



# Effects of gamma irradiation on the measurement of hepatitis B virus DNA in dentin harvested from chronically infected patients

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**Background:** The manufacturing of the demineralized dentin matrix (DDM) has been proven to extensively reduce the presence of human hepatitis B viral DNA (HBV DNA). This study measured and compared HBV DNA in fresh dentin to that in gamma radiation (GR)-sterilized dentin extracted from HBV-infected patients. The application of GR as a means of terminal sterilization is hypothesized to inactivate or eliminate HBV within the dentin matrix.

**Methods:** Dentin from 18 HBV-infected patients was collected and divided into three fragments. The first fragment was unaltered and used as the control group; the remaining two fragments were sterilized with gamma radiation doses of 15 or 25 kGy. DNA was extracted and purified from each fresh (control), and the GR-sterilized (experimental) dentin specimen and HBV DNA copy numbers were evaluated on the basis of the real-time polymerase chain reaction. The copy numbers were used to assess GR efficacy as a means of terminal sterilization for HBV inactivation or elimination.

**Results:** HBV DNA was detected in 66.67% of the fresh dentin specimens. The differences in HBV DNA levels between the fresh dentin and the GR-sterilized dentin were confirmed by the Wilcoxon signed-rank test for the doses of 15 and 25 kGy with P value of 0.012 and 0.010, respectively. Among the twelve HBV-DNA-positive fresh dentin samples, HBV DNA persisted in eleven after GR sterilization, yet the copy number was reduced to <10 (except for a single sample within each experimental group).

**Conclusions:** The results suggest that 15 and 25 kGy of GR significantly reduced the HBV DNA levels in the fresh dentin matrix. Expansion of the possible clinical applications of allogenic grafts with the irradiated DDM will require additional studies, including validation of viral load inactivation to prevent infectious transmission and examination of GR exposure effects on the osteoinductivity of the matrix.

**Keywords:** Gamma irradiation sterilization; hepatitis B viruses (HBV); hepatitis B viruses DNA (HBV DNA)

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## Introduction

The demineralized dentin matrix (DDM) has become a commonly used bone-graft substitute that is fabricated from the discarded extracted teeth. DDM consists of acid-insoluble type I collagenous scaffolds that contain non-collagenous proteins such as bone morphogenetic protein. Even though dentin is remarkably similar to bone with regard to its chemical composition and biological behavior, dentin is an acellular matrix without blood vessels, while bone includes osteocytes and blood vessels. Since DDM is an acellular and avascular matrix that displays antigenicity, human DDM can be biorecycled as an alternative to an autogenous bone graft, familial graft, or allograft (1). In several *in vivo* and clinical studies involving biological family members, DDM facilitated successful outcomes as an allogenic bone substitute without any immunological complications (2,3). However, similar to allogenic bone grafts, certain viral diseases (hepatitis B and C, and human immunodeficiency viruses) may be transmitted through infected human-based implantation materials, necessitating valid viral clearance (4).

Various allograft processing procedures aimed to ensure virus inactivation have been introduced and evaluated, including demineralization, use of detergents, freeze-drying, chemical sterilization, and gamma irradiation. These methods provide an exponential reduction in the potential graft contamination and disease transfer (5,6). The process of bone demineralization has been shown to drastically reduce the infectivity of all RNA and DNA viruses with an overall sterility assurance level greater than one million ( $10^{-6}$ ) for all viruses and as much as one trillion ( $10^{-12}$ ) for poliovirus (7). Similarly, processed DDM obtained from a Hepatitis B virus-infected (HBV-infected) donor showed complete viral inactivation after demineralization, whereas unprocessed DDM from the same HBV-infected donor showed active HBV DNA copy numbers (4). A low-dose gamma irradiation process is known to inactivate both enveloped and non-enveloped viruses containing either DNA or RNA, thus providing an additional assurance against the occurrence of viral transmission during bone graft procedures (8). According to the guidelines of the European Association of Tissue Banks (EATB) and the International Atomic Energy Agency (IAEA), the permissible dose for gamma irradiation is 25 kGy, whereas the American Association of Tissue Banks (AATB) suggests a dose of 15 kGy (8,9).

