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Original Article

Effects of continuous exposure to low concentration of ClO_2 gas on the growth, viability, and maintenance of undifferentiated MSCs in long-term cultures

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ABSTRACT

Introduction: Hygienic management is more important in the manufacturing of cell products than in the production of chemical agents, because cell material and final product cannot be decontaminated. On the other hand, especially in the selection of hygienic agent, the adverse effects on the cells must be considered as well as the decontamination effect. ClO_2 is a potent disinfectant, which is now expected as a safe and effective hygienic agent in the field of cell production. In this study, we investigated the effects of low dose ClO_2 gas in the atmosphere of CO_2 incubator on the characteristics of MSCs cultured in it.
Methods: First, we installed a ClO_2 generator to a CO_2 incubator for cell culture in which a constant level of ClO_2 can be maintained. After culturing human cord derived MSCs in the CO_2 incubator, the characteristics of cells were analyzed.
Results: Continuous exposure to 0.05 ppmv of ClO_2 gas did not affect cell proliferation until at least 8th passage. In the FACS analysis, antigens usually expressed on MSCs, CD105, CD90, CD44, CD73 and CD29, were positively observed, but differentiation markers, CD11b and CD34, were little expressed on the MSCs exposed to 0.05 ppmv or 0.1 ppmv of ClO_2 gas just as on the control cells. Also in the investigation for cell death, 0.05 ppmv and 0.1 ppmv of ClO_2 gas little affected the viability, apoptosis or necrosis of MSCs. Furthermore, we assessed senescence using SA-β-gal staining. Although the frequency of stained cells cultured in 0.1 ppmv of ClO_2 gas was significantly increased than that of not exposed cells, the stained cells in 0.05 ppmv were rare and their frequency was almost the same as that in control.
Conclusions: All these results indicate that, although excessive concentration of ClO_2 gas induces senescence but neither apoptosis nor cell differentiation, exposure to 0.05 ppmv of ClO_2 gas little affected the characteristics of MSCs. In this study we demonstrate that continuous exposure to appropriate dose of ClO_2 gas can be safely used as decontamination agent in cell processing facilities.

Abbreviations: ClO_2, chlorine dioxide; H_2O_2, hydrogen peroxide; HEPA, high efficiency particulate air; PMD Act, Pharmaceuticals and Medical Devices Act; MSCs, mesenchymal stem cells; WHO, World Health Organization; EPA, Environmental Protection Agency; FDA, Food and Drug Administration; TWA, time weight average; OSHA, Occupational Safety and Health Administration.

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1. Introduction

Various products are manufactured in the field of regenerative medicine, and numerous laws and guidelines govern the manufacturing of cell products all over the world. In Japan, two laws established in November 2014 [1–4]—the Act on the Safety of Regenerative Medicine and the PMD Act—were implemented, and legal regulations both in clinical research and in production of regenerative products were devised. However, because no well-established guidelines exist for the production of cell products at the bench in cell processing facilities (CPFs) [5], each individual person at each facility operates production under their own judgment regarding specific issues and situations.

Hygienic management is more important in the manufacturing of cell products than in the production of chemical agents. Many rules accepted in the field of chemical manufacturing cannot be directly applied for cell products because of the specific characteristics of cells. Because cells are raw and living and are sensitive to temperature and most disinfectants, finished products cannot be chemically or physically sterilized.

When selecting decontamination agents for CPFs, the adverse effects on staff and cell materials must be considered along with their antimicrobial effects. Hygiene management in CPFs is mainly conducted by air current through HEPA filters and by establishing air pressure gradients between cleanrooms with different classes. However, some types of microbes entering clean rooms together with persons and goods and sometimes with minute insects cannot be eliminated in such a manner. Especially, in CO_2 incubators with a high humidity and habitable temperature, only a few surviving and proliferating microbes can be enough to be harmful. Thus, ways to disinfect the whole space are desirable. Although, formerly, formaldehyde was used for atmosphere decontamination in various facilities, various studies have reported its cancer-causing effects [6,7]. Currently, the use of formaldehyde is restricted by the WHO and other environmental regulatory authorities. In contrast, hydrogen peroxide (H_2O_2) is widely used for decontamination in pharmaceutical plants and CPFs [8,9] because of its wide-spectrum antimicrobial efficacy against various microbes including bacterial spores, viruses, and yeasts. However, we have reported that the post-decontamination residual H_2O_2 in the atmosphere or surface of equipment has unignorable effects on the viability and growth of mesenchymal stem cells over an extended period [10].

