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Hydrogen Peroxide Gas Plasma Sterilizer Combined with Dielectric Barrier Discharge and Corona Discharge Inactivates Prions

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Abstract: Prions are highly resistant to physical or chemical damage, although previous studies have shown that STERRAD[®], a hydrogen gas plasma sterilizer using radiofrequency (RF) discharge, has an inactivation effect. Here, the effect of hydrogen peroxide gas combined with dielectric barrier discharge (DBD) plasma and corona discharge plasma using a RENO-S130 sterilizer on scrapie prions was examined. Scrapie prion-infected mouse brain homogenate was air-dried on a cover glass, sealed in a Tyvek pouch, and subjected to RENO-S130 treatment using either non-lumen mode (28 min) or Eco mode (45 min) with hydrogen peroxide gas derived from 50% hydrogen peroxide. Control (untreated) samples were prepared on a cover glass using the same procedure but without exposure to RENO-S130. PrPres (proteinase K (PK)-resistant prion protein), an index of the conformational variant of prion protein (PrP^{Sc}), was decreased by treatment with RENO-S130 under both modes of operation. Specifically, PrPres was identified after the 1st and 2nd cycles of protein misfolding cyclic amplification (PMCA) in control samples but was below the detection limit in RENO-S130-treated samples. A bioassay showed that treatment of prions with RENO-S130 (non-lumen or Eco mode) significantly prolonged mouse survival time. Taken together, these findings show hydrogen peroxide gas combined with DBD/corona discharge plasma can inactivate prions by reducing prion propagation and prion infectivity. This treatment is potentially applicable to the sterilization of prion-contaminated heat-sensitive medical devices.

Keywords: corona discharge; dielectric barrier discharge; hydrogen peroxide gas plasma; prion; sterilization



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1. Introduction

Transmissible spongiform encephalopathies (TSEs), also referred to as prion diseases, are responsible for deadly neurodegenerative disorders including human prion diseases such as Creutzfeldt–Jakob disease (CJD), fatal familial insomnia (FFI), and Gerstmann–Sträussler–Scheinker syndrome (GSS) as well as animal prion diseases such as bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, and scrapie in goat and sheep [1]. Prion diseases are transmissible and can be caused by infection or inheritance, while most human prion diseases (85% to 90%) are sporadic and of unspecified origin [1,2]. The prion agent is highly resistant to inactivation and therefore poses a risk to public health. Indeed, environmental and food contamination with prions may play a role in its transmission.

Prion diseases are a type of proteopathy caused by an infectious particle (prion), whose main component comprises an abnormal isoform of prion protein (PrP^{Sc}) [1]. Prion transmission can be achieved by the conformational change of the cellular isoform of prion protein (PrP^C), which is mainly found in neurons, into PrP^{Sc} [3]. The accumulation of

PrP^{Sc} in brain tissue causes neurological symptoms such as a type of dementia, which eventually results in death [3]. Because prions are infectious, the conversion of PrP^C to PrP^{Sc} is a crucial step in the transmission of prion diseases [4,5]. Protein misfolding cyclic amplification (PMCA) is a laboratory based procedure that mimics the PrP^C-PrP^{Sc} conversion to amplify PrP^{Sc} in vitro [6,7]. As such, PMCA can be used to evaluate the efficiency of prion decontamination methods [8–10].

Prions are notoriously difficult to inactivate using standard disinfection methods such as exposure to ultraviolet (UV) light, γ -rays, or autoclaving at 121 °C for 20 min [11–13]. There are some effective reagents against prions including treatment with 1 M NaOH, 2% NaOCl, or sodium dodecyl sulfate (SDS), as well as autoclaving at 134 °C for 18 min [14]. Unfortunately, these procedures can damage medical devices, especially heat-sensitive pieces of equipment. Thus, a safe and efficient inactivation method for prions is needed that is suitable for decontaminating medical instruments to protect public health.

STERRAD[®] (Advanced Sterilization Products (ASP), Johnson & Johnson) is a hydrogen peroxide gas plasma sterilizer that is widely used for prion inactivation of heat-sensitive medical instruments [13]. Although STERRAD[®] generates gas plasma by radio frequency (RF) discharge, RF plasma is only used for the removal of hydrogen peroxide gas, and not for sterilization purposes [15,16]. Indeed, the presence or absence of RF gas plasma did not affect the inactivation effect of the instrument [16–18].

Hydrogen peroxide gas and/or plasma are known to be effective for the sterilization of heat-sensitive medical devices [19–21]. Studies using hydrogen peroxide gas sterilizers and gas plasma instruments have evaluated the inactivation effects against a range of pathogens [22–27], including prions [28–30]. However, to date, no studies have investigated the effect of hydrogen peroxide gas plasma combined with dielectric barrier discharge (DBD) and corona discharge on the inactivation of prion agents.

These considerations prompted us to study the effectiveness of hydrogen peroxide gas plasma combined with DBD and corona discharge as a novel inactivation method for prions. Here, we investigated whether mouse scrapie prion can be inactivated by using the hydrogen peroxide gas plasma instrument RENO-S130 (Renosem Co., Ltd., Gyeonggi-do, Korea). PMCA was used to analyze the proliferation activity of PrP^{Sc} and a mouse bioassay was used to assess prion infectivity following treatment with the device.

