

Inactivation Effect of Standard and Fractionated Electron Beam Irradiation on Enveloped and Non-Enveloped Viruses in a Tendon Transplant Model

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Keywords

Allograft · Irradiation · Ebeam · Tissue sterilization · Tissue bank

Summary

Background: For increasing allograft tendon safety in reconstructive surgery, an effective sterilization method achieving sterility assurance including viruses without impairing the grafts properties is needed. Fractionated Electron Beam (Ebeam) has shown promising in vitro results. The proof of sufficient virus inactivation is a central part of the process validation. **Methods:** The Ebeam irradiation of the investigated viruses was performed in an optimized manner (oxygen content < 0.1%, –78 °C). Using principles of a tendon model the virus inactivation kinetics for HIV-2, HAV, pseudorabies virus (PRV) and porcine parvovirus (PPV) were calculated as TCID₅₀/ml and D₁₀ value (kGy) for the fractionated (10 × 3.4 kGy) and the standard (1 × 34 kGy) Ebeam irradiation. **Results:** All viruses showed comparable D₁₀ values for both Ebeam treatments. For sufficient virus titer reduction of 4 log₁₀ TCID₅₀/ml, a dose of 34 kGy of the fractionated Ebeam irradiation was necessary in case of HIV-2, which was the most resistant virus investigated in this study. **Conclusion:** The fractionated and the standard Ebeam irradiation procedure revealed comparable and sufficient virus inactivation capacities. In combination with the known good biomechanical properties of fractionated Ebeam irradiated tendons, this method could be a safe and effective option for the terminal sterilization of soft tissue allografts.

Schlüsselwörter

Allograft · Bestrahlung · Ebeam · Gewebesterilisation · Gewebebank

Zusammenfassung

Hintergrund: Zur weiteren Erhöhung der Sicherheit von Sehnenallografts in der rekonstruktiven Sporttraumatologie und Orthopädie wurde mit der Elektronenstrahlbehandlung (Ebeam) eine effektive Sterilisationsmethode entwickelt, die höchstmögliche biologische Sicherheit ohne klinisch relevante Beeinträchtigung der Transplantateigenschaften bieten könnte. Der Nachweis einer hinreichenden Virusinaktivierung ist dabei zentraler Bestandteil der Prozessvalidierung. **Methoden:** In einem aus der Sehnenbestrahlung abgeleiteten Testmodell wurden folgende Viren untersucht: HIV-2, HAV, Pseudorabies-Virus (PRV) und Porcines Parvovirus (PPV). Die Elektronenstrahlbehandlung der untersuchten Virusstämme erfolgte unter optimierten Bedingungen (Sauerstoff-Gehalt von weniger als 0,1%, –78 °C). Die fraktionierte Ebeam-Bestrahlung (10 Zyklen à 3,4 kGy) wurde mit der Ebeam-Standardbestrahlung (1 Zyklus à 34 kGy) hinsichtlich der Virusabreicherungskapazität (TCID₅₀/ml) und der D₁₀-Werte (kGy) verglichen. **Ergebnisse:** Alle Viren zeigten vergleichbare D₁₀-Werte für beide Ebeam-Bestrahlungen. Für eine ausreichende Virustiterreduktion von 4 log₁₀ TCID₅₀/ml war für das resistensteste Virus (HIV-2) eine Dosis von 34 kGy fraktionierter Ebeam-Bestrahlung notwendig. **Schlussfolgerung:** Die fraktionierte Ebeam-Bestrahlung weist im Vergleich zum Standard-Ebeam-Verfahren eine vergleichbare und hohe Virusabreicherungskapazität auf. In Kombination mit den bekannten guten biomechanischen Eigenschaften von fraktioniert Ebeam-bestrahlten Sehnen könnte dieses Verfahren eine sichere und wirksame Option für die terminale Sterilisation von Sehnen- und Bandallografts sein.

Introduction

Human allogenic soft tissue has many indications in reconstructive surgery and its use has increased in many countries, especially for anterior cruciate ligament (ACL) reconstruction [1–3]. Compared to autografts, the main advantage is the lack of any donor side morbidity and the faster return to normal activity [4]. However, especially in Europe, the use of allografts is limited mainly due to the potential risk of disease transmission from the donor to the recipient [5] law restrictions, and an insufficient availability.

