repeated three times. The collected supernatant was combined and filtered (0.45 μm). A portion of the obtained filtrate was tested immediately.

To evaluate the effectiveness and robustness of the gamma irradiation in inactivating viruses, 1 ml of virus stock solution was spiked with 1 g of porcine bone material obtained before gamma irradiation. The virus-spiked samples were incubated at room temperature for 10 min to allow the adsorption of the virus solution. The virus-spiked samples were treated with gamma irradiation at the intensities of 5, 15, and 25 kGy. As a hold control, one of the virus-spiked samples was kept at 4°C during gamma irradiation in order to determine precisely the amount of virus titer lost during the virus inactivation study. To the gamma irradiated samples, 5 ml of virus culture media was added, vigorously shaken with a vortex mixer in order to withdraw viruses from the virus-spiked porcine bone material, and then centrifuged. The supernatant was collected. The above procedure was performed three times. The collected supernatant was combined and filtered (0.45 μm). A portion of the obtained filtrate was tested immediately. All virus inactivation experiments were carried out in duplicate and mean values are given.

### Calculation of virus reduction factors

The virus log reduction factor was defined as the $\log_{10}$ of the ratio of the virus loads in the spiked starting and post process materials, as previously described (International Conference on Harmonisation, 1998; Bae *et al*., 2010). The formula takes into account the titers and volumes of the materials before and after the processing step.

$$10^{Ri} = (v^{I})\,(10^{aI})/(v^{II})\,(10^{aII})$$

where : Ri = the reduction factor for a given stage, $v^{I}$ = the volume of the input material, aI = the titer of the virus in the input material, $v^{II}$ = the volume of the retained output material, aII = the titer of the virus in the output material.

## Results

### Morphological characterization of porcine bone HA

The morphological characteristics of porcine bone HA (THE Graft®) prepared from this study were compared with those of a commercial bovine bone HA (Bio-Oss®). SEM was used for the surface structure observation. Figure 2 shows the macro-porous nature of the two graft materials and similar structures. Also, the TEM images were very similar for Bio-Oss® and THE Graft® (Fig. 3). The TEM image clearly showed the micro-porous nature of the two graft materials and similar structures. The major pore size distributions of THE Graft® and Bio-Oss® are similar. They are evenly distributed from 0.03 mm to 1.0 mm (Fig. 4).

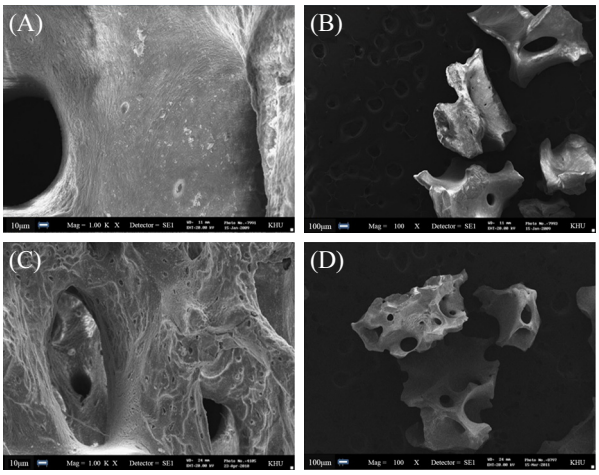


**Fig. 2. Comparative SEM images of Bio-Oss® (A, B) and THE Graft® (C, D).** Characterization of surface structures of both materials was carried out through SEM (LEO SUPRA55, Carl Zeiss) at 20 kV of electron acceleration.

