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# Novel process development of pandemic avian influenza viral safe bone tissue engineering biomaterial using chicken by product

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**ABSTRACT**
A novel process for manufacturing viralsafe chicken bone hydroxyapatite (CBH) has been developed to serve as advancedxenograft material for bone applications. Chicken bone pieces were defatted with successive treatments of 30% hydrogen peroxide and 70% ethyl alcohol. The defatted chicken bone pieces were heat-treated in an oxygen atmosphere box furnace at 300°C to removecollagen and organic compounds. The bone pieces were ground with a grinder and then the bone powder was sterilized by gammairradiation. Morphological characteristics such as SEM (Scanning Electron Microscopy) and TEM (Transmission Electron Microscopy)images of the resulting CBH were similar to those of a commercial bovine bone hydroxyapatite (Bio-Oss®). In order toevaluate the efficacy of 300°C heat treatment and gamma irradiation at a dose of 25 kGy for the inactivation of chicken viruses during themanufacture of CBH, a variety of experimental avian influenza viruses including H1N1 (Spanish flu), H2N2 (Asian flu), H3N2 (Hong Kong flu), and H5N1 (Bird flu) viruswere chosen. H1N1, H2N2, H3N2, and H5N1 virus werecompletely inactivated to undetectable levels during the 300ºC heat treatment. The mean log reduction factors achieved were ≧4.27for H1N1, ≧5.63 for H2N2, ≧6.32 for H3N2, and ≧5.18 for H5N1. Gamma irradiation was also very effective to inactivate the viruses. H1N1, H2N2, H3N2, and H5N1 virus were completely inactivated to undetectable levels during the gamma irradiation. The mean log reduction factorsachieved were ≧4.57 for H1N1, ≧5.79 for H2N2, ≧6.12 for H3N2, and ≧4.83 for H5N1. The cumulative log reduction factors achieved usingthe two different virus inactivation processes were ≧9.27 for H1N1, ≧11.52 for H2N2, ≧12.27 for H3N2, and ≧10.15 for H5N1. These resultsindicate that the manufacturing process for CBH from chicken by product bone material has sufficient virusreducing capacity toachieve a high margin of virus safety.

**Keywords** :bone tissue engineering biomaterial, chicken by product bone material, hydroxyapatite, avian influenza viruses, virus safety

## I. INTRODUCTION

Bone grafting is a surgical procedure that replaces missingbone. Autograft, allograft, xenograft, and synthetic bone graftsubstitute materials play an important role in reconstructiveorthopaedic and periodontic surgery (Damien and Parsons,1991; Bauer and Muschler, 2000; Venkataraman et al., 2015).Autogenous bone, with its osteogenic, osteoinductive, andosteoconductive properties, has long been considered as the idealgrafting material for bone reconstructive surgery. However,drawbacks with autogenous bone include morbidity, availabilityand unpredictable graft resorption (Hallman and Thor, 2008).Allografts are graft materials harvested from different humanindividuals and require processing in order to lessen antigenicityand disease transfer. They are osteoconductive and osteoinductive(Khan et al., 2005). Xenografts are obtained from the bones ofindividuals of other species with composition and biomechanicalproperties that are almost similar to human bone. Two illustrationsof xenografts used in dentistry are i) coral-derived bonesubstitutes with geometry similar to that of human cancellousbone interconnected macropores (200–600 μm) and ii) demineralizedanimal bone grafts, which are biocompatible andosteoconductive (Jensen et al., 2009). Synthetic bone graftsubstitute materials are osteoconductive alloplastic materialssuch as calcium phosphate and bioactive glass (Välimäki andAro, 2006).

In particular, the use of xenografts has increased in recent years becauseadequate amounts can be easily obtained. However, they alsohave a limitation as the risk of transmission of zoonoticdiseases is possible. The best known demineralized xenograftis bovine bone hydroxyapatite(Bio-Oss®), which has a porous structuresimilar to human osseous tissue as well as a long resorptiontime, serving as an ideal scaffold for osteogenesis (Pinholt etal., 1991; Haas et al., 1998).