Although transplantation associated infections are rare, cases are indicated [6]. Simonds et al. [7] reported transmission of HIV-1 by organ and tissue transplantation in 7 cases. Buck [5] estimated the risk of HIV transmission from an unrecognized infected donor was approximately one in 1.6 million. Without any precautions like donor screening etc., the risk was estimated to be 1 in 161. Because the danger increases with an increasing number of infected persons in a community, the possibility to transplant a tissue from an unrecognized donor for other infections might be much higher. For hepatitis C it is estimated to be 1 in 421,000 in the USA [8]. With the implementation of EU directives 2004/23/EC and 2006/17/EC basic requirements of viral safety in tissue donation were defined in general. While serological tests (anti-HIV-1/2, anti-HCV, HBsAg, anti-HBc, TPHA) are mandatory, the nucleic acid testing (NAT) for the HIV, HBV and HCV is not explicitly required [11, 12]. Additional NAT screening in blood products shortens the diagnostic window period (the time from exposure to positivity) for HCV by 41–60 to 15–22 days. An analogous reduction of the diagnostic window is also possible for HIV, the most feared viral agent in blood banking. Investigations carried out by the Interorganizational Task Force on Nucleic Acid Amplification Testing of Blood Donors in the USA showed that implementing HIV NAT reduced the diagnostic window for HIV by 10–15 days to approximately 10 days [13, 14]. But even serology and NAT in combination cannot close the window period completely. Furthermore, there is the risk of bacterial contamination during the procedure. In 2002 the Center for Disease Control in the USA reported 26 cases of bacterial infection associated with transplanted tissues from a particular donor which resulted in the death of 1 patient [15, 16].

In the past, many terminal sterilization methods for soft tissue transplants were tested to overcome these problems without finding a solution. Chemical treatment with ethylene oxide caused intra-articular inflammatory reactions, graft-to-host reactions, and clearly reduced biomechanical strength of the grafts. Thus, the ethylene oxide treatment was discarded for tendon sterilization [17, 18]. Although the peracetic acid treatment is an established sterilization method of bone, dermis, amnion and fascia lata transplants with no evidence to impair the transplants properties, in ACL tendon grafts it has

caused significantly reduced biomechanical strength and decreased remodeling activity in ACL tendon grafts in an in vivo animal model [19]. However, it is used for tendon sterilization in Germany, but less data are available of the clinical outcome of such treated grafts.

Currently, low-dose gamma irradiation (≤ 25 kGy) is used mainly for ACL graft sterilization in the USA and parts of Europe which eliminates bacteria, fungi, and sensitive viruses, but not all pathogens [3]. For sufficient pathogen inactivation, including resistant viruses like parvovirus or HIV, a dose of more than 30 kGy is needed [20, 21]. However, many studies demonstrated a dose-dependent reduction of the biomechanical properties if grafts were gamma irradiated with a dose beyond 20 kGy [22–24]. Clinical studies found significantly increased failure rates even if grafts were sterilized with middle dose (20–25 kGy) of gamma irradiation [25, 26].

Electron Beam (Ebeam) is a high energy electron treatment which is currently used for sterilization of medical devices and in radiation therapy [27]. The impact should be comparable to gamma irradiation [28]. The main advantage compared to gamma irradiation is the shorter irradiation time and reduced dose variances during the processing. Thus, the dose application could be better controlled [29].

