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Original Article

Evaluation of cleaning methods for change-over after the processing of cell products to avoid cross-contamination risk

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ABSTRACT

Introduction: Cell-processing facilities face the risk of environmental bacteria contaminating biosafety cabinets during processing, and manual handling of autologous cell products can result in contamination. We propose a risk- and evidence-based cleaning method for cross-contamination, emphasizing proteins and DNA.

Methods: The transition and residual risks of the culture medium were assessed by measuring both wet and dried media using fluorescence intensity. Residual proteins and DNA in dried culture medium containing HT-1080 cells were analyzed following ultraviolet (UV) irradiation, wiping, and disinfectant treatment.

Results: Wet conditions showed a higher transition to distilled water (DW), whereas dry conditions led to higher residual amounts on SUS304 plates. Various cleaning methods for residual culture medium were examined, including benzalkonium chloride with a corrosion inhibitor (BKC + I) and DW wiping, which demonstrated significantly lower residual protein and DNA compared to other methods. Furthermore, these cleaning methods were tested for residual medium containing cells, with BKC + I and DW wiping resulting in an undetectable number of cells. However, in some instances, proteins and DNA remained.

Conclusions: The study compared cleaning methods for proteins and DNA in cell products, revealing their advantages and disadvantages. Peracetic acid (PAA) proved effective for nucleic acids but not proteins, while UV irradiation was ineffective against both proteins and DNA. Wiping emerged as the most effective method, even though traceability remained challenging. However, wiping with ETH was not effective as it caused protein immobilization. Understanding the characteristics of these cleaning methods is crucial for developing effective contamination control strategies.

1. Introduction

Cell processing facilities harbor risks, notably environmental bacteria [1], which can potentially contaminate biosafety cabinets during processing [2–5]. Predominantly, the processing of these cell products is manually performed [6], increasing the likelihood of unintended contamination in the aseptic handling area (Fig. 1). Specifically, the culture medium used for processing autologous cells contains patient-specific proteins, DNA, and in the absence of proper cleaning processes, these residues of culture medium may continue to remain on the floor surfaces of biosafety cabinets.

Abbreviations: BKC+I, benzalkonium chloride with corrosion inhibitor; DW, distilled water; DNA, deoxyribonucleic acid; ETH, ethanol; FBS, fetal bovine serum; PAA, peracetic acid; SUS, stainless steel; UV, ultra violet.

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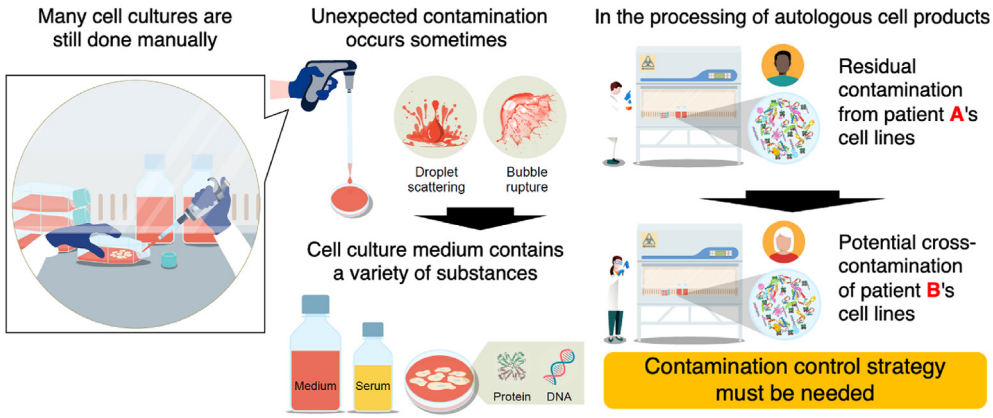

Fig. 1. Risk of cross-contamination during the processing of autologous cell products. Despite advances, cell processing is mostly performed manually, which can lead to inadvertent contamination of biosafety cabinet surfaces. Failure to properly address this contamination during production line switches can result in cross-contamination.

Therefore, even Grade A isolators could contaminate subsequent lots, which may pose unknown pathogenic risks from the previous lot or result in allergic reactions after transplantation. Nevertheless, optimal methods for removing residual contamination from culture media and cells have not been sufficiently explored.