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It is consisted of hydroxyapatite(HA) prepared byalkaline treatment and thermal treatment at 300°C to removeorganic components of medullar bovine bone. Another bovinexenograft is Gen-Ox®obtained through deproteinization athigh temperatures (between 950 and 1,000°C) (Accorsi-Mendonçaet al., 2008). However, a unique safety issue with using bovinematerials, namely the risk of bovine spongiform encephalopathy(BSE) transmission, is rarely addressed in literature andappears to be ignored by practitioners. BSE is a type of transmissiblespongiform encephalopathy (TSE) or prion disease,which is a group of fatal neurodegenerative diseases affectinghumans and a broad spectrum of animal species (Kim et al.,2016). Therefore, chicken bone is considered to an alternative tobovine bone (Korean Rural Development Administration Report, 2019).

KJMbioLTD. in Korea is currently producing a chicken bone hydroxyapatite (CBH) using chicken by products. CBHis a resorbable bone substitute material that consists ofHA. It is prepared by removing organic compounds fromchicken bone pieces using 30% hydrogen peroxide and 70%ethyl alcohol solutions for 12 h each. After rinsing withdistilled water, bone pieces are dried at 100°C for 24 h andheat-treated in an oxygen atmosphere box furnace at 300°Cto remove collagen and organic compounds. The bone piecesare ground with a grinder. Finally, gamma irradiation isperformed (Kim et al., 2014a, 2014b, Korean Rural Development Administration Report, 2019).

The medical grafting products, developed from chickentissues, also have a risk of viral contamination (Hodde andHiles, 2002). Therefore, the ability to remove and/or to inactivateviral contaminants during the manufacturing of animal-derivedbone material has become an important parameter for assessingthe safety of the products (Forest et al., 2007; InternationalOrganization for Standardization, 2007). The manufacturing process for CBHcontains anintentional viral inactivation procedure through gamma irradiation.Also it involves a 300°C heat treatment procedurewhich can potentially inactivate viral contaminants.

In this study, we have developed a manufacturing processfor a pandemic avian influenza viral safe bone tissue engineering biomaterial usingchicken bone andevaluated the efficacy of viral inactivation procedures. For thisstudy, the morphological characteristics of the CBH were compared with those of a commercialBio-Oss®. Also, four pandemic avian influenza viruses, H1N1 (Spanish flu), H2N2 (Asian flu), H3N2 (Hong Kong flu), and H5N1 (Bird flu)virus, were chosen as the model viruses for theevaluation of virus safety for chickenderived medical products(Table 1). The viruses used in this study were selected torepresent viruses with a range of biophysical and structuralfeatures, which might also present themselves as unknown orunidentified contaminants in the starting material, and displaya significant resistance to physical or chemical agents (International Organization for Standardization, 2007).

## II. MATERIALS AND METHODS

Manufacturing process for chicken bone HA

Chicken bone HA(CBH) was prepared from chicken by product bone slaughtered forhuman consumption in commercial abattoirs (Harim Co. Ltd, Korea). Chickenbone was cut into slices 1 cm thick. Defatting and deproteinizationwere achieved by chemical and heat treatment. The chicken bone was immersed in distilled water and degreasedwith 30% hydrogen peroxide solution (Sigma Aldrich, St. Louis, MO, USA) for 12 hand 70% ethyl alcohol solution (Sigma Aldrich) for 12 h. Afterrinsing with distilled water, bone pieces were dried at 100°C for24 h and heat-treated in an oxygen atmosphere box furnace at 300°C to remove collagen and organic compounds. The bonepieces were ground with a grinder. Finally, glass vials that werepackaged with bone powder were sterilized with gamma irradiationat a dose of 25 kGy according to the ISO 11137-2:2006(International Organization for Standardization, 2006). Gammairradiation was performed using the continuous type gammairradiator from Greenpia Tech Inc. using the radio nuclidecobalt 60 (60Co) (Fig. 1).

Morphological characterization of chicken bone HA

The morphological characteristics of the CBHprepared from this study were compared with those of agrafting material of bovine origin, Bio-Oss®. Morphologicalcharacterization of the materials was carried out through scanningelectron microscopy (SEM, LEO SUPRA55, Carl Zeiss) at 20kV 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 atvarious magnifications. Pore size was defined as the longestdistance across a single pore.

