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Effectiveness of hydrogen peroxide and electron-beam irradiation treatment for removal and inactivation of viruses in equine-derived xenografts

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A B S T R A C T

Bone grafting is a common procedure for bone reconstruction in dentistry, orthopedics, and neurosurgery. A wide range of grafts are currently used, and xenografts are regarded as an interesting alternative to autogenous bone because all mammals share the same bone mineral component composition and morphology. Antigens must be eliminated from bone grafts derived from animal tissues in order to make them biocompatible. Moreover, the processing method must also safely inactivate and/or remove viruses or other potential infectious agents. This study assessed the efficacy of two steps applied in manufacturing some equine-derived xenografts: hydrogen-peroxide and e-beam sterilization treatments for inactivation and removal of viruses in equine bone granules (cortical and cancellous) and collagen and pericardium membranes. Viruses belonging to three different human viral species (*Herpes simplex virus type 1*, *Coxsackievirus B1*, and *Influenzavirus type A* H1N1) were selected and used to spike semi-processed biomaterials. For each viral species, the tissue culture infective dose ($TCID_{50}$) on cell lines and the number of genome copies through qPCR were assessed. Both treatments were found to be effective at virus inactivation. Considering the model viruses studied, the application of hydrogen peroxide and e-beam irradiation could also be considered effective for processing bone tissue of human origin.

1. Introduction

Bone grafting procedures are quite common in orthopedic and dental surgeries. They entail the use of implantable biomaterials such as bone substitutes, as well as protective barriers (Bauer and Muschler, 2000). Among graft materials, autologous bone is regarded as the gold standard because of its osteoconductive, osteoinductive, and osteogenic properties (Khan et al., 2005). However, the quantity that can be harvested from intraoral sites is limited. Harvest from extraoral sites avoids this limitation, but the need for a second surgery increases morbidity (Buser et al., 1999; Nkenke et al., 2001; Nkenke et al., 2004). Moreover, depending on the collection site, a surgical suite and personnel may be necessary, increasing the cost.

The alternative of allografts is limited by the availability of donors and the existence of a well-functioning tissue banks system managing donor screening, that must be implemented in order to minimize the risk of disease transmission (Buck and Malinin, 1994), and well-organized tissue collection. Availability of donors may vary across different countries in relation to culture and religion. Moreover, depending on different tissue banking processing systems, not all allografts are provided in a form that allows for long term room temperature storage.

Abbreviations: Cox-B1, Coxsackievirus B1; CPE, Cytopathic Effect; DMEM, Dulbecco's Modified Eagle Medium; dsDNA, double-stranded DNA; EQA, External Quality Assessment; FBS, Fetal Bovine Serum; FDA, Food and Drugs Administration; H1N1, influenza A virus subtype H1N1; HSV-1, Herpes simplex virus type 1; MDCK, Madin-Darby Canine Kidney; PBS, Phosphate Buffered Saline; qPCR, quantitative Real-Time PCR; RT-qPCR, Reverse-Transcriptase qPCR; SAL, Sterility Assurance Level; ssRNA, single-stranded RNA; $TCID_{50}$, Tissue Culture Infective Dose 50; TSE, Transmissible Spongiform Encephalopathies.

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Several bone substitutes such as heterologous bone and a variety of natural and synthetic materials have been proposed as alternatives to autogenous grafts and allografts (Gazdag et al., 1995). Some bone-grafting procedures, mainly in oral and maxillofacial surgery, imply also using protective barriers/membranes to contain the graft material, prevent soft-tissue cell in-growth, and guide new tissue formation (Bunyaratavej and Wang, 2001). Membranes can be either non-absorbable or absorbable. Usually the latter are preferred because no second surgery is needed to retrieve them (Fragiskos, 2007).

The use of bone substitutes and membranes derived from mammals other than humans (so-called xenografts) could theoretically be of some advantage, since mammalian tissues share significant biological and mechanical features (Weiner and Wagner, 1998) that, if preserved in the grafts, could be highly beneficial in clinical applications. As far as mammalian bone tissue is concerned, both morphology and chemical composition of the bone mineral component are highly preserved among all mammalian species. Equally, the bone collagen amino acid sequence shows minimal variations among mammals (Eastoe, 1955). A material preserving these features could be beneficial by providing surgeons with a bone substitute whose biological and mechanical behavior is at least partially similar to human bone.

In those cases when barriers are needed, fast-resorptive collagen membranes are widespread, since the extraction and purification of dermal or tendon mammalian collagen is a simple and proven process (Bowes, 1950; Bowes and Kenten, 1950a,b; Mokrejs et al., 2012). Decellularized pericardium or peritoneum membranes are also employed, preserving the native structure of the natural serous membrane of which they are composed; such membranes have a longer resorption time, and they provide excellent mechanical resistance (Bunyaratavej and Wang, 2001). Xenograft materials are therefore widely used (Al Ghamdi et al., 2010; Kao and Scott, 2007).

In preparing xenografts, the origin tissues have to be made biocompatible through a process aimed at eliminating any immunogenic molecules (Chappard et al., 1993). Antigen elimination can be achieved with different techniques, including the alternative or joint application of high temperature (Lin et al., 1999; Lussi and Geistlich, 1999), chemicals (Grooms and Mills, 1999; Maatz, 1957), or enzymes (Pagnutti et al., 2007). Preferable processes preserve the mechanical resistance and biological features of the original tissues (i.e. the physiological interaction of the graft with the receiving tissue cells being either fibroblasts, osteoblasts, or osteoclasts), thus improving graft efficacy (Best et al., 1997).