Considering there are no international guidelines for this process changeover, each cell-processing facility must establish its own changeover method. Because the Japanese guidelines target only bacteria and fungi, there are no indicators of proteins or nucleic acids. Although various cleaning techniques exist for bacteria and fungi, such as wiping [4], ultraviolet (UV) irradiation [5], and fogging with disinfectants [7], which are common techniques for surface sterilization, no information is available regarding proteins and DNA. However, owing to the characteristics of cell products that cannot be sterilized, these substances must be removed in advance to prevent cross-contamination with residual materials.

In the present study, to assess the risk of cross-contamination, we initially analyzed the transition amounts and residual culture media of wet and dry condition. Subsequently, to evaluate cleaning methods for the dried medium, we utilized the protein and DNA contained in fetal bovine serum as an indicator and examined the changes in protein and DNA resulting from UV irradiation and wiping with disinfectants. Finally, we measured the residual amounts of cells using various cleaning methods. Based on these findings, we proposed a risk- and evidence-based cleaning method for mitigating cross-contamination in biosafety cabinets, focusing on proteins and DNA, which have received limited attention. Developing appropriate contamination control strategies can enhance the safety and quality of cell products that cannot be sterilized.

2. Materials and methods

2.1. Transition and residual experiments

To evaluate transition and residual risk (Figs. 1), 200 μL of culture medium was dispensed onto a SUS304 stainless steel plate (5 × 5 cm; AS ONE Co., Osaka, Japan) using a micropipette and allowed to either remain wet or air-dry. The chosen culture medium was minimum essential medium α (MEM-α, Thermo Fisher Scientific, MA, USA), including 10% fetal bovine serum (Thermo Fisher Scientific). Three different experimenters wore rubber gloves, touched the wet and dry media with their fingertips, and placed their fingertips in distilled water (DW; Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan). Wiping condition in the transition and residual experiments, 2 mL of 70% disinfectant ethanol (ETH, ethanol for disinfection; 76.9–81.4% w/v ethanol and 3.7% w/v isopropyl alcohol, Yamazen Pharm Co., Osaka, Japan) was utilized with 7 × 7 cm cutting of BEMCOT (Asahi Kasei Co., Tokyo, Japan) and 500 g of one-way force was applied at 50 mm/s as in previous studies [4]. For a 500-g load, a weight scale (AS ONE Co.) was placed under the SUS plate and wiped while measuring. A wiping speed of 50 mm/s was defined as wiping a 50-mm SUS304 plate in 1 s. This experiment was repeated four times. The amount of medium transferred into the DW was measured by the fluorescence intensity of phenol red (excitation: 485 nm, emission: 535 nm) using a plate reader (Infinite M200; Tecan, Männedorf, Switzerland). Concentrations were calculated using standard curves. The recovered solution for analysis from the SUS304 plate after each treatment was collected by swab method using a micropipette with 200 μL of DW. The validity of this detection method was ensured by prior validation before experiments (Fig. S1).

2.2. UV irradiation, disinfectant treatment, and wiping conditions

UV irradiation was conducted in a biosafety cabinet equipped with a 15 W UV-C germicidal lamp (Sankyo Denki Co., Kanagawa, Japan). The irradiation dose was measured using a UV intensity meter (UVC-254SD; SATOTECH, Kanagawa, Japan) at 254 nm (range: 220–280 nm). The equipment used received an irradiation dose of 200 mJ/cm^2 for 20 min. Disinfectant treatment included DW, benzalkonium chloride with corrosion inhibitor (BKC + I, Zalkonin N solution; 0.1% w/v benzalkonium chloride containing 0.5% w/v dicyclohexylamine nitrite as an anticorrosive and 8% w/v ethanol as a preservative, Kenei Pharmaceutical Co., Ltd., Osaka, Japan), ETH and peracetic acid (PAA, Acecide; 0.3% w/v peracetic acid, Saraya Co., Ltd, Osaka, Japan) were utilized in 200 μL quantities. Two mL of DW, BKC + I, and ETH were applied simultaneously while wiping using a 7 × 7 cm cutting of BEMCOT with the relevant solution immediately after applying 500 g of one-way force at 50 mm/s as in previous studies [4]. Additionally, PAA was allowed to stand for 10 min to replicate a foggy environment.

2.3. Protein and DNA measurement

Proteins were quantified using a Qubit 4 fluorometer (Life Technologies, Thermo Fisher Scientific). Each was examined using a Qubit Protein Assay Kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions.