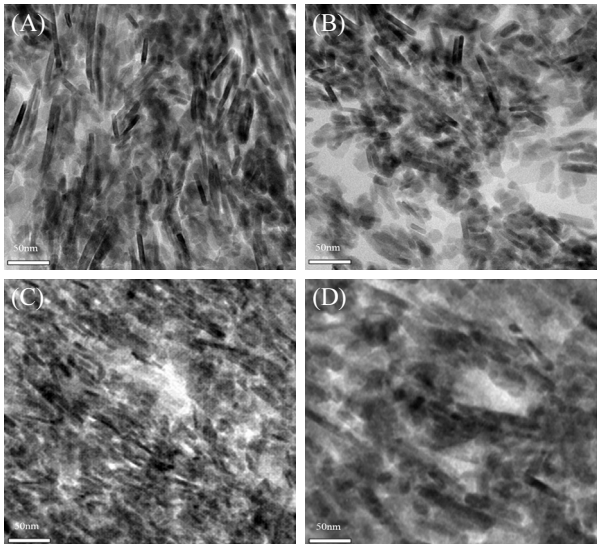


**Fig. 3. Comparative TEM images of Bio-Oss® (A, B) and THE Graft® (C, D).** Micro-porous structures of both materials were observed using TEM (JEM-3010, JEOL) at 300 kV of electron acceleration.

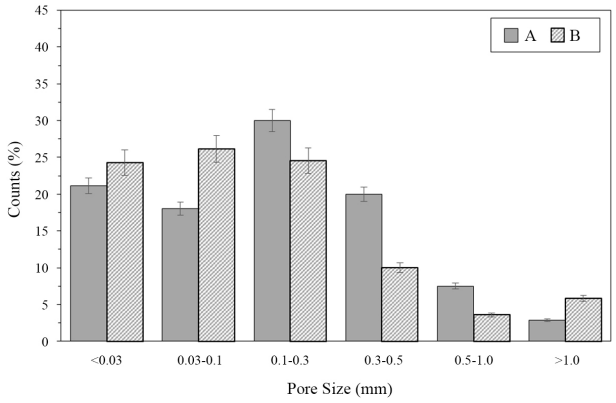


**Fig. 4. Comparative pore sizes of Bio-Oss® (A) and THE Graft® (B).** Pore size analysis was performed on SEM images taken at various magnifications.

## Virus inactivation by heat treatment

Heat treatment at 1,300°C was extremely effective at inactivating all the viruses tested (Table 2). All the viruses were completely inactivated to undetectable levels within 30 min of heat treatment. The log reduction factors achieved were ≥4.81 for TGEV, ≥4.49 for PRV, ≥6.28 for PRoV, and ≥5.21 for PPV.

## Virus inactivation by gamma irradiation

Gamma irradiation was also very efficient at inactivating all the viruses tested (Table 3). TGEV was rapidly inactivated from an initial titer of 6.35 $\log_{10}$ $TCID_{50}$ to 2.07 $\log_{10}$ $TCID_{50}$ after 5 kGy irradiation and then to undetectable levels after 15 kGy irradiation. PRV was completely inactivated from an initial titer of 7.57 $\log_{10}$ $TCID_{50}$ to undetectable levels after 5 kGy irradiation. PRoV was also rapidly inactivated from an initial titer of 7.77 $\log_{10}$ $TCID_{50}$ to 2.42 $\log_{10}$ $TCID_{50}$ after 5 kGy irradiation and then to undetectable levels after 15 kGy

**Table 2. Inactivation of viruses through heat treatment**

| Exposure time in minutes | Total virus titer ($Log_{10}TCID_{50}$) | | | |
|---|---|---|---|---|
| | TGEV | PRV | PRoV | PPV |
| Spiked starting material | 6.45 | 7.52 | 8.04 | 6.92 |
| 30 min after 1,300°C heat treatment | ND[a] (≤1.64)[b] | ND (≤1.71) | ND (≤1.75) | ND (≤1.69) |
| 1 h after 1,300°C heat treatment | ND (≤1.64) | ND (≤1.71) | ND (≤1.74) | ND (≤1.71) |
| 2 h after 1,300°C heat treatment | ND (≤1.64) | ND (≤1.71) | ND (≤1.76) | ND (≤1.71) |
| Reduction factor ($log_{10}$) | ≥4.81 | ≥4.49 | ≥6.28 | ≥5.21 |

[a] No infectious virus was detected.
[b] These values were calculated using a theoretical minimum detectable level of infectious virus with a 95% confidence level.

