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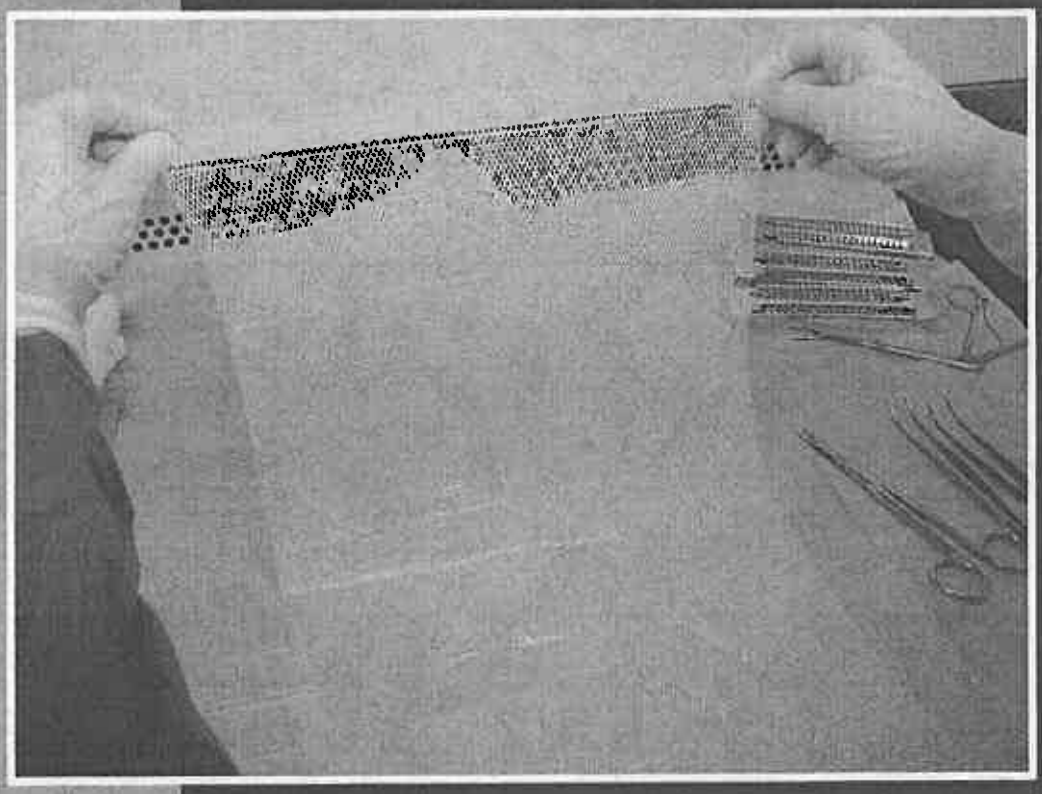

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Bone tissue, lyophilized and stored at room temperature for 15 days or more, is not capable of transmitting HIV, HCV or HBV

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Abstract Over the past 57 years, 17 recipients of frozen bone have been infected with: HIV (Centers for Disease Control and Prevention in Morb Mortal Wkly Rep MMWR 37(39):597–599, 1988; Li et al. in J Formos Med Assoc 100(5):350–351, 2001; Simonds et al. in NEJM 326(11):726–732, 1992; Schratt et al. in Unfallchirurg 99(9):679–684, 1996); HCV (Eggen and Nordbo in NEJM 326(6):411, 1992; Conrad et al. in J Bone Joint Surg Am 77:214–224, 1995; Trotter in J Bone Joint Surg Am 851(11):2215–2217, 2003; Tugwell et al. in Ann of Internal Med 143(9):648–654, 2005); or HBV (Shutkin in J Bone Joint Surg Am 36:160–162, 1954). However, bone, lyophilized and stored at room temperature, has never transmitted these viral diseases. A literature review was undertaken to determine whether there is any evidence that lyophilized bone is capable of transmitting HIV, HCV and HBV.

Keywords Bone · Frozen · Lyophilized · Enveloped virus · Transmission

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All references to bone tissue allografts is abbreviated by the use of the word "bone".

The use of the word "lyophilized" will designate biologic products that have been freeze-dried, without the addition of cryopreservative agents, and thereafter stored at room temperature.

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Methods

The literature review was initiated as a result of five enquiries: (1) What are the reports of bone transmitting HIV, HCV and HBV?; (2) How long do HIV, HCV and HBV, dried and at room temperature, remain infectious on fomites?; (3) How do enveloped viruses replicate?; (4) How long do enveloped viruses survive following lyophilization?; and, (5) What lyophilization processes have succeeded in preserving viruses and proteins? The discussion below treats these inquiries in the order listed.