Recently, we investigated the impact of a high-dose Ebeam irradiation (34 kGy) on the biological remodeling and the biomechanical properties of soft tissue allografts used for ACL reconstruction in an in vivo sheep model and found significantly reduced biomechanical properties up to 12 weeks (unpublished data). To reduce the detrimental effects, we tried to modify the irradiation procedure and investigated the fractionated application of the required overall dose in 10 cycles of 3.4 kGy instead of 34 kGy in one cycle in vitro. Fractionation is a common procedure in radiotherapy and results in a reduced damage of healthy tissue surrounding a tumor tissue. Thus, we analyzed the biomechanical properties of human patellar tendons after standard Ebeam (34 kGy), standard gamma (34 kGy) and fractionated Ebeam (10×3.4 kGy) irradiation and found significantly higher biomechanical strength in the fractionated Ebeam treated grafts compared to standard Ebeam or gamma irradiated tendons [1]. This effect might be related to the reduced density of free radicals generated during the fractionated Ebeam procedure compared to the standard Ebeam irradiation. These radicals are suspected to cause protein and collagen damage and, therefore, to be responsible for the impairment of biomechanical properties of irradiated tissue [30–32]. These effects are called the secondary or indirect irradiation effects. Although, it is well known from radiotherapy that fractionation does not influence the efficiency of tumor cell elimination it is unclear, whether the fractionation of the dose input influences the inactivation curve of viruses.

To our knowledge, this is the first study investigating the virus inactivation by standard and fractionated Ebeam treat-

ment. We hypothesized to find no differences in virus inactivation curves for both Ebeam procedures and proclaimed a comparable inactivation dose (D_{10}) as for standard gamma irradiation.

Material and Methods

Viruses

We investigated the following viruses: HIV-2, enveloped, Retroviridae, genus Lentivirus, strain lymphadenopathy-associated virus type 2 (LAV-2) propagated in CEM cells [33], and HAV, non-enveloped, Picornaviridae, genus Hepatovirus; pseudorabies virus (PRV), Aujeszky's disease virus, enveloped, Herpesviridae, genus Varicellovirus) strain Bartha was used as a model virus for the human herpesviruses and porcine parvovirus (PPV, non-enveloped, Parvoviridae) as a model for the human parvovirus B19 (table 1).

Cell Lines and Culture Media

The following virus/cell systems were used; briefly: PRV / mink lung cells (ML); PPV / pig kidney cells (pK13); HAV / embryonic rhesus monkey kidney cells (Frhk-4) and HIV-2 /human T lymphocyte cells (Molt4/8). Dulbecco's modified Eagle's medium high glucose (Gibco) supplemented with 10% fetal calf serum (FCS) and glutamine (0.5 mg/ml) was used for all cells except for the Molt 4/8 cells which were grown in RPMI 1640 medium supplemented with 10% FCS and glutamine (0.5 mg/ml). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂. All cell lines and viruses were obtained from stocks by the Robert Koch Institute, Berlin, and are registered and documented there.

Preparation of Virus Stocks

Virus stocks were prepared from the supernatant of cultured infected cells as described elsewhere [34]. Briefly, subconfluent cell monolayers in tissue culture flasks T 75 were infected with the respective virus using a multiplicity of infection (moi) of 0.01–0.1. Cell cultures showing a prominent cytopathogenic effect (CPE) were scraped off and subjected to 1–3 freeze-thaw cycles. In the case of HIV-infected MOLT 4/8 cells, 1 week after the infection cells were mixed with uninfected cells (1:1) and co-cultivated for 7 days. Cell debris was removed by centrifugation (2,000 rpm, 10 min, 4 °C, Varifuge 3.0. R, Heraeus, Hanau, Germany), and aliquots of virus supernatant of 1 ml were prepared. Tubes were completely filled with virus suspension and stored at –70 °C.

Irradiation Procedure

Following the biomechanical experiments with tendon transplants, all samples were placed on a height-adjusted stage in a dry ice-filled styro-foam box, maintaining a temperature of about –78 °C during transport and irradiation (fig. 1). This specific sample packaging fulfills the requirement of minimum overdose ratio during the electron treatment with 10 MeV electrons in order to reduce the maximum applied dose during sterilization as well as the undesired impairing of biomechanical properties of the graft. The overdose ratio, the minimum dose, and the place of minimum dose were determined during a dose mapping study using alanine dosimeters. These alanine dosimeters were calibrated against the National Standard of National Physical Laboratory (Teddington, UK). The samples were transported in a transport container according to EN 829 (Deutsches Institut für Normung 1996) to the irradiation facility GSE 80 of Gamma Service Produktbestrahlungs GmbH (Radeberg, Germany). The sterilization was performed in two different ways. In the case of standard Ebeam, an absorbed dose of 3.4, 6.8, 10.2 kGy etc. up to

