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Prion inactivation using a new gaseous hydrogen peroxide sterilisation process

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Summary Prions pose a challenge to decontamination, particularly before the re-use of surgical instruments. They have relatively high resistance to standard decontamination methods and require extreme chemical and/or heat-based treatments for devices used in known or suspected cases of disease. This study investigated the effectiveness of a new gaseous hydrogen peroxide sterilisation process for prions as an alternative low-temperature method. Gaseous peroxide, in addition to known antimicrobial efficacy, was shown to inactivate prions both in in-vitro and in-vivo assays. In contrast to the gas form, liquid peroxide was not effective. The mechanism of action of gaseous peroxide suggested protein unfolding, some protein fragmentation and higher sensitivity to proteolytic digestion. Hydrogen peroxide liquid showed a degree of protein clumping and full resistance to protease degradation. The use of gaseous peroxide in a standard low-temperature sterilisation process may present a useful method for prion inactivation.

Introduction

Decontamination of contaminated, re-usable devices is usually a two-step process consisting of cleaning followed by microbial inactivation (disinfection and/or sterilisation). Micro-organisms vary in their responses to various inactivation techniques and can be classified based on their intrinsic resistance.[1] Bacterial spores, for example, are the most resistant to biocidal methods, while enveloped viruses are considered more sensitive. This classification system is the basis for the recommendations on the safe reprocessing of re-usable

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devices but it has been recently challenged in cases of known or suspected transmissible spongiform encephalopathy (TSE) diseases.[1–3] TSE diseases such as Creutzfeldt–Jakob disease (CJD) and variant CJD (vCJD) are caused by a misfolded form of the prion protein (PrP), so-called PrP^{res}, derived from a host-encoded cellular precursor PrP^{c}. These agents pose a challenge in device reprocessing as they are known to demonstrate high resistance to standard reprocessing methods, and have been shown experimentally and clinically to be transmitted via contaminated surfaces/materials of animal/human origin.[2] In one case, CJD was shown to be transmitted to two patients as a result of contact with contaminated intracerebral electrodes previously used in the brain of a person with the disease. Although the initial reprocessing of the device may not have been optimal, the electrodes were shown to transmit the disease some years later when introduced into an experimental animal, despite multiple cleaning and sterilisation cycles.[4] Retrospective studies have proposed at least four other cases of iatrogenic transmission.[5] It is presumed in these cases that the routine reprocessing procedures were also insufficient to eliminate infectivity.

In 2000 the World Health Organization (WHO) published infection control guidelines for known or suspected TSE cases.[2] These guidelines include the identification of risk groups, special consideration of surgical procedures associated with high-risk tissues (e.g. brain and spinal cord), vigorous cleaning and specialised decontamination practices (e.g. extended immersion in 1 N NaOH). However, more recent reports on prion disease and surface decontamination would suggest further considerations are warranted. Although device re-use can be avoided in known cases of TSEs, asymptomatic cases are a concern, considering their particularly long incubation times.[3] In the case of the TSE disease Kuru, the incubation periods in men range from 39 to 56 years and may even be up to 7 years longer.[6] Tissue infectivity and the detection of the prion protein is also known to vary depending on the disease, with particular concern in many peripheral tissues and even blood.[7] It is also estimated that the average occurrence of the most prevalent TSE in humans, CJD, is in the order of 1.5 per million population worldwide and more than 80% of these occur sporadically.

Overall, the risks of disease transmission remain low and guidelines are employed on the prevention of iatrogenic transmission to further reduce this risk. In hospitals, these universal measures consist of an emphasis on effective cleaning. The efficiency of prion cleaning from contaminated surfaces has been investigated only recently, with some surprising results. Prion infectivity has been shown to have high affinity and to bind tightly to surfaces, making it difficult to remove.[8,9] Despite this, some cleaning methods have been shown to reduce infectivity, including some that degrade the prion protein, but conversely others have been shown to increase the resistance to subsequent inactivation.[8–10] Further, despite routine cleaning of devices, studies have shown that residual levels of protein, below visual detection, can remain on device surfaces.[11,12] Other methods, such as immersion in 1 M NaOH or 2% NaOCl or autoclaving at 134 °C for 18 min, can damage many instruments and are unregulated.