2. Materials and Methods

2.1. Brain Homogenates Derived from Mice Infected with Prion

C57BL6J mice were infected with mouse-adapted scrapie (Chandler strain) and the brains of terminally diseased mice were collected and suspended in phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, CA, USA) (final concentration, 10% (*w/v*)). The suspension was then put through a 28-G injection needle before freeze-thawing at −80 °C.

2.2. Hydrogen Gas Plasma Instrument RENO-S130

The RENO-S130 instrument (Renosem Co., Ltd., Gyeonggi-do, Korea) is composed of a DBD plasma box, sterilization chamber, and corona discharge plasma box (Figure 1) and performs three processes: (1) a depressurization procedure, where the chamber is depressurized from 760 Torr to 0.5 Torr; (2) sterilization process, consisting of a hydrogen peroxide injection step, a DBD plasma step at 760 Torr (non-lumen mode) and at 760–988 Torr (Eco mode), a corona discharge plasma step at 0.5–760 Torr, and a further depressurization step; and (3) ventilation. In total, there are two modes of sterilization process: non-lumen mode and Eco mode, both of which use hydrogen peroxide gas derived from non-concentrated 50% hydrogen peroxide [31], while the time of sterilization process of Eco mode is longer than the non-lumen mode. One cycle of Reno-130 treatment includes two initial depressurization processes, and two sterilization processes below 60 °C, followed by one ventilation process. The total process time is 28 min (two initial depressurization processes: total 5 min; two sterilization processes: total 18 min; one ventilation process: total 5 min) for non-lumen

mode and 45 min (two initial depressurization processes: total 5 min; two sterilization processes: total 35 min; one ventilation process: total 5 min) for Eco mode.

