Hydrogen Peroxide Gas and Ozone Gas Synergistically Inactivate Prion Infectivity on Stainless Steel Wire

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Abstract

Prions are infectious agents causing prion diseases, which include Creutzfeldt-Jacob disease (CJD) in humans. Several cases have been reported to be transmitted through medical instruments that were used for preclinical CJD patients, raising public health concerns on iatrogenic transmissions of the disease. Since preclinical CJD patients are currently difficult to identify, medical instruments need to be adequately sterilized not to transmit the disease. In this study, we investigated the sterilizing activity of two oxidizing gases, ozone gas and hydrogen peroxide gas, against prions fixed on stainless steel wires using a mouse bioassay. Mice intracerebrally implanted with prion-contaminated stainless steel wires treated with ozone gas or hydrogen peroxide gas developed prion disease later than those implanted with control prion-contaminated stainless steel wires, indicating that ozone gas and hydrogen peroxide gas could reduce prion infectivity on wires. Incubation times were further elongated in mice implanted with prion-contaminated stainless steel wires treated with ozone and hydrogen peroxide-mixed gas, indicating that ozone-mixed hydrogen peroxide gas inactivates prions on these wires more potently than ozone gas or hydrogen peroxide gas. Taken together, these results suggest that ozone-mixed hydrogen peroxide gas may be useful for prion sterilization of medical instruments.

Introduction

Prion diseases are a group of fatal neurodegenerative disorders in humans and animals, caused by accumulation of infectious protein aggregates, or prions, in the brain. They manifest as sporadic, genetic, and acquired diseases in humans. Sporadic Creutzfeldt-Jakob disease (CJD) are most common, accounting for about 85% of human prion disease cases. Genetic prion diseases, which include familial CJD, fatal familial insomnia, and Gerstmann-Sträussler-Scheinker syndrome, are linked to pathogenic mutations in the prion protein gene Pmp, and account for 10-15% of human cases. The remaining cases are those of acquired prion diseases, including kuru, variant CJD, and iatrogenic CJD. Kuru is a disease prevailed among Fore people in Papua New Guinea through cannibalism. Variant CJD is believed to be transmitted from bovine spongiform encephalopathy (BSE) through consumption of BSE-contaminated beef. Fortunately, BSE cases have been dramatically reduced due to the meat and bone meal ban in ruminant feed and therefore the transmission risk of BSE to humans has become very low at present. However, iatrogenic transmission of CJD still remains as potential public health concerns. People have been reported to develop CJD after receiving corneal or dura mater grafts, growth hormone, gonadotropin, and red blood cells that were contaminated with prions. Medical instruments that were used in preclinical or clinical CJD patients have been also shown to transmit the disease. Two patients were reported to die of CJD after stereo-electroencephalographical examinations using the electrodes that were previously used for other CJD patient. Neurosurgical instruments that were used for preclinical CJD patients have been also reported to transmit the disease. There is also an epidemiological report suggesting that surgeries for peripheral vessels, digestive system, spleen, and female genital organs might be associated with CJD risk. Adequate prion decontamination procedures...
are therefore required for medical instruments, particularly surgical instruments, not to potentially transmit the disease.

Prions consist of the misfolded, amyloidogenic isoform of prion protein, designated PrP\textsubscript{Sc}, which is produced through conformational conversion of the normal cellular counterpart, PrP\textsubscript{C}, a membrane glycoprotein expressed most abundantly in the brain, particularly by neurons\textsuperscript{1,2}. Consistent with prions being made of protein alone, protein denaturants including urea, guanidine-hydrochloride, sodium dodecyl sulfate, and sodium hydroxide have been shown to effectively reduce prion infectivity whereas nucleic acid-damaging procedures, which are capable of inactivating conventional pathogens such as bacteria and viruses, did not\textsuperscript{14-17}. Autoclaving is also recommended for inactivation of prion infectivity\textsuperscript{14,18}. However, medical instruments or devices are so complicated and delicate that they may be susceptible to the damages that the chemicals or autoclaving may cause. Effective reduction of prion infectivity has been also reported with gas sterilizations, including hydrogen peroxide gas sterilization\textsuperscript{19,20}. Mixing of ozone solution in prion-infected brain homogenates was also shown to reduce prion infectivity in the homogenates\textsuperscript{21}. These facts intrigued us to explore if hydrogen peroxide gas could synergistically function with ozone gas to inactivate prion infectivity.