**Table 3. Inactivation of viruses through gamma irradiation**

| Exposure time in minutes | Total virus titer ($Log_{10}TCID_{50}$) | | | |
|---|---|---|---|---|
| | TGEV | PRV | PRoV | PPV |
| Spiked starting material | 6.35 | 7.57 | 7.77 | 6.59 |
| 5 kGy gamma irradiation | 2.07 | ND (≤1.70) | 2.42 | 2.16 |
| 15 kGy gamma irradiation | ND[a] (≤1.69)[b] | ND (≤1.71) | ND (≤1.74) | ND (≤1.69) |
| 25 kGy gamma irradiation | ND (≤1.70) | ND (≤1.70) | ND (≤1.72) | ND (≤1.71) |
| Reduction factor ($log_{10}$) | ≥4.65 | ≥5.87 | ≥6.05 | ≥4.88 |

[a] No infectious virus was detected.
[b] These values were calculated using a theoretical minimum detectable level of infectious virus with a 95% confidence level.

irradiation. PPV was rapidly inactivated from an initial titer of 6.59 $log_{10}$ $TCID_{50}$ to 2.167 $log_{10}$ $TCID_{50}$ after 5 kGy irradiation and then to undetectable levels after 15 kGy irradiation. The log reduction factors achieved were ≥4.65 for TGEV, ≥5.87 for PRV, ≥6.05 for PRoV, and ≥4.88 for PPV.

## Discussion

Surface reactivity is one of the common characteristics of bone bioactive materials. It contributes to their bone bonding ability and their enhancing effect on bone tissue formation. Especially, the surface structure, particle size, and size range are very important, as they directly affect the surface area available to react with cells and biological fluid (Ducheyne and Qiu, 1999). Bio-Oss®, bovine bone HA, is prepared from cow bones by heating them at relatively low temperature (300°C) to remove organic substances using alkaline chemicals and by sterilization with dry heat (Concannon *et al*., 1997). It is known as an ideal scaffold for osteogenesis because it has a porous structure similar to human osseous tissue (Pinholt *et al*., 1991; Haas *et al*., 1998). Therefore, the surface structure and pore size of porcine bone HA (THE Graft®) were compared with those of Bio-Oss®. Although the origin and manufacturing process of THE Graft® were different from those of Bio-Oss®, the two graft materials showed similar macro/micro-porous nature as well as similar pore size distribution (Figs. 2, 3, and 4). In the comparative animal efficacy studies of THE Graft® and Bio-Oss® using rat calvarial defects and rabbit calvarial defects models, there were no differences between the bones formed by the two graft materials (Yoo *et al*., 2010; Park *et al*., 2012). Therefore THE Graft® was confirmed as an effective bone graft material with biocompatibility and abilities in osteogenesis and space maintenance.

For evaluation of the viral inactivation efficacy of the manufacturing process for porcine bone HA, four common porcine relevant viruses, TGEV, PRV, PRoV, and PPV were chosen as the experimental model viruses. Based on the International Organization for Standardization guideline (2007), the viral inactivation efficacy of 1,300°C heat treatment and gamma irradiation were evaluated. Both processes were extremely effective at inactivating all the viruses tested (Tables 2 and 3). All the viruses were completely inactivated to undetectable levels within 30 min of heat treatment. Also, all the viruses were completely inactivated to undetectable levels after 15 kGy gamma irradiation.