Preparation and titration of viruses

For the propagation and titration of H1N1 (Spanish flu), H2N2 (Asian flu), H3N2 (Hong Kong flu),and H5N1 virus (bird flu), ST cells (ATCC CRL-1746), Vero cells(ATCC CCL-81), MA-104 cells (ATCC CRL-2378), and MPKcells (ATCC CCL-166) were used, respectively. All the virusesand cells were

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obtained from American Type Culture Collection.Prior to viral propagation, all the host cells were grown in highglucose 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 DMEMcontaining 2% fetal bovine serum for the propagation andtitration of H1N1, H2N2, and H5N1,respectively. Meanwhile,for the propagation and titration of H3N2, MA-104 cells weregrown in DMEM plus 0.1% trypsin (Gibco).

An aliquot from each sample used in the virus inactivationstudies and an appropriate control were titrated immediatelyafter being collected in 7-fold serial dilutions to the end pointusing a quantal 50% tissue culture infectious dose (TCID50)assay (Kärber, 1931). For titration of the viruses, indicator cellmonolayers in 24-well culture plates were infected using atleast eight replicates of 0.25 ml of the appropriate dilution ofeach sample or the positive control. Negative control wellswere mock-infected using at least eight replicates of 0.25 ml ofthe culture medium. The plates were then incubated at 35°C forapproximately 1 h, and the wells were fed with 1 ml of the tissueculture medium. After 7–14 days incubation, the wells wereexamined for cytopathic effect (CPE).

As a part of the virus validation protocol, cytotoxicity,interference and load titer tests were performed. The cytotoxicitytests were performed on those samples generated for virustitration in virus spiking experiments to control for any possiblecytotoxic effects on the indicator cells that might interfere withthe virus titration. The interference tests were performed todetermine whether the starting materials for virus spikingstudies exerted an inhibitory effect on the ability of the celllines to permit the detection of the virus. The load titer assayswere performed to determine precisely the point at whichspiking level leads to a loss in the virus titer.

Virus inactivation studies

Virus inactivation studies were conducted using the validatedscaledown processes. To evaluate the effectiveness androbustness of the 300°C heat treatment in inactivating viruses,1.5 ml of virus stock solution was spiked with 1 g of chicken by product bone material before heat treatment. The virusspiked sampleswere incubated at room temperature for 10 minutes to allow theadsorption of the virus solution. The virusspiked samples weretreated at 300°C for different durations (0, 0.5, 1, and 2 h). Asa hold control, one of the virusspiked samples was kept at 4°Cduring heat treatment in order to determine precisely theamount of virus titer lost during the virus inactivation study.Samples were collected at different times. Each sample wasmixed with 5 ml of virus culture media, vigorously shaken witha vortex mixer in order to withdraw viruses from the virusspikedchicken by product bone material, and then centrifuged. The supernatantwas collected. The above procedure was repeated threetimes. The collected supernatant was combined and filtered (0.45μm). A portion of the obtained filtrate was tested immediately.To evaluate the effectiveness and robustness of the gammairradiation in inactivating viruses, 1 ml of virus stock solutionwas spiked with 1 g of chicken by product bone material obtained beforegamma irradiation. The virusspiked samples were incubatedat room temperature for 10 min to allow the adsorption of thevirus solution. The virusspiked samples were treated withgamma irradiation at the intensities of 5, 15, and 25 kGy. As ahold control, one of the virusspiked samples was kept at 4°Cduring gamma irradiation in order to determine precisely theamount of virus titer lost during the virus inactivation study. Tothe gamma irradiated samples, 5 ml of virus culture media wasadded, vigorously shaken with a vortex mixer in order towithdraw viruses from the virusspiked chicken by product bone material, and then centrifuged. The supernatant was collected.The above procedure was performed three times. The collectedsupernatant was combined and filtered (0.45 μm). A portion ofthe obtained filtrate was tested immediately. All virusinactivation experiments were carried out in duplicate andmean values are given.