Recent studies have suggested that prions may be more susceptible than previously thought to gentler methods, including enzyme degradation, heating under hydrated conditions and gaseous hydrogen peroxide.[9,13] Hydrogen peroxide is widely used in liquid and gaseous form for biocidal applications. Previous studies showed that peroxide could be effective in reducing prion infectivity, but only in true gaseous form.[8,9] In this report, we extend the study of gaseous peroxide in a new vacuum sterilisation process for efficient prion inactivation.

Methods

Infectious materials and surface contamination

The hamster-adapted scrapie strain 263 K was stabilised and propagated as previously described.[9] Similarly, bovine spongiform encephalopathy (BSE) strain adapted to conventional mice (6PB1 strain) was also studied.[14] After two serial passages of 6PB1 in transgenic mice overexpressing murine PrP (Tga20), the strain was so-called TGB1 and was also evaluated. Brain homogenates of various regions of the brain were used for studies isolated from BSE in cattle- and human-derived samples from vCJD and sCJD cases.[15] All of them were previously confirmed as positive cases in biochemistry analysis.

The protocol of contamination of stainless steel wires as a test model was previously described.[9,13]

In-vitro methods

Most in-vitro studies were conducted with the 263 K and 6PB1 strains. Methods used for liquid or gas exposures were previously described.[9]

For suspension studies, liquid hydrogen peroxide at 0.2, 3, 30 or 60% v/v were mixed in a ratio 4:1

with 20% brain homogenate at room temperature for 15 min. Samples were then immediately purified. It should be noted that no chemical neutralisation was conducted in these studies due to potential interference with the test method. PrP^{res} was purified with the Bio-Rad kit.[9] After purification (digestion by proteinase K (PK) and concentration through centrifugation according to the manufacturer), the resulting pellet was resuspended in Laemmli buffer before denaturation at 100 °C for 5 min. After a second centrifugation, the supernatant was collected (corresponding to the solubilised protein), separated by 12% sodium dodecyl sulphate—polyacrylamide gel electrophoresis and prion proteins detected by western blot analysis. Immunoblotting was performed with various mouse monoclonal antibodies raised against the PrP protein: Bar-210, N-terminal (codons 26—34); SAF-37 octarepeat region (codons 59—89); SHA31 (codons 145—152), SAF-60 (codons 157—161) and SAF-70 (codons 156—162) detecting the protein core; and Pri-917 (codon 216—221) the C terminal region. Immunoreactivity was visualised with a peroxidase-conjugated goat anti-mouse antibody (Southern Biotechnology Associates, Birmingham AL, USA) followed by chemiluminescence (ECL, Amersham, Orsay, France) and autoradiography.

Gaseous hydrogen peroxide sterilisation

A gaseous peroxide sterilisation process was developed and tested for the reprocessing of re-usable medical devices (STERIS Corporation, Mentor, OH, USA). The process was designed to sterilise devices under vacuum in a dedicated chamber. For in-vivo studies, stainless steel wires contaminated with prion—brain homogenates were placed into plastic plates and exposed to the vacuum process at 30 °C for three or six pulses.[9] Each pulse consisted of pulling a vacuum (to 0.4 kPa), vaporisation of 1.2 g of 35% (w/w) hydrogen peroxide, exposure for 5 min and exhausting to atmospheric pressure. The peroxide gas concentration was ~2 mg/L. Following exposures, the chamber was aerated by drawing a vacuum to 1.3 kPa for 1 min and exhausting in triplicate.

