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Original Article

Effects of residual H_2O_2 on the growth of MSCs after decontamination

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ABSTRACT

Introduction: Regenerative therapy is a developing field in medicine. In the production of cell products for these therapies, hygienic management is even more critical than in the production of a chemical drug. At the same time, however, care is required with the use of decontamination agents, considering their effects on cell viability and characteristics. To date, hydrogen peroxide (H_2O_2) is most widely used for decontamination in pharmaceutical plants and cell processing facilities.
Methods: In this study, we examined the effects of residual H_2O_2 in the atmosphere of cell processing units after decontamination on the viability and proliferation of mesenchymal stem cells derived from human bone marrow.
Results: We detected residual H_2O_2 sufficient to affect cell proliferation and survival even more than 30 h after decontamination ended. Our results suggest a longer time period is required before starting operations after decontamination and that the operating time should be as short as possible.
Conclusions: Here we show the effects of post-decontamination residual H_2O_2 on the viability and proliferation of mesenchymal stem cells derived from human bone marrow, which may provide us with important information about the hygienic management of cell processing facilities.

1. Introduction

Regenerative medicine is currently a focus of global attention, and countries worldwide are actively working to create these products as new treatment modalities through pioneering research and novel discoveries of technologies [1]. In the production of cell products for clinical administration, hygienic management is even more important than in the production of chemical agents. Materials and products in cell/tissue production cannot be sterilized chemically or physically, and contaminating pathological microbes can proliferate during cell processing. In Japan, two laws were established in November 2014 [2–5]: the Act on the Safety of Regenerative Medicine and the Act on Pharmaceuticals and Medical Devices (PMD Act) were implemented, and legal regulations both in clinical research and in production of regenerative products were prepared. Under these regulations, sanitary control and prevention of contamination in cell processing facilities (CPFs) are major components. Although detailed and global guidance and standards of hygiene management are available regarding the production of chemical agents, no well-established guidance exists for the production of cell/tissue products at the bench in CPF [6]. In most situations, guidelines for good manufacturing practices or others for sterile pharmaceutical products and foods are applied for the production of cell/tissue processing, but each individual person at each facility operates production under their own judgment regarding the specific issues in regenerative products.

In the selection of decontamination agents for CPF, the adverse effects on staff and cells must be considered along with antimicrobial effectiveness. In the regenerative therapy field, because we use various cell types and amounts of cells for different conditions, the types and quantities of agents for decontamination should be

Abbreviations: H_2O_2, hydrogen peroxide; CPF, cell processing facility; BSC, biological safety cabinet; MSC, mesenchymal stem cell; HEPA, high efficiency particulate air.

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selected in accordance with the specific factors involved. Formerly, formaldehyde was generally used for atmospheric decontamination in pharmaceutical facilities. However, with the cancer-causing effects of formaldehyde identified [7,8] as well as acute toxicities such as mucosal irritation and cutaneous inflammation [9], it was classified as a probable human carcinogen by the World Health Organization (WHO) International Agency for Research on Cancer. Currently, its use is seriously restricted by WHO and other environmental regulations. Thus, the establishment of proper guidance for the sanitation of CPF atmosphere using decontamination agents other than formaldehyde is an emergent issue.

Hydrogen peroxide (H_2O_2), chlorine disinfectants, and their mixtures now are widely used for decontamination in pharmaceutical plants and CPFs [10,11]. Above all, H_2O_2 is mostly a sterilizing agent for sanitation in CPFs [12]. H_2O_2 is a reactive oxygen species, which can degrade organic compounds. Because it demonstrates broad-spectrum antimicrobial efficacy, including against bacterial spores, viruses, and yeasts, and has insecticidal power, it is applied to sterilize various surfaces [13], such as surgical tools [14], and can also be used as mist for room sterilization [15,16]. However, highly concentrated H_2O_2 should be considered hazardous because it is an aggressive oxidizer and will corrode many materials, including human skin [17]. The American Conference of Governmental Industrial Hygienists (ACGIH) has also classified H_2O_2 as a "known animal carcinogen, with unknown relevance on humans" and has established a permissible exposure limit of 1.0 ppm calculated as an 8-h time-weighted average [18].

In this study, we first examined the effects of residual H_2O_2 after decontamination on the viability and proliferation of cells. Although the effects of low concentration of H_2O_2 in the atmosphere will be different depending on the cell types, in this study, we selected MSCs as cell type, which can be a representative example because they are insulated from the influence of other conditions, such as operation technique, cell lot, and all.