Table 1. Viruses and cell lines

Virus	Cell lines
HIV-2, Retroviridae, enveloped, approximately 100 nm	human T lymphocyte cells, (Molt 4 clone 8)
PRV, Herpesviridae, enveloped, approximately 170 nm	mink-lung cells (ML)
HAV, Picornaviridae, non-enveloped, approximately 30 nm	embryonal rhesus monkey kidney cells (Frhk-4)
PPV, Parvoviridae, non-enveloped, approximately 10 nm	pig kidney cells (pK13)

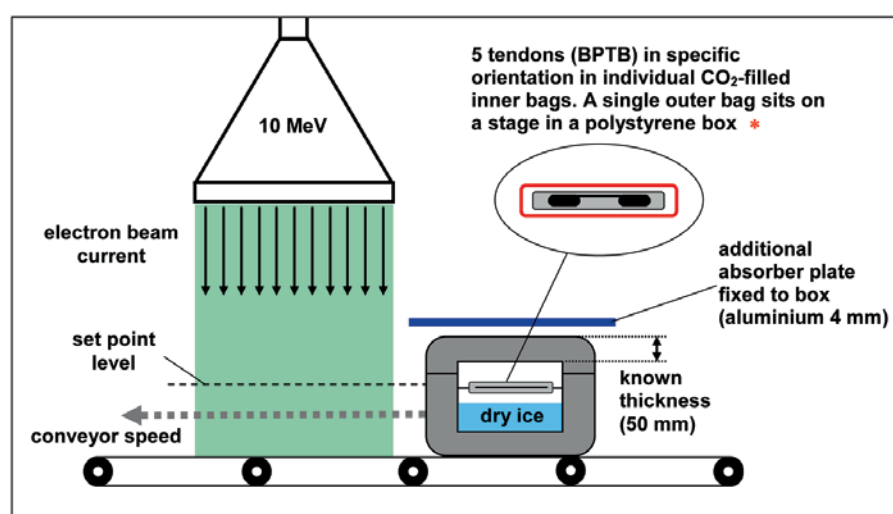


Fig. 1. Schematic overview of sample packaging and Ebeam irradiation process.

*instead of BPTB vials with virus suspensions were used, tubes were placed vertical in the box to ensure an equal irradiation exposure, each box contains two tubes of each virus