Liquid hydrogen peroxide exposures

For surface in-vitro exposures, brain homogenates dried onto glass slides were immersed in dilutions of liquid peroxide at 20 °C for 15 min, rinsed twice in water, extracted and analysed as described above (using PK at 8 μg/mL). [9]

For liquid in-vivo exposures, contaminated wires were directly exposed to 6% v/v hydrogen

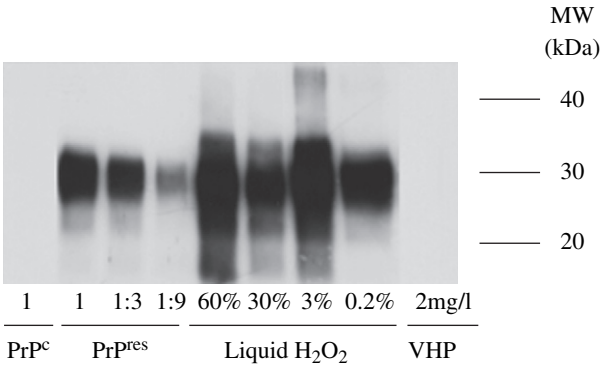

Figure 1 In-vitro analysis of the effects of hydrogen peroxide liquid or gas on prion proteins (PrP) adsorbed onto surfaces. Note: 2 mg/L of VHP is ~1500 ppm and 1% liquid peroxide is ~10 000 ppm. MW, molecular weight; VHP, vaporised hydrogen peroxide.

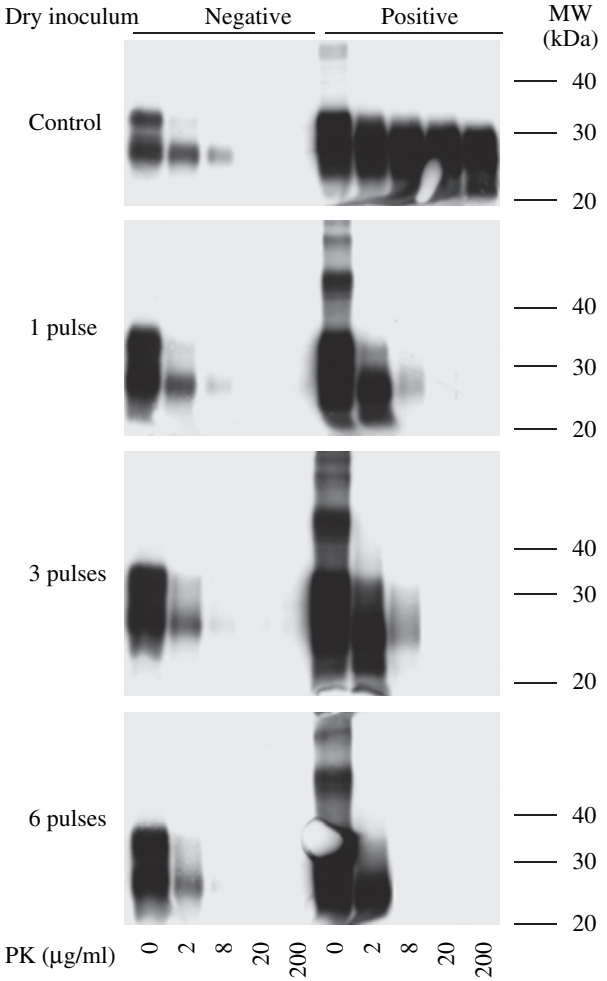

Figure 2 In-vitro comparison of the effects of gaseous peroxide under vacuum. Each exposure pulse equals 5 min contact time. Positive and negative columns refer to prion-contaminated or uninfected homogenates, respectively, dried onto test surfaces. MW, molecular weight; PK, proteinase K.

peroxide at 20 °C for 1 h and rinsed with water as previously described.[9]

Results

In-vitro investigations

The effects of hydrogen peroxide liquid and gas on the prion protein (scrapie 263 K) were investigated by western blot analysis (Figure 1). Exposure to liquid hydrogen peroxide (at 60, 30, 3 and 0.2%) demonstrated a marked increase of PK resistance and the detection of supplementary higher molecular weight PrP^{res}, suggesting clumping on exposure to liquid peroxide. Clumping was particularly evident on exposure to higher concentrations, but none of the liquid exposures significantly decreased the detection of PrP^{res}, suggesting little or no inactivation. In contrast, exposure to VHP (corresponding to three peroxide pulses under vacuum at ~2 mg/L) rendered the infectious material fully sensitive to PK treatment.