In this study, we show that, compared to ozone gas alone or hydrogen peroxide gas alone, ozone-mixed hydrogen peroxide gas was highly effective in inactivating RML scrapie prions fixed on the surface of stainless steel wires in a mouse bioassay, indicating that ozone gas and hydrogen peroxide gas could synergistically inactivate prion infectivity on the surface of these wires. Thus, compared to sterilization with ozone gas alone or hydrogen peroxide gas, sterilization with ozone-mixed hydrogen peroxide gas may be useful for prion decontamination of medical instruments or devices.

**Results**

**Ozone gas and hydrogen peroxide gas synergistically inactivate prion infectivity on stainless steel wires**

To investigate if ozone gas and hydrogen peroxide gas could synergistically inactivate prion infectivity, we exposed ozone gas, hydrogen peroxide gas, and ozone-mixed hydrogen peroxide gas to RML scrapie prions fixed on the surface of stainless steel wires in the ET sterilization mode of the gas sterilizer (Fig. 1A). The treated wires were then implanted into the brains of ICR mice. We also similarly implanted gas-unexposed, prion-contaminated stainless steel wires into ICR mice as a control. Mice implanted with the control wires developed disease at 176 ± 8 days post-implantation (dpi, 10 diseased/10 implanted mice) (Fig. 1B). However, compared to those in the control mice, incubation times were prolonged in mice implanted with ozone gas-exposed, prion-contaminated wires (190 ± 16 dpi, 10 diseased/10 implanted mice, \(p<0.0001\)), further elongated in mice implanted with hydrogen peroxide gas-exposed, prion-contaminated wires (>236 ± 16 dpi, 9 diseased/10 implanted mice, \(p<0.0001\)), and greatly extended in mice implanted with ozone-mixed hydrogen peroxide gas-exposed, prion-contaminated wires (>315 ± 68 dpi, 4 diseased/10 implanted mice, \(p<0.0001\)) (Fig. 1B). To confirm that the diseased mice succumbed to prion disease, we biochemically and pathologically investigated the brain of these implanted mice. We
first investigated accumulation of PrP<sup>Sc</sup> in the brains of these mice by Western blotting with 6D11 anti-PrP antibody, which recognizes residues 93-109 of mouse PrP. The PK-resistant PrP, or PrP<sup>Sc</sup>, with similar glycosylation banding patterns was observed at similar levels in the brains of all the diseased mice examined (Fig. 2, Supplementary Fig. 1). In contrast, no PrP<sup>Sc</sup> was observed in the brains of non-diseased mice sacrificed at 365 dpi, except for mild accumulation of PrP<sup>Sc</sup> detected in one mouse implanted with ozone-mixed hydrogen peroxide gas-exposed, prion-fixed wires (Fig. 2, Supplementary Fig. 1). We also examined prion disease-specific vacuolation in the brains of diseased mice. Many vacuoles were observed in various brain regions, particularly in the cerebral cortex, hippocampus, thalamus, and cerebellum, of diseased mice, but not in prion-uninfected control brains (Fig. 3). Taken together, these results show that, while ozone gas and hydrogen peroxide gas alone could effectively inactivate prion infectivity, they could synergistically inactivate prion infectivity so strongly that more than half of the mice implanted with prion-fixed wires exposed to ozone-mixed hydrogen peroxide gas remain disease-free.

**Prion-inactivating activity of ozone-mixed hydrogen peroxide gas is correlated to exposure time but not to hydrogen peroxide gas concentration**

To investigate the effects of the exposure time of ozone-mixed hydrogen peroxide gas on its inactivating activity of prion infectivity, we exposed RML prion-contaminated stainless steel wires to ozone-mixed hydrogen peroxide gas in two different time windows using the short and the standard sterilization modes (Fig. 4A) and implanted them into the brains of ICR mice. Mice implanted with the standard mode-treated wires developed disease with significantly longer incubation times than those implanted with the short mode-treated wires [>309 ± 62 dpi (6 diseased/11 implanted mice) vs >261 ± 68 dpi (10 diseased/11 implanted mice), p=0.0363] (Fig. 4B), indicating that longer exposure time could increase the prion-inactivating activity of ozone-mixed hydrogen peroxide gas.