HBV has been detected in virtually all bodily secretions and excretions. However, only blood, visibly bloody body fluids, semen, and vaginal secretions pose a risk of transmission (10). HBV is transmitted by percutaneous and mucosal exposure to either infective blood or body fluids (10). HBV is stable on environmental surfaces for at least seven days and is 100 times more infectious than the human immunodeficiency virus (HIV). Several factors such as the viral load of the source can influence the risk of infective HBV transmission (10). In addition to its clinical feasibility, the use of DDM as an allogenic bone-graft substitute is hindered by the possibility that the allogenic dentin from infected patients can facilitate the transmission of viral diseases. Moreover, if a potential risk of viral transmission exists, it is necessary to determine whether terminal sterilization via gamma irradiation (GR) provides a sufficient reduction of the risk of transmission.

This study aimed to estimate the capability of GR using the gamma irradiation doses of 15 and 25 kGy to eliminate or inactivate HBV in dentin obtained from chronically HBV-infected patients by measuring the levels of HBV DNA contained within the pre- and post- irradiated dentin. It was hypothesized that GR sterilization of the fresh dentin matrix could completely inactivate or eliminate any HBV present within the matrix, preventing infectious transmission of HBV during transplantation.

## Methods

This study was approved by the Seoul National University Bundang Hospital Institutional Review Board (SNUBH IRB No. B-1705/395-308), and all of the participants provided informed consent. All participants were chronically infected with HBV and were negative for HIV and hepatitis C virus antibodies, and vertically infected infants and child patients were excluded. 18 teeth were extracted from the participants, and all of the extractions were clinically justified. The HBV infection of each patient was verified by serological examinations exhibiting titers over 1,000 international units (IU)/mL (*Table 1*). After extraction, the teeth were placed in sterile bottles and stored in a refrigerator. Then, the teeth were sealed without contamination and transferred on dry ice to a manufacturer (Korea Tooth Bank, Seoul, Korea). To prevent the degradation of the HBV DNA, each tooth was packaged in a new sterile bottle and stored at  $-20^{\circ}\text{C}$  while awaiting HBV measurements.

**Table 1** Demographic information and serological status of the patients from whom the teeth used in this study were collected

Patient No.	Sex/age (years)	Tooth number	HBV examination date	Tooth extraction date	HBV examination
					Serology [HBsAg (IU/mL)]/anti-HBs
1	M/63	27	12/22/2017	05/31/2017	Positive/<10.0
2	M/63	22	12/22/2017	05/31/2017	Positive/<10.0
3	F/77	14	10/06/2016	06/01/2017	Positive/<10.0
4	F/58	29	02/23/2018	06/05/2017	Positive/<10.0
5	F/58	28	02/23/2018	06/05/2017	Positive/<10.0
6	M/45	32	09/05/2017	11/09/2017	Positive/<10.0
7	M/45	31	09/05/2017	01/09/2017	Positive/<10.0
8	M/57	13	11/06/2017	12/18/2017	Positive/<10.0
9	M/71	19	03/09/2018	12/21/2017	Positive/<10.0
10	M/71	5	03/09/2018	12/21/2017	Positive/<10.0
11	M/55	1	08/13/2018	03/21/2018	Positive/<10.0
12	M/34	17	10/16/2017	10/18/2017	Positive (2,661.34)/<10.0
13	F/39	17	10/10/2017	11/02/2017	Positive (4,857.49)/<10.0
14	F/62	13	05/04/2018	01/29/2018	Positive/<10.0
15	F/62	15	05/04/2018	01/29/2018	Positive/<10.0
16	M/54	14	04/15/2015	03/26/2018	Positive (4,497.63)/<10.0
17	F/72	25	03/08/2010	04/02/2018	Positive (6,540.00)/<10.0
18	F/72	32	03/08/2010	04/02/2018	Positive (6,540.00)/<10.0

HBV, hepatitis B virus; F, female; M, male; HBsAg, hepatitis B surface antigen; Anti-HBs, antiviral antibody titer; IU, international unit.