Chlorine dioxide (ClO_2) is also a potent disinfectant, which is very different from elemental chlorine in that ClO_2 does not produce trihalomethane or other halogenated organic compounds with carcinogenic potency [11,12]. It is considered as relatively safe. Aqueous ClO_2 is approved for control of pathogenic and spoilage microorganisms in drinking water and food including fruits, vegetables, and table birds if used under the proper regulations and concentrations provided by the WHO Guidelines for Drinking-water Quality, the EPA and the FDA [12,13]. Gaseous ClO_2 also has powerful antimicrobial activities and is widely used in hospitals and other healthcare environments to sterilize medical equipment, rooms and tools [14]. Although carcinogenicity in long-term exposure to ClO_2 has been denied by studies in vivo and in vitro, at high concentrations, ClO_2 has lung, oral, and ocular toxicities [15]. Therefore, EPA has set a maximum level of 0.8 mg for ClO_2 in drinking water, and the OSHA, an agency of the United States Department of Labor, has set a permissible exposure limit of 0.1 ppmv in air for people working with ClO_2 [16]. ClO_2 gas has antimicrobial activities even at low concentrations. Recent studies have reported that 0.05 ppmv of ClO_2 gas, which is half of the 8-h TWA concentration, has antibacterial effects and can inhibit hyphal growth of fungi [17,18]. Furthermore, It is reported that 0.03 ppmv of gaseous ClO_2 completely inhibited the infection by aerosol-induced influenza A virus in mice [19].

Considering these factors, we hypothesize that continuous exposure to ClO_2 gas can be a safe and useful method for prevention of contamination and hygienic control in CPF. In this study, we investigated the effects of low-dose ClO_2 gas in the atmosphere of a CO_2 incubator on the characteristics of MSCs, such as cell proliferation, cell death, maintenance of stemness and senescence, repeatedly subcultured several times in the incubator and found the optimum gas concentration in incubators for cell culture.

2. Materials and methods

2.1. Cell culture

MSCs derived from human umbilical cord matrix were purchased from PromoCell (Heidelberg, Germany). The cells were seeded on 100 mm culture dishes (Corning, Rochester, NY, USA) at a density of 5000 cells/cm^2, cultured with Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) and 1% (v/v) antibiotic-antimycotic mixed stock solution (Nacalai Tesque), and cultivated in a CO_2 incubator (PHC, Osaka, Japan) filled with or without 0.05–0.1 ppmv of ClO_2 gas. The culture medium was replaced every 2 days, and cells were subcultured every 5 days.

2.2. Generation of ClO_2 gas and the control of gas emission

ClO_2 gas was obtained from an electrochemical system as previously described [14]. Briefly, sodium chlorite solution and electrolyzed gaseous chlorine can produce high-purity chlorine dioxide gas with sodium chloride. ClO_2 gas emission can be stabilized by on-off intermittency of the electrical current. ClO_2 gas concentration in the incubator was continuously measured every minute using a ClO_2 analyzer (model GD-70D, Riken Keiki, Tokyo, Japan) and was maintained at 0.05 and 0.1 ppmv, respectively, with errors within 30%.

2.3. Cell proliferation analysis

The population doubling level (PDL) was calculated using the following equation:

$$PDL = \log_{10}(X_1/X_0)/\log_{10}2$$

where X_0 is the initial seeded cell number, and X_1 is the final cell number at each passage. The estimated growth efficiency and proliferation potential of the MSCs were determined based on the total cumulative population doubling level (CPDL) [20].

2.4. Flow cytometry analyses

Expression of cell surface markers was assessed using a flow cytometer (FACS Canto II, BD Bioscience, San Jose, CA, USA). Mouse monoclonal anti-human CD90-FITC, CD34-FITC, CD11b-FITC, CD44-FITC, CD29-PE, and CD73-PE antibodies were purchased from BioLegend (Cambridge, UK), and mouse anti-human CD105-PE antibody was purchased from Miltenyi Biotec (Bergisch-Gladbach, Germany). Cells were washed with PBS and were detached with TrypLE select (Thermo Fisher Scientific); then, 200,000 MSCs were resuspended in 200 μL of Dulbecco's modified phosphate-buffered saline (D-PBS) (Nacalai Tesque) including 1% FBS, and the anti-

bodies were added. For isotype controls, goat anti-mouse IgG1 conjugated with fluorescein isothiocyanate (FITC) and PE were used. The samples were then run and analyzed on flow cytometer, and data were analyzed using FlowJo Software Ver. 10 (BD Bioscience).

For evaluation of cell death, cells were stained with propidium iodide (PI) and FITC conjugated annexin V using Annexin V-FITC Apoptosis Detection Kit (Nacalai Tesque), according to the manufacturer's protocol. The populations of apoptosis and necrosis were analyzed via flow cytometry.