We thus assumed that further longer exposure with an increasing concentration of hydrogen peroxide gas could enhance the prion-inactivating activity of ozone-mixed hydrogen peroxide gas. To investigate this, we exposed prion-contaminated stainless steel wires to ozone-mixed hydrogen peroxide gas in the long sterilization mode and implanted them into the brains of ICR mice. In the long mode, 45% hydrogen peroxide was used for the second injection of hydrogen peroxide and vaporized for about 13 min, instead of 3% hydrogen peroxide that was vaporized for about 9 min in the standard mode and 4 min in the short mode (Fig. 4A). Paradoxically, incubation times of mice implanted with the long mode-treated wires (225 ± 36 dpi, 11 diseased/11 implanted mice) were shortened to those of mice implanted with the short mode-treated wires (p=0.1206) (Fig. 4B). These results indicate that long exposure to high concentration of hydrogen peroxide gas could diminish the synergistic effects of ozone-mixed hydrogen peroxide gas on inactivation of prion infectivity.

**Ozone-mixed hydrogen peroxide gas reduces prion infectivity more than 2.6 log in mouse bioassay**
To assess the prion-inactivating activity of ozone-mixed hydrogen peroxide gas, we determined prion titers (50% infectious dose, ID$_{50}$) retained on the stainless steel wires after exposed to ozone-mixed hydrogen peroxide gas using a mouse bioassay. For this bioassay, we used transgenic mice, Tg(MoPrP)/Prnp$^{0/0}$ mice, since they are highly sensitive to prion infection due to overexpression of mouse PrP$^C$ in their brains on the Prnp$^{0/0}$ genetic background$^{22}$. We first created a standard curve between prion titers and incubation times for RML prions, by intracerebrally inoculating Tg(MoPrP)/Prnp$^{0/0}$ mice with serially diluted brain homogenates from RML prion-infected, ill mice. The ratio of diseased mice against total mice inoculated and their incubation times are shown in Table 1.

According to the method of Reed and Muench$^{23}$, prion titers were calculated as $10^{8.3}$ ID$_{50}$ in the original brain homogenate inoculum. The standard curve was then given by $\log_{10}(ID_{50}) = 18.57 - 0.166X$, where $X$ is incubation time (days), 72$<X<$101. Since the ET mode- and the standard mode-treated wires reduced prion infectivity to similar levels, we determined prion titers retained on the ET mode- and the standard mode-treated wires as well as on the untreated control wires. All Tg(moPrP) mice implanted with the control wires developed disease at 96 ± 7 dpi, indicating that prion titers on the control wires are 2.6 $\log_{10}(ID_{50})$ according to the standard curve (Table 2). However, after implantation of the ET mode- or the standard mode-treated wires, only 2 out of 7 Tg(moPrP) mice succumbed to disease (ET mode: $>173$ dpi, 2 diseased/7 implanted mice; Standard mode: $>171$ dpi, 2 diseased/7 implanted mice), implying that prion titers retained on the ET mode- or the standard mode-treated wires are less than 1 $\log_{10}(ID_{50})$ (Table 2). These results indicate that ozone-mixed hydrogen peroxide gas in the ET mode or the standard mode could reduce RML prions fixed on the stainless steel wires more than 2.6 $\log_{10}(ID_{50})$.

**Discussion**

In the present study, we compared ozone gas, hydrogen peroxide gas, and ozone-mixed hydrogen peroxide gas for their activities to inactivate prion infectivity, by exposing RML prions contaminated on stainless steel wires to each gas and then implanting the treated stainless steel wires into the brains of mice. Consistent with the results previously reported by others showing that ozone and hydrogen peroxide gas could inactivate prion infectivity$^{19-21,24,25}$, we found that mice implanted with ozone gas- or hydrogen peroxide gas-exposed, RML prion-contaminated wires developed the disease significantly longer than those implanted with gas-unexposed, RML prion-contaminated wires. However, incubation times of mice implanted with ozone-mixed hydrogen peroxide gas-exposed, RML prion-contaminated wires were much longer than those of mice implanted with ozone gas alone- or hydrogen peroxide gas alone-exposed, RML prion-contaminated wires. These results indicate that ozone gas and hydrogen peroxide gas could synergistically function to inactivate prion infectivity, suggesting that ozone-mixed hydrogen peroxide gas may be more useful for prion decontamination of medical instruments or devices than ozone gas alone or hydrogen peroxide gas alone.