The effect of VHP on the protein was further tested by exposure to one to six pulses (Figure 2). A similar effect was observed for all conditions

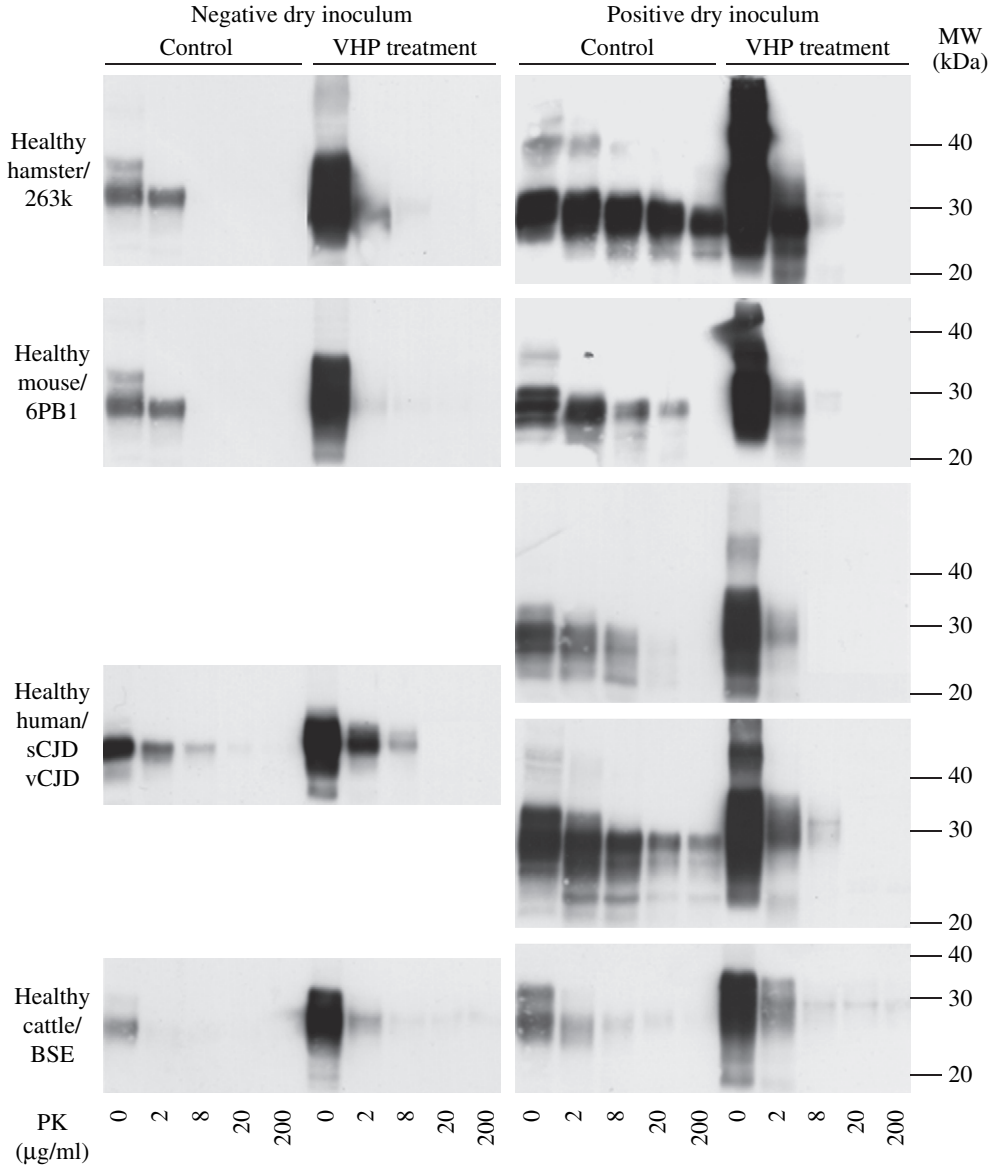

Figure 3 In-vitro analysis of the effects of VHP against various prion protein (PrP) strains. 263 K (scrapie, hamster model), 6PB1 [bovine spongiform encephalopathy (BSE), mouse model], sporadic (S) Creutzfeldt—Jakob disease (CJD) (human), variant (v) CJD (human) and BSE (cattle). All strains were detected with the SAF-60 antibody, which detects the central (and resistant) core of the PrP. Positive and negative columns refer to prion-contaminated or uninfected homogenates, respectively, dried onto test surfaces. VHP, vaporised hydrogen peroxide; MW, molecular weight.

tested, with an increase of immunoreactivity and PrP^{res} sensitivity to proteolytic digestion. This was also observed with other prion strains, including brain homogenates from a laboratory mouse-adapted BSE strain (6PB1), samples from BSE-infected cattle, a human sCJD and a human vCJD case (Figure 3). It was interesting to note that the 263 K scrapie strain showed the greatest resistance to PK treatment in these experiments whereas loss of resistance to proteolytic degradation was equal for all strains after VHP exposures (Figure 3, control).

Mechanisms of action

To better understand the mechanism of action against prion (hydrophobic) proteins, various antibodies were used to study the effects of vacuum exposures with the 263 K (scrapie) and 6PB1 (BSE) strains. These antibodies bind to various sections of the protein, from the N- to the C-terminal regions (Figure 4). In the positive (untreated) controls, PrP^{res} was still visible and demonstrated a consistent signal from the SAF-60 region to the C-terminal part to at least at 8 μg/ml concentration of PK, while the