To ensure the microbial safety of xenografts, a final sterilization step is routinely added, usually involving gamma or electron-beam (e-beam) irradiation (Block, 1991). Ethylene oxide (EtO) treatment is usually not applied because of the difficulty in eliminating residual chemicals from porous tissues (Buben et al., 1999), with related biosafety risks (Jackson et al., 1990; Vangsness et al., 2006). E-beam irradiation may be preferred to gamma because of the more precise dose control it allows and the shorter irradiation time, that makes it more respectful of tissue features of interest (Mitchell et al., 2004; Pelker et al., 1983; Silindir and Özeray, 2009). Tissue-cleaning processes aim also to reduce effectively the microbial load of the origin tissues, in order to obtain a lower, less-destructive, radiation dose to be applied for sterilizing. In this context, hydrogen peroxide is widely used both as an antigen-inactivating/eliminating and as a disinfecting agent (Eckmayer et al., 2009; Grooms and Mills, 1999). Its oxidative properties are exploited both to disrupt lipids (Grooms and Mills, 1999) and lower the bacterial contamination (Klapes and Vesley, 1990). Its capacity to inactivate possible viral contamination was well documented also when used as a vapor for several substrates (Berrie et al., 2011; Heckert et al., 1997; Pottage et al., 2010; Roberts and Antonoplos, 1998). To the best of the authors' knowledge, no studies have been done regarding the efficacy of hydrogen peroxide alone when used as a solution on biological tissues for viral inactivation. Similarly, while there are several studies regarding viral inactivation properties of gamma irradiation on biological tissues for human surgeries (Campbell and Li, 1999; Grieb et al., 2006, 2005; Moore, 2012; Pruss et al., 2002), the antiviral efficacy of e-beam irradiation at different dosages is less evaluated (Preuss et al., 1997) and mainly in food industry (Espinosa et al., 2012; Praveen et al., 2013; Sanglay et al., 2011). No data appear to be available on the effectiveness of a combined hydrogen peroxide/e-beam irradiation treatment for inactivating possible viral contamination of mammal tissues used to manufacture implantable xenografts for bone regeneration.

Recently, a set of equine-derived bone substitutes and membranes have become available for bone regeneration surgeries in orthopedic medicine and dentistry (Di Stefano et al., 2012, 2013; Gigante et al., 2011; Pistilli et al., 2013; Santini et al., 2011; Sessa et al., 2010; Stievano et al., 2008). The process applied to make such set of equine-derived implantable devices free of immunogenic components preserve the osteoclastic adhesion and remodeling properties of the origin bone (Perrotti et al., 2009), as well as the mechanical properties of both the origin bone and membranes. It is based on the action of hydrolytic enzymes, and comprises using a solution of hydrogen peroxide to dissolve and eliminate cells, proteins and lipid components from the tissue of origin.

Equine-derived biomaterials may be preferred to those of bovine or porcine origin for reasons that span from religious issues to factors concerning Transmissible Spongiform Encephalopathies (TSE) safety. It is well known, in fact, that horses are not affected by TSE given the intrinsic stability of equine prions (Qing et al., 2014; Zhang, 2011). Yet, concerning possible viral contaminations of the origin tissues, it is known that horses may suffer from several viral infections. The majority of them are regarded as having the capability to infect humans (Bender and Tsukayama, 2004) because the same viral species (or some close variants) may be transmitted from other mammal species different than horse to humans, but there's no proof of a direct horse-to-human transmission. This is the case of *Vesicular Stomatitis virus* (Webb et al., 1987), *Nipah virus* (Hooper and Williamson, 2000), *Borna Disease virus* (Kinnunen et al., 2013), *Equine Foamy virus* (Kehl et al., 2013), *Influenza A virus* (Langley and Morris, 2009; Webby et al., 2007) and *Rabies virus* (Bender and Tsukayama, 2004; Langley and Morris, 2009). *Hendra virus* and *Venezuelan Equine Encephalomyelitis virus* are the only proofed cause of equine-derived zoonosis in humans (Go et al., 2014; Hooper and Williamson, 2000; Tulsiani et al., 2011). These have been reported to occur sporadically, with time-limited outbreaks and in well-defined and limited geographic areas. Both zoonoses, even if showing seldom outbreaks and affecting a limited number of patients, may cause severe symptoms and be fatal.

The purpose of this article is therefore to evaluate the antiviral efficacy of the manufacturing process applied for the production of the equine-derived biomaterial described above focusing, as indicated by Food and Drugs Administration (FDA) guidelines (FDA, 1998), on the two steps that can be considered of major relevance in viral inactivation: hydrogen peroxide treatment and electron-beam irradiation, alone and in combination. For this purpose, as described in literature (Grieb et al., 2005; Hodde and Hiles, 2002; Larzul, 1999; Pruss et al., 1999) and specified by the European Community (Commission, 1991) and FDA guidelines (FDA, 1998), a set of "model" or "relevant" viruses have to be evaluated as they may represent the potential viral contaminant in the starting materials or in manufacturing intermediates.

Since equine viruses are not readily available commercially, the robustness of the manufacturing processes to remove and/or inactivate them was estimated by characterizing the clearance of human viruses modeling the equine viral pathogens. Thus, a set of human viral species closely related to those potentially infecting the ani-