Due to the limited amounts of RML prions fixed on the surface of stainless steel wires, we only observed that ozone-mixed hydrogen peroxide gas in the ET or the standard mode reduced prion infectivity more than 2.6 $\log_{10}(ID_{50})$ in a mouse bioassay. However, it was reported that vaporized hydrogen peroxide
alone reduced hamster-adapted 263K scrapie prion infectivity contaminated onto the wires about 4.5 Log$_{10}$ in hamster bioassay$^{20}$. Ozone (13.7 mg/L) treatment of 263K-infected brain homogenates was also shown to reduce prion infectivity more than 4.1 Log$_{10}$ in bioassay$^{21}$. Given that ozone-mixed hydrogen peroxide gas was more potent in inactivating prion infectivity on wires than ozone gas alone or hydrogen peroxide gas alone, it can be conceivable that ozone-mixed hydrogen peroxide gas could reduce prion infectivity more than 4.5 Log$_{10}$ at least. Studies using stainless steel wires fixed with higher amounts of RML prions could provide a more accurate estimation of how much ozone-mixed hydrogen peroxide gas can inactivate prion infectivity on wires.

Prion infectivity is believed to be enciphered in the protein conformation of PrP$^{\text{Sc}}$. Upon prion infection, PrP$^{\text{Sc}}$ interacts with PrP$^{\text{C}}$ and induces conformational changes in the interacting PrP$^{\text{C}}$ to adopt the conformation of PrP$^{\text{Sc}}$, eventually causing prion disease$^{26,27}$. Ozone and hydrogen peroxide are powerful oxidants, oxidizing susceptible amino acids such as cysteine, methionine, tryptophan, tyrosine, histidine, and phenylalanine and thereby causing the structural changes of target proteins$^{28,29}$. Thus, ozone gas and hydrogen peroxide gas oxidize PrP$^{\text{Sc}}$, modulating its protein conformation leading to destruction of the activity to convert PrP$^{\text{C}}$ into PrP$^{\text{Sc}}$, or prion infectivity. Ozone-mixed hydrogen peroxide gas may oxidize PrP$^{\text{Sc}}$ more robustly than ozone gas alone or hydrogen peroxide gas alone, thereby inactivating prion infectivity more strongly than ozone gas alone or hydrogen peroxide gas alone.

Mice implanted with the standard mode-treated, RML prion-contaminated stainless steel wires developed disease with significantly longer incubation times than those implanted with short mode-treated, RML prion-contaminated wires, indicating that the standard mode is more effective in inactivating prion infectivity than the short mode. Since the exposure time of ozone-mixed hydrogen peroxide gas in the standard mode is longer than that in the short mode, these results indicate that exposure time is an important factor for ozone-mixed hydrogen peroxide gas to effectively inactivate prion infectivity. However, mice implanted with the long mode-treated wires developed disease with shortened incubation times, which were similar to those of mice implanted with the hydrogen peroxide gas alone-treated wires. In the long mode, RML prions-fixed wires were exposed to an increasing concentration of hydrogen peroxide in a long exposure period, suggesting that higher concentration of hydrogen peroxide gas in a long period might reduce the synergistic oxidative effects of ozone-mixed hydrogen peroxide gas on inactivation of prion infectivity. This might be explained by the fact that high concentration of hydrogen peroxide could act as a scavenger for hydroxyl radicals generated during decomposition processes of ozone$^{30}$. We also found that incubation times of mice implanted with the standard mode-treated wires were not significantly elongated, compared to those of mice implanted with the ET mode-treated wires, although total exposure time is longer in the ET mode than in the standard mode. It is thus possible that the exposure time used in the standard mode might be long enough for inactivation of prion infectivity by ozone-mixed hydrogen peroxide gas. However, further studies are required to optimize prion sterilization conditions, including exposure time and hydrogen peroxide concentration, for ozone-mixed hydrogen peroxide gas to be in practical use as a prion decontamination tool for medical instruments or devices.
Materials And Methods

Ethics statement

The Ethics Committees of Animal Care and Experimentation of Tokushima University approved this study (approval no. T30-103). Animals were cared for in accordance with The Guiding Principle for Animal Care and Experimentation of Tokushima University and with Japanese Law for Animal Welfare and Care. Mice were housed under specific pathogen-free conditions in cages of 5-6 animals with water and food ad libitum. Cages were provided with a standard softwood bedding. Mice were kept on a standard 12:12 light:dark cycle. Every effort was made to reduce distress and the number of animals used according to the ARRIVE guidelines.