2.5. Quantitative real-time PCR

Quantitative analyses of mRNA expression were performed with StepOne Real-Time PCR System (Thermo Fisher Scientific) using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Extracted RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). Sequences of each primer set are listed in supplemental Table 1 (TaKaRa, Tokyo, Japan).

2.6. Senescence associated-β-Gal staining

The expression of pH-dependent senescence associated β-galactosidase (SA-β-gal) activity was analyzed using the Cellular Senescence Assay Kit (CELL BIOLABS, San Diego, CA, USA) according to the manufacturer's guidelines. Briefly, after washing twice with D-PBS, cells were fixed with the fixing solution and were incubated with cell staining solution under light shielded conditions at 37 °C overnight in 1X cell staining solution. The morphology and cell staining of adhered cells were observed by phase-contrast light microscopy.

2.7. Data analysis

The data were expressed as mean ± standard deviation of at least three independent experiments. The obtained data were statistically analyzed using the Student's t test. $p < 0.05$ was considered significantly different.

3. Results

3.1. The effects of continuous exposure to ClO_2 on the proliferation of MSCs

To evaluate the effects of continuous exposure to low concentration of ClO_2 on the proliferation of MSCs, we installed a ClO_2 generator to a CO_2 incubator for cell culture in which a constant level of ClO_2 was maintained. In previous reports, 0.075 ppmv of ClO_2 gas has shown antibacterial and antiviral effects and has been found to inhibit the hyphal growth of fungi [17,18]. In this study, we confirmed the anti-fungal effects of 0.05 ppmv ClO_2 gas in the atmosphere of a CO_2 incubator. As shown in Fig. 1a, the proliferation and hyphal growth of fungi *Cladosporium herbarum* (NBRC 31006) (Institute of Environmental Biology, JDC Corporation, Kanagawa, Japan) on the indicator were inhibited almost perfectly.