### Experimental design

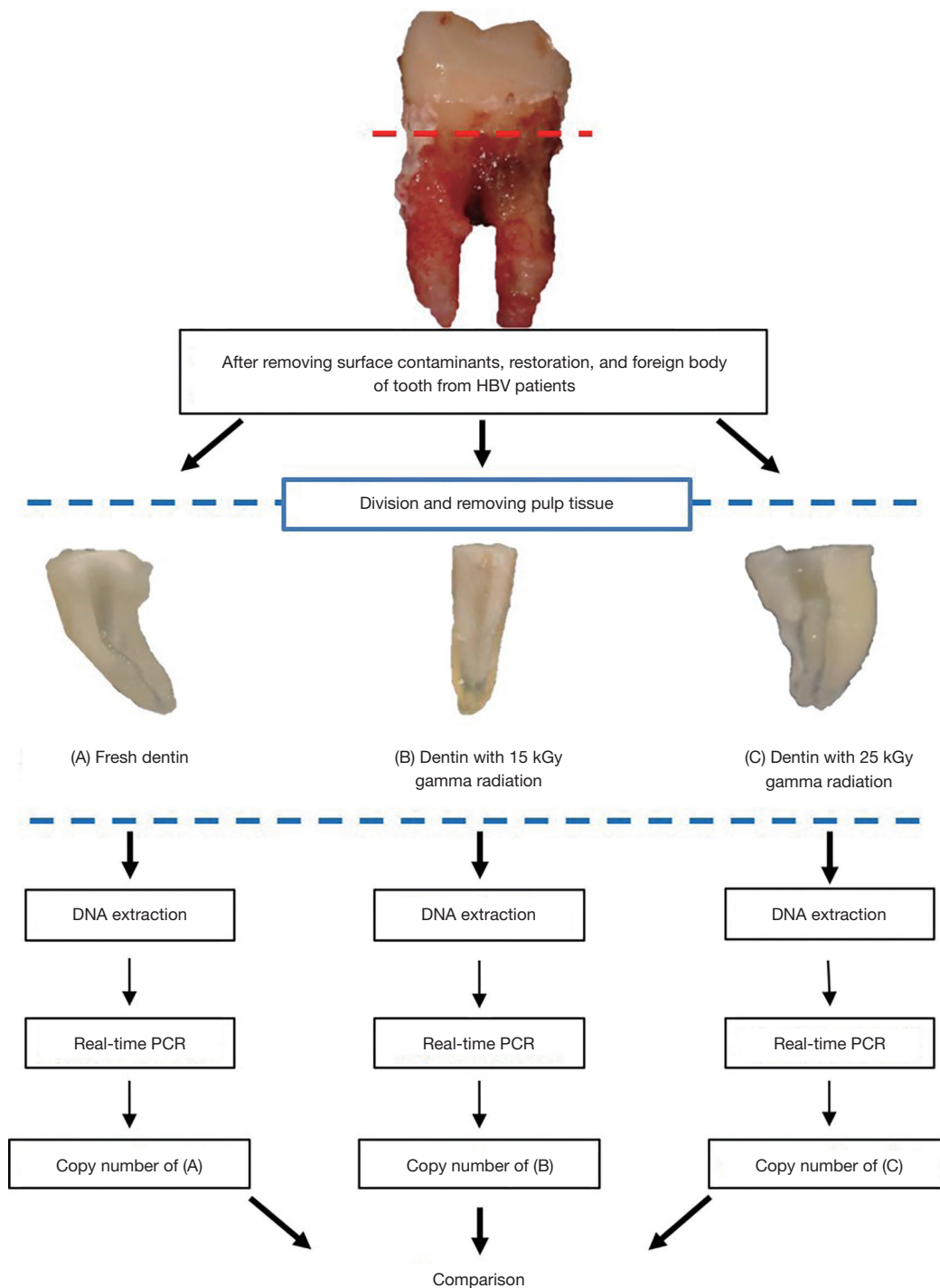
The dentin harvested from each HBV-infected patient was divided into three samples. The first sample was examined in HBV DNA measurements; these samples were not processed and were used as the control group. The second sample underwent gamma irradiation with a dose of 15 kGy (experimental group), and the third sample underwent gamma irradiation with a dose of 25 kGy (experimental group). After each experimental group underwent GA sterilization, the samples were analyzed for HBV DNA. The permissible dose of 15 and 25 kGy GR were determined by the guidelines of AATB, EATB, and IAEA (8,9).