Calculation of virus reduction factors

The virus log reduction factor was defined as the log10 of theratio of the virus loads in the spiked starting and post processmaterials, as previously described (International Conferenceon Harmonisation, 1998). The formula takesinto account the titers and volumes of the materials before andafter the processing step.

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

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

## III. RESULTS

Morphological characterization of chicken bone HA

The morphological characteristics of chicken bone HA(CBH)prepared from this study were compared withthose of a commercial bovine bone HA (Bio-Oss®). SEM wasused for the surface structure observation. Figure 2 shows themacro-porous nature of the two graft materials and

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similarstructures. Also, the TEM images were very similar forBio-Oss® and CBH(Fig. 3). The TEM image clearlyshowed the micro-porous nature of the two graft materials andsimilar structures. The major pore size distributions of CBHand Bio-Oss®are similar. They are evenly distributedfrom 0.03 mm to 1.0 mm (Fig. 4).

Virus inactivation by heat treatment

Heat treatment at 300°C was extremely effective at inactivatingall the viruses tested (Table 2). All the viruses werecompletely inactivated to undetectable levels within 30 min ofheat treatment. The log reduction factors achieved were ≧4.27 for H1N1, ≧5.63 for H2N2, ≧6.32 for H3N2, and ≧5.18 for H5N1.

Virus inactivation by gamma irradiation

Gamma irradiation was also very efficient at inactivating allthe viruses tested (Table 3). H1N1 was rapidly inactivatedfrom an initial titer of 6.35 log10 TCID50 to 2.07 log10 TCID50after 5 kGy irradiation and then to undetectable levels after 15kGy irradiation. H2N2 was completely inactivated from aninitial titer of 7.57 log10 TCID50 to undetectable levels after 5kGy irradiation. H3N2 was also rapidly inactivated from aninitial titer of 7.77 log10 TCID50 to 2.42 log10 TCID50 after 5 kGyirradiation and then to undetectable levels after 15 kGyirradiation. H5N1 was rapidly inactivated from an initial titer of6.59 log10 TCID50 to 2.167 log10 TCID50 after 5 kGy irradiationand then to undetectable levels after 15 kGy irradiation. The logreduction factors achieved were ≧4.57 for H1N1, ≧5.79 for H2N2, ≧6.12 for H3N2, and ≧4.83 for H5N1.

## IV. DISCUSSION

Surface reactivity is one of the common characteristics ofbone bioactive materials. It contributes to their bone bondingability and their enhancing effect on bone tissue formation.Especially, the surface structure, particle size, and size rangeare very important, as they directly affect the surface areaavailable to react with cells and biological fluid (Ducheyne andQiu, 1999). Bio-Oss®, bovine bone hydroxyapatite(HA), is prepared from cowbones by heating them at relatively low temperature (300°C) toremove organic substances using alkaline chemicals and bysterilization with dry heat (Concannon et al., 1997). It is knownas an ideal scaffold for osteogenesis because it has a porousstructure similar to human osseous tissue (Pinholt et al., 1991;Haas et al., 1998). Therefore, the surface structure and pore sizeof chicken bone HA (CBH) were compared with those ofBio-Oss®. Although the origin and manufacturing process of CBH were different from those of Bio-Oss®, the twograft materials showed similar macro/micro-porous nature aswell as similar pore size distribution (Figs. 2, 3, and 4). In thecomparative animal efficacy studies of CBH and Bio-Oss®using rat calvarial defects and rabbit calvarial defects models,there were no differences between the bones formed by the twograft materials(Yoo et al., 2010; Park et al., 2012; Kim, 2016; Korean Rural Development Administration Report, 2019). Therefore CBH was confirmed as an effective bone graft materialwith biocompatibility and abilities in osteogenesis and spacemaintenance.