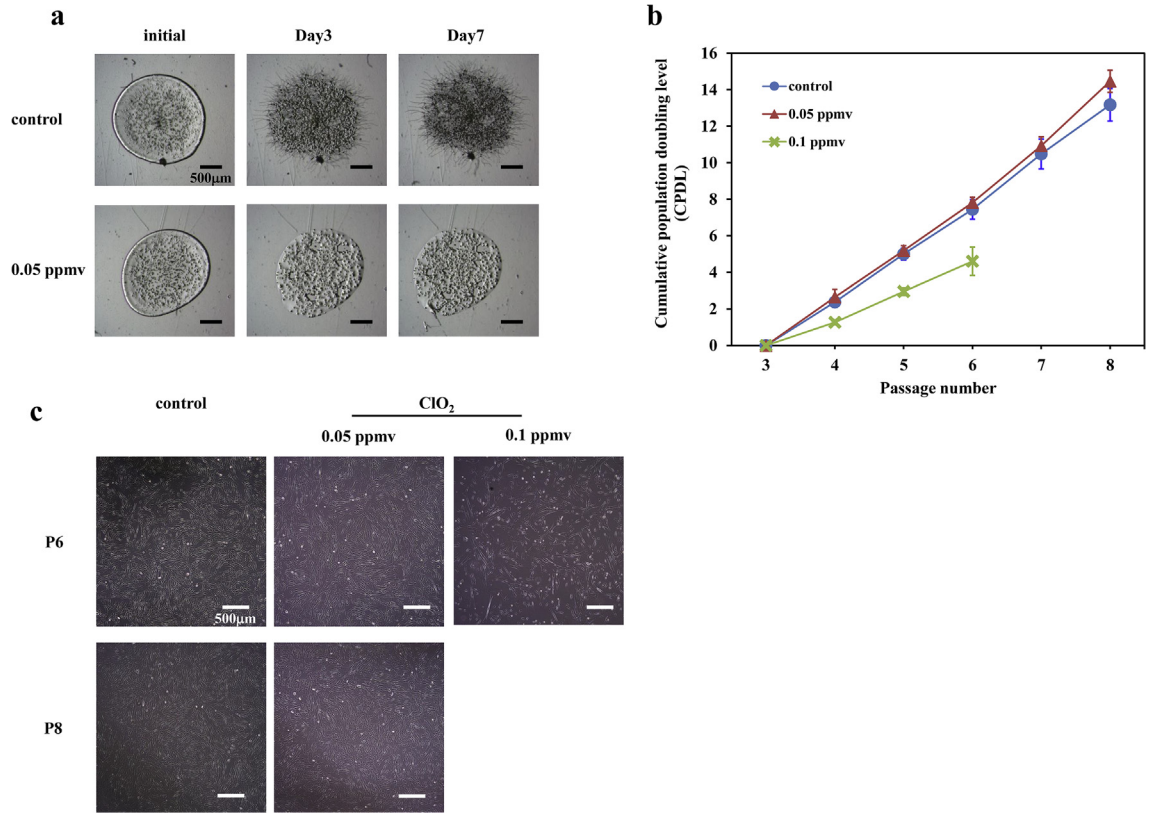

Fig. 1. Effects of ClO_2 gas in the atmosphere of CO_2 incubator on the proliferation of MSCs. (a) Microscopy images of biological indicators for *Cladosporium herbarum*. Indicators were settled in the CO_2 incubator filled with or without 0.05 ppmv of ClO_2 gas. On Day 3 and Day 7, the growth of fungi was observed by microscopy (40 × magnification). Length of the black scale bars: 500 μm. (b) Cumulative population doubling levels of MSCs cultivated at various ClO_2 gas conditions (blue circles: 0 ppmv (control); red triangles: 0.05 ppmv; green crosses: 0.1 ppmv) from P4 to P8 (to P6 in 0.1 ppmv). On the x axis, one passage means 5 days. As MSCs cultured with 0.1ppmv of ClO_2 came unstuck at P7, we could perform no more successive culture and green line plot stop at P6. Data are expressed as the mean ± standard deviation (n = 3). (c) Microscopy images at P6 (0, 0.05 and 0.1 ppmv, upper panels) and P8 (0 and 0.05 ppmv, lower panels) of MSCs cultivated without (control) or with ClO_2 gas (40 × magnification). Length of the white scale bars: 500 μm.