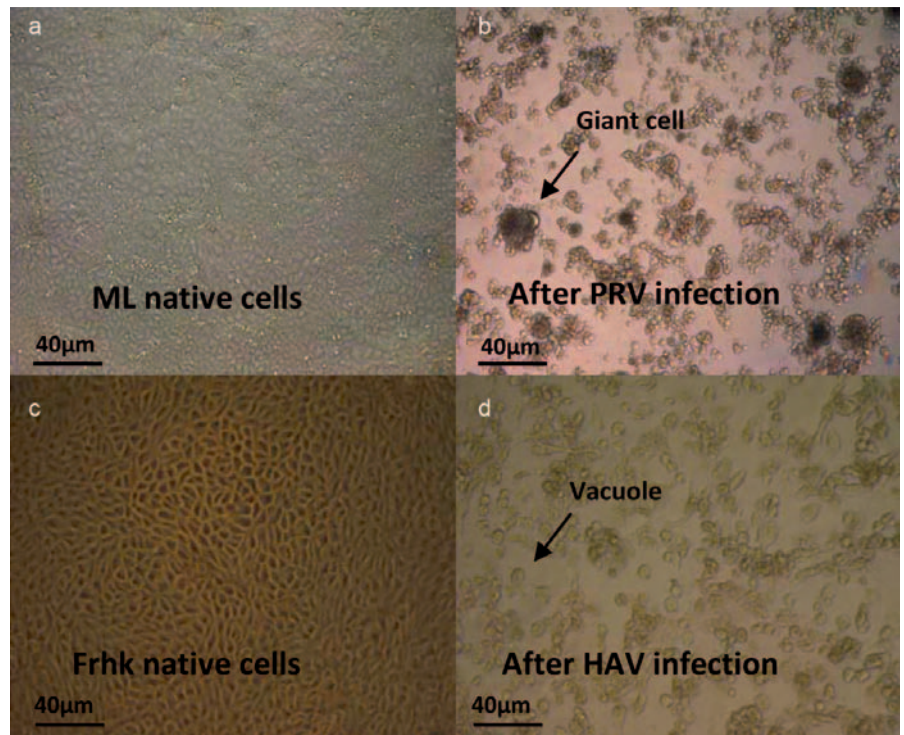


Fig. 2. Example of CPEs caused in cell culture by two of the investigated viruses evaluated by light microscopy. **a** ML native cells and **c** Frhk native cells in cell culture; typical CPE caused by virus infection with **b** PRV and **d** HAV such as cell lysis, vacuoles and giant cells (arrow).

Table 2. D_{10} values and dosages for $4 \log_{10}$ reduction for standard and fractionated ebeam irradiation

Virus	D_{10} value, kGy		$4 \log_{10}$, kGy	
	standard Ebeam	fractionated Ebeam	standard Ebeam	fractionated Ebeam
PRV	5.6 ± 0.4	5.8 ± 0.4	20.8–24.0	21.6–24.8
HAV	6.5 ± 0.2	5.9 ± 0.2	25.2–26.8	22.8–24.4
PPV	8.6 ± 0.6	7.5 ± 0.6	32.0–36.8	27.6–32.4
HIV-2	9.0 ± 0.5	8.0 ± 0.5	34.0–38.0	30.0–34.0

34 kGy was applied in one treatment cycle. In the case of fractionated Ebeam, the required absorbed dose was applied in 1, 2, 3 etc. up to 10 cycles. The average dose of each cycle amounted to about 3.4 kGy. During the sterilization procedure, the absorbed dose was controlled via conveyor speed and electron current. The maximum deviation from the required minimum dose amounted to $\pm 1.4\%$ for fractionated Ebeam and $\pm 3.0\%$ for standard Ebeam. After sterilization, the samples were stored and transported in the polystyrene boxes with dry ice at -78°C to the virological laboratory and stored at -80°C until virus titration was performed. As titration and incubating control, one sample of each virus was transported and stored in same way as the other samples without being irradiated.

Virus Titration

Virus titer was determined as described elsewhere [34, 35]. Briefly, cells were seeded 1 day before the viral assay in 96-well plates containing 1.5×10^4 cells in cell culture medium per well. 10-fold serial dilutions were made from the samples in cell culture medium. 100 μl of each dilution were pipetted into each of 12 wells in quadruplicate. The plates were incubated at 37°C (5% CO_2 , saturated H_2O atmosphere) and observed by transmitted light microscopy over several days from always the same investigator until the virus control showed a CPE. An overview of some of the relevant CPEs in this study is given in figure 2. Before concluding evaluation, results were confirmed by a second qualified person. For

HIV-2, 100 μl of cell suspension were transferred to new 96-well micro-titer plate after 7 days and 100 μl of fresh medium were added to each culture. The infectivity titers expressed as tissue culture infectious dose ($\text{TCID}_{50}/\text{ml}$) were calculated according to Spearman and Kärber and Münch [36, 37]. The virus reduction factor (Ri) was calculated as described by Löwer [38].

Determination of Inactivation Dose D_{10}

The inactivation of viruses often approximates to an exponential relationship:

$$N[D] = N_0 \times 10^{-\frac{D}{D_{10}}} \quad (1)$$

N_0 is the virus titer before and $N[D]$ the virus titer after irradiation with the absorbed dose D . D_{10} describes the required absorbed dose to reduce the initial virus titer to 10%. This dose-response curve based on the assumption of a single-hit-single-target model

Results

The virus inactivation was evaluated in frozen suspensions to imitate the real production process in this experiment. The titer