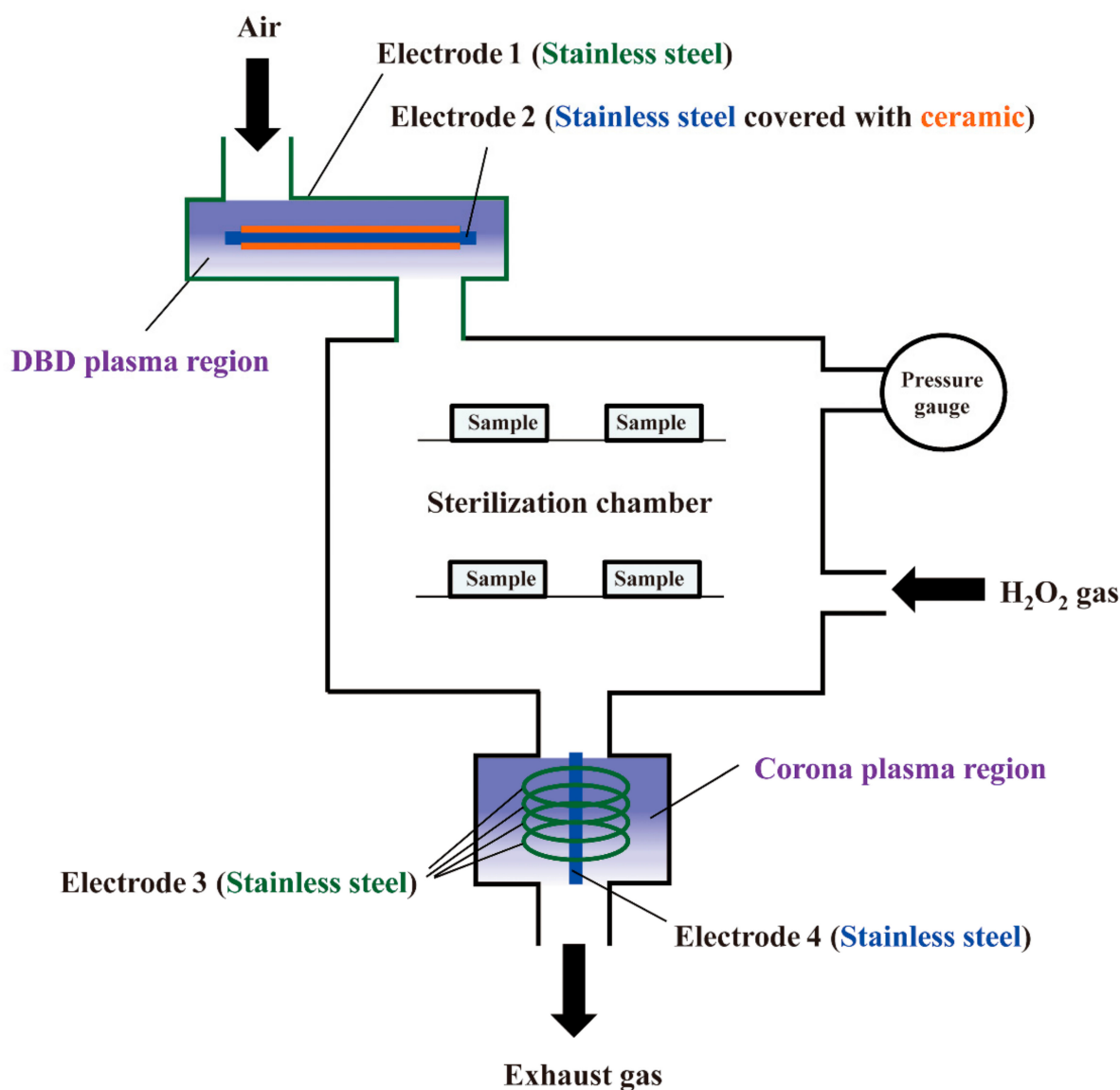


Figure 1. Schematic illustration of the hydrogen peroxide gas plasma instrument (RENO-S130) used to investigate prion inactivation. Electrode 1 (stainless steel) and electrode 2 (stainless steel covered with ceramic) are located in the dielectric barrier discharge (DBD) plasma region and air input flow (flow rate of 5~10 L/min) was applied. The sterilization chamber box is located next to the DBD plasma region. The pressure of the sterilization chamber box was monitored using a pressure gauge. The discharge voltage V_{pp} (peak-to-peak voltage) was set to 22 kV and a frequency 15 kHz was applied. For the corona discharge plasma, the discharge voltage V_{pp} was 14~34 kV and the frequency was 15 kHz. An aliquot (10 μ L) of 10% scrapie prion (Chandler strain)-infected mouse brain homogenate was air-dried on a cover glass and sealed in a Tyvek pouch. Samples were then placed in the sterilization chamber. H_2O_2 : hydrogen peroxide.

2.3. DBD Plasma Generation

The DBD plasma box comprises two electrodes and a high voltage power supply. Electrode 1 is made of stainless steel (Figure 1). Electrode 2 is made of stainless steel covered with ceramic and positioned at an interval of 3~7 mm from electrode 1. For the DBD plasma step, a high voltage was applied using an alternating power supply (V_{pp} (peak-to-peak voltage) of 34 kV; frequency of 15 kHz; NTO-1501, NT Electronics).

2.4. Corona Discharge Plasma Generation

The corona discharge plasma box comprises two electrodes and a high voltage power supply. Stainless steel electrode 3 and electrode 4 were positioned 3~6 mm apart (Figure 1). In the corona plasma step to generate an alternating current (AC) corona discharge, a high voltage electrode was given from an alternating power supply (V_{pp} of 34 kV; frequency of 15 kHz; NTO-1501, NT Electronics). The RENO-S130 eliminate hydrogen peroxide gas was below 1 ppm by corona discharge with a filter made of charcoal and aluminosilicate. Exhaust gas was collected using $TiOSO_4$ in a midjet fritted-glass impinger and measured by a standard method (No. ID-126-SG) of Occupational Safety and Health Administration (OSHA), part of the United States Department of Labor [32]. The resulting hydrogen peroxide concentration was below 0.02 ppm.

2.5. Measurement of Voltage and Current Waveforms

Voltage and current waveforms were measured using an oscilloscope (MSO-X 2012A, Agilent Technologies, Santa Clara, CA, USA) with a high-voltage probe (B025369, Tektronix, Beaverton, OR, USA).

2.6. Treatment of Prions with the RENO-S130 Device

An aliquot (10- μ L) of 10% (*w/v*) prion-infected mouse brain homogenate was air-dried on a cover glass (Matsunami Glass Ind., Ltd., Osaka, Japan). The cover glass was put into a Tyvek Pouch (Renosem Co., Ltd.) and positioned in the RENO-S130 hydrogen peroxide gas plasma sterilizer. Control (untreated) samples were prepared using the same procedure but without exposure to RENO-S130. Following treatment, the resultant samples were solubilized in 100 μ L of PBS.

2.7. Prion Inoculation

Mouse bioassays were performed to investigate the changes in infectivity after RENO-S130 treatment (non-lumen or Eco mode). An aliquot (20- μ L) of sample retrieved from each dried spot on the cover glass in 100 μ L of PBS after RENO-S130 treatment were intracerebrally injected into C57BL/6J mice using a microsyringe. Mice were subsequently monitored for signs of prion disease (tremors and ataxia) during the maintenance period. The mice were housed in accordance with standard animal care protocols and sacrificed according to guidelines of the University of the Ryukyus.

2.8. Western Blot Analyses

The protein concentration of each sample retrieved from the cover glass was determined using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples were normalized to equivalent protein concentrations (100 μ g protein per 50 μ L) prior to Western blot analysis. Briefly, the samples were incubated with 20 μ g/mL of proteinase K (PK) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 60 min at 37 °C for the detection of PrPres (PK-resistant prion protein), which is an indicator of PrP^{Sc} [33]. Untreated samples (no PK) were analyzed to estimate total prion protein (PrP), comprising PrP^C and PrP^{Sc}. Samples were diluted 1:1 with 2 $\times$ SDS loading buffer (90 mM Tris/HCl pH 6.8, 2% (*w/v*) SDS, 20% (*v/v*) glycerol, 0.02% (*w/v*) bromophenol blue, and 10% (*v/v*) 2-mercaptoethanol). The PK reaction was quenched by incubating the samples at 100 °C for 10 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in a 12% acrylamide gel as described previously [34]. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ, USA) using a semidry blotter (Bio-Rad Laboratories, Inc.). Membranes were incubated with 5% (*w/v*) skimmed milk (Wako Pure Chemical Industries, Ltd.) for 1 h at 37 °C and then for 1 h at 37 °C with anti-PrP antibody SAF83 (SPI Bio, Paris, France) in PBS-Tween (0.1% (*v/v*) Tween 20) containing 0.5% (*w/v*) skimmed milk. Blots were then thoroughly rinsed (3 $\times$ 10 min) in PBS-Tween and immersed in horseradish peroxidase (HRP)-labeled anti-mouse immunoglobulin 2nd antibody (Jackson ImmunoResearch, West Grove, PA,