DNA quantification was performed using a fluorometric assay with a Quantus Fluorometer (Promega, WI, USA). Each sample was examined using the QuantiFluor ONE dsDNA System Assay Kit (Promega), following the manufacturer's instructions.

2.4. Cell culture and viability evaluation

HT-1080 cells (a human sarcoma cell line obtained from JCRB Cell Bank; JCRB9113) were cultured in a cell culture incubator at 37 °C and 5% CO_2 in α-minimum essential medium (α-MEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific). To assess cell viability, double staining with acridine orange for live cells and propidium iodide for dead or apoptotic cells was conducted using a live/dead assay kit (Logos Biosystems). The obtained cell counts were adjusted for dilution concentrations to accurately calculate total cell counts. From the obtained cell count, cells were diluted to 2.0×10^4 cells/200 μL, seeded onto SUS304 stainless steel plates, and dried. Cell viability was rapidly re-measured after employing each cleaning method.

2.5. Statistical analysis

Statistical analyses were conducted using Prism version 9 (GraphPad Inc., La Jolla, CA, USA) and R software (R Foundation for Statistical Computing, Vienna, Austria). Data were presented as medians and interquartile ranges (IQR). Each statistical test is detailed in the corresponding figure legend. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Risk of transition and residual in and cross-contamination from biosafety cabinets

A simulated drop of culture medium was tested to evaluate the risk of transition of the cell culture medium remaining on the floor surface of the biosafety cabinet. The transition to DW using wet and dry culture media was measured by the fluorescence intensity of the amount of culture medium adhering to the fingertip (Fig. 2A). Wet conditions (median, 26.4 μL; IQR, 12.8 to 63.2) were significantly higher than dry conditions (median, 0.97 μL; IQR, 0.54 to 1.08), as shown in Fig. 2B. Conversely, the residual amount on the SUS304 plate, representing the floor surface of the biosafety cabinet (Fig. 3A), was significantly higher in the dry condition (median, 25.1 μL; IQR, 16.3 to 81.2) than in the wet condition (median, 2.10 μL; IQR, 1.72 to 3.92) (Fig. 3B).

3.2. Cleaning methods for biosafety cabinet and risk of residual culture medium

To evaluate the cleaning method for the residual dry medium, air-dried 200 μL culture medium on SUS304 was treated by various cleaning methods, and protein and DNA concentrations were determined (Fig. 4A). The amount of recovered medium was roughly determined by the phenol red color; however, the recovered solution could not be quantified because it was acidified after the 10 min PAA treatment (Fig. 4B). Therefore, the amounts of proteins and DNA were measured.

Residual protein amounts on SUS304 plates were similar in dry condition (median, 116 μg; IQR, 110 to 121) and UV-irradiated groups (median, 115 μg; IQR, 109 to 118). Similarly, the results of ETH wiping (median, 42.8 μg; IQR, 39.4 to 46.2) and PAA treatment (median, 42.2 μg; IQR, 34.9 to 45.8) were comparable, demonstrating a significant reduction of residual proteins (Fig. 4C) compared with the dry condition. The largest decrease was observed in BKC + I wiping (median, 1.10 μg; IQR, 0.00 to 2.78), followed by DW wiping (median, 3.43 μg; IQR, 2.29 to 4.70).

The residual DNA amount on SUS304 plates was higher after UV irradiation (median, 41.0 ng; IQR, 32.2 to 47.0) with no significant difference, while ETH wiping (median, 20.6 ng; IQR, 17.8 to 25.8) was not reduced compared to that of the dry condition (median, 21.9 ng; IQR, 19.7 to 24.4). DNA tended to decrease after PAA treatment (median, 4.48 ng; IQR, 3.11 to 7.77), even though the difference was not significant compared to dry condition. However, both BKC + I (median, 0.00 ng; IQR, 0.00 to 0.40) and DW wiping (median, 0.00 ng; IQR, 0.00 to 0.00) showed significantly lower levels.