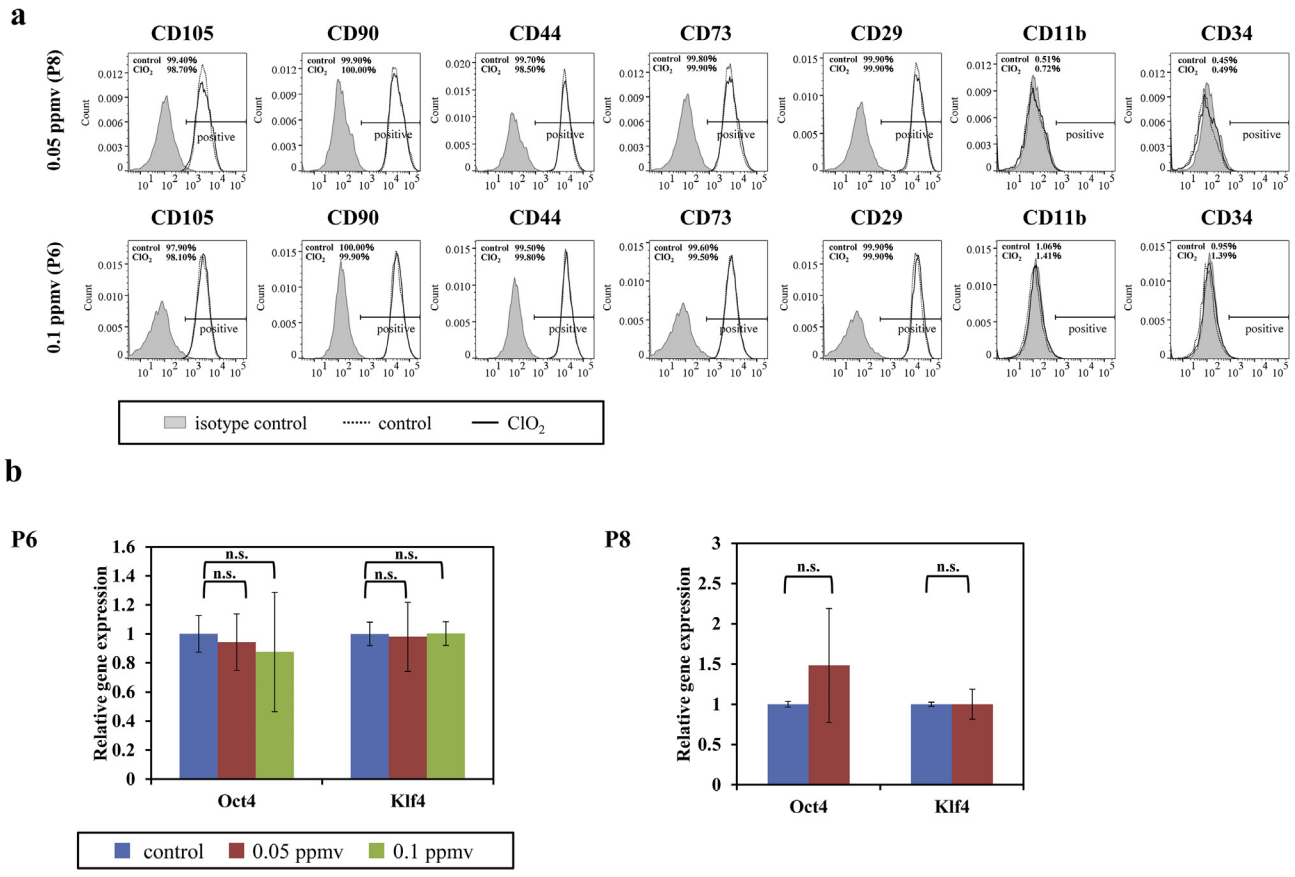

Fig. 2. Expression profiles of differentiation/undifferentiation markers. (a) Expressions of surface markers at passage 8 on MSCs cultivated with or without 0.05 ppmv of continuous ClO_2 gas (upper panels) and at P6 with 0.1 ppmv (lower panels) were analyzed by FACS analysis: surface markers expected to be positive (CD105, CD90, CD44, CD73, and CD29) and negative (CD11b and CD34) in MSCs. The percentages of cells stained positively with each respective marker are indicated. In each panel, solid lines indicate cells exposed to gas, dotted lines indicate cells not exposed to gas, and the grey histogram represents isotype-matched negative control cells. (b) Expressions of stemness-related genes (Oct4 and Klf4) in MSCs cultivated at various ClO_2 gas concentrations at P6 (0, 0.05 and 0.1 ppmv, the left side) and P8 (0 and 0.05 ppmv, the right side). Relative expression levels compared with control are shown. Data are expressed as the mean ± standard deviation (n = 3). n.s. means not significantly different.