intensity of the band decreased when using antibodies directed against the N-terminal and octapeptide regions of the protein. Indeed, the N-terminal region seems to be more sensitive than the core and the C-terminal part of the protein to enzyme digestion. After VHP treatment, the signal of PrP^{res} totally disappeared at 8 μg/ml of PK. Interestingly, after VHP exposure and treatment at 2 μg/ml PK, both the N-terminal and C-terminal regions of the protein were more sensitive to protease degradation compared to the core of the protein. Moreover, immunoreactivity in the absence of PK treatment was only observed when using antibodies against the core of the protein (SHA31 or SAF-60 or SAF-70) (Figure 5). These results correlate with previous studies showing that bovine serum albumin (a hydrophilic protein) was fragmented on exposure to VHP.[15] Again the 263 K prion strain appeared to be the more resistant compared to 6PB1 under the test conditions used.

In-vivo investigations

Prion inactivation with the gaseous peroxide process was confirmed in vivo using different animal infectivity models (Table I). Gaseous peroxide was equally effective in three- and six-pulse cycles, demonstrating no infectivity in any of the animals tested in the scrapie (hamster) model. The results were identical for the BSE models tested in a three-pulse cycle. The process was effective without any pre-cleaning, considering that the test devices were directly contaminated with 10% infected brain homogenate under worst-case conditions. In contrast, exposure to 6% liquid peroxide demonstrated little to no reduction in infectivity ($\leq$1 $\log_{10}$) in the scrapie model. Overall, the in-vivo results correlated well with the in-vitro observations, suggesting loss of infectivity due to protein changes and some fragmentation on exposure to gaseous peroxide.