Results and discussion

What are the reports of bone transmitting HIV, HCV and HBV?

In the 1950's, reports recognized freezing both as a storage method for bone, and as a way to reduce antigenicity (Joyce MJ et al. (Feb. 17–18, 2007) Musculoskeletal Allograft Tissue Safety. American Academy of Orthopedic Surgeons, 74th Annual

Meeting.). As a result, freezing contributed to a resurgence in surgeries utilizing bone (Carter et al. 1989).

The first case of the transmission of viral disease by frozen bone was reported by Shutkin (1954). The donor had undergone an above the knee amputation. After recovery, the donor bone was immediately cut into small portions under aseptic conditions, placed in double sterile containers, and frozen. Five months later, the bone was implanted into a medical student. This article is acknowledged to be the first reported case of HBV transmitted by frozen bone tissue (Tomford 1995; Pruss et al. 2005). In reporting this case of hepatitis transmission Shutkin (1954) noted: "The virus [hepatitis] has been shown to survive temperatures of −10° to −20° centigrade for 4-1/2 years, but to become inactive after 5 years at this temperature."

Subsequently, there have been reported 9 cases of HIV (Centers for Disease Control and Prevention 1988; Li et al. 2001; Simonds et al. 1992; Schratt et al. 1996) and 7 cases of HCV (Eggen and Nordbo 1992; Conrad et al. 1995; Trotter 2003; Tugwell et al. 2005). In every case, frozen bone transmitted the disease. Two of these articles report inadvertent human experiments in the context of frozen versus lyophilized bone. In Simonds et al. (1992), the donor bone was infected with HIV. The recipients of frozen bone acquired HIV, whereas the recipients of lyophilized bone did not become infected with HIV. In Tugwell et al. (2005), the donor bone was infected with HCV. Those recipients receiving frozen bone acquired HCV, whereas those receiving lyophilized bone did not acquire HCV. The bone was subjected to differing processes in both Simonds et al. (1992), and Tugwell et al. (2005). In Simonds et al. (1992), frozen bone transmitted HIV in three out of three cases, whereas, the bone that was lyophilized and treated with ethanol did not transmit disease in any of the twenty five recipients. In Tugwell et al. (2005), frozen bone lavaged with sterile water and then soaked in a solution of isopropyl alcohol, antibiotics, and sterile water transmitted disease in three out of three cases whereas bone that was lyophilized, subjected to the same soaking, and irradiated did not transmit disease to any of the sixteen recipients.

These unplanned human experiments recall an observation made years previously, namely, that although lyophilized bone has been in use since 1951 there have been no known cases of HIV or any virus transmitted through lyophilized bone (Asselmeier et al. 1993). This position has been reaffirmed numerous times by many authorities including the CDC (Centers for Disease Control and Prevention. What is the Risk of Disease Transmission with Bone Allografts? http://www.cdc.gov/OralHealth/infectioncontrol/faq/allografts.htm 2006).

How long do HIV, HCV and HBV, dried and at room temperature, remain infectious on fomites?

There are numerous studies which confirm that enveloped viruses, when dried and stored at room temperature, lose their infectivity within days.

Outside the body HIV loses its infectivity by 90–99% within several hours. There has not been a single case of HIV transmission reported via environmental exposure. (Centers for Disease Control and Prevention 2006 *How Well Does HIV Survive Outside the Body?*. http://www.cdc.gov/hiv/resources/qa/qa35.htm.) Laboratory studies have shown that HIV survives when dried and at room temperature at most for 7 days (Kurth et al. 1986; Sattar and Springthorpe 1991; van Bueren et al. 1994).

The Kamili et al. (2007) study demonstrates that HCV dried at room temperature can remain infectious for more than 16 h but less than 4 days. In Kamili et al. (2007), three HCV infectious aliquots were allowed to dry for 16 h, 4 days, and 7 days, respectively. The aliquot dried for 16 h, injected into a chimpanzee, transmitted HCV, whereas, the aliquot dried for 4 days, injected into a chimpanzee, did not transit HCV.

HBV, dried on a stainless steel surface, has been shown to survive for as long as 14 days (Favero and Maynard 1974; Sattar and Springthorpe 1996).