3.3. Cleaning methods for biosafety cabinet and risk of residual culture medium containing cells

To assess the cleaning method for residual dry medium and cells containing rich protein and DNA, air-dried 200 μL culture medium containing 2×10^4 HT-1080 cells on SUS304 were treated with various cleaning methods, and protein and DNA concentrations were determined (Fig. 5A). Cells were counted immediately after each cleaning procedure (Fig. 5B). A few cells were detected under

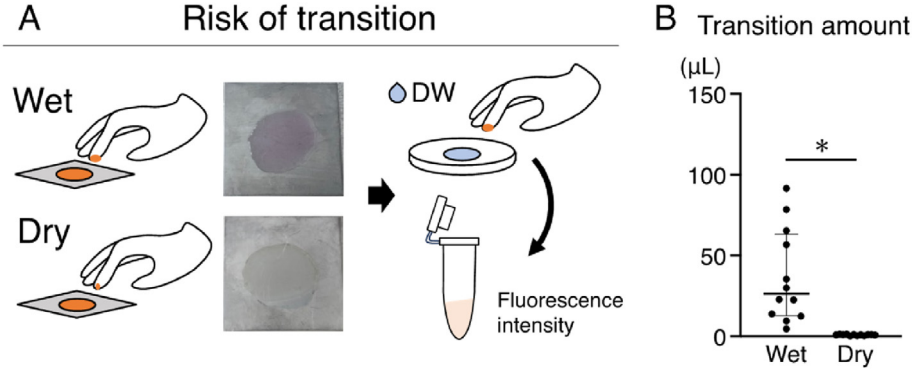

Fig. 2. Risk of transition and cross-contamination within biosafety cabinets. (A) Experimental setup depicting wet and dry conditions of culture medium on SUS304 plates. (B) Quantification of medium transition to DW. Medium amounts were assessed based on phenol red fluorescence intensity (Ex: 485 nm, Em: 535 nm). Data are presented as Median with IQR. *$P < 0.05$. P values were calculated using the Mann–Whitney test.

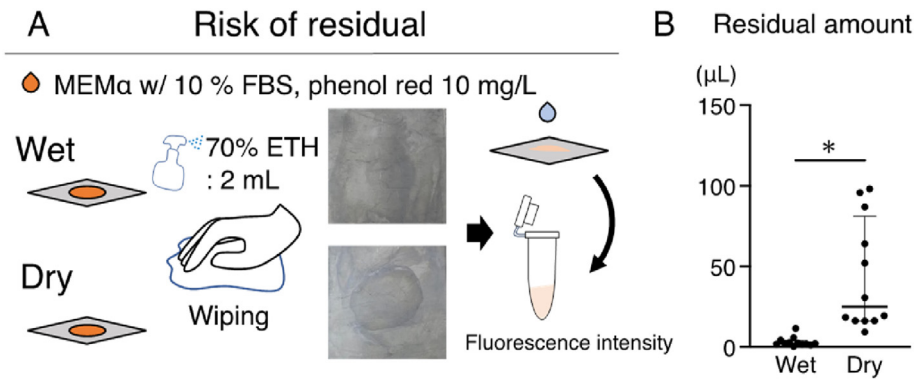

Fig. 3. Risk of residuals and cross-contamination within biosafety cabinets. (A) Experimental setup illustrating wet and dry conditions of culture medium on SUS304 plates after wiping with 2 mL spraying of 70% ETH. (B) Residual amounts of medium on SUS304 plates. Medium quantities were determined based on phenol red fluorescence intensity (Ex: 485 nm, Em: 535 nm). Data are presented as Median with IQR. *$P < 0.05$. P values were calculated using the Mann–Whitney test.