Discussion

Hydrogen peroxide, in liquid and gaseous form, is one of the most widely used biocidal chemicals.[16]

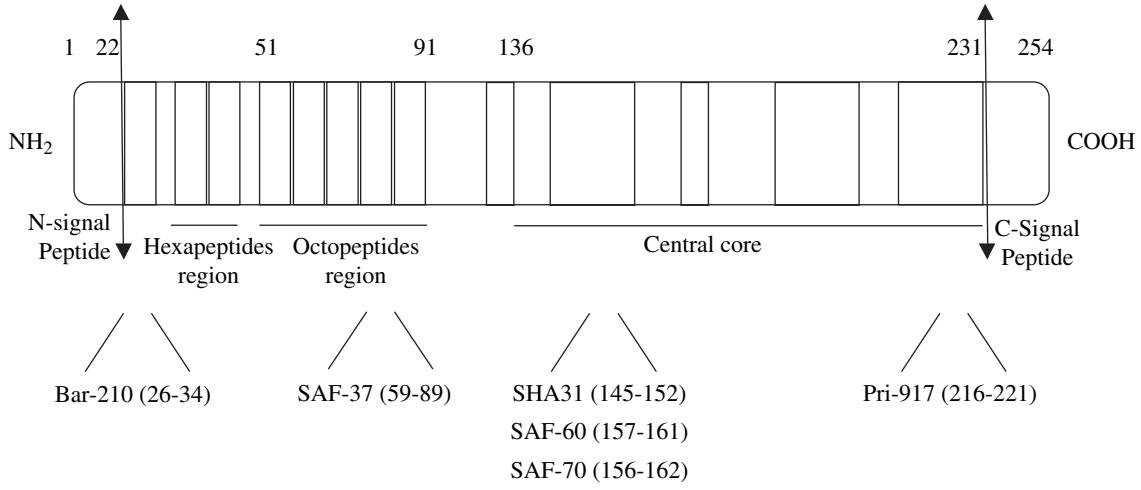

Figure 4 Mechanism of action of gaseous peroxide using various antibodies on prion laboratory strains 263 K and 6PB1. Schematic of the primary structure of the prion protein (PrP) with the estimated antibody binding sites.