For evaluation of the viral inactivation efficacy of themanufacturing process for CBH, four pandemic avian influenza viruses, H1N1 (Spanish flu), H2N2 (Asian flu), H3N2 (Hong Kong flu), and H5N1 (Bird flu)virus were chosen as the experimental model viruses (Kim, 2004; Monto, 2005; Yuen et al., 2005; Hsieh et al., 2006, Damienet al., 2020). Based on the InternationalOrganization for Standardization guideline (2007),the viral inactivation efficacy of 500°C heat treatment andgamma irradiation were evaluated. Both processes were extremelyeffective at inactivating all the viruses tested (Tables 2and 3). All the viruses were completely inactivated to undetectablelevels within 30 min of heat treatment. Also, all theviruses were completely inactivated to undetectable levels after15 kGy gamma irradiation.The cumulative virus reduction factor for a manufacturingprocess is determined from the sum of the individual virusreduction factors based on an individual process involvingdifferent physicochemical methods (International Conferenceon Harmonisation, 1998). The cumulative virus reduction factorsachieved for the different viruses using the process stepsevaluated in this study are presented in Table 4. The cumulativelog reduction factors, ≧9.27 for H1N1, ≧11.52 for H2N2, ≧12.27 for H3N2, and ≧10.15 for H5N1, are several magnitudesgreater than the potential virus load of current chicken by product bone material. Accordingly, these results indicate that the processsteps for manufacturing chicken by product bone material arecapable of inactivating a wide range of viruses that represent abroad spectrum of physicochemical attributes. Regulatoryguidelines recommend incorporating multiple orthogonalmethods for viral clearance; that is, methods that have independent(unrelated) clearance mechanisms. Therefore, sincethe mechanisms of virus inactivation in each of these steps aredifferent from one another, it is concluded that the overallprocess of chicken by product bone material production is robustin reducing the virus load. This is the first systematic evaluationof virus inactivation

during the process of manufacturing HAfrom chicken by product bone.

## V. ACKNOWLEDGEMENT

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**Figure Legends**

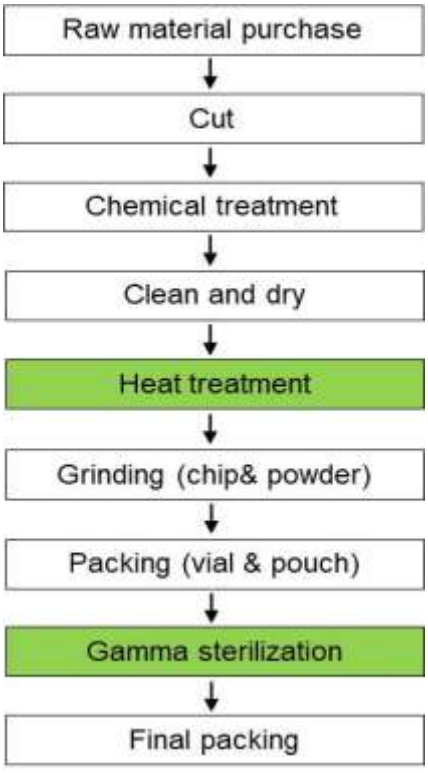


**Figure 1.**Manufacturing process for chicken bone hydroxyapatite(CBH). The green boxes indicate the validation stepsemployed for virus inactivation.