USA) in PBS-Tween containing 0.5% (*w/v*) skimmed milk for 60 min at 37 °C. After that, the blots were thoroughly rinsed in PBS-Tween (3×10 min). Reacting bands were detected with enhanced chemiluminescence (ECL) reagent (Amersham Biosciences) and Ez-Capture MG (ATTO Corp., Tokyo, Japan).

2.9. PMCA

PMCA amplification was carried out as described previously [34]. Briefly, samples retrieved from the treated spots on the cover glass were analyzed by PMCA in a capped and sealed polystyrene tube using 10% C57/BL6J mouse brain homogenates in PBS containing 1% Triton X-100, 4 mM EDTA, and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) as the PrP^C substrate. Each round of PMCA comprised 40 cycles of sonication (5×3 s pulse oscillations separated by 1 s), prior to a final step at 37 °C for 1 h with slight shaking. The resultant products after the 1st round of PMCA were diluted 1:10 with the PrP^C substrate, and then subjected to a further 2nd round of PMCA. Samples were treated with or without PK and then subjected to Western blotting to detect PrPres and total PrP, respectively.

2.10. Statistical Analyses

The statistical analysis of Kaplan-Meier survival curves was accomplished by using the log-rank test in the prion-inoculated animal bioassay. A level of statistical significance was 0.01. GraphPad Prism version 7.02 (GraphPad Prism Software Inc., La Jolla, CA, USA) was employed for the statistical analysis.

3. Results

Samples derived from prion (Chandler strain)-infected mouse brain homogenate on a glass surface were subjected to RENO-S130 treatment (Figure 1). RENO-S130 is composed of DBD plasma region, sterilization chamber, and corona plasma region. In DBD plasma step, the voltage waveforms had a V_{pp} of 22 kV (maximal peak of +11 kV; minimal peak of −11 kV) and frequency of 15 kHz at 760 Torr (Figure 2a), while the current waveforms showed $I = 23$ mA (Figure 2d). In corona plasma step, the voltage waveforms showed a V_{pp} of 14 kV (maximal peak of +7 kV; minimal peak of −7 kV) and frequency of 15 kHz at 760 Torr (Figure 2b), while the current waveforms showed $I = 31$ mA (Figure 2e). The voltage waveforms showed a V_{pp} of 3 kV (maximal peak of +1.5 kV; minimal peak of −1.5 kV) and frequency of 15 kHz at 10 Torr (Figure 2c), while the current waveforms showed $I = 68$ mA (Figure 2f). The RENO-S130 instrument operates in two modes: a non-lumen mode that is suitable for medical devices that require surface sterilization only, or an Eco mode suitable for medical devices with an inner space or lumen [35]. The sterilization time for the non-lumen mode was 28 min, while that for the Eco mode was 45 min. Next, the RENO-S130-treated and untreated samples were subjected to PK treatment followed by Western blot analysis using an anti-PrP antibody (SAF83) (Figure 3). For the untreated sample (Control), a prominent band corresponding to PrPres (an indicator of PrP^{Sc}), which is resistant to PK digestion, was detected. However, the intensity of the PrPres signal was markedly reduced for the RENO-S130-treated samples using either of the two process modes (non-lumen mode or Eco mode) compared with the untreated sample (Control). Similarly, the level of total PrP, including PrP^{Sc} and PrP^C, was below the detection limit by Western blotting after treatment with the RENO-S130 device. These results suggest that both PrP^{Sc} and PrP^C were degraded by RENO-S130 treatment under both non-lumen mode and Eco mode conditions.