2. Materials and methods

2.1. Experimental design

The effects of residual H_2O_2 on cell growth after decontamination were evaluated by exposing human MSCs in a biological safety cabinet (BSC) in an experimental vinyl chamber within a cleanroom. After the decontamination with H_2O_2 vapor, we waited until the atmospheric concentration of H_2O_2 adequately fell down. And then MSCs were exposed in the BSC for some periods, and their proliferation rates were evaluated.

2.2. Construction of a vinyl chamber in clean room

The chamber was composed of vinyl sheets (PVC Film Achilles flare FU-RE04; Achilles Corporation, Tokyo, Japan), including a BSC and H_2O_2 generator (DryDeco mobby MHPE-0411TK02; Taikisha Ltd., Tokyo, Japan) to produce H_2O_2 mist. It was constructed within a clean room to prevent H_2O_2 from leaking out and to keep the atmospheric concentration of H_2O_2 constant. The size of chamber was 9 m^3.

2.3. Measurement of H_2O_2 concentration in water and in atmosphere

Concentrations of H_2O_2 dissolved in water were measured using water analysis products (DIGITALPACKTEST-MULTI DPM-MT and PACKTEST WAK-H_2O_2; Kyoritsu Chemical-Check Lab Corp., Tokyo, Japan) for H_2O_2 (high concentration, 3–700 mg/L; low concentration, 0.05–5 mg/L).

Atmospheric concentrations of H_2O_2 were measured by H_2O_2 sensors (DragerPolytron700, 8317610; Drager Sensor H_2O_2 HC, 6809675, for 30–300 mg/L and Drager Sensor H2O2LC, 6809705 for 3–30 mg/L). We also used a detector tube (gas detector tube measurement system GV-110S and H_2O_2 detector tube No. 32; Gastec Corporation, Kanagawa, Japan) to confirm that H_2O_2 concentration was lower than 1.0 mg/L.

2.4. Cell preparation and cell culture

MSCs derived from human bone marrow were purchased from PromoCell GmbH (Heidelberg, Germany, Cat. C12974, Lot 4031804.5) and cultured in Dulbecco's Modified Eagle Medium (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (Nacalai Tesque, Kyoto, Japan) and 1% antibiotic-antimycotic mixed stock solution (100×), including penicillin 10,000 units/mL, streptomycin 10,000 μg/mL, and amphotericin-B 25 μg/mL (Nacalai Tesque). At experiment start, MSCs subcultured in three passages were seeded at a density of 2.2 × 105 cells/100 mm dish and cultured in a highly humidified incubator maintained with 5% CO2 at 37 °C. H2O2 for supplementation in culture medium was purchased form Fujifilm Wako Pure Chemical Corporation (Osaka, Japan), and sodium pyruvate solution was from Nacalai Tesque.

2.5. Cell calculation

To quantify the proliferation rate of cultured MSCs, detached MSCs were stained with trypan-blue solution. Cell numbers were counted using Countess® II FL (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.6. Statistical analyses

Statistical analyses were carried out using standard Student *t* tests, and error bars indicate standard deviation. A P value ≤ 0.05 was considered to represent a statistically significant difference.

3. Results

3.1. Decontamination with H_2O_2 in the working unit in CPF and its concentration in the atmosphere

The effects of residual H_2O_2 on cell growth after decontamination were evaluated using human MSCs in an experimental vinyl chamber installed within a cell processing clean room. As shown in Fig. 1a, two types of H_2O_2 sensors, for high concentration (30–300 mg/L; sensors A and B) and for low concentration (3–30 mg/L; sensor c), were set in the chamber. Sensors A and B were used during generation of H_2O_2, and sensor c was used for concentration monitoring after generation of H_2O_2 was stopped.

In the chamber, H_2O_2 was generated to keep the concentration in the atmosphere at approximately at 200 mg/L for 5 h. During this time, the BSC continued running, and the H_2O_2 of the atmosphere within the cabinet was kept about 200 mg/L, just below the concentration outside the cabinet. For concentrations during and after the generation of H_2O_2, values detected by sensors are shown in Fig. 1. After stopping the generation, the cracking unit was started, and H_2O_2 concentrations at sensors A and B were rapidly degraded to 50 mg/L. However, after that, the decrease rate of H_2O_2 became slower and slower. It was not until 25 h and 40 min after stopping generation of H_2O_2 that the sensitive detector tube showed 1.0 mg/L, which is the time-weighted average threshold limit value of H_2O_2 by ACGIH [18]. At that time point, defined as Time A, we started the clean room ventilation and removed the vinyl sheets.

