Elimination of transmissible spongiform encephalopathy infectivity and decontamination of surgical instruments by using radio-frequency gas-plasma treatment

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It has now been established that transmissible spongiform encephalopathy (TSE) infectivity, which is highly resistant to conventional methods of deactivation, can be transmitted iatrogenically by contaminated stainless steel. It is important that new methods are evaluated for effective removal of protein residues from surgical instruments. Here, radio-frequency (RF) gas-plasma treatment was investigated as a method of removing both the protein debris and TSE infectivity. Stainless-steel spheres contaminated with the 263K strain of scrapie and a variety of used surgical instruments, which had been cleaned by a hospital sterile-services department, were examined both before and after treatment by RF gas plasma, using scanning electron microscopy and energy-dispersive X-ray spectroscopic analysis. Transmission of scrapie from the contaminated spheres was examined in hamsters by the peripheral route of infection. RF gas-plasma treatment effectively removed residual organic residues on reprocessed surgical instruments and gross contamination both from orthopaedic blades and from the experimentally contaminated spheres. In vivo testing showed that RF gas-plasma treatment of scrapie-infected spheres eliminated transmission of infectivity. The infectivity of the TSE agent adsorbed on metal spheres could be removed effectively by gas-plasma cleaning with argon/oxygen mixtures. This treatment can effectively remove ‘stubborn’ residual contamination on surgical instruments.

INTRODUCTION

Current concerns about the levels of residual contamination found on surgical instruments, reusable anaesthetic equipment and ophthalmic and dental instruments after conventional cleaning and sterilization have been the subject of a number of recent studies (DesCoteaux et al., 1995; Miller et al., 2001; Dinakaran & Kayarkar, 2002; Smith et al., 2002). Problems of nosocomial infection have also been raised with respect to Helicobacter pylori on endoscopes (Nürnberg et al., 2003) and the bacterial contamination of Doppler probes (Kibria et al., 2002). The resistance of the transmissible spongiform encephalopathy (TSE) infective agent to conventional decontamination procedures is of particular concern. There is clear evidence of cases of iatrogenic Creutzfeldt–Jakob disease (CJD) arising from surgery where neurosurgical-instrument contamination has been implicated (Brown et al., 2000). Moreover, by using animal models, it has been shown that transmission of infectivity can occur via stainless-steel surfaces (Zobeley et al., 1999; Flechsig et al., 2001; Fichet et al., 2004; Yan et al., 2004).

To date, there have been five authenticated incidents of surgical patients suffering from undiagnosed CJD, where the reuse of instruments from these surgical procedures represents a potential risk to other patients. Four of these incidences, in the UK (Mayor, 2003), the USA (Belkin, 2003) and Australia (Zinn, 2000), have exposed individuals within a total cohort of 43 patients to the risk of sporadic CJD (sCJD). A single instance in Canada exposed individuals within a cohort of 71 patients to a risk of variant CJD (vCJD) (Tapp, 2003). The risk of iatrogenic transmission of CJD posed by neurosurgical interventions and invasive procedures involving the lymphoreticular system cannot be
considered insignificant. These studies highlight the ongoing concerns about the level of residue removal that can be achieved by the types of decontamination methods currently employed and indicate that there is a need to develop more effective methods. Recent studies have suggested that a combination of protease and detergent treatments reduce TSE infectivity significantly. The infectivity of bovine spongiform encephalopathy (301V) mouse brain is reduced by a factor of 3 log doses by solution digestion with the modified subtilisin Properase (McLeod et al., 2004). Reduction of steel-bound infectivity has proved more difficult. A three-stage process involving sequential digestion with protease K, pronase digestion and denaturation with hot SDS solution has recently been evaluated by using the mouse intracerebral wire-transmission model. This has been shown to reduce infectivity bound to stainless steel substantially (Jackson et al., 2005).