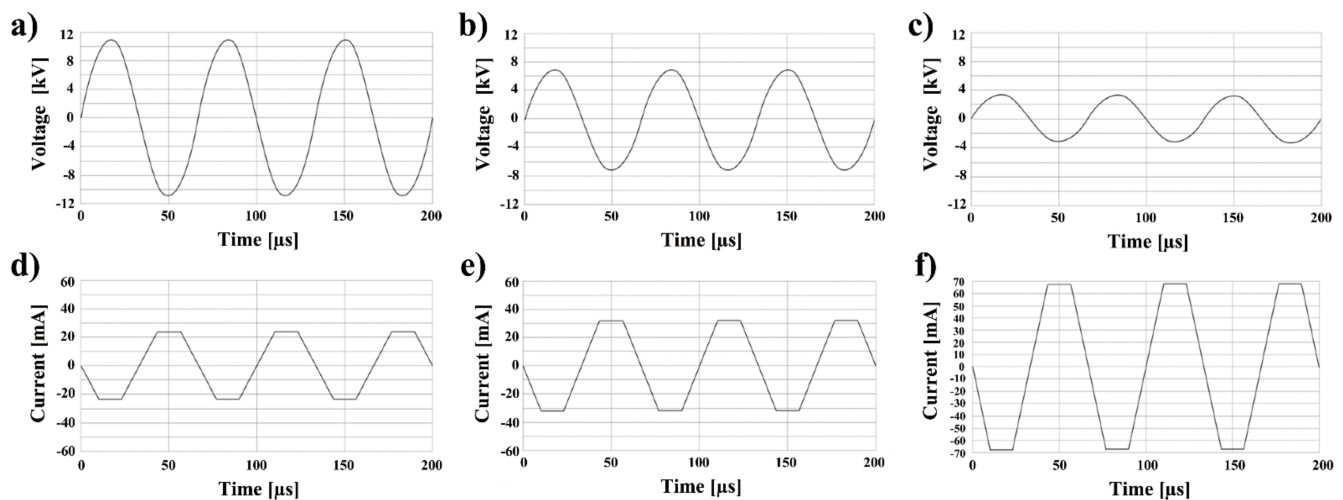


Figure 2. Voltage and current waveforms during operation of the DBD plasma at atmospheric pressure conditions (a,d), the corona discharge plasma at atmospheric pressure conditions (b,e), and the corona discharge plasma at vacuum conditions (c,f) in the RENO-S130 device. Voltage waveforms (a) and current waveforms (d) during operation of the DBD plasma in the condition at 760 Torr, voltage waveforms (b) and current waveforms (e) during the operation of the corona discharge plasma in the condition at 760 Torr, and voltage waveforms (c) and current waveforms (f) during the operation of the corona discharge plasma in the condition at 10 Torr were measured using an oscilloscope with a high-voltage probe.

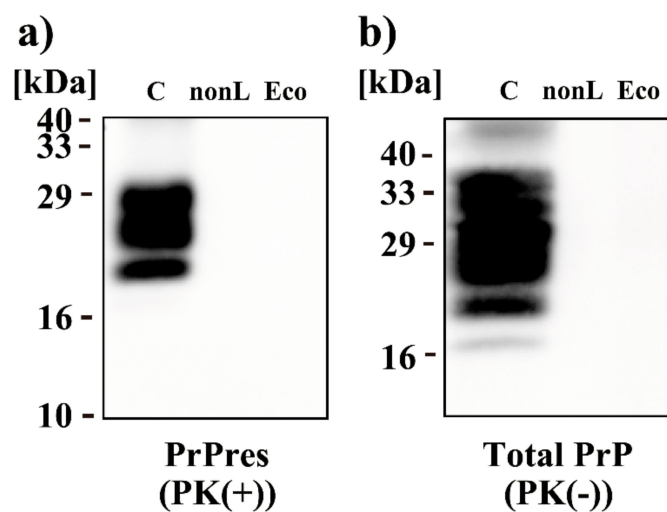


Figure 3. Western blotting of prions after treatment with the RENO-S130 device. Scrapie prion (Chandler strain) was treated with the RENO-S130 device using two process modes: non-lumen mode (nonL) and Eco mode (Eco). An untreated sample (C) was used as a negative control. Samples were retrieved from the spots on the cover glass (100 μ g protein per 50 μ l) and incubated at 37 $^{\circ}$ C for 60 min with or without 20 μ g/mL of proteinase K (PK) prior to Western blotting to detect PrPres (a) and total prion protein (PrP) (b), respectively. Western blotting was conducted using SAF83, an anti-PrP antibody. Size markers (kDa) are shown on the left-hand side of each blot.

Next, PMCA analysis was performed to investigate the change of in vitro proliferation activity of Chandler prion after treatment with the RENO-S130 device (Figure 4). In PMCA, uninfected C57BL6/J mouse brain homogenate was used as a source of PrP^C substrate. The prion samples were diluted (1:10) with PrP^C substrate and analyzed in the 1st round of PMCA to compare the change of amplification efficiency of PrP^{Sc} induced by treatment with the RENO-S130 device. This procedure was then repeated using the resulting 1st round samples to generate the 2nd round of PMCA. Aliquots from the different samples were then incubated with (PK(+)) or without PK (PK(-)). Western blot analysis using an

anti-PrP antibody (SAF83) was performed. In the control sample, PrPres was detected after the 1st round of PMCA followed by digestion with PK (PK(+)), but no such band was detected in the RENO-S130-treated samples (non-lumen or Eco mode). Likewise, PrPres was also found in control samples that had been analyzed in a 2nd round of PMCA, prior to digestion with PK (PK(+)). However, PrPres was not found in RENO-S130-treated samples (non-lumen or Eco mode) after the 2nd round of PMCA in PK(+). Total PrP, comprising mainly PrP^C substrate, was present in all PK(-) samples at similar intensities following the 1st and 2nd rounds of PMCA. Taken together, these results show that the *in vitro* proliferation activity of the prion was decreased by both types of RENO-S130 treatment (non-lumen or Eco mode). These observations suggest that the two modes of RENO-S130 treatment eliminate PrP^{Sc} amplification capability.