### Preparation of dentin for measurement of HBV DNA.

Each tooth was debrided of all cavities, calculus, soft tissues, previous restoration material, and root canal materials with a dental high-speed burr (FG 6856 018 Diamond Bur;

Gebr. Brasseler GmbH & Co, Lemgo, Germany) and was irrigated with saline. Above the cemento-enamel-junctions, the enamel was removed using an FG700 010 Cutting Burr (Gebr. Brasseler GmbH & Co, Lemgo, Germany). All of the attached hard tissues, dental pulp, and soft tissues were debrided with a dental handpiece. The root cementum portion was eliminated by scraping with an FG 6856 018 Diamond Burr and #15 surgical blade. The remaining root matrix was split into three pieces using a FG700 010 Cutting Burr (Gebr. Brasseler GmbH & Co, Germany).

To identify the level of HBV DNA, the control group fragments were cleaned with sterile water and dried in a chamber at 56 °C for 2 h (Figure 1A). The experimental group fragments were gamma-irradiated with the doses of 15 or 25 kGy (Figure 1B,C) at Greenpia Technology (Greenpia Technology INC. R&D Center, Yeosu, Korea) using a Gamma-ray (Co-60) survey facility instrument (JS-8900 MDS Nord International Co. Ltd, Ottawa, ON, Canada).



**Figure 1** Experimental design flow diagram. Preparation of fresh dentin for the measurement of hepatitis B virus (HBV) DNA. The teeth from the chronically-infected HBV patients were severed at the cemento-enamel junction. (A) A segment of the root (control group) cleaned from soft and hard tissues was used as fresh dentin to measure the HBV DNA level. (B) Second segment of dentin (experimental group) was used to measure the HBV DNA levels after gamma irradiation with 15 kGy. (C) Third segment of dentin (experimental group) was used to measure the HBV DNA levels after gamma irradiation with 25 kGy. The HBV DNA level of fresh dentin (control group) measured as copy number by quantitative polymerase chain reaction was compared to those of the gamma-irradiated dentin samples (experimental groups).

The total absorbed dose of GR was obtained at room temperature ( $14\pm 1$  °C) at a dose rate of 1 kGy per unit time. A dosimetry system (Harwell RED 4034 Dosimeters, Harwell Dosimeters LTD, Oxfordshire, UK) was used following the IAEA standards to verify the standardization of the absorbed doses, with the total absorbed dose error obtained as less than 8%.

### *DNA isolation and purification*

In preparation for the quantitative polymerase chain reaction, each sample of the fresh and irradiated dentin (0.5 g) underwent digestion and decalcification using a TBONE EX Kit (DNA Chip Research Inc., Tokyo, Japan) via phenol-chloroform extraction. Following the manufacturer instructions, total DNA was evaluated as follows. The samples were placed into Solution A (30 mL) in 50-mL tubes. After soaking in Solution, A at 23 °C for 12 h, Solution B (1.8 mL) was added into each tube. Then, the mixture was agitated gently at 37 °C for 2 h, followed by 5 min of centrifuging at  $13,000\times g$ . After processing, Solution C (400  $\mu$ L) and Proteinase K (20 mg/mL, 50  $\mu$ L) were added to the resulting supernatant, and the mixture was incubated under gentle agitation at 56 °C for 3 h (Table S1).