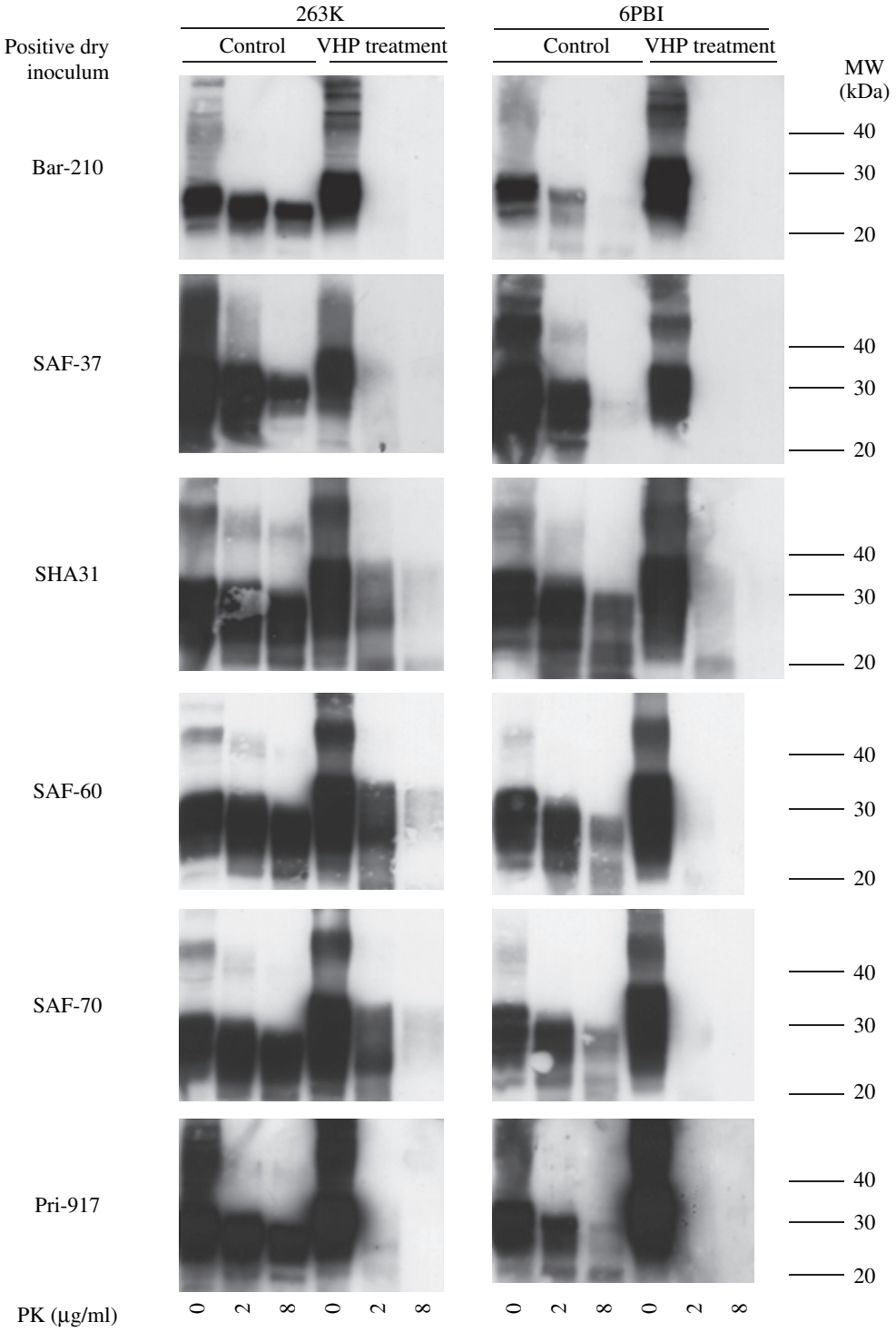

Figure 5 In-vitro analysis of the effect of VHP along the protein from 263 K and 6PB1 prion strains. VHP, vaporised hydrogen peroxide; MW, molecular weight.

It is considered relatively stable, can demonstrate broad-spectrum efficacy and readily degrades into water and oxygen. For liquid applications, it is used directly on surfaces (including the eye and skin) or in formulation (e.g. hard surface disinfectants). More recently, many liquid-based fogging or condensed hydrogen peroxide-based systems have also been used for hospital fumigation.[17] Hydrogen peroxide antimicrobial activity is markedly increased when in a gaseous phase. Gaseous peroxide is rapidly effective at concentrations as low as 0.1 mg/L against various pathogenic micro-

Table I The in-vivo decontamination results with gaseous and liquid hydrogen peroxide using the contaminated-wire model and various prion strains

Positive control dilution	263K scrapie model				6PB1 BSE (conventional) model				TGB1 BSE (transgenic) model			
	Transmission rate (%)	Total deaths/ total number	Duration of disease[a] (days)	Log_{10} reduction factor[b]	Transmission rate (%)	Total deaths/ total number	Duration of disease[a] (days)	Log_{10} reduction factor[b]	Transmission rate (%)	Total deaths/ total number	Duration of disease[a] (days)	log_{10} reduction factor[b]
1×10^{-3}	100	12/12	117 (6)		100	7/7	303 (44)		100	10/10	230 (26)	
1×10^{-5}	92	11/12	201 (60)		71	5/7	278 (50)		56	5/9	379 (75)	
1×10^{-7}	0	0/12	>540		0	0/2	>540		12.5	1/8	198	
1×10^{-1} wire-contaminated and treated												
6% liquid H_2O_2 (1 h, 20 °C)	100	11/11	114 (13)	1	—	—	—	—	—	—	—	—
2 mg/L gaseous H_2O_2, 3 pulses (30 °C)	0	0/8	>540	>5.5	0	0/9	>540	>5.5	0	0/9	>540	>5.3
2 mg/L gaseous H_2O_2, 6 pulses (30 °C)	0	0/8	>540	>5.5	—	—	—	—				

[a] Only animals found dead in the study were taken into account in the calculation of the disease duration.