These reports examine the infectivity of these viruses subjected to air drying as opposed to lyophilization. Since typical exposure to these blood borne pathogens is "dried" and not lyophilized, it is appropriate to review "dried" when guiding safety measures for hospital personnel and the general public. Both processes trigger rapid loss of infectivity. In Uhlenhaut et al. (2005), an enveloped virus was two to four logs lower compared with the original virus stock less than 1 h following lyophilization. Air drying for 24 h reduced the titer, of the same enveloped virus, by five logs.

It is possible to extend infectivity beyond the limited number of days reported. This may be done by: (1) lowering the temperature, for example, from room temperature to refrigerated temperatures (Levy and Fieldsteel 1982); (2) changing the condition from dried to liquid medium (van Bueren et al. 1994; Sattar and Springthorpe 1996); and, (3) adding cryopreservative agents or supportive medium (Scott and Woodside 1976; Levy and Fieldsteel 1982).

How do enveloped viruses replicate?

In order to replicate, a fusion event must occur between the virus's lipid envelope and the cell's lipid membrane. This fusion event results in the building of a bridge enabling the viral DNA or viral RNA to enter the cell (Tscherne et al. 2006). Water is an integral part of the maintenance of the virus's lipid envelope. Lacking water, the lipid membrane will collapse from a lamellar bilayer shell into hexagonal arrays and sheets (Bode and Read 2000). The lipid membrane contains viral proteins needed for infection of host cells, and disrupting the viral lipid envelope renders the virus non-infectious ((FDA, U.S. Food and Drug Administration. Statement of Kathryn C. Zoon, Ph.D. Director, before the Subcommittee on Human Resources and Intergovernmental Relations Committee on Government Reform and Oversight U.S. House of Representatives; July 31, 1997).

These publications support the hypothesis set forth in Kamili et al. (2007). Kamili et al. (2007) showed that HCV survives for less than 4 days dried and at room temperature. From these data, the authors conclude there could be a substantial decay of the infectious virus, "... perhaps due to loss of integrity of the viral envelope, while the concentration of viral genomic material (i.e. RNA) remains stable."

Removal of water by air drying or by lyophilization inactivates enveloped viruses by destroying the lipid envelope. When water is removed from the enveloped virus, the ability to replicate is lost.

How long do enveloped viruses survive following lyophilization?

Two laboratory studies show that enveloped viruses survive lyophilization. In Crawford et al. (2004), feline leukemia virus (FeLV) was determined to survive lyophilization. However, the test revealed a minimal viral load and the authors did not opine as to the level of viral activity which rendered the virus sub-infectious. In a separate study, Uhlenhaut et al. (2005), the enveloped virus, vesicular stomatitis virus (VSV), was tested following lyophilization and a three to four log reduction was noted.

Neither Crawford et al. (2004) nor Uhlenhaut et al. (2005) report the actual length of time that lapsed between lyophilization and testing. In the Crawford et al. (2004) study the time elapsed was less than 7 days. In researching HIV, some scientists use FeLV as a substitute. The report in Crawford et al. (2004) that FeLV survived for a period of less than 7 days, compares with the maximum range of the duration of the viability of HIV as reported by Sattar and Springthorpe (1996). In Uhlenhaut et al. (2005), the time elapsed between lyophilization and testing of VSV was less than 1 h.

These two studies, Crawford et al. (2004) and Uhlenhaut et al. (2005), augment the information supporting the rapidity of the decay in viral infectivity. By injecting chimpanzees, Kamili et al. (2007) established a rough approximation of more than 16 h but less then 4 days for HCV. Crawford et al. (2004) and Uhlenhaut et al. (2005) provide more precise information about the presence of infectious virus by testing dried aliquot at a specified point in time. Crawford et al. (2004) and Uhlenhaut et al. (2005) demonstrate the rapid loss of viral load after lyophilization, yet, the remaining infectivity of enveloped viruses is in keeping with the reports in the other topic areas. Just as Kamili et al. (2007) proved air dried HCV remained infectious, so did Crawford et al. (2004) and Uhlenhaut et al. (2005) prove that enveloped viruses survive lyophilization. However, all three articles establish a window of infectivity that does not extend beyond 7 days. Although the viruses studied in Crawford et al. (2004) and Uhlenhaut et al. (2005) are not HIV, HCV or HBV, the survival of the enveloped viruses VSV and FeLV after drying is consistent with the survival of HIV, HCV and HBV dried and stored at room temperature.

What lyophilization processes have succeeded in preserving viruses and proteins?