A more radical generic approach to decontamination than enzymic digestions and chemical treatments would be one relatively unaffected by the chemical complexity or tenacity of adhesion of the contaminants, which could be used to remove all organic deposits. Radio-frequency (RF)-generated gas plasmas are commonly used for surface cleaning and decontamination in the electronics industry and can, in principle, degrade complex biomolecules completely to gaseous products without exposing the metal surfaces to high temperatures or corrosive chemicals (Sugawara et al., 1998). In a preliminary study using a low-pressure Ar:O2 plasma (procedure 1), we demonstrated that tenaciously bound organic material, which resisted conventional decontamination treatments, could be efficiently removed from the metal surfaces of contaminated endodontic files (Whittaker et al., 2004).

In this study, we addressed two related questions. The first was whether RF gas-plasma treatment of surfaces contaminated with a TSE agent would effectively reduce the amount of TSE infectivity. To assess the method, we used intraperitoneally implanted stainless-steel spheres as surrogate surgical instruments, contaminated with the 263K strain of scrapie, to transmit the infection by the peripheral route in hamsters. We elected to use this route of infectivity transmission as it is arguably more relevant to transmission with hot SDS solution has recently been evaluated by using the mouse intracerebral wire-transmission model. This has been shown to reduce infectivity bound to stainless steel substantially (Jackson et al., 2005).

Surgical instruments vary in structural complexity and in the degree and localization of soiling in use. The second question was whether RF gas-plasma treatment could indeed be practical as a routine method for the removal of contamination of instruments in a hospital setting. We evaluated the method on a series of reprocessed surgical instruments. These were intercepted directly after conventional cleaning and sterilization, and examined both before and after gas-plasma treatment by scanning electron microscopy (SEM) and energy-dispersive X-ray analysis (EDX). Our results here showed that a gas-plasma cleaning step, as an adjunct to detergent cleaning, can achieve significant reductions in the amount of biological material adhering to instrument surfaces.

METHODS

Surgical instruments. We examined five used, surgical, stainless-steel, disposable, power-driven orthopaedic bone-saw blades (Howmedica). These disposable blades were not cleaned by a hospital sterile-services department (SSD), but were autoclaved at 121 °C for 15 min, washed in TriGene disinfectant (Medichem International) and rinsed before examination.

We also examined surgical instruments in regular use in a teaching hospital surgical unit. The instruments had been cleaned stringently by conventional hospital procedures, were fully compliant with Quality Management System EN ISO 9002 and judged suitable for reuse for surgical procedures. On examination by SEM, out of a total of 17 randomly selected instruments from a single tray of instruments, 14 showed significant levels of contamination. A further 35 instruments were selected from random trays for detailed examination.

Surface contamination of stainless-steel spheres. The inoculum was prepared as a 20 % (w/v) brain homogenate of the 263K strain of hamster scrapie in 0-32 M sucrose, and had a titre of 107 infectious units in 50 μl of a 1:100 dilution by the intercranial route (Kimberlin & Walker, 1978), a pH of 7.5 and a total protein concentration of 22.5 mg ml⁻¹.

Pre-weighed stainless-steel spheres (type 316, 2 mm diameter; Alfa Aesar) were immersed in 20 μl volumes of freshly prepared inoculum and allowed to dry at room temperature to a constant weight for approximately 3 days. The mean weight of the homogenate dried onto the spheres was 1.1 mg.

Treatment of the stainless-steel spheres. Spheres in group 1 were left untreated. In group 2, the spheres were autoclaved at 157 °C for 18 min, followed by a TriGene disinfectant wash and then rinsed with water. In group 3, they were washed rigorously in TriGene disinfectant and then rinsed in water. In group 4, they were subjected to the RF gas-plasma treatment defined in procedure 3 (see below).

Bioassays. Single spheres were implanted intraperitoneally into individual 6-week-old female hamsters by using an implant needle. Each group comprised five hamsters. The inoculated hamsters were monitored for symptoms of scrapie infection and euthanized once clinical scrapie disease was established (Marsh & Kimberlin, 1975).

Gas-plasma decontamination procedures. Gas-plasma treatments were carried out by using a Plasma-Etch PE-200 (Plasma Etch). Three separate gas-plasma procedures were used in this study. In procedure 1, the instrument temperature was held at 25 °C and an Ar:O2 (1:2) mixture (at 66-7 Pa) was subjected to RF excitation (13.5 MHz) at a power density of >6 mW cm⁻² for 1 h. In procedure 2, the SSD-cleaned instruments were subjected to procedure 1 and then sonicated in distilled water. In procedure 3, the SSD-cleaned instruments were soaked in distilled water for 30 min and subjected to procedure 1 while still wet (Baxter et al., 2005).