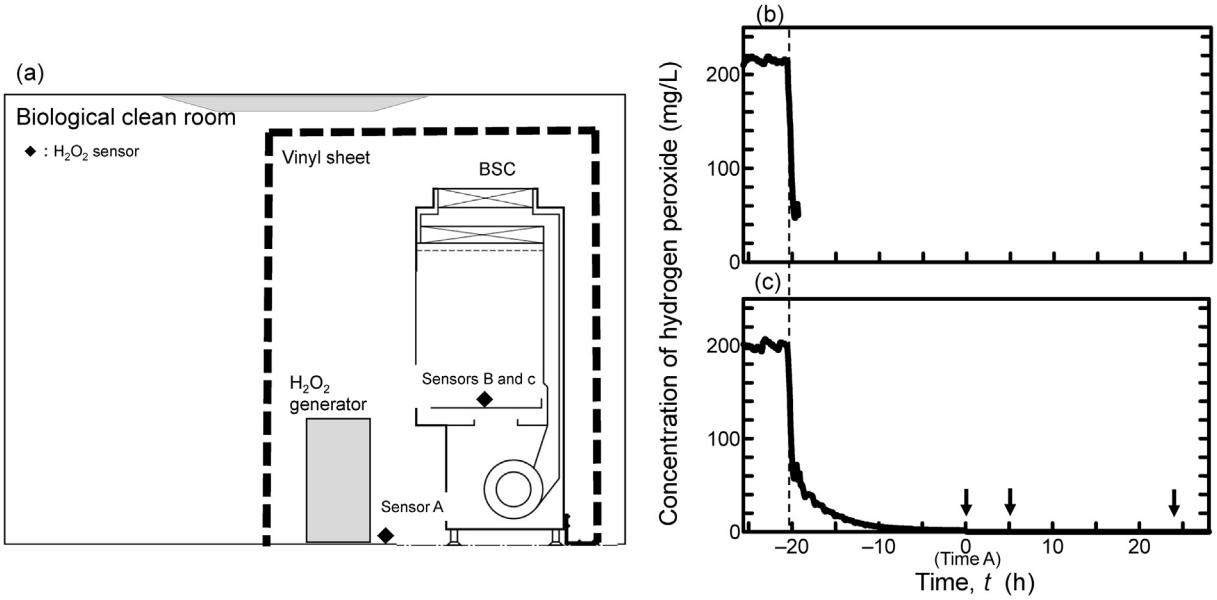

Fig. 1. The construction of experimental chamber in clean room and H_2O_2 concentrations. (a) Schematic illustration of the experimental environments. An experimental chamber with vinyl sheet was set in a biological clean room including a BSC and H_2O_2 generator. Filled squares indicate H_2O_2 sensors, and the dotted line indicates the vinyl sheet. (b) Time profile of H_2O_2 concentration. Curved line shows the concentration of H_2O_2 in the vinyl sheet measured by the sensor A in Fig. 1 during the decontamination. (c) Line shows the concentration of H_2O_2 in the BSC measured by sensor B and c in Fig. 1 during and after decontamination. The minimum detection limit of the sensors was 3 mg/L. Time A: $t = 0$ is defined as the time point at which H_2O_2 concentration reached 1 mg/L, as measured by a detection tube. The dotted line indicates the end of 5 h of decontamination. Each arrow indicates the time when the respective dish was uncovered in the subsequent experiments.

To confirm the efficacy of H_2O_2 against microbes, biological indicators covered with over 1×10^6 of spore bacteria were placed at three points in the BSC before H_2O_2 generation. We could identify no growth in any of the biological indicators after a 7-day incubation at 55 °C (data not shown).

3.2. Concentrations of integrated H_2O_2 in the water exposed to atmosphere

To investigate how much H_2O_2 residual in the atmosphere after decontamination could be integrated into water, 100-mm dishes filled with distilled water to 10 mm depth were exposed covered or uncovered in the BSC from Time A, 5 h and 24 h after Time A, respectively. Dishes were retrieved after 1, 2, or 3 h of exposure in the cabinet, and the concentrations of H_2O_2 in the water of dishes were measured.