The preservation of proteins undergoing lyophilization is a difficult task requiring cryoprotectants that

"may protect proteins by... preferential interaction, replacement of water, formation of a glass, hydrogen bonding, and steric hindrance." (Wang 2000). In the preservation of viruses numerous cryopreservative agents have been identified, including: bovine serum albumin; low-molecular-weight dextrans; polyvinylpyrrolidone; poly-ethylene glycol; dimethyl sulfoxide; skim milk; glucose; sucrose; lactose; sucrose PG additive of Bovarnick, et al.; mannitol; inositol; sorbitol; sodium glycerophosphate; sodium glutamate; and, calcium lactobionate (Berge et al. 1971). Dextrose and albumin act as efficient cryoprotective agents by providing a protective coat for membranes (Scott and Woodside 1976), an effect presumably triggered also by these other agents. Consequently, it is understandable that the use of a sucrose stabilizer and gelatin has been cited as retaining the maximum quantities of infectious virus (Levy and Fieldsteel 1982).

In addition, as noted by Zhai et al. (2004) "Sugars stabilize membranes and proteins... working as a water substitute ... the concentrated sugar solution lowers the nucleation temperature of the water inside the virus membrane and prevents large ice crystal formation within both the virus and the external medium." Enveloped viruses have been cited as responsive to sucrose stabilizers and gelatin in order to retain infectivity (Greiff and Rightsel 1967; Levy and Fieldsteel 1982). Thus, enveloped viruses can maintain infectivity if cryopreservative agents are added to protect the lipid envelope prior to the drying process. Conversely, chemical processes inactivate enveloped viruses by disrupting the lipid envelope (FDA, U.S. Food and Drug Administration. Statement of Kathryn C. Zoon, Ph.D. Director, before the Subcommittee on Human Resources and Intergovernmental Relations Committee on Government Reform and Oversight U.S. House of Representatives; July 31, 1997).

Conclusions and implications

Formerly, it was believed that minimally processed bone retained insufficient blood to transmit the blood borne viruses, HIV, HCV, and HBV (Tomford 1995). This belief was dismissed following reports of disease transmission. While the incidents reported for bone have been much less than for other biologic products (Boyce et al. 1999), nevertheless, these few cases reinforce the fact that even minimal copies of these viruses, presumably contained in very small quantities of blood on bone, can transmit these viral diseases (Katayama et al. 2004; Hsia et al. 2006; Komiya et al. 2008).

Freezing preserves the infectivity of enveloped viruses presumably by keeping their lipid membranes intact for years. The process of drying and storing at room temperature, without the addition of cryopresevative agents, results in the collapse of the lipid membrane. This collapse occurs over a period of days (HCV—4 days; HIV—7 days; and the larger DNA virus, HBV—14 days). Once the lipid envelope is disrupted the virus is rendered non-infectious. Consequently, the evidence leads to the conclusion that bone, lyophilized and stored at room temperature for 15 days or more, is not capable of transmitting HIV, HCV and HBV. In other words, enveloped viruses do not survive forever. If steps are taken to protect the lipid envelope the dissent to inactivity can be delayed for quite some time. Protecting the lipid envelope by freezing, or by adding cryopreservative agents prior to drying, can extend the life of the lipid envelope. However, drying without cryopreservative agents or adding detergents to disrupt the lipid envelope accelerates the progression toward inactivity. While air drying and lyophilization do not inactivate enveloped viruses, the process of water removal using either method limits infectivity of the viruses to less than 15 days.

These observations could have some relevance for the procedures adopted in routine tissue banking to reduce the danger of transmission of infection after transplantation of allografts. For example, the careful steps now taken to reduce the contaminant load might be augmented by adding an additional routine quarantine storage for several weeks at room temperature following lyophilization. These observations also may have an implication in the debate over the level of gamma radiation needed for viral disease inactivation (Pruss et al. 2005). The International Atomic Energy Code of Practice for the Radiation Sterilization of human tissues deals adequately with bacterial infections and if properly implemented can guarantee a Sterility Assurance Level of 10^{-6} (IAEA 2007). Careful serological testing and processing procedures reduce the risk of viral transmitted infections. Low-dose radiation has been recognized as accomplishing

bacterial sterility (Brockbank and Siler 2001). Augmenting these steps with routine quarantine at room temperature following lyophilization could lead to the adoption of relatively low-dose gamma radiation to accomplish sterility assurance levels of 10^{-6} for both bacteria and enveloped viruses.

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