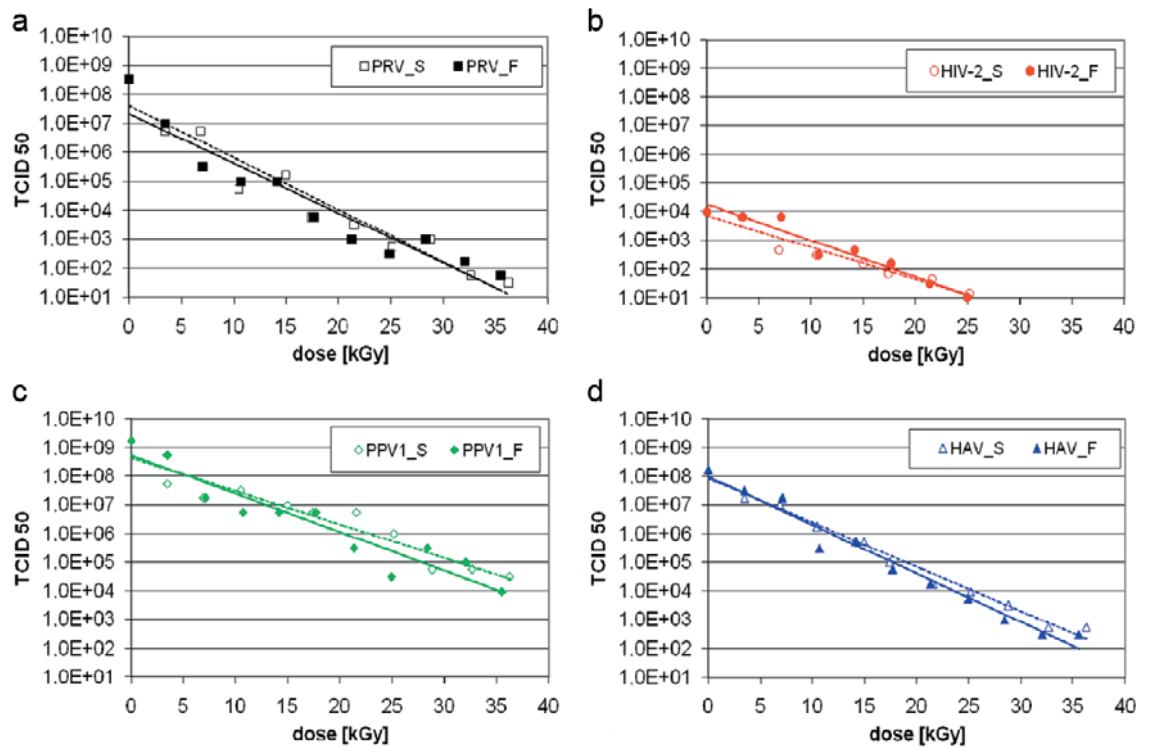


Fig. 3. Inactivation curves for standard and fractionated Ebeam treatment of the investigated viruses showed a linear relationship between the reduction factor and the observed dose. **a** PRV, **b** HIV-2, **c** PPV, **d** HAV.

of the titration and incubation control served as reference. As expected, a linear relationship between the reduction factor and the absorbed dose was observed (fig. 3). The D_{10} values for the different viruses were calculated from the regression curve; from the D_{10} values, the irradiation dose needed to reduce the initial titer by $4 \log_{10}$ was determined (table 2).

We found no differences in the virus inactivation comparing fractionated and standard Ebeam treatment. For all investigated viruses, a reduction of the virus titer of at least $4 \log_{10}$ could be demonstrated. PRV showed the highest sensitivity (for standard Ebeam $D_{10} = 5.6 \pm 0.4$ kGy and for fractionated Ebeam $D_{10} = 5.8 \pm 0.4$ kGy) to irradiation. HIV-2 was the most resistant virus investigated in this study (for standard Ebeam $D_{10} = 9.0 \pm 0.5$ kGy and for fractionated Ebeam $D_{10} = 8.0 \pm 0.5$ kGy). Our results suggest that an irradiation dose of 34.0 kGy of fractionated Ebeam irradiation achieves a sufficient reduction of the initial virus titer by $4 \log_{10}$ steps also for the most resistant virus.