Detection and analysis. SEM inspection of the instruments was carried out by using a Philips XL30CP Microscope, operating at 20 kV, with resolution of greater than 5 nm. Secondary electron (SE) and backscatter electron (BE) imaging enabled regions with a mean atomic number difference >0.1 to be resolved.
EDX was carried out in the SEM by using an Oxford Instruments Isis 300 X-ray analyser, capable of detecting elements of atomic number greater than 6. The imaged area (5 µm) was subjected to elemental analysis to a depth of approximately 3 µm ($V = 6 \times 10^{-17} \text{ m}^3$) by using an X-ray fluorimeter capable of detecting elements comprising $\geq 0.1\%$ of the mass of the sample volume.

RESULTS

TSE infectivity

Fig. 1(a and b) shows an SEM image of a stainless-steel sphere that was contaminated experimentally with a brain homogenate of the 263K strain of scrapie. Spheres were coated to a depth of approximately 100 µm corresponding to a mean dry-tissue weight of 1.1 mg per sphere. As expected, EDX analysis of the contamination indicated material that contained carbon, nitrogen, oxygen, sulfur, calcium and magnesium. Analysis by bioassay of the untreated 263K-contaminated spheres implanted intraperitoneally into Syrian hamsters resulted in terminal disease after 92 ± 3 days (Table 1, group 1).

The different cleaning methods used on the control spheres were shown by SEM imaging to result in significantly different levels of decontamination. SEM images of the spheres that had been autoclaved before being washed showed randomly dispersed clean patches, with large areas of residual contamination (Fig. 1c and d). Cleaning by this method resulted in the most inconsistent disinfection and,
consequently, the incubation period of the disease varied appreciably (202 ± 28 days; Table 1, group 2). Detergent washing of spheres resulted in only a few small areas of residual contamination remaining, each of approximately 20–30 μm in diameter (Fig. 1e and f), and there was no transmission of disease (Table 1, group 3).

No residual contamination on the RF gas-plasma treated spheres could be detected by SEM (Fig. 1g and h). EDX analysis with a detection limit of 5–6 fmol carbon (0.5 amol of a typical 30 kDa protein) within the sample volume indicated that the treatment had removed all of the experimental contamination, and analysis by bioassay showed no transmission of infectivity (Table 1, group 4).

**Surgical instruments**

To test the feasibility of using gas plasma alone as a cleaning method for heavily contaminated instruments, we examined a number of single-use, disposable orthopaedic bone-saw blades. These had been used in conventional surgery and, for reasons of safety of handling within the laboratory, the instruments were autoclaved before being washed stringently in a manner similar to that used for washing the stainless-steel spheres. By using SE imaging, we observed large deposits of material (Fig. 2a). EDX analysis indicated that the material contained carbon, nitrogen, oxygen, sulfur, calcium and phosphorus. As calcium and phosphorus were detected only as trace components of residues on the reprocessed instruments, it seemed likely that this was due to the mineral matrix of microscopic bone fragments adhering to the blades. The RF gas-plasma treatment removed all of the organic deposits, although calcium, phosphorus and some carbon remained in some surface residues (Fig. 2b). This was reasonable, as we would expect a residue of calcium carbonate and phosphates to remain after oxidation of the bone matrix. These salt deposits were removed easily by brief sonication of the instruments in sterile water (Fig. 2c), although sonication alone, without a prior plasma treatment, had little effect (data not shown).

We subsequently examined a total of 52 stringently cleaned surgical instruments that had been reprocessed by conventional SSD procedures. Forty-nine of these instruments, which are listed in Table 2 (columns 1 and 2), were subjected to increasingly rigorous RF gas-plasma decontamination procedures (procedures 1–3). Of 17 instruments examined from a single tray, prior to plasma treatment, 14 (82%) were shown by SEM to have multiple areas of contamination, varying from small spots of <1 mm in diameter to substantial areas of over several square millimetres. Typical examples of the instruments found to be harbouring residual contamination, which were subjected to detailed study by SEM and EDX both prior to and after the plasma treatment, were the Allis tissue forceps (B. Braun Medical Ltd) and the De Bakey vascular clamps (Ackermann Instrumente GmbH).