Antibodies

The antibodies used in this study are: 6D11 mouse anti-PrP Ab (SIG-399810; BioLegend, San Diego, CA), mouse anti-ß-actin Ab (M177-3; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan), anti-mouse IgG horseradish peroxidase (HRP)-linked Ab (NA931; GE Healthcare, Little Chalfont, England).

Animals

Crl:CD1(ICR) mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). Tg(MoPrP)/Prnp<sup>0/0</sup> mice were obtained elsewhere by intercross between the backcrossed Prnp<sup>0/0</sup> mice and Tg(MoPrP) mice with a FVB background.<sup>22</sup>

Fixation of RML prions onto stainless steel wires

Stainless wires (751267; SUS 304; diameter 0.2 mm; The Nilaco Corporation, Tokyo, Japan) were cut to 5 mm in length. Batches of 10 wires were sterilized in 1.5 mL microtube containing 1 mL of 70% (vol/vol) ethanol in deionized water at room temperature (RT) overnight. After air drying, the stainless steel wires were incubated in a microtube containing 400 µL of 10% (wt/vol) RML-infected brain homogenates at 4˚C overnight. Brain homogenates were prepared as described previously.<sup>31</sup> The wires were then washed three times in a microtube containing 1 mL of phosphate-buffered saline (PBS) (11482-15; Nakalai Tesque, Osaka, Japan) and air-dried in Petri dish at RT overnight.

Inactivation of RML prions on stainless steel wires

Ozone-mixed hydrogen peroxide gas sterilizer (Miura Co., Ehime, Japan) was used to inactivate RML prions fixed onto stainless steel wires in four different sterilization modes, the endotoxin (ET), the standard, the short, and the long modes.

Intracerebral inoculation with prion-infected brain homogenates

1% RML prions-infected brain homogenates in PBS were prepared using the brains from RML prion-infected, terminally ill mice and serially diluted with PBS. These homogenates were intracerebrally
inoculated into 5- to 6-week-old ICR or Tg(MoPrP)/Prnp<sup>0/0</sup> mice (20 µL/mouse) using a 30-gauge injection needle (HS-2739A; Dentronics Co., Ltd., Tokyo, Japan). Mice were diagnosed as sick when they developed more than five of the following features: emaciation, decreased locomotion, ruffled body hair, ataxic gait, kyphosis, priapism, upright tail, crossing leg, hind leg paresis, and foreleg paresis.

**Intracerebral insertion of prion-contaminated stainless steel wires**

The wire was inserted intracerebrally into 5- to 6-week-old mice (1 wire/mouse), using a 25-gauge injection needle (NN-2516R; Terumo Corporation, Tokyo, Japan).

**Western blotting**

Western blotting was performed as described previously. Total proteins were run on a SDS-polyacrylamide gel and transferred to an Immobilon-P PVDF membrane (IPVH00010, Millipore, Billerica, USA). After blocking with 1% non-fat dry milk in TBST (10 mM Tris-HCl, pH7.4, containing 0.05% Tween-20, and 150 mM NaCl) at RT for 1 h, the membranes were incubated with 6D11 anti-PrP antibody (BioLegend) at 4°C overnight in TBST containing 0.5% non-fat dry milk, washed 3 times with TBST at RT for 5 min, and incubated with anti-mouse IgG HRP-linked Ab (GE Healthcare) at RT for 2h in TBST containing 0.5% non-fat dry milk. After washing 3 times with TBST at RT for 5 min, signals were visualized using Immobilon Western Chemiluminescent HRP substrate (WBKLS0500, Millipore) and detected by LAS-4000 mini chemiluminescence imaging system (Fuji Film, Tokyo, Japan).