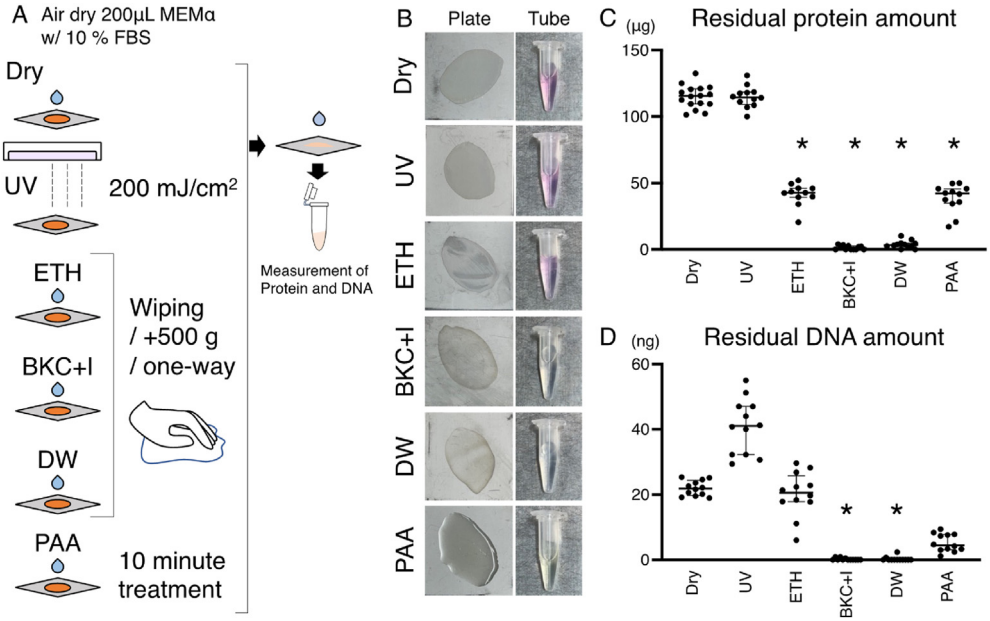

Fig. 4. Cleaning methods for biosafety cabinet and risk of residual culture medium.
(A) Experimental setup for cleaning methods. (B) Air-dried 200 μL MEMα with 10% fetal bovine serum (FBS) on SUS304 plates after each cleaning method, collected by 200 μL DW into 1.5 mL tubes. (C) Measurement of residual protein amount (μg) (n = 12). *P < 0.05. P values were calculated using the Kruskal–Wallis test with Steel-Dwass's multiple comparison test. (D) Measurement of residual DNA amount (ng) (n = 12). *P < 0.05. P values were calculated using the Kruskal–Wallis test with Steel-Dwass's multiple comparison test.

the other conditions, while no cells were detected after wiping with BKC + I or DW (Fig. 5C). However, certain conditions were confirmed for the detection of proteins and DNA in the recovered solution. Under conditions containing cells, comparable amounts were detected from dry condition (median, 132 μg; IQR, 107 to 167), UV-irradiated (median, 122 μg; IQR, 109 to 158), and ETH wiping (median, 98.7 μg; IQR, 89.6 to 126). Proteins tended to decrease after PAA treatment (median, 51.0 μg; IQR, 43.2 to 99.4), even though the difference was not significant compared to that of the dry condition.

Comparable amounts were detected from dry condition (median, 24.2 ng; IQR, 23.0 to 30.4), UV-irradiated (median, 29.1 ng; IQR, 27.6 to 38.5), and ETH wiping (median, 20.5 ng; IQR, 18.7 to 23.6) for DNA. DNA levels were significantly decreased after BKC + I wiping (median, 0.00 ng; IQR, 0.00 to 0.48), DW wiping (median, 0.00 ng; IQR, 0.00 to 0.03), and PAA treatment (median, 2.41 ng; IQR, 1.47 to 3.36) compared to dry condition.

4. Discussion

In this study, we examined the cleaning methods emphasizing residual proteins and DNA, which represent significant risks during the processing of autologous cell products but have been overlooked. While numerous cleaning methods examined in our study are commonly utilized by many facilities, comprehensive side-by-side comparisons have been lacking. The novel insights gained from our investigation unveil the various advantages and disadvantages of different cleaning methods concerning proteins and DNA. These findings demonstrates crucial insights into the distinctive attributes of cell products, characterized by their abundance in proteins and DNA.

Numerous stages in the processing of autologous cell products entail manual procedures [6]. Despite the potential for these manual processes to introduce inadvertent contamination into the aseptic cell processing environment, the Japanese guidelines