The resultant pure DNA solution from each sample was obtained using an QIAamp DNA Investigator Kit (Qiagen, Hilden, Germany, Table S1). Using a silica membrane, the DNA samples were purified, as described in detail in Table S1. The purified DNA was filtered in an adipose tissue extracts buffer (100  $\mu$ L, Buffer ATE; 10 mM Tris-HCl, 0.1 mM EDTA, 0.04% sodium azide, Table S1).

### *Quantitative polymerase chain reaction*

A Hepatitis B Virus polymerase chain reaction (PCR) Kit (MyBiosource, Inc., San Diego, CA, USA) with oasis lyophilized 2X qPCR Mastermix (Primerdesign, Ltd., Camberley, UK) and a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) were used for quantitative real-time PCR (qPCR). As per the manufacturer's instructions, the PCR reactions were performed by aliquoting the master mix (15  $\mu$ L) into the individual reaction wells of a 96-well reaction plate (Applied Biosystems, Foster City, CA, USA), followed by the addition of each experimental DNA sample (5  $\mu$ L) to obtain the final volume of the reaction well (20  $\mu$ L), as described in detail in Table S1. The 'standard DNA' was set

up from six concentrations of a reference DNA sample that were prepared by serial dilution and analyzed in triplicate for each of the 96-well plates in the study. All of the DNA samples were analyzed in triplicate. The thermal cycling profile included two stages, namely Stage 1 of 2 min at 95 °C; and Stage 2 of 50 cycles of 10 s at 95 °C and 60 s at 60 °C followed by signal acquisition (Table S1).

### *Data and statistical analysis*

A real-time PCR thermal cycler software (SDS 2.4.1 for the 7900HT) was used to analyze the data. Fluorescence data were normalized using the ROX (6-Carboxyl-X-Rhodamine,  $\lambda_{max}$  =610 nm) signal, and both the threshold and baseline signals were activated automatically. The HBV DNA copy numbers in the radiation sterilized dentin were compared to those of the fresh dentin harvested from the same tooth. Due to the limited number of samples, neither cut-off levels nor specificity could be determined.

All statistical analyses were performed using the R statistical software (version 3.5.1; R Foundation for Statistical Computing, Vienna, Austria). The Wilcoxon signed-rank test was used for the comparison of the HBV DNA copy numbers of the control and experimental groups, and a difference was considered to be significant if the observed P value were less than 0.05.

## **Results**

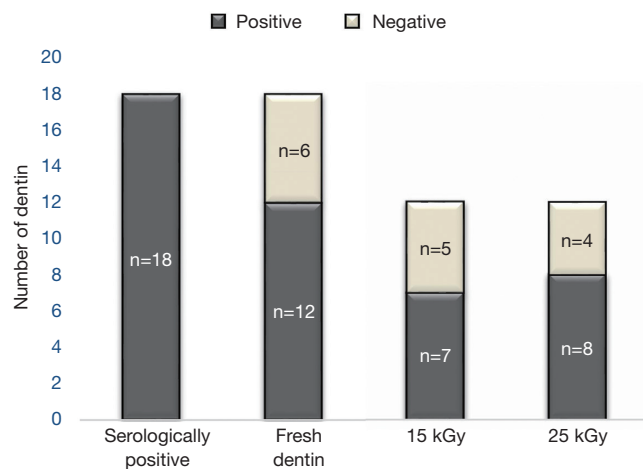
### *HBV DNA in fresh dentin*

Among the 18 fresh dentin samples from the HBV patients, 12 specimens (66.67%) were found to contain HBV DNA. In the other six fresh dentin samples, HBV DNA was not measured either due to insufficient DNA quality or due to insufficient amounts for analysis (Figure 2).