**Hematoxylin-eosin staining**

Paraffin-embedded samples were sectioned at 5 µm. The sectioned samples were deparaffinized, rehydrated, and stained with Mayer’s hematoxylin solution (131-09665; Wako Pure Chemical Industries) at RT for 5 min and 1% eosin Y solution (051-06515; Wako Pure Chemical Industries) at RT for 2 min. After washing, the samples were mounted with Softmount (192-16301; Wako Pure Chemical Industries). Images of sample were visualized using BZ-810 (Keyence, Osaka, Japan) and analyzed with BZ-800 analyzer software (Keyence).

**Statistical analysis**

Incubation times were analyzed using the log rank (Mantel-Cox) test.

**Data availability**

All data generated or analyzed during this study are included in this published article.

**Declarations**

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Author Contributions

H.H., J.C., A.D.P., K.U., J.Y., and H.K. performed experiments. T.N., Y.T., J.Y., and H.K. financially supported this study. Y.K. and S.S. designed this study and S.S. supervised this study. H.H., J.C., and S.S. wrote the manuscript. All authors reviewed the manuscript.

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Competing interests

T.N. and Y.T. are employees of Miura Corporation and J.Y. and H.K. are employees of IHI Corporation. Other authors declare that they have no conflict of interest.

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**Tables**
Table 1. Incidence rate and incubation times in Tg(MoPrP) mice intracerebrally inoculated with serial 10-fold dilutions of RML prions.

| Dilution of RML inoculum (log_{10} dilution) | Incidence rate^{1} (Symptomatic mice/Total mice) | Incubation times^{2} (Mean ± SD, days) |
|---------------------------------------------|-------------------------------------------------|---------------------------------------|
| -2                                          | 3/3                                             | 72 ± 2                                |
| -3                                          | 5/5                                             | 78 ± 5                                |
| -4                                          | 6/6                                             | 89 ± 2                                |
| -5                                          | 6/6                                             | 95 ± 4                                |
| -6                                          | -                                               | -                                     |
| -7                                          | 4/4                                             | 101 ± 6                               |
| -8                                          | 3/5                                             | 167 ± 54                              |
| -9                                          | 1/4                                             | 134                                   |
| -10                                         | 0/5                                             | >269                                  |
| -11                                         | 0/5                                             | >269                                  |

^{1} Infectious titers were calculated as 8.3 ID_{50} in the original RML inoculum of 20 mL according to the method of Reed and Muench (23).

^{2} The relationship between infectious titers and incubation times was indicated as Log_{10}(ID_{50}) = 18.57-0.166X (X, incubation times; 72<X<101).

Table 2. Incubation times in Tg(MoPrP) mice intracerebrally implanted with RML prion-contaminated stainless steel wires exposed to ozone-mixed hydrogen peroxide gas in the ET or standard mode.

| Gas exposure mode | Diseased mice /Total mice | Incubation times^{1} (days) | Infectious titers^{2} (ID_{50}) |
|-------------------|---------------------------|----------------------------|---------------------------------|
| Unexposed         | 8/8                       | 96 ± 7                     | 2.6                             |
| Standard mode     | 2/7                       | >171 (106, 106)            | <1                              |
| ET mode           | 2/7                       | >173 (113, 116)            | <1                              |

^{1} Implanted mice were observed by 197 dpi. Two of mice implanted with the standard- and the ET mode-treated, prion-contaminated stainless steel developed disease at indicated dpi in parenthesis.

^{2} Infectious titers on the implanted stainless steel wires were calculated using ID_{50} = 18.57-0.166X (X, incubation times; 72<X<101).
Figures

A

O₂-mixed H₂O₂ gas

O₃ pre-treatment (8 min)

1 cycle

45% H₂O₂ (10 min)

O₃ (5 min)

3% H₂O₂ (5 min)

× 6 cycles

O₃ gas

1 cycle

H₂O (10 min)

O₃ (5 min)

H₂O (5 min)

× 6 cycles

H₂O₂ gas

1 cycle

Air

45% H₂O₂ (10 min)

Air (5 min)

3% H₂O₂ (5 min)