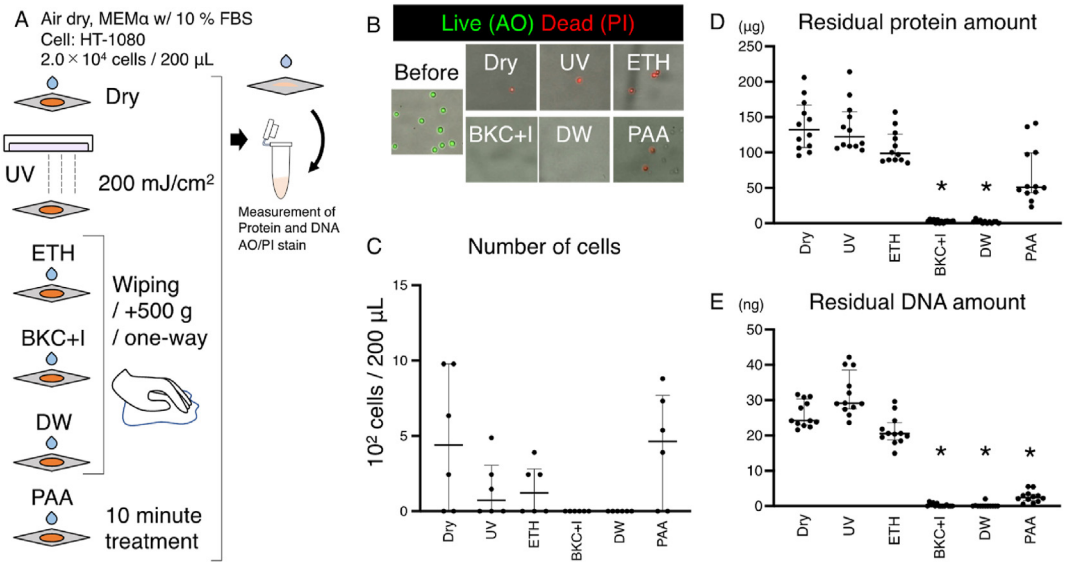

Fig. 5. Cleaning methods for biosafety cabinets and risk of residual culture medium containing cells. (A) Experimental setup for cleaning methods. Air-dried 200 μL MEMα with 10% FBS containing 2 × 10^4 HT-1080 cells on SUS304 plates after each cleaning method, collected by 200 μL DW into 1.5 mL tubes. (B) Cell counts performed after each treatment; live cells are stained green by AO and dead cells are stained red by PI. Wet represents the count before treatment and is the value used to adjust for cell count. (C) Number of cells were detected by live/dead assay. (D) Measurement of residual protein amount (μg) (n = 12). *P < 0.05. P values were calculated using the Kruskal–Wallis test with Steel–Dwass's multiple comparison test. (E) Measurement of residual DNA amount (ng) (n = 12). *P < 0.05. P values were calculated using the Kruskal–Wallis test with Steel–Dwass's multiple comparison test.

primarily address bacterial or fungal contamination. However, the raw materials used in cell products contain high levels of proteins and DNA. Unanticipated contamination encompasses human serum within the culture medium and proteins and DNA within cells and other materials. For instance, when transitioning from production line A to production line B within the same biosafety cabinet, operators must acknowledge the likelihood of proteins and DNA from line A contaminating line B. This underscores that the risk of cross-contamination exists not only within biosafety cabinets but also in the utilization of closed isolators. Thus, there is a pressing need to reassess previous assumptions regarding the risk of cross-contamination during processing. Investigating the distinct hazards associated with cell products, with emphasis on residual proteins and DNA, is imperative.

Initially, it remains unclear to what extent contamination can occur within biosafety cabinets. Notably, culture media can diffuse up to approximately 50 cm from a height of 30 cm via droplet or bubble rupture [5], indicating the challenge of completely tracing contamination by these media in most instances. Moreover, these contaminants are initially in a wet state upon scattering and gradually transition to a dry state over time. A key disparity between these two contamination states highlighted in this study is that while the wet medium is readily transferable, it is also easily removed by cleaning. Conversely, medium in the dry state, although less prone to transfer, poses difficulty in removal by cleaning. Given that contamination resulting from droplets ranges from a few to several tens of microliters and dries within 10 min, contamination in the dry state is presumed to lead to cross-contamination issues at actual processing sites. Furthermore, the accumulation of these contaminants can foster the formation of biofilms, which are notoriously challenging to eradicate. In environments with higher levels of soiling, such as those encountered with endoscopes, routine cleaning procedures often struggle to prevent biofilm formation [8,9]. Hence, there is a pressing need to establish robust methods for the removal of residual proteins and DNA. However, an optimal cleaning method for ensuring safe cabinets remains elusive.