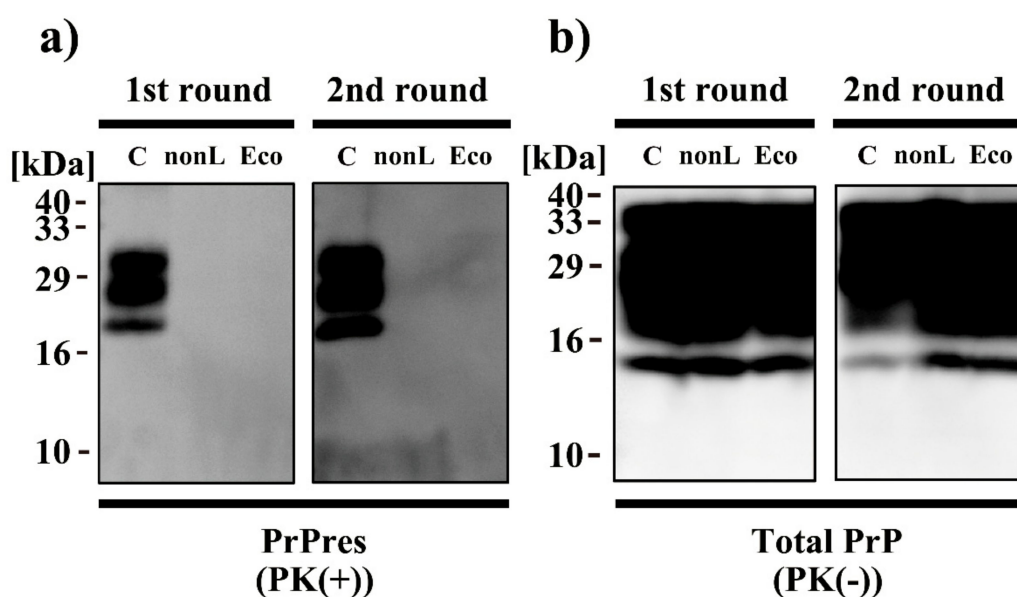


Figure 4. Protein misfolding cyclic amplification (PMCA) was used to assess the effectiveness of inactivation of scrapie prion after treatment with the RENO-S130 device. Uninfected mouse brain homogenate was used as PrP^C substrate. Untreated control (C) or RENO-S130-treated (nonL and Eco) samples retrieved from the spots on the cover glasses were diluted 1:10 with PrP^C substrate and analyzed in a 1st round of PMCA. Thereafter, a 2nd round was performed following an additional 1:10 dilution with PrP^C substrate. An aliquot taken from the various samples was then incubated with or without PK (20 µg/mL) at 37 °C for 60 min to reveal PrPres (a) and total PrP (b), respectively. To detect PrPres and total PrP, samples were subject to Western blot analysis with SAF83, an anti-PrP antibody. Size markers (kDa) are shown on the left-hand side of the blots.

Next, Chandler prion-infected mouse brain homogenate, either treated with the RENO-S130 device (non-lumen or Eco mode) or untreated (Control), were intracerebrally injected into C57BL/6J mice and their behavior and survival monitored over time (Figure 5). All six mice in the Control group showed unusual behavior, including tremors and ataxia, and died before 227 days [30]. Of the six mice in each of the two RENO-S130 treatment groups (non-lumen and Eco mode), five showed no clinical symptoms of prion disease up to the end of the study period (576 days). One mouse in the non-lumen group died on day 401 and one mouse in the Eco group died on day 181, although neither displayed abnormal behavior during the study period. A log-rank test demonstrated that the survival curve was significantly different between the Control group and the non-lumen group or Eco group. No significant differences were observed between the non-lumen group and Eco group.

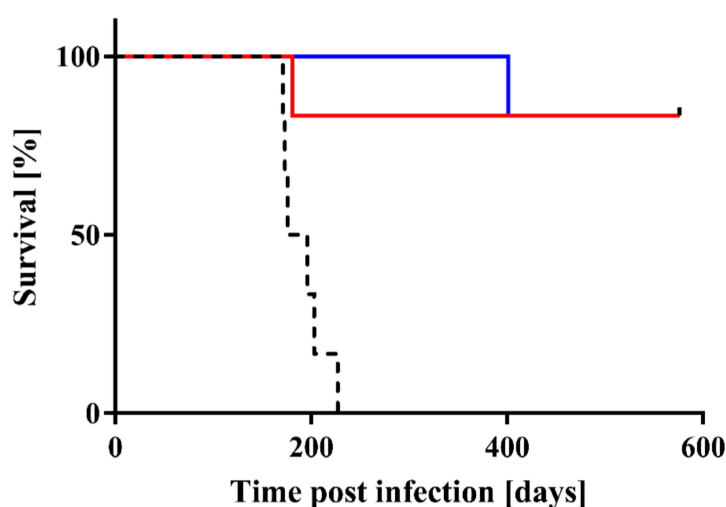


Figure 5. Mice injected with scrapie prion following exposure to RENO-S130 survived much longer than those incubated with untreated scrapie prion. The mice were injected intracerebrally with Chandler prion that had been either exposed to the RENO-S130 device (blue line: non-lumen (N = 6); red line: Eco (N = 6)) or was untreated (dotted black line: Control (N = 6)). Survival curves were statistically compared by log-rank test (where $p < 0.01$ was defined as significant). The survival curve for Control mice was reported previously [30].