× 6 cycles

B

Symptom-free, %

1% BH, N=9
Gas-unexposed wires, N=10
O₃-exposed wires, N=10
H₂O₂-exposed wires, N=10
O₃-mixed H₂O₂ gas-exposed wires, N=10

Incubation time (days)

Figure 1
Ozone gas and hydrogen peroxide gas synergistically inactivate prion infectivity of stainless steel wires. (A) Schematic representations of the sterilization protocols for ozone-mixed hydrogen peroxide gas, ozone gas alone, and hydrogen peroxide gas alone in the ET mode. For sterilization with ozone-mixed hydrogen peroxide gas, RML prion-contaminated stainless steel wires were pre-treated by injection of 25,000 ppm ozone gas for 3 min and followed by 6 cycles of a 10-min sterilization process, which comprises injection of 45% hydrogen peroxide followed by injection of 25,000 ppm ozone gas and 3% hydrogen peroxide 5 min later. For the treatment with ozone gas alone or hydrogen peroxide gas alone, water was used instead of hydrogen peroxide or ozone gas, respectively. The sterilization process of each mode is terminated by injection of air. (B) The percentage of symptom-free mice after intracerebral inoculation with 1% brain homogenate (1% BH) from RML-infected, diseased mice and intracerebral implantation with gas-unexposed, ozone gas-exposed, hydrogen peroxide gas-exposed, and ozone-mixed hydrogen peroxide gas-exposed, RML prion-contaminated wires. O3, ozone; H2O2, hydrogen peroxide; **, p<0.01.
**Figure 2**

Western blotting for PrPSc in the brains of mice implanted with various gas-treated, RML prion-contaminated stainless steel wires. Brain homogenates from mice uninfected with RML prions (n=4), implanted with gas-untreated, RML prion-contaminated stainless steel wires (n=6), ozone gas-treated, RML prion-contaminated stainless steel wires (n=6), hydrogen peroxide gas-treated, RML prion-contaminated wires (n=5), and ozone-mixed hydrogen peroxide gas-treated, RML prion-contaminated...
wires (n=7) were treated with or without proteinase K (PK) and subjected to Western blotting with 6D11 anti-PrP antibody. β-actin is an internal control for Western blotting. O3, ozone; H2O2, hydrogen peroxide.

**Figure 3**

Brain vacuolation in mice implanted with various gas-treated, RML prion-contaminated stainless steel wires. Representative pictures of HE-stained brain sections from mice uninfected with RML prions (n=4), implanted with gas-untreated, RML prion-contaminated stainless steel wires (n=4), ozone gas-treated, RML prion-contaminated stainless steel wires (n=4), hydrogen peroxide gas-treated, RML prion-contaminated wires (n=4), and ozone-mixed hydrogen peroxide gas-treated, RML prion-contaminated wires (n=3) are shown. O3, ozone; H2O2, hydrogen peroxide. Cx, cerebral cortex; Hp, hippocampus; Th, thalamus; Cb, cerebellum. Bar, 100 μm.
Sterilization of RML prions fixed on stainless steel wires in different sterilization modes. (A) Schematic representations of the sterilization protocols of ozone-mixed hydrogen peroxide in the standard, the short, and the long modes. RML prions-fixed wires were pre-treated by injection of 25,000 ppm ozone gas for 3 min and followed by 2 cycles of a 14-min sterilization process comprising injection of 45% hydrogen peroxide followed by injection of 25,000 ppm ozone gas and 3% hydrogen peroxide 5 min later in the standard mode, a 7-min sterilization process comprising injection of 45% hydrogen peroxide followed by
injection of 25,000 ppm ozone gas and 3% hydrogen peroxide 5 min later in the short mode, and a 19.5-min sterilization process comprising injection of 45% hydrogen peroxide followed by injection of 25,000 ppm ozone gas and 45% hydrogen peroxide 6.5 min later in the long mode. The sterilization process of each mode is terminated by injection of air. (B) The percentage of symptom-free mice after intracerebral implantation with RML prion-contaminated stainless steel wires exposed to ozone-mixed hydrogen peroxide gas-exposed in the standard, the short, and the long modes. O3, ozone; H2O2, hydrogen peroxide; *, p<0.05; **, p<0.01; n.s., not significant.

**Supplementary Files**

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- [SupplementaryFigure1.pdf](#)