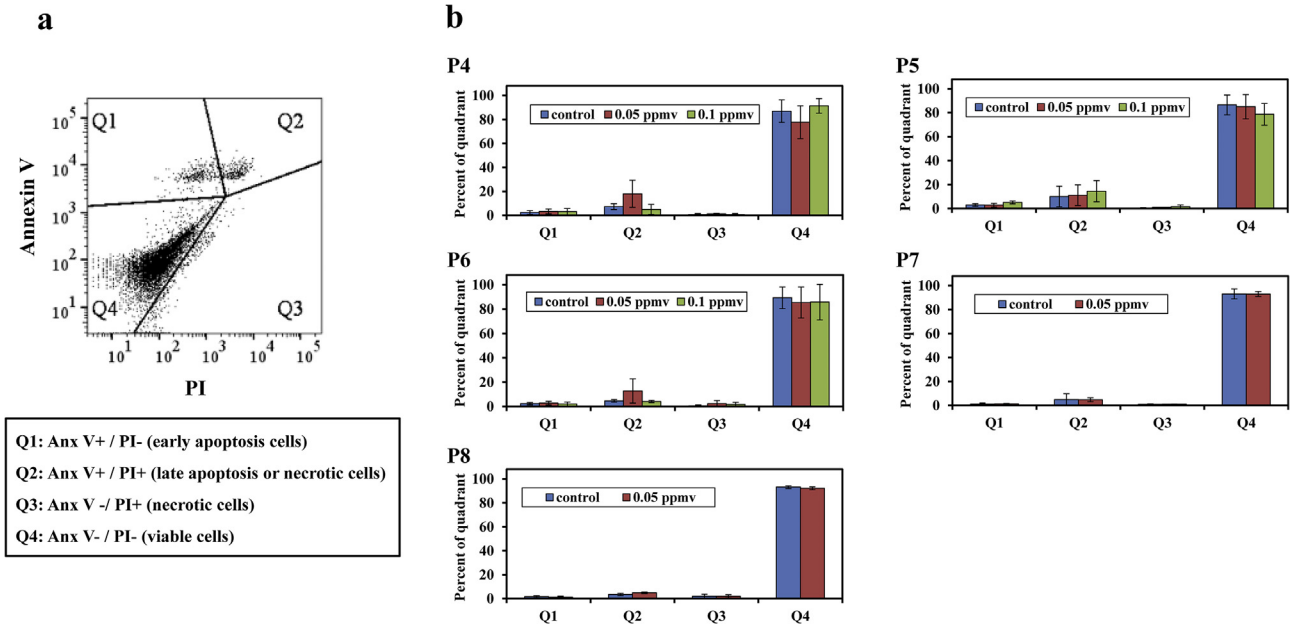

Fig. 3. Evaluation of cell death. (a) MSCs cultured under various conditions (without or with 0.05 or 0.1 ppmv of ClO_2 gas) were double-stained with FITC-conjugated annexin V and PI and were subjected to FACS analysis. In the dot plot analysis, cells can be classified into four areas: early apoptosis cells (Q1), late apoptotic or necrotic cells (Q2), necrotic cells (Q3), and viable cells (Q4). (b) Populations in each area at the respective passages are shown and are expressed as the mean ± standard deviation (n = 3). ✳ = $p < 0.05$, Blue bars indicate control, red bars 0.05 ppmv of ClO_2, and green bars 0.1 ppmv. As MSCs with 0.1ppmv could not be subcultured, green bars are only in P4–P6.

MSCs from human umbilical cord subcultivated for four passages were prepared and serially cultivated to the 8th passage in the CO_2 incubator with or without ClO_2. We evaluated CPDL values of MSCs cultivated under each condition to compare their proliferation potential. As shown in Fig. 1b, although the CPDL of the cells exposed to 0.1 ppmv of ClO_2 gas was significantly lower than that of the control (without ClO_2 gas) at 6th passage (4.52 vs. 7.42, $p < 0.05$), continuous presence of ClO_2 gas at 0.05 ppmv did not influence CPDL during the entire examined passages.

Fig. 1c shows the microscopic images of MSCs. On the 6th passage, cells cultured with 0.1 ppmv ClO_2 gas had a significantly smaller density than the control, and some of cells had a spread shape as typically observed in senescent cells. After the 6th passage, cells were detached and neither their CPDL nor morphology could be observed. On the other hand, cells exposed to 0.05 ppmv ClO_2 had almost the same density and the same shape as those of the control both at the 6th and 8th passage.

These results suggest that ClO_2 gas at 0.05 ppmv in the atmosphere hardly affects cell proliferation until at least the 8th passage.

3.2. Evaluation of stemness by flow cytometry and real-time PCR

To examine the influence of the long-term exposure to ClO_2 gas on the maintenance of stemness of MSCs, we carried out flow cytometry analysis using antibodies for several surface markers positively and negatively specific for MSCs [21] at 6th passage (0.1 ppmv) and at 8th passage (0.05 ppmv). All examined antigens usually expressed on MSCs (CD105, CD90, CD44, CD73 and CD29) were positively expressed on more than 90% of cells exposed to 0.05 ppmv and 0.1 ppmv ClO_2 just as on those without ClO_2, but the expression of differentiation markers, CD11b (macrophages) and CD34 (endothelial/hematopoietic progenitor cells), was low on the cells in all three groups (Fig. 2a and Supplemental Table 1).

To further examine the influence of exposure to ClO_2 gas on the stemness-related gene expression, mRNA expressions of Oct4 and Klf4 in MSCs were analyzed using real-time PCR analysis (Fig. 2b). At the 6th passage, the expression of these mRNAs in MSCs cultured both with 0.05 and 0.1 ppmv ClO_2 gas was not significantly different from those in control MSCs. Similarly, at the 8th passage, the expression in MSCs cultured with 0.05 ppmv ClO_2 gas did not show significant difference compared with control.

These results strongly suggest that continuous ClO_2 gas exposure at 0.05 and 0.1 ppmv hardly affects the maintenance of undifferentiated states of MSCs till 8 passages.

3.3. Analysis of cell death

Next, to investigate the effects of the exposure to ClO_2 gas on the mortality of MSCs, the MSCs cultured under various conditions were double-stained with FITC-conjugated annexin V and