The HBV DNA of fresh dentin was significantly different from that of the radiation-sterilized dentin. Confirming the statistical significance of the results, for the experimental groups exposed to the GR doses of 15 and 25 kGy, the Wilcoxon signed-rank test found P value of 0.012 and 0.010, respectively. There was no observed significant difference between the radiation doses of 15 and 25 kGy ( $P=0.721$ ).

### *HBV DNA in dentin gamma-irradiated with doses of 15 and 25 kGy*

The HBV DNA in 5 (41.67%) and 4 (33.33%) of the 12



**Figure 2** Number of dentin samples from the serologically hepatitis B virus-positive patients. The number of hepatitis B virus (HBV)-DNA-positive fresh dentin samples, and the number of HBV-DNA-positive gamma-irradiated dentin samples. Among 18 dentin samples from the HBV-infected patients, twelve fresh dentin samples (66.67%) contained detectable HBV DNA. HBV DNA of the remaining six fresh dentin samples (33.33%) was either degraded or of insufficient level for detection. After GR irradiation with 15 kGy, 5 (41.67%) of the twelve experimental group dentin samples corresponding to the twelve fresh dentin samples that were positive for HBV DNA had degraded or eliminated viral load levels. After GR irradiation with 25 kGy, four (33.33%) of the twelve experimental dentin samples corresponding to the twelve fresh dentin samples that were positive for HBV DNA had degraded or eliminated viral load levels.

dentin samples known to be positive for HBV DNA was observed to be eliminated or degraded by GR doses of 15 and 25 kGy, respectively. HBV DNA copy numbers of 3.51, 11.34, and 1.43 were observed in the radiation-sterilized dentin from patients #15, #11, and #9, respectively; these samples corresponded to copy numbers greater than 10 (53.43, 46.67, and 16.35, respectively) in the fresh dentin samples, demonstrating that the viral DNA in these samples underwent extensive degradation by GR at a dose of 15 kGy. After the GR exposure of 25 kGy, the samples obtained from patients #15, #11, and #9 also demonstrated extensive degradation of the HBV DNA.

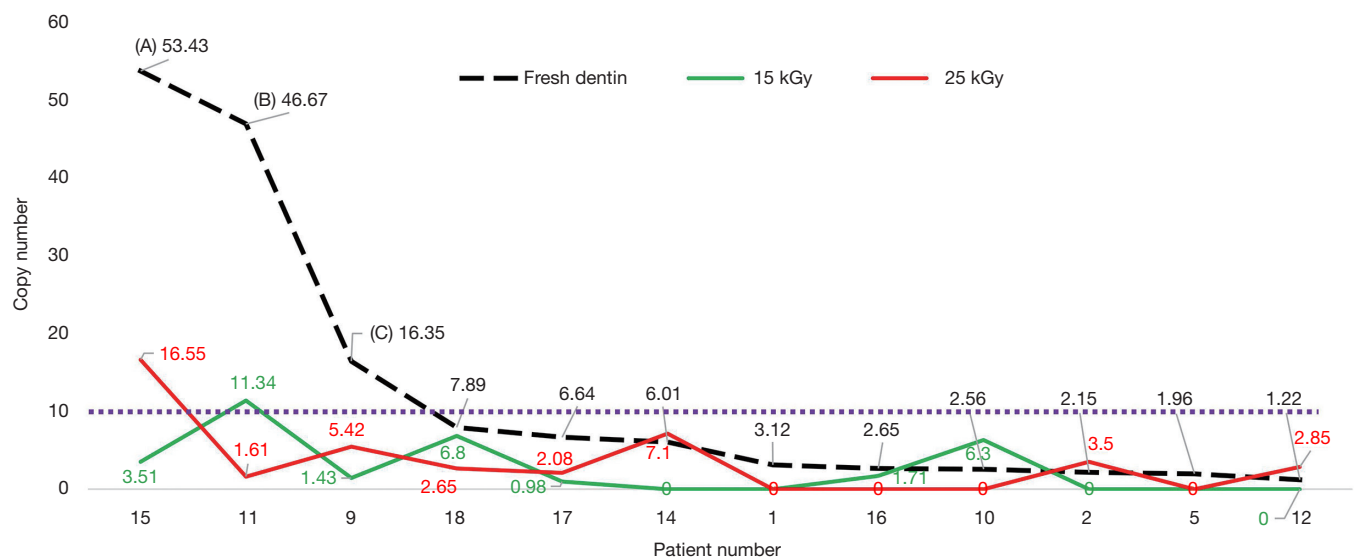
Eleven of the twelve (with the exception of patient #11 for irradiation with 15 kGy and of patient #15 for irradiation with 25 kGy) fresh dentin samples (91.67%) with the HBV DNA copy numbers of less than 10.0 were correlated with the samples that showed negative results for