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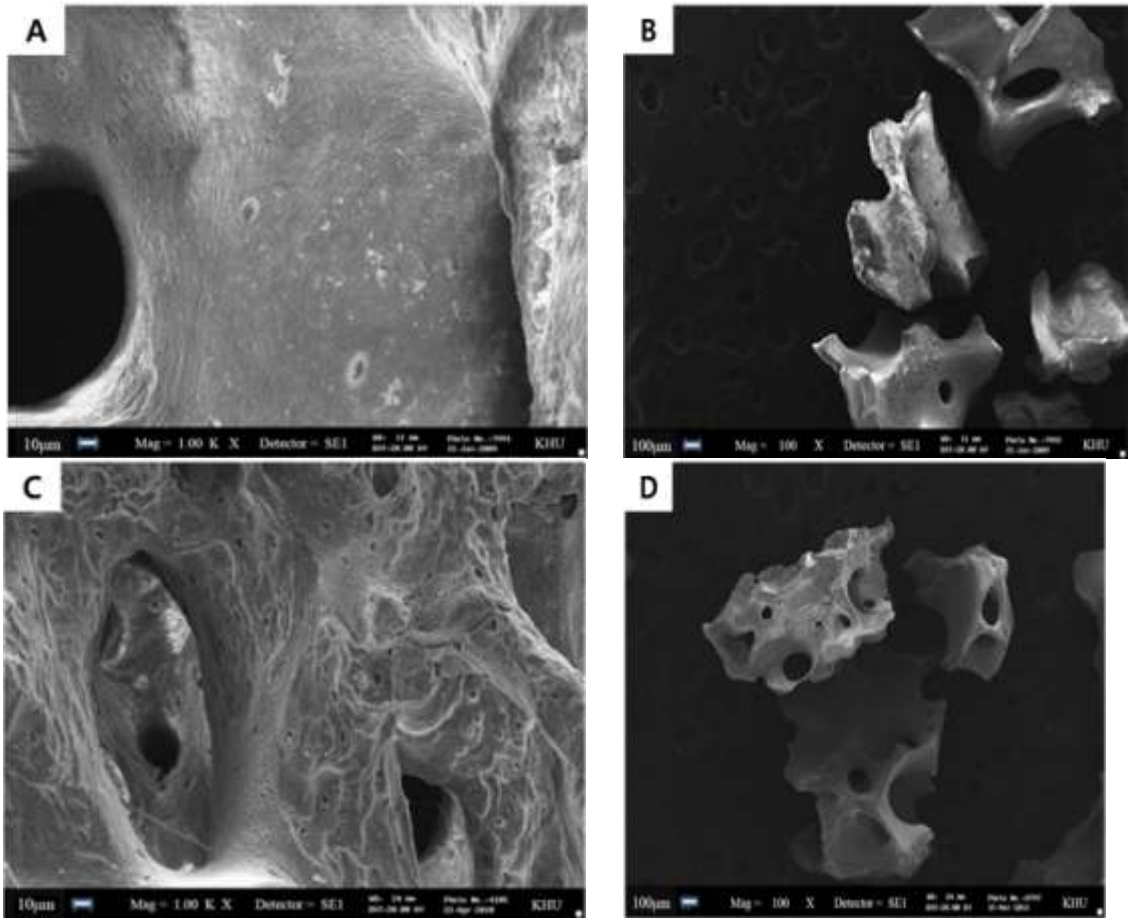
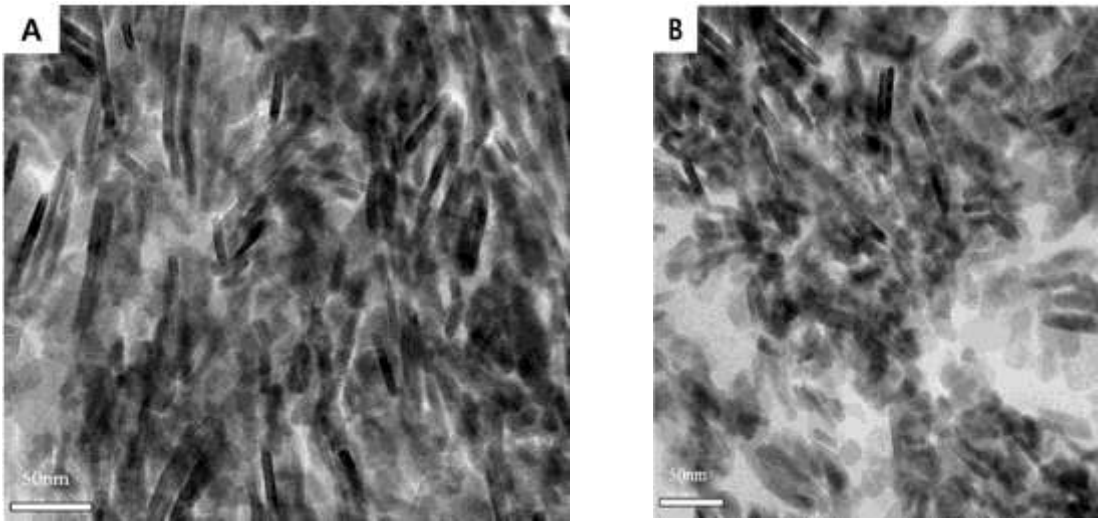



**Figure 2.**Comparative SEM images of Bio-Oss®(A, B) and CBH (C,D). Characterization of surface structures of both materials was carriedout through SEM (LEO SUPRA55, Carl Zeiss) at 20 kV of electronacceleration.