Discussion

The use of allograft tendons for ACL replacement has significantly increased during the last decades due to their advantages compared to autografts, e.g. lack of any donor side morbidity, a faster return to daily living activities, a shorter operation time, and a reduced scarring [3]. However, the main disadvantage is the danger of disease transmission from the donor to the recipient.

Although basic safety procedures like serological and NAT screening for markers of virus infections and a donor risk analysis are performed [3] the danger of disease transmission cannot be excluded because of the window period in which an early infection, for example with HIV, could not be detected although tissue might be infectious.

Existing sterilization methods like gamma irradiation show dose-dependent tissue damaging effects [26].

The development of a terminal sterilization method which achieves a sufficient pathogen inactivation without impairing the biomechanical properties of the graft would provide an added safety to allograft tissue for transplantation. As an additional safety tool we investigated Ebeam irradiation in vitro and found favorable biomechanical results which could be improved further if the required absorbed dose of 34 kGy was applied in 10 cycles of 3.4 kGy [1, 12].

This study aimed to investigate the virus inactivation by a standard and a fractionated Ebeam sterilization. We found no differences between these two proceedings. Both methods achieved a sufficient $4 \log_{10}$ titer reduction for all 4 viruses investigated in this study if a dose of 34–38 kGy was used. Fractionation of the required minimum absorbed dose seemed to have no influence on the virus inactivation. Grieb et al. [32] assumed that pathogen inactivation is caused mainly by the direct disruption of the genetic material because of the applied irradiation energy, the so called the direct or primary irradiation effects. In contrast protein/collagen damage is mainly caused by interactions with indirectly formed free radicals during the irradiation procedure what is called the indi-

rect or secondary irradiation effects [31, 32]. That supports our findings of the improved biomechanical properties of the grafts and the unchained virus inactivation after fractionated Ebeam irradiation compared to standard Ebeam treatment.

Furthermore, the evaluated D_{10} values coincide with the results of other studies, which investigated the virus inactivation by gamma irradiation. It confirms the findings from Seto et al. [28, 40] and Dzedzic-Goclawska et al. [31] who proclaimed a comparable impact of Ebeam and gamma irradiation. HIV-2 was the most resistant virus in this study what seems to be contrary to the results of Pruss et al. [20] where the investigated parvovirus was the most resistant virus. Taking into account the experimental uncertainties of the inactivation dose D_{10} in both studies, comparable values for all viruses were determined. Minor changes could depend on the different models. Pruss et al. [20] placed the virus directly in a cortical bone diaphysis, and instead of porcine parvovirus they investigated the bovine parvovirus [31].

The main disadvantage of Ebeam irradiation is the limited penetration depth, which depends on the used energy, the atomic number of the absorbing product, and its density. In water (density 1 g/cm³) the penetration depth of 10 MeV electrons amounts to about 6 cm which is deep enough for soft tissue grafts. Therefore, Ebeam irradiation is useful for tissue grafts. In the case of sterilization of larger bone grafts, two-sided sterilization should be used in order to fulfill the requirements of penetration depth

One limitation of this study is that we investigated virus suspensions. Hence, protecting interaction with organic tissue matrix could not be evaluated. Grieb et al. [41], however, found no differences in virus inactivation if pulverized tissue mixed with pathogen or virus suspension was used. They con-

cluded that the organic matrix did not protect the pathogens from inactivation. Nevertheless, it remains unclear what happens if pathogen is incorporated in vital tissue as in a tissue graft from an infected donor. Further studies should focus on that to exclude that sterilization assurance level is influenced by organic matrix-pathogen interactions.

Conclusion

To conclude, comparable virus inactivation and inactivation dose values (D_{10}) were determined for both Ebeam procedures. The superior biomechanical in vitro results using the fractionated Ebeam process compared to standard Ebeam or gamma treatment suggest that this novel procedure is a safe and effective terminal sterilization method which achieves full pathogen inactivation without impairing the biomechanical properties of soft tissue grafts like tendons and ligaments. However, the biological effects must be confirmed in an animal model before it can be used for human graft sterilization.

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Disclosure Statement

The authors declared no conflict of interest.

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