[b] Estimated based on comparison with a series of positive control dilutions for each strain. All the treated wires were exposed at the highest positive dilution (1×10^{-1}). Calculation of LD_{50} according to the method of Reed and Muench indicates that infectivity of wires was 5.5 LD_{50} in the 263K scrapie model, 5.5 LD_{50} in the 6PB1 bovine spongiform encephalopathy (BSE) (conventional) model and 5.3 LD_{50} in the TGB1 BSE (transgenic) model. Previous testing (not shown) determined that the initial level of wire contamination was identical when using undiluted or 10% brain homogenates; therefore, it is expected that the levels of reduction are at least one order of magnitude higher than those indicated above.

organisms.[18] It also demonstrates good material compatibility, including on plastics, elastomers and electrical components.[17,19] The technology is widely used for various industrial, pharmaceutical applications, and in research on surface disinfection and sterilisation.[20]

Hydrogen peroxide is an oxidising agent that reacts with various microbial components (including proteins, lipids and nucleic acids), resulting in a loss of structure and function and microbial death.[16,18] Peroxide gas may be more effective than liquid for many reasons, including increased instability of the biocide with greater reactivity (e.g. presence of other oxidising species such as hydroxyl radicals) and greater penetration to target molecules. This certainly plays a role in the efficacy against proteins (such as toxins and prions), with unfolding and degradation observed with gaseous but not liquid peroxide. Direct exposure to liquid peroxide (including condensation under saturated vapour conditions) causes formation of multimers and other soil constituents, which may protect target pathogens and prevent penetration of the biocide. Indeed, this mechanism has been shown to protect viable micro-organisms under some liquid/condensed peroxide process conditions (G. McDonnell, unpublished results). These effects may partially explain decreased efficacy in the presence of soil and little or no efficacy against prions on exposure to a condensed hydrogen peroxide—plasma sterilisation processes.[8,21] In contrast, the efficacy of true gaseous peroxide processes under atmospheric and vacuum conditions has now been verified.[9] Atmospheric exposures (at 1—1.5 mg/L for 3 h) did show a dramatic reduction in infectivity ($\sim$4.5 $\log_{10}$ reduction) and only completely effective when combined with enzymatic pre-cleaning.[9] Exposures under vacuum conditions were found to be more efficient, demonstrating no infectivity in vivo in the absence of cleaning. This may be expected as the gas demonstrates greater penetration under vacuum and efficacy at higher concentrations (2—2.5 mg/L). It is important to note that the efficacy of liquid peroxide could be improved in combination with other formulation chemicals and deserves further investigation.

The efficacy of peroxide gas was verified against various prion strains, both in vivo and in vitro. The intrinsic resistance of different strains of prions in inactivation processes has been investigated; some reports suggest variable strain resistance, others suggest differences from host factors, including the brain material itself.[13,22] Gaseous hydrogen peroxide was shown to be equally effective in vitro against five different strains of prions in various brain homogenate preparations from cattle, human, hamster and mouse sources, although differences in sensitivity to protease digestion were observed; in this case, the scrapie 263 K strain was the most resistant and a similar phenomenon was also observed with other treatments.[23] In-vivo infectivity studies confirmed the efficacy of peroxide gas against both the scrapie and BSE strains.

Overall, the low-temperature hydrogen peroxide gas process may be a useful technology for reducing the risks associated with prion-contaminated devices and other surfaces. As for all infectious agents, the goal should be to employ universal precautions, in particular when associated with prion contamination. In the past, this has not been possible due to the harsh thermal and/or chemical decontamination processes recommended. However, with the development of new processes that are effective at removing and inactivating prions, it would seem likely that such precautions will be practical and widely used in the future.

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Conflict of interest statement
All authors had full access to all data and had responsability for the decision to submit for the publication.

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