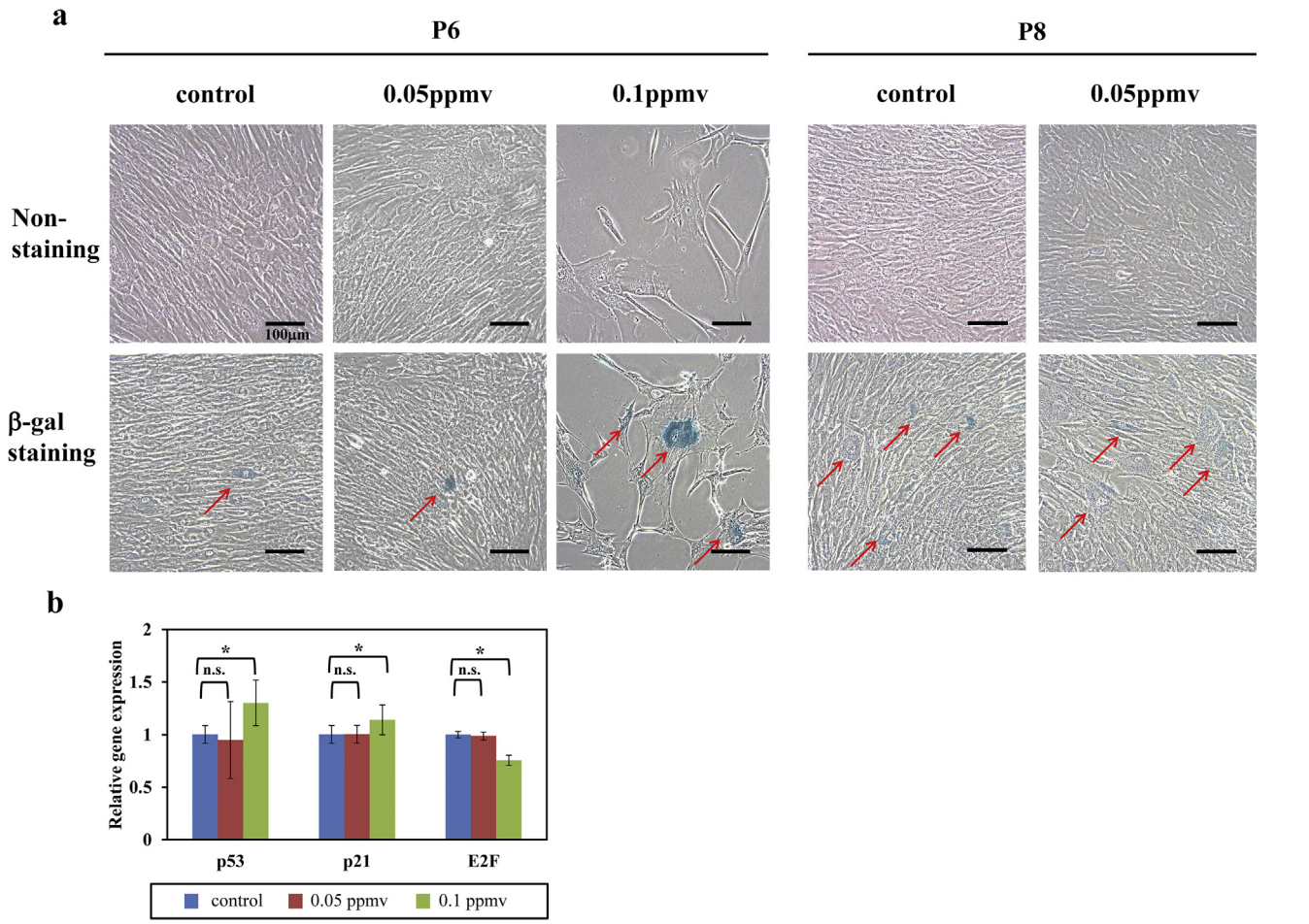

Fig. 4. Evaluation of senescence of MSC. (a) Morphological changes of non-staining and SA-β-gal staining (red arrows) on MSCs cultivated with and without ClO_2 gas at passage 6 (0, 0.05, and 0.1 ppmv) and 8 (0 and 0.05 ppmv). Length of the black bars corresponds to 100 μm. Red arrows indicate green-stained senescent cells. (b) Expressions of senescence related genes (p53, p21, and E2F) in MSCs cultivated at various ClO_2 gas concentrations at P6 (0 as control, 0.05 and 0.1 ppmv). Relative expression levels compared with control are shown. Data are expressed as the mean ± standard deviation ($n = 3$). * means $p < 0.05$, and n.s. "not significantly different" when compared with control.

propidium iodide (PI) and were subjected to FACS analysis. Using two-dimensional dot plot, cells can be split into four groups—FITC-single positive: early apoptotic, FITC- and PI-double positive: late apoptotic or necrotic, PI-single positive: necrotic, and double negative: viable cells [22] (Fig. 3a).

As shown in Fig. 3b, from the 4th to 6th passage, the ratios of viable MSCs were larger than 80%, and those of viable, apoptotic, and necrotic cells had little differences between the three groups. For the other two passages, we subsequently observed cell death and viability of MSCs cultured with or without 0.05 ppmv of ClO_2 gas. The populations in any of the fields were almost the same in the two groups. These results suggest that the exposure to ClO_2 gas even at the concentration of 0.1 ppmv hardly affects the viability, apoptosis, and necrosis of MSCs.