The cumulative virus reduction factor for a manufacturing process is determined from the sum of the individual virus reduction factors based on an individual process involving different physicochemical methods (International Conference on Harmonisation, 1998). The cumulative virus reduction factors achieved for the different viruses using the process steps evaluated in this study are presented in Table 4. The cumulative log reduction factors, ≥9.46 for TGEV, ≥10.36 for PRV, ≥12.33 for PRoV, and ≥10.09 for PPV, are several magnitudes greater than the potential virus load of current porcine bone material. Accordingly, these results indicate that the process steps for manufacturing porcine-derived bone material are capable of inactivating a wide range of viruses that represent a broad spectrum of physicochemical attributes. Regulatory guidelines recommend incorporating multiple orthogonal methods for viral clearance; that is, methods that have independent (unrelated) clearance mechanisms. Therefore, since the mechanisms of virus inactivation in each of these steps are

**Table 4. Cumulative log reduction factors of viruses achieved during the manufacturing processes for porcine bone**

| Process step | Reduction factor ($Log_{10}$) | | | |
|---|---|---|---|---|
| | TGEV | PRV | PRoV | PPV |
| 1,300°C heat treatment | ≥4.81 | ≥4.49 | ≥6.28 | ≥5.21 |
| Gamma irradiation | ≥4.65 | ≥5.87 | ≥6.05 | ≥4.88 |
| Cumulative log reduction factor | ≥9.46 | ≥10.36 | ≥12.33 | ≥10.09 |

different from one another, it is concluded that the overall process of porcine-derived bone material production is robust in reducing the virus load. This is the first systematic evaluation of virus inactivation during the process of manufacturing HA from porcine bone.

## 적 요

동물유래성분을 원재료로 사용하는 의료기기는 원료물질 유래 바이러스가 오염될 가능성이 있기 때문에 생산과정 중 바이러스가 오염되지 않도록 하여야 한다. 또한 생산공정은 오염될 가능성이 있는 바이러스들을 불활화하거나 제거하는 공정을 포함하여야 한다. 본 연구를 통해 돼지유래 이종골을 원재료로 사용한 바이러스 안전성이 보증된 치과용 골이식재 (THE Graft®) 제조공정을 개발하였다. THE Graft® 제조공정은 30% 과산화수소수와 80% 에탄올을 각각 처리하여 지방을 제거하는 공정과 1,300°C 열처리 공정을 통해 콜라겐과 유기물을 제거하는 공정을 포함한다. 또한 최종적으로 생산된 hydroxyapatite 성분의 골이식재에 25 kGy의 감마선을 조사하여 감염성 위해인자를 불활화하는 공정을 포함한다. THE Graft®의 형태학적 특성을 소유래 hydroxyapatite 성분의 골이식재인 Bio-Oss와 SEM과 TEM을 이용하여 비교한 결과 구조적 특성이 유사함을 확인하였다. 1,300°C 열처리 공정과 25 kGy 감마선 조사 공정의 바이러스 불활화 효과를 평가하기 위해 transmissible gastroenteritis virus (TGEV), pseudorabies virus (PRV), porcine rotavirus (PRoV), porcine parvovirus (PPV)를 모델 바이러스로 선정하였다. 1,300°C 열처리 공정에서 TGEV, PRV, PRoV, PPV 모두 검출한계 이하로 불활화되었으며, 바이러스 로그 감소 값은 각각 ≥4.65, ≥5.81, ≥6.28, ≥5.21이었다. 또한 감마선 조사 공정에서도 TGEV, PRV, PRoV, PPV 모두 검출한계 이하로 불활화되었으며, 바이러스 로그 감소 값은 각각 ≥4.65, ≥5.87, ≥6.05, ≥4.89이었다. 두 공정에서 TGEV, PRV, PRoV, PPV의 누적 바이러스 로그 감소 값은 각각 ≥9.30, ≥11.68, ≥12.33, ≥10.10이었다. 이상의 결과에 의하면, THE Graft® 제조공정은 바이러스 안전성 보증을 위한 충분한 바이러스 불활화 능력을 가지고 있는 것으로 판단된다.

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