**Allis tissue forceps.** An initial examination of the surface of this instrument by using BE imaging clearly showed distinct areas of contamination of the order of 1–30 μm in diameter (Fig. 3a and b). EDX showed that

Table 1. *In vivo* analysis of infectivity of 263K-contaminated stainless-steel spheres before and after decontamination treatments

<table>
<thead>
<tr>
<th>Treatment of contaminated spheres</th>
<th>No. terminally infected hamsters/total no.</th>
<th>Incubation time ± SD (days)</th>
</tr>
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<tbody>
<tr>
<td>Group 1: no decontamination treatment</td>
<td>5/5</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>Group 2: autoclave followed by detergent wash</td>
<td>5/5</td>
<td>202 ± 28</td>
</tr>
<tr>
<td>Group 3: detergent wash</td>
<td>0/5</td>
<td>466*</td>
</tr>
<tr>
<td>Group 4: RF gas plasma (procedure 3)</td>
<td>0/5</td>
<td>466*</td>
</tr>
</tbody>
</table>

*All animals in these groups were clinically sound when euthanized at 466 days.*

![Fig. 2. SE images of an orthopaedic bone-saw blade. (a) Thick residual deposits on the cutting edge of the blade remaining after washing. (b) Surface of the blade after RF gas-plasma treatment (procedure 1). (c) Decontaminated surface after RF gas-plasma treatment followed by sonication in water (procedure 2).](image-url)
these were organic material containing carbon, nitrogen, oxygen and sulfur. This atomic composition suggested that they consisted principally of proteinaceous material. There was no phosphorus signal, indicating little nucleic acid contamination. Plasma treatment (procedure 1) of the forceps resulted in complete removal of all of the patches of organic contamination (Fig. 3c). EDX of these areas and of random spots on the surface gave no indication of any residual contaminating material.

Table 2. Surgical instruments examined by SEM both before and after decontamination by one of the three RF gas-plasma decontamination procedures

<table>
<thead>
<tr>
<th>Instruments</th>
<th>RF gas-plasma procedure used*</th>
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</thead>
<tbody>
<tr>
<td>Stainless steel (n)</td>
<td>Other metals (n)</td>
</tr>
<tr>
<td>Scissors (2)</td>
<td>Bone saw (1)</td>
</tr>
<tr>
<td>Forceps (1)</td>
<td></td>
</tr>
<tr>
<td>Skin hooks (1)</td>
<td></td>
</tr>
<tr>
<td>Gillies needleholder (4)</td>
<td>Forceps (titanium) (1)</td>
</tr>
<tr>
<td>Forceps (3)</td>
<td></td>
</tr>
<tr>
<td>Toothed clip piece (1)</td>
<td></td>
</tr>
<tr>
<td>Scissors (9)</td>
<td>Bone saw (2)</td>
</tr>
<tr>
<td>Vascular clamps (5)</td>
<td>Scalpel handle (brass) (1)</td>
</tr>
<tr>
<td>Jefferson cannula (4)</td>
<td></td>
</tr>
<tr>
<td>Drill piece (6)</td>
<td></td>
</tr>
<tr>
<td>Sponge holders (5)</td>
<td></td>
</tr>
<tr>
<td>Forceps (5)</td>
<td></td>
</tr>
<tr>
<td>Skin hooks (2)</td>
<td></td>
</tr>
<tr>
<td>Scalpel handle (2)</td>
<td></td>
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</tbody>
</table>

*For details of the RF gas-plasma procedures, see Methods.

De Bakey vascular clamps. The instruments showing the highest levels of residual contamination were all complex implements, where residues build up in sites that are difficult to access by conventional cleaning methods. SE imaging (Fig. 4a and b) and EDX analysis of the vascular clamps clearly showed significant areas that were contaminated by organic residues (containing carbon, nitrogen, oxygen and sulfur) and areas in the crevices and teeth of the instrument where the contamination was from both

Fig. 3. BE images of a pair of Allis tissue forceps. (a) Residual contamination on the surface of the instrument before gas-plasma treatment. (b) Magnified image of the surface shown in (a), showing protein residues in the range of 1–30 μm in diameter. (c) Decontaminated surface after treatment with RF gas plasma (procedure 3).
organic residues and inorganic salts (comprising sodium, calcium, magnesium and chlorine). Soaking alone did not remove any of the debris from the instrument (image not shown); however, when the instrument was soaked and then subjected to the RF gas-plasma treatment (procedure 3), both the organic and inorganic residues were removed completely (Fig. 4c and d).