Several methods are available for cleaning biosafety cabinets, including UV irradiation, wiping, and spraying fogging disinfectants. Among these, the use of disinfectants is categorized as low, moderate, or advanced based on their antimicrobial spectrum [10]. Peracetic acid is commonly considered an advanced disinfectant capable of achieving decontamination within biosafety cabinets, since it is effective against endospores and various other organisms; however, its application demands more time and manpower [11,12]. Nevertheless, this study demonstrates that the use of disinfectants may not suffice for the removal of proteins and DNA. Our experiment, wherein a 10-min treatment with PAA was assumed to simulate fogging, revealed effects consistent with the mechanism of action of peracetic acid, namely, the destruction and denaturation of nucleic acids and proteins. While the reduction in DNA, a nucleic acid, was notable, the reduction in protein content was not significant enough to confirm substantial differences. Moreover, peracetic acid may not be the preferred cleaning method for cell products due to its high irritancy and corrosiveness to metals [13,14]. However, it is anticipated to be highly effective against pathogenic microorganisms, such as viruses, which were not investigated in this study [15]. Therefore, the optimal choice of cleaning method should be made, considering the residual risk to the manufacturing site, such as the potential for virus contamination in processing items, and whether open work involving flasks and dishes or closed work utilizing bags is required to complete the process.

UV irradiation exhibits excellent efficacy in killing bacteria and fungi [5,16,17]. While proper irradiation is essential for achieving short-term effects [18], biosafety cabinets serve as suitable processing sites for cell products because they are not shielded. UV irradiation, known for denaturing proteins and nucleic acids, effectively eliminates bacteria and fungi. However, it only denatures proteins and DNA and does not eliminate them. Although DNA concentration was measured using a detection system that emits fluorescence intensity by binding to the double helix structure of DNA, the amount of DNA in the medium exceeded that of the dry condition, suggesting nucleic acid denaturation. The risk

associated with UV irradiation differs from that of other cleaning methods. Nonetheless, UV irradiation offers numerous advantages as prolonged exposure to UVC can deactivate viruses [19]. Decisions based on this information will necessitate specific verifications, such as determining whether it is safe to overlook trace amounts that may persist at the manufacturing site.

Clean wiping is considered the most effective method used in the experiment. However, managing the procedure alone is challenging due to the difficulty in obtaining traceability in terms of reliability assurance, which is the foundation of good manufacturing practices [4]. Therefore, establishing and evaluating a technology to accurately wipe from edge to edge are necessary. For example, ensuring traceability through image analysis and designing robots that can accurately track the wiping processes are likely candidates.

Although the combination of ethanol and wiping is considered the most common cleaning method, it is not highly effective due to the protein immobilizing capacity of ethanol [4]. Because the raw materials of cell products are rich in proteins, wiping with ethanol alone may not be the best option. Meanwhile, the highly effective BKC + I and DW are nonvolatile and may remain, which is also not an optimal option. A candidate method that can be practically employed is wiping with DW followed by wiping with ETH for cleaning protein-rich culture media; however, this would require additional labor. ETH has excellent properties in terms of dissolving lipids [20] (lipids are also present in large quantities in raw materials for cell products), which was not verified in this study and could be an important alternative for formulating a contamination control strategy.

5. Conclusion

In conclusion, the findings of this study are novel as they focus on proteins and DNA in terms of human-to-human transmission within biosafety cabinets. The results obtained will contribute to a comprehensive understanding of the composition of raw materials, the residual risks they carry, and the necessary cleaning methods (i.e., contamination control strategies). However, protein and nucleic acid contaminations do not necessarily entail a risk of morbidity beyond the cell product lot. Medical treatments, such as blood transfusions, exemplify this, where proteins and nucleic acids from other individuals are transplanted under controlled risks. The crucial aspect of cell processing, where risks are highly variable [21,22], is recognizing that residues of proteins and nucleic acids, which have received little attention, pose a risk, understanding what that risk may entail, and assessing whether it warrants mitigation. Compared to previous studies, we believe that this study can serve as an important and practical reference for developing a contamination control strategy for cell products.

Authors' contributions

MM, MK, and NI performed data acquisition. MM performed data analysis, interpretation, and contributed to the study conception and design. Manuscript drafting was done by MM, YK, TT, TY, MN, and IS. Manuscript revision for important intellectual content was carried out by all authors, who have read and approved the final manuscript.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships that may be considered potential competing interests: Mitsuru Mizuno reports the financial support provided by Terumo Corporation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.07.002.

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