HBV after the irradiation with both 15 kGy and 25 kGy. This is considered to indicate a complete elimination or inactivation of HBV (Figure 3, Table S2).

## Discussion

The authors hypothesized that GR exposure with a dose of either 15 or 25 kGy inactivated HBV by degrading its nucleic acids, rendering it noninfectious. The viral load in the dentin harvested from infected patients may be the principal factor for determining the methods used in terminal sterilization to prevent the transmission of any viral infections. Based on the results of the current study, dentin may be a potential carrier of HBV, as indicated by the fact that 66.67% (12/18) of the fresh dentin samples tested positive for HBV DNA by qPCR. GR with the doses of either 15 or 25 kGy was shown to significantly reduce the HBV DNA in fresh dentin. A statistically significant reduction in the measured quantity of HBV DNA was achieved in five samples with 15 kGy (41.67%) and in four samples with 25 kGy (33.33%) within a total of twelve samples. Among the twelve HBV-DNA-positive fresh dentin samples, HBV DNA persisted in eleven of the post-irradiation dentin samples (91.67%) that were reduced to below copy number of 10, except for the dentin in sample #11 after 15 kGy of GR and the dentin in sample #15 after 25 kGy of GR. The highest copy numbers of 53.43, 46.67, and 16.35 in fresh dentin samples were reduced to 3.51, 11.34, and 1.43 after 15 kGy and 16.55, 1.61, and 5.42 after 25 kGy, respectively. Even though the actual cut-off levels and specificity were not determined in the current experiment, a copy number of less than 10 is generally considered to be negative for viral infection. Therefore, the validation of the viral inactivation by GR with either 15 or 25 kGy doses in dentin performed in this work may enable the expanded allogenic application of DDM from the GR-sterilized dentin. In this work, we observed no sterilization difference as measured by the HBV DNA levels between the radiation dose sizes of 15 and 25 kGy.

The DDM derived from dentin consists of osteoconductive type 1 collagen and non-collagenous proteins including the osteoinductive bone morphogenetic proteins. The procedure used to generate DDM involves washing, demineralization with organic solvents, freeze-drying, and sterilization, and should result in a significant level of viral inactivation in a similar fashion to processing of a demineralized bone matrix (DBM) (5). In 2018, it was proven that the procedure for producing DDM also



**Figure 3** Change in the copy number of hepatitis B virus DNA between fresh and gamma-irradiated dentin. The highest (A), second highest (B), and third highest (C) copy numbers (53.43, 46.67, and 16.35, respectively) of hepatitis B virus (HBV) DNA in fresh dentin were degraded to 3.51, 11.34, and 1.43 after a 15 kGy GR dose and were degraded to 16.55, 1.61, and 5.42 after a 25 kGy GR dose (patients #15, #11, and #9). Five members (out of nine) of the 15 kGy group (55.56%) and four (out of nine) in the 25 kGy group (44.44%) for which the corresponding fresh dentin samples had HBV DNA copy numbers of less than 10.0 were negative for HBV DNA (inactivated or eliminated HBV). The HBV DNA of fresh dentin varied significantly from that in dentin after GR doses of 15 or 25 kGy, and the statistical significance was determined by Wilcoxon signed-rank test obtaining P value of 0.012 and 0.010, respectively. There was no significant observed difference between the outcomes of the 15 kGy dose and the 25 kGy dose groups ( $P=0.721$ ).