**DISCUSSION**

Although there is some correlation between incidence of CJD and frequency of surgical intervention (Zerr & Poser, 2004), there has been no solid evidence implicating instruments used in peripheral surgery. However, as the pathogenesis of vCJD differs from that of sCJD and lymphoreticular tissues are involved, it is timely to monitor the efficiency with which TSE disease could be transmitted by contaminated devices via the peripheral route of infection and to examine more exacting methods for the decontamination of surgical instruments.

In this study, we have shown that both stringent washing procedures and RF gas-plasma treatment (using procedure 3) removed contamination from spheres coated experimentally with the hamster-adapted strain of scrapie, reducing the infectivity to below the level detectable by this bioassay. However, by using backscatter SEM and EDX analysis, we found that the washed spheres still showed visible levels of residual contamination and that traces of organic material still adhered to the surfaces. Conversely, the plasma-cleaned spheres appeared, at least to the detection limits of our analysis, to be clear of all traces of contamination. We concluded that, whilst both procedures removed the TSE infectivity, RF gas-plasma treatment appeared to be more effective in the removal of residual debris from stainless steel.

As the washing protocol that we used here for the decontamination of the spheres was effectively that currently in use in clinical environments for the decontamination of surgical instruments, we conducted a study on a sample set of 49 reprocessed instruments (Table 2). These instruments were removed from clinical use after conventional hospital decontamination and examined by SEM and EDX both prior to and after Ar : O₂ plasma treatment. Our results indicated that a significant amount of adhered organic residue remained on the instruments after conventional cleaning. Typically, these residues varied from spots of <1 μm in diameter to irregular areas of several square millimetres. Some indication of the significance of the level of contamination observed may be gleaned from the approximation that a 1 μm² spot of a monolayer of a pure globular protein with a molecular mass of 30 kDa (roughly that of PrP⁰⁰) would contain in the order of 10⁴ protein molecules. This gives cause for concern, particularly for instruments that are reused in neurosurgery and...
ophthalmic procedures, as an estimate for a PrPSc ‘unit of infectivity’ measured by end-point titration is approximately 10^5 molecules (Prusiner, 1991). When the instruments were subjected to the first RF gas-plasma cleaning procedure (Table 2), a single exposure to low-pressure Ar:O₂ plasma substantially diminished the levels of contaminating organic residues on exposed surfaces, but the RF gas plasma alone did not remove inorganic residues. However, when instruments were treated by using RF gas-plasma procedure 3 (Table 2) where the residual contaminants were hydrated by soaking prior to plasma treatment, removal of the debris to below the level of our detection methods could readily be achieved. Studies are currently under way to determine the nature of the physical interaction of the RF gas plasma with the hydrated residues.

This work has shown that gas-plasma treatment can be used effectively to remove residual contamination from the surfaces of metal surgical instruments without apparent damage to the instruments themselves. At present, there is no simple, practical, cost-effective way of distinguishing between PrPSc and other proteins on the surfaces of surgical damage to the instruments themselves. At present, there is no simple, practical, cost-effective way of distinguishing between PrPSc and other proteins on the surfaces of metal surgical instruments without apparent

Debris on processed surgical instruments. We have demonstrated convincingly, both experimentally and on surgical instruments, that soaking together with gas-plasma treatment can decrease residual contamination to a level undetectable by SEM and EDX. Furthermore, gas-plasma treatments may prove especially valuable in the routine cleaning of complex instruments where a build-up of tissue and salt residues in areas inaccessible to normal cleaning processes may create protective sites that could render routine sterilizing procedures ineffective. In this context, we conclude that it is timely to consider the implementation of gas-plasma treatment for the effective decontamination of surgical instruments.

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