The incubation time of the disease mirrored survival, with a marked dissimilarity between the Control group and the non-lumen group or Eco group. The mean incubation time of mice injected with untreated prion was 191.0 ± 9.0 days [30]. By contrast, one mouse in the non-lumen group died on day 401, while 5 mice survived more than 576 days. Likewise, one mouse in the Eco group died on day 181, while 5 mice survived more than 576 days. Overall, these findings suggest that the incubation time of mice injected with RENO-S130 treated (non-lumen or Eco mode) prion-infected brain tissue was longer than for the Control group.

Western blotting detected PrPres in all mice that died of prion disease in the untreated (Control) group, but not in the non-lumen and Eco mode group including the two mice that died within the 576 day period following injection with the treated brain tissue (Figure 6). These results indicated that all of the untreated mice (Control) had succumbed to prion disease. However, the two mice that died within the study period in the non-lumen and Eco mode group did not appear to have developed prion disease.

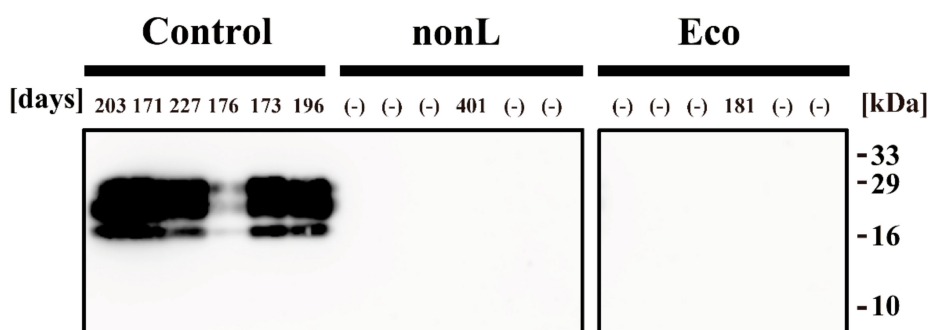


Figure 6. PrPres was detected in the brains of mice that died of prion disease. Samples of prion-infected mouse brain homogenate, either exposed to the RENO-S130 device (non-lumen or Eco modes) or untreated samples (Control), were intracerebrally injected into mice (six mice per group). Mouse brains were subsequently isolated either when the mice displayed clinical symptoms of prion disease, such as tremors and ataxia, or at 577 days (-). To detect PrPres, samples were treated with PK and then analyzed by Western blotting with an anti-PrP antibody (SAF83). Brain samples of untreated control mice were previously obtained [30]. Size markers (kDa) are shown on the right-hand side of the blot.

Thus, treatment of the prion infected samples with a hydrogen peroxide gas plasma sterilizer prior to subsequent injection into mice prolonged survival and delayed the clinical onset of prion disease. These results suggest that scrapie prion can be inactivated by treatment with the RENO-S130 device using either the non-lumen or Eco mode of operation.

4. Discussion

The prion agent is a pathogen that is highly resistant to inactivation using standard chemical and physical disinfection methods. Prion-related iatrogenic disease can be caused by prion-contaminated medical devices [13,17]. Thus, there is a requirement to develop effective methods to inactivate prion agents from medical instruments. The usefulness of a novel hydrogen peroxide gas plasma sterilizer RENO-S130 for prion inactivation has been evaluated.

Several studies have reported that gas plasma has an inactivating effect on prions [36]. For example, gas plasma generated by corona discharge in open air was shown to inactivate scrapie prions [37]. In the study, Julák et al. used a cell infectivity assay and Western blot analysis and found a reduction of both PrP^{Sc} and PrP^C after exposure to gas plasma. In addition, Baxter et al. [38] contaminated stainless steel spheres with scrapie prion before treatment with RF plasma generated using an Ar/O₂ gas mixture for 1 h, which prolonged the incubation time of hamsters infected with the scrapie prion. In a study of the European project BIODICON, prions were shown to be inactivated by Ar-plasma more efficiently than a mixed Ar/N₂ plasma [39].

At present, the hydrogen peroxide gas plasma sterilization device STERRAD[®] is primarily employed to disinfect medical instruments that cannot be exposed to extreme heat [40]. An earlier study demonstrated that a stainless steel wire contaminated with Scrapie 263K could be efficiently sterilized by treatment with a hydrogen peroxide gas plasma sterilizer such as STERRAD[®] NX or STERRAD[®] 100S [41]. The results showed that STERRAD[®] NX (90% hydrogen peroxide gas, 53 °C, 7 min) had an inactivating effect i.e., none of the 8 test animals exposed to the treated prion developed the disease, and the infectious titer was reduced by at least 5 to 6 logs. However, STERRAD[®] 100S (59% hydrogen peroxide gas, 50 °C, 20 min) showed almost no prion inactivating effect. This observation indicated that hydrogen peroxide gas may only have a prion inactivating effect under specific conditions (e.g., incubation temperature, concentration). However, other studies suggested that the sterilization effect of STERRAD[®] occurs irrespective of the presence or absence of gas plasma and that only hydrogen peroxide gas appears to contribute to the inactivation [16,18]. This hypothesis is supported by the results of experiments that indicate hydrogen peroxide gas itself elicits a prion inactivating effect [29,30]. Thus, it is necessary to analyze the prion inactivation effect of each device in turn because conditions, such as the sterilization process, hydrogen peroxide concentration, and incubation temperature, differ depending on the specific hydrogen peroxide gas plasma sterilizer.