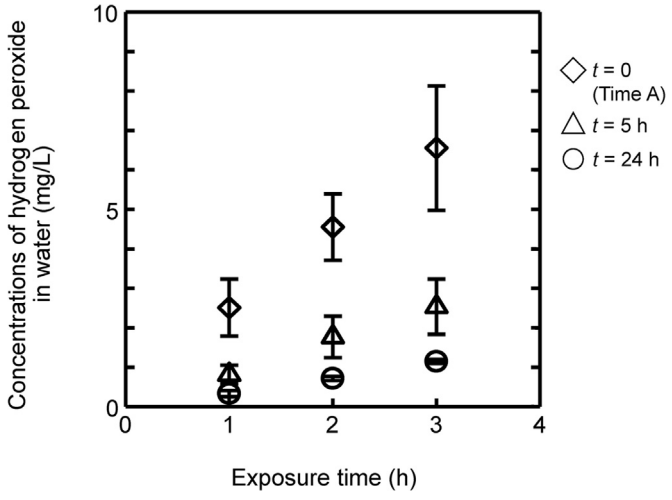

Fig. 2. Concentrations of H_2O_2 in water. Uncovered dishes of distilled water at 10 mm of depth were placed in the BSC for 1, 2, and 3 h from 0, 5, and 24 h after Time A, respectively. Vertical bars indicate the standard deviations (n = 3).

Concentrations of H_2O_2 in the water of dishes are shown in Fig. 1b and c. When the dishes were placed uncovered in the cabinet at Time A, the H_2O_2 concentrations in the water were 2.5, 4.6, and 6.6 mg/L after 1, 2, and 3 h of exposure, respectively, even though the concentration in the air flow in cabinet was no more than 1.0 mg/L. For the same measurements performed with dishes placed at 5 h after Time A, each concentration was 0.8, 1.6, and 2.5 mg/L, respectively, and at 24 h after Time A, they were 0.3, 0.7, and 2.5 mg/L, respectively (Fig. 2). These results suggest that the residual H_2O_2 in the BSC was integrated into the water exposed to the air flow even after some substantive waiting time and that this integration rate depended on the exposure period.

3.3. The correlation of H_2O_2 concentration dissolved in the medium with the proliferation of MSCs

To elucidate the effects on cell growth of H_2O_2 dissolved in the culture medium, we observed cell proliferation following addition of known doses of liquid H_2O_2 directly in the medium. MSCs were seeded in the medium supplemented with H_2O_2 at the concentrations of 0, 0.1, 0.2, 0.5, 1.0, and 5.0 mg/L, respectively, and cultured in the CO_2 incubator for 48 h. Cell proliferation and survival rates were significantly reduced dose dependently with more than 0.5 mg/L of H_2O_2 (Fig. 3), and MSCs were killed off with 5.0 mg/L of H_2O_2. These results, together with those in Fig. 2, suggest that during operation of the BSC, residual H_2O_2 could dissolve in the culture medium and affect later cell proliferation.

3.4. The effects of residual H_2O_2 in the cabinet on the proliferation of MSCs

To examine the effects of residual H_2O_2 in BSC on the proliferation of cells, we seeded defrosted MSCs in 100-mm dishes at a

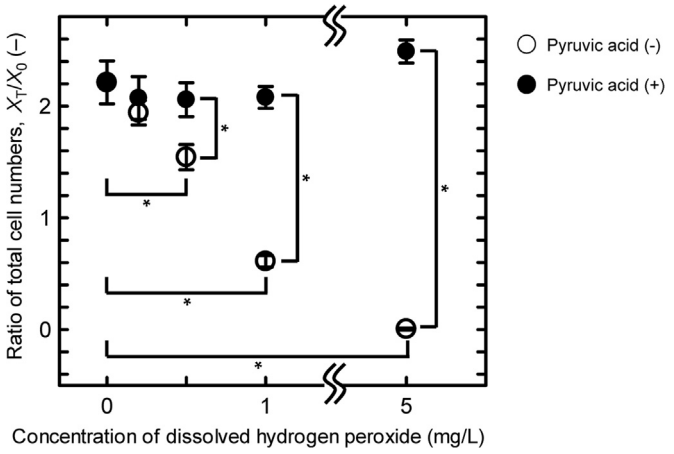

Fig. 3. Ratio of total cell numbers of MSCs cultured in the medium mixed with H_2O_2. MSCs were cultured for 48 h in the medium supplemented with H_2O_2 at a concentration of 0, 0.2, 0.5, 1.0, and 5.0 mg/L, respectively, with (filled circle) or without (open circle) pyruvic acid. Vertical bars indicate the standard deviations (n = 3). Statistical significance based on the student's *t*-test (*P < 0.01).