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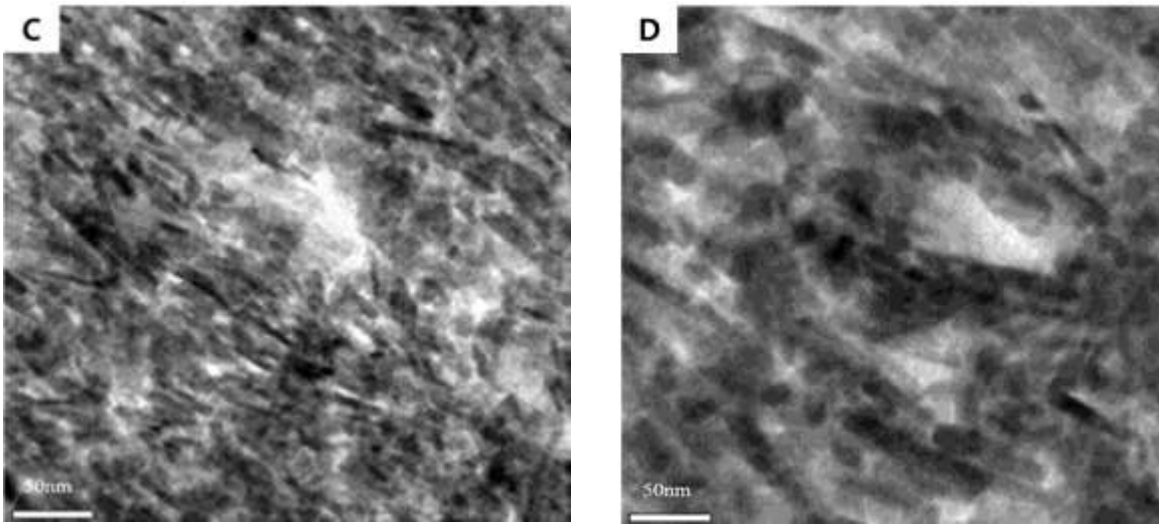


**Figure 3.**Comparative TEM images of Bio-Oss®(A, B) and CBH (C,D). Micro-porous structures of both materials were observed using TEM(JEM-3010, JEOL) at 300 kV of electron acceleration.