3.4. Effects of exposure to low concentration of ClO_2 in the atmosphere on the cellular senescence of MSCs

Finally, we examined the cellular senescence in MSCs. After the CO_2 incubator was filled with 0.1 ppmv or 0.05 ppmv of ClO_2 gas or without ClO_2 gas and cells were cultured for 6 passages, MSCs were supplemented with X-gal at an acidic condition (pH 6.0). Then, only cells in the senescent state were stained blue-green. Just as observed in Fig. 1b, although cells cultured in 0.1 ppmv ClO_2 gas had significantly smaller density than that in control and some of cells had a spread shape as typically observed in senescent cells, MSCs cultured in 0.05 ppmv of ClO_2 had almost the same density and shape as those without gas both at the 6th and 8th passages. Furthermore, the spread shaped cells in 0.1 ppmv ClO_2 were strongly stained in blue-green, but the stained cells in 0.05 ppmv of ClO_2 were rare and their frequency was almost the same as that in control (Fig. 4a). To further clarify the molecular mechanism for the senescence, the expressions of p53 and its downstream p21 and E2F were examined using real time PCR. As shown in Fig. 4b, senescence related molecules, p53 and p21 were significantly increased and growth factor, E2F, which is inhibited by p53 and p21, was decreased only in MSCs exposed with 0.1 ppmv of ClO_2 but not in those control or 0.05 ppmv, which is compatible with the morphological results in Fig. 4a.

All these results indicate that the inhibition of cell proliferation observed in MSCs cultured continuously under 0.1 ppmv of ClO_2 gas is supposed to be caused by their senescent state. However, 0.05 ppmv of ClO_2 gas had little effect on the senescence of MSCs during long culture.

4. Discussion

ClO_2 is widely used as a disinfectant both in liquid and gaseous matter. Recently, it was reported that low-concentration of ClO_2 gas, within the concentration harmless to humans, can kill or inactivate viruses, bacteria, and fungi [17,18], which has created an expectation for it to be used in many fields such as the food and medicine industries.

The antiseptic effects of ClO_2 are caused by its oxidizing properties. In our data, MSCs cultured with 0.1 ppmv of ClO_2 gas were revealed to be in more senescent state compared to control. Recently, cellular senescence has been reported to be induced by oxidative stress [23], as well as telomere shortening [24], DNA damage [25], and activated oncogenes [26]. It is believed that reactive oxygen species (ROS) induce some DNA damage, which sequentially induces activation of p53 and thus cellular senescence [23]. In our data, in MSCs cultured with 0.1 ppmv of ClO_2, the expressions of p53 and p21 were elevated along with the increase of senescent cells. However, 0.05 ppmv of ClO_2 did not induce premature senescence or increase of p53 or p21, which means, at this concentration, the DNA damage in MSCs is not larger than that in MSCs cultured without ClO_2.

In contrast, although our data showed that 0.1 ppmv of ClO_2 gas was harmful to in vitro culture of MSCs, another previous paper reported that rats continuously exposed to 0.1 ppmv ClO_2 gas for 6 months showed no adverse events during the experiment [27], and American OSHA states that the 8-h TWA of permissible exposure level of the ClO_2 gas in workspace atmosphere is limited at 0.1 ppmv. The main reason for this divergence can be that the concentration of ClO_2 dissolved in liquid can be much higher than that in the atmosphere because of its very high solubility. In our study, the concentration of ClO_2 in water exposed to 0.1 ppmv of ClO_2 gas in the atmosphere elevated in a time dependent manner within 48 h (Supplemental Figure). Another reason can be that cells cultured in vitro are more susceptible to environmental stimulation than those in living organisms because in vivo, there are various functions to remove unfavorable factors for cells in their environment.

In this study, we evaluated various biological effects of continuous exposure to low-concentration of ClO_2 gas on MSCs to examine the safety for cell culture. From the result that the continuous exposure to 0.05 ppmv of ClO_2 gas is not harmful to MSCs, together with the fact that 0.05 ppmv of ClO_2 gas can inactivate microbes in the atmosphere, low-concentration of ClO_2 gas can be a convenient disinfection agent for cell culture environments, which will provide us a new strategy for supporting the development of regenerative medicine.

Declaration of Interest

Koushirou Sogawa, Kenji Yachiku, Takanori Miura, and Takashi Shibata belong to Taiko Pharmaceutical Co., Ltd, and are getting salary from the company.

Ryoma Okawa, Motoko Shiozaki, Hiroshi Takayanagi, and Sachiko Ezoe belong to the joint collaborative research laboratory with Taiko Pharmaceutical Co., Ltd.

Taiko Pharmaceutical Co., Ltd. had no control over the interpretation, writing, or publication of this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2019.12.007.

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