concentration of 5000 cells/cm^2, i.e., 275,000 cells/dish. After 24 h of incubation, these dishes were placed covered or uncovered for an hour in the cabinet decontaminated with H_2O_2 or in the cabinet not exposed to H_2O_2. After that, MSCs in each dish were cultured in CO_2 incubator for 48 h and their viability assessed. When the MSCs were placed uncovered in the BSC at Time A, almost all the MSCs were killed off after 48-h culture. However, when the dish was covered, the proliferation rate of MSCs was not significantly different from those in an uncovered dish in the cabinet not exposed to H_2O_2. When MSCs were placed in the cabinet after 5 h of waiting time from Time A, the proliferation rate of cells in the uncovered dish was also significantly reduced compared to that of cells in the covered dish or not exposed to residual H_2O_2. The 24 h of waiting time from Time A, i.e., 50 h from stopping decontamination, could almost entirely cancel the effects of residual H_2O_2 on the cell proliferation rate (Fig. 4). These results suggest that H_2O_2 remaining in the equipment could affect cell proliferation and survival for more than 30 h after decontamination.

3.5. Cancellation of H_2O_2 toxicity by pyruvic acid

We also examined the effects of pyruvic acid, which has been reported to protect cells against reactive oxygen species such as H_2O_2 [9,19]. MSCs were cultured in the medium supplemented with 1 mM of pyruvic acid along with liquid H_2O_2 at concentrations of 0, 0.1, 0.2, 0.5, 1.0, and 5.0 mg/L, respectively, and their proliferation evaluated. As shown with filled circles in Fig. 3, the proliferation of MSCs cultured in medium including pyruvic acid was not affected even by 5.0 mg/L of H_2O_2. Thus, the effects of residual H_2O_2 after decontamination can be avoided by a deoxidizing composition of medium as well as the waiting time after decontamination.

4. Discussion

Sterilization of BSCs in the clean room is now mainly achieved with wiping using decontamination agents. However, because fungi or bacterial spores trapped in HEPA filters and other equipment can diffuse into the atmosphere, we should develop a safe and effective way to decontaminate the whole environmental space, based on valid evidence. We studied the effects of residual H_2O_2 on the cell viability and proliferation, and our results will provide some important information in achieving new methods of decontamination for the entire space.

We detected residual H_2O_2 that was sufficient to affect cell proliferation and survival at more than 30 h after stopping H_2O_2 generation. Today, in cell/tissue engineering fields as well as in pharmaceuticals, H_2O_2 vapor is gaining in importance as a decontamination agent of cell processing equipment, especially of cell processing isolators [20]. Some studies have reported that the effects on microbes of some fixed concentrations of H_2O_2 are influenced by the atmospheric humidity [21,22]. Because H_2O_2 is highly stable in liquid form, it can be easily condensed in water, so that we have to consider that exposure to a low concentration of H_2O_2 in atmosphere can mean a higher concentration in culture medium. It is probable that cells processed in isolator systems have been damaged because of residual H_2O_2 without noticed, because cells from organs have their own individual features and strict specifications cannot be applied across all cell/tissue products. Although clean rooms and isolator systems in cell culture engineering have been widely developed, H_2O_2-inactivation time for changeover has