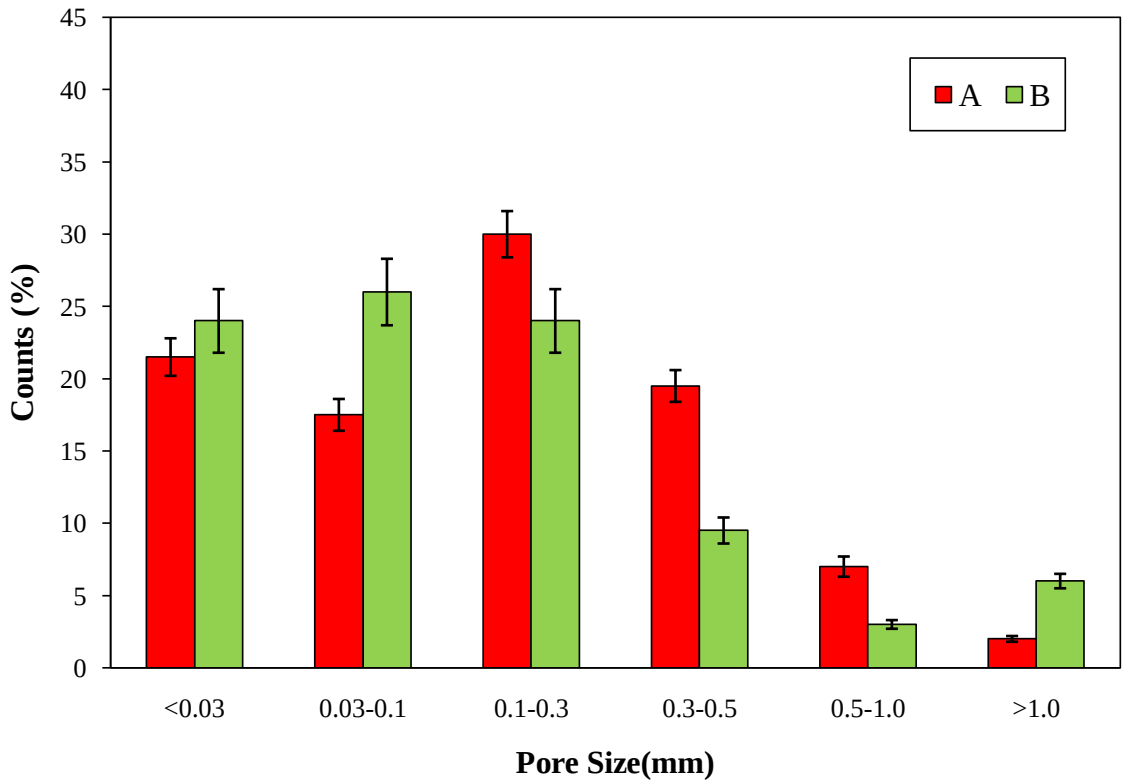


**Figure 4.**Comparative pore sizes of Bio-Oss®(A) and CBH (B). Poresize analysis was performed on SEM images taken at various magnifications.

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**Table Legends**

**Table 1.**Features of avian influenza viruses used for the evaluation of virus clearance.

| Virus | Family | Host | shape | Genome |
|---|---|---|---|---|
| H1N1 | Orthomyxoviridae | Avian | Circular | ds-DNA |
| H2N2 | Orthomyxoviridae | Avian | Circular | ds-DNA |
| H3N2 | Orthomyxoviridae | Avian | Circular | ds-DNA |
| H5N1 | Orthomyxoviridae | Avian | Icosahedral | ss-RNA |

**Table 2.**Inactivation of avian influenza viruses through 300°C heat trestment.

| Exposure time in times | Total virus titer (Log10TCID50) | | | |
|---|---|---|---|---|
| | H1N1 | H2N2 | H3N2 | H5N1 |
| Spiked starting material | 6.15 | 7.32 | 8.13 | 6.85 |
| 30 min | ND[a](≤1.89)[b] | ND (≤1.69) | ND (≤1.78) | ND (≤1.65) |
| 1 hr | ND (≤1.89) | ND (≤1.69) | ND (≤1.79) | ND (≤1.67) |
| 2 hr | ND (≤1.89) | ND (≤1.69) | ND (≤1.81) | ND (≤1.67) |
| Reduction factor (log10) | ≥4.26 | ≥5.63 | ≥6.32 | ≥5.18 |

[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 avian influenza viruses through gamma irradiation.

| Exposure time in times | Total virus titer (Log10TCID50) | | | |
|---|---|---|---|---|
| | H1N1 | H2N2 | H3N2 | H5N1 |
| Spiked starting material | 6.27 | 7.49 | 7.81 | 6.56 |
| 5 kGy | 1.98 | ND (≤1.71) | 2.37 | 2.18 |
| 15 kGy | ND[a](≤1.68)[b] | ND (≤1.72) | ND (≤1.71) | ND (≤1.71) |
| 25 kGy | ND (≤1.70) | ND (≤1.70) | ND (≤1.69) | ND (≤1.73) |
| Reduction factor (log10) | ≥4.57 | ≥5.79 | ≥6.12 | ≥4.83 |

[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 4.**Cumulative log reduction factors of avian influenza viruses achieved during the manufacturing processes.

| Process step | Reduction factor(Log10) | | | |
|---|---|---|---|---|
| | H1N1 | H2N2 | H3N2 | H5N1 |
| 300°C heat treatment | ≥4.72 | ≥4.37 | ≥6.15 | ≥5.13 |
| Gamma irradiation | ≥4.55 | ≥7.15 | ≥6.12 | ≥5.02 |
| Reduction factor (log10) | ≥9.27 | ≥11.52 | ≥12.27 | ≥10.15 |