Here, we examined the effect of hydrogen peroxide gas plasma generated by the combination of DBD and corona discharge using a new sterilizer RENO-S130 as a potential disinfection/sterilization method for the scrapie prion agent and confirmed the inactivation effect. In the RENO-S130 device, hydrogen peroxide is vaporized as a sterilizing agent and plasma is generated for processing [35]. RENO-S130 differs from other hydrogen peroxide gas plasma sterilizers in terms of temperature, humidity, hydrogen peroxide gas concentration, etc. during sterilization. The temperature of the sterilization chamber was maintained below 55 °C. The RENO-S130 sterilizer has a decompression process in which the inside of the chamber is vacuum depressurized to 0.5 Torr. Hydrogen peroxide (50%) is injected into the chamber, and gas plasma is generated by DBD for sterilization at 760 Torr (non-lumen mode) or at 760~988 Torr (Eco mode). The gas plasma generated by corona discharge degrades the hydrogen peroxide gas during decompression to 0.5 Torr, which is then further replaced with air to completely remove any residual hydrogen peroxide gas. The RENO-S130 sterilizer can operate in non-lumen mode, which is suitable for medical

devices that require surface sterilization, or Eco mode, which is used for medical devices containing an inner compartment.

The results from the present study suggest the RENO-S130 device is effective at inactivating scrapie prion. There are, however, a few limitations to this study. Firstly, quantitative PMCA methods such as real-time quaking-induced conversion (qRT-QuIC) should be performed to fully elucidate the effect of RENO-S130 treatment on prions. Secondly, the observed reduced levels of infectivity caused by treatment with the RENO-S130 device should be clarified in a quantitative manner. Additional analyses, including endpoint titration [42] and an incubation time interval assay [43], would be required to determine the reduced levels of infectivity resulting from RENO-S130 treatment. Nonetheless, because the observed inactivation effect of RENO-S130 was considerable, it can be concluded that treatment with the RENO-S130 device clearly does inactivate scrapie prion. Noteworthy, the brain sample homogenates comprised about 2 mg of proteins. It is estimated that protein contamination levels resulting from surgical procedures are typically much lower (8 to 91 µg per medical device) [44]. In addition, pretreatment washing of medical devices using enzyme or alkaline detergents will achieve 4–6 log reduction in microorganisms and proteins [45]. Hence, pretreatment alongside exposure to the RENO-S130 device in the sterilization process may enhance the total reduction of prion infectivity to achieve complete inactivation.

Our previous studies have shown that reactive chemical species generated by the gas plasma may be major contributing factors responsible for the inactivation of pathogens including prions [34,46,47]. Nevertheless, the main reactive chemical species besides hydrogen peroxide gas contributing to the inactivation of prions by the RENO-S130 device remains to be clarified. Our preliminary studies using an ozone analyzer UV-100 (Eco Sensors Division of KWJ Engineering Inc., Santa Fe, NM, USA) have shown that the concentration of ozone in the sterilization chamber box of RENO-S130 was 10–50 ppm. Ozone is generated when air is introduced into the DBD plasma region and then moved into the sterilization chamber. A recent study has shown that hydrogen peroxide gas and ozone gas synergistically decrease the infectivity of prion [48], and ozone generated by the DBD plasma in RENO-S130 may contribute to the enhanced sterilization effect. Further studies are required to ascertain which of these factors contribute to reducing the *in vitro* propagation activity and infectivity of prions. Additional studies using optical emission spectroscopy (OES) and electron spin resonance (ESR) spectroscopy would be also necessary to determine the reactive chemical species in hydrogen peroxide gas plasma generated by the RENO-S130 device that are involved in the mechanism of prion inactivation.

Taken together, our results indicate that scrapie prion can be rendered harmless by treatment with a hydrogen peroxide gas plasma sterilizer combined with DBD and corona discharge using a RENO-S130 device. PMCA analysis and mouse bioassays demonstrate that the RENO-S130 treatment decreased the *in vitro* propagation activity of scrapie prion as well as the infectivity of the prions. Therefore, this sterilization procedure is potentially applicable to the decontamination of prion-contaminated medical devices. However, for medical applications, this disinfection/sterilization method will need to be fully evaluated using human prions to establish whether it is effective at preventing iatrogenic transmission. Susceptibility to physical and chemical treatment may vary between different animal prions [11]. Indeed, the heat susceptibility of prion depends on the specific prion strains [49]. Therefore, prions derived from different strains and animals may behave differently to the treatment with the RENO-S130 device. Thus, additional studies on the treatment of a variety of prions using the RENO-S130 device, together with an analysis of the sterilization mechanism, is needed before it can be used in a clinical setting.

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