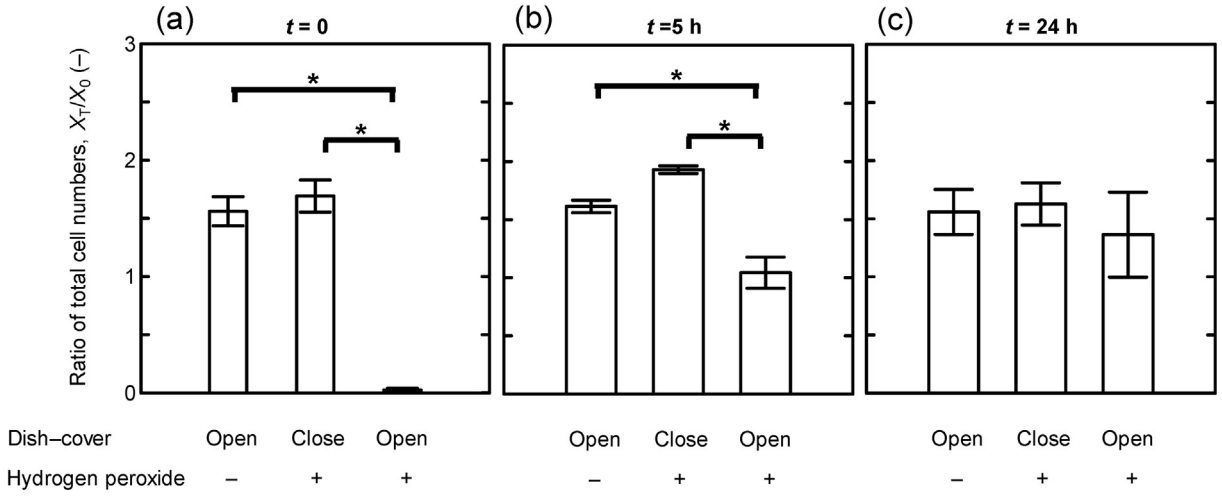

Fig. 4. Ratio of total cell numbers of MSCs exposed to the atmosphere after decontamination. MSCs were cultured for 72 h after exposure to the atmosphere in the BSC for 1 h from the indicated points ($t = 0$ (a), 5 (b), and 24 (c) hours after Time A, respectively). At each time, cell dishes were placed uncovered in the not-decontaminated BSC (left bar), covered in the decontaminated BSC (middle bar), or uncovered in the decontaminated BSC with H_2O_2 (right bar). Vertical bars indicate the standard deviations (n = 3). Statistical significance based on the student's *t*-test (*P < 0.01).

not established yet. More data are needed to establish guidance for the use of clean room and isolators for cell processing.

The reason H_2O_2 vapor remained so long in the atmosphere in the BSC may be mainly that a non-negligible amount of H_2O_2 was absorbed onto the high efficiency particulate air (HEPA) filter attached to the BSC, which flowed down into the culture dish along with the blowing air from the filter. How much H_2O_2 can be absorbed in a HEPA filter is unknown and should be our next area of focus. In our data in Fig. 1, H_2O_2 concentration in atmosphere in the BSC was a little lower than that outside of the BSC during the generation of H_2O_2, and this gap might represent absorbed and accumulated H_2O_2 in the HEPA filter or in other parts of the BSC throughout the decontamination period.

In the case of a covered culture dish or flask (data not shown), H_2O_2 in the atmosphere had little effect on cell proliferation, even though the inside gas was exchanged effectively and maintained gas balance with the outer air. This fact suggests that in the event of an uncovered dish, the surface of the culture medium was contacting the air flow including H_2O_2 but at a very low concentration and was continuously blending and condensed into the culture medium. On the other hand, because the ventilation velocity of the covered dish or flask is slower than the dissolving speed of H_2O_2 into culture medium, the inside H_2O_2 concentration would have become lower and lower. When we use containers with a wide contact area with the outer air such as a culture dish, we invest in shortening the operating time.

Vapor phase H_2O_2 has effective broad-spectrum antimicrobial properties and inactivates bacteria, fungi, viruses, and highly resistant spores by its oxidizing functions [15,23]. However, several endogenous or exogenous molecules in the organisms, such as cysteine, glutathione, methionine, ascorbic acid, and α-keto acids, including pyruvic acid, alter oxidation of proteins and important cellular components caused by H_2O_2 and reactive oxygen species [24]. The presence of catalase or other peroxidases in these organisms can increase tolerance in the presence of lower H_2O_2 concentrations. In this case, higher concentrations and longer contact times would be required for sporicidal activity, and the amount of residual H_2O_2 in the atmosphere or in the detail of device is non-negligible.

Pyruvic acid is a main player in energy metabolism in cells. It supplies energy to cells through the citric acid cycle (also known as the Krebs cycle) under aerobic conditions and produces lactate when oxygen is lacking. In the citric acid cycle, pyruvic acid is carboxylated into oxaloacetate, which reduces NADH to NAD. Thus, pyruvic acid is an excellent scavenger of oxidant species through its own decarboxylation [19]. By supplementing culture medium with pyruvic acid, the effect of H_2O_2 on cultured cells in culture medium can be canceled without undermining its antiseptic effects in the atmosphere.

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