extensively reduced the level of HBV DNA (4). The major differences between DDM and DBM are that bone contains viable osteocytes and blood vessels (haversian canals and endothelial cells), while there are neither cells nor blood vessels in dentin that may be a potential source of transmissible viruses (1). Because dentin is obtained from patients during dental treatment, the possibility of disease transmission via a dentin allograft may be lower than that from a bone allograft. Based on these reasons, a DDM allograft may be a safer alternative to a bone allograft in terms of viral disease transmission (11,12). Recent animal studies have shown promising results for the xenografts of DDM with the absence of any immunological complications (13,14). Furthermore, some clinical trials reported the successful results of nonpathogenic allografts derived from within a biological family (2,3). However, little evidence has been reported regarding the effective sterilization processes for infectious pathogens in DDM allografts derived from individuals with various medical conditions.

GR is a simple, cost-effective, and popular sterilization technique in bone allografts that is known to inactivate

both enveloped and non-enveloped viruses. GR will fully penetrate tissue and thus has no exposure dependence on the tissue size or dimensions (15,16). Hilmy *et al.* showed that the clinical efficacy of long bones after a GR irradiation process with 25 kGy shows no prominent difference from the efficacy of the aseptically prepared bones (17). Various guidelines have been suggested for the optimal minimum dose of GR to ensure sterilization, with a 35 kGy dose suggested in some studies to attain sterility assurance levels (18).

One of the disadvantages of GR is its hazardous effects on the osteoinduction properties of bone allografts tissues such as the DBM. Glowacki *et al.* reported that a 35–50 kGy GR dose at the ambient temperature can eliminate the osteoinductive properties of demineralized bone powder. Additionally, it was found that a 20 kGy GR dose resulted in a 20 percent decrease in osteoinduction (19). Even though the effects of gamma irradiation on the osteoinductivity of DDM could be extrapolated from DBM, more studies are required to determine the specific effects of the doses of 15 and 25 kGy on the osteoinductivity of DDM (20–22).

As previously noted, it is currently not known whether or not the HBV DNA in fresh dentin is infectious because we did not determine the associated cutoff levels of the HBV DNA in the present experiments. Another limitation of the present study is the small number of dentin samples procured from the chronically HBV-infected patients. It is also noteworthy that the relatively low transmissible virus density in chronically infected dentin may be closely related to the acellular and avascular characteristics of the dentin matrix, and may suggest that doses in the 15–25 kGy range may be excessive for low viral load densities but necessary for the inactivation of high-density HBV.

## Conclusions

The results of this study suggest that GR dose of 15–25 kGy significantly reduced the levels of HBV DNA. Despite the adverse effects of GR on bone osteoinductivity, the terminal sterilization of human DDM by GR appears to provide an expanded margin of safety against both HBV and the viruses that are currently not tested or are unknown for allogenic application. To achieve completely virus-free grafting DDM, additional studies, including both the validations of viral load inactivation and the investigations of the effects of GR exposure on the osteoinductivity of DDM, are necessary. In addition, despite its low copy number, testing of the infective potential of the HBV DNA that persisted after the GR sterilization may be required.

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## Footnote

**Conflicts of interest:** The authors have no conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted following the World Medical Association

Helsinki Declaration and ethical principles Committee of the Seoul National University Bundang Hospital Institutional Review Board approved the study (SNUBH IRB No. B-1705/395-308). All participants signed informed written consent.

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