Note

Comparison of the Cytotoxicity of High-Level Disinfectants by the MTT Assay and Direct Contact Assay

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Most critical instruments are not designed for heat sterilization and autoclaving. These items are usually treated with chemical agents such as peracetic acid (PAA), glutaraldehyde (GA) and ortho-phthalaldehyde (OPA). MTT assay is often used to evaluate the *in vitro* cytotoxicity of these chemical agents. In this study, disinfectants were allowed to come in direct contact with cells. Their cytotoxicity was evaluated based on cell viability and adhesive properties. The results obtained from the direct contact method were compared with those obtained from the conventional MTT assay wherein the disinfectants were added into a nutrient medium. It was found that the two methods yielded very different results, especially when aldehyde- and halogen-containing disinfectants were tested, and that toxicity may be underestimated in the MTT assay. Hence, it can be assumed that the direct contact assay is more accurate when evaluating the cytotoxicity of residual chemicals. It was also observed that the cytotoxicity of PAA was lower than that of GA and OPA.

Key words : High level disinfectants/Endoscopes/Cytotoxicity assay/Peracetic acid/Ortho-phthalaldehyde.

High-level disinfectants are used to disinfect non-invasive medical devices that come in direct contact with the skin and mucous membranes. Examples of such devices are endoscopes, which are disinfected by approved medical drugs like glutaraldehyde (GA), ortho-phthalaldehyde (OPA) and peracetic acid (PAA). In disinfecting complex structured devices such as endoscopes, problems regarding the toxicity of residual disinfectants arise. With poor rinsing considered to be the culprit, discoloration of the skin and mucous membrane, primary irritation, anaphylaxis and other kinds of allergic reactions have been reported especially in conjunction with OPA (Abdull and Adams, 2007; Ayaki et al., 2007; Cooper et al., 2008; Fujita et al., 2006; Sokol, 2004; Suzukawa et al., 2007; Suzukawa et al., 2006; Venticinque et al., 2003) and GA (Arrandale et al., 2012; Rozen et al., 1994; Stein et al., 2001; West et al., 1995). In assessing residual disinfectants after disinfection, the amount of rinsing required would differ depending on the parameters of the no-effect concentration (NEC). In terms of safety, the

lowest value obtained in the toxicity tests should be applied.

The MTT assay is generally used in evaluating the *in vitro* cytotoxicity of disinfectants (De Souza et al., 2007; Hirsch et al., 2009; Muller and Kramer, 2006). With this method, it is possible to evaluate the toxicity of disinfectants in terms of their residual concentration using the NEC as a base. With this method, disinfectants were added into the nutrient medium. The problem is that test chemicals have the potential to react with the components of the medium, resulting in loss of their efficacy, leading to the possibility that toxicity may be underestimated. Hence, it is crucial to use a method that is capable of measuring the direct toxic effect of the test chemical on the cells without the influence of medium components.

In this study, disinfectants were allowed to come in direct contact with cells. Their cytotoxicity was then evaluated according to the measured viability and adhesive rates of cells. The obtained measurements were then compared with the results from the conventional MTT assay. The reactivity between the disinfectants and nutrient medium was also investigated.

HeLa Cells (JCRB) were utilized with Eagles's MEM

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(EMEM, Dainippon Sumitomo Pharma Co. Ltd.) containing 10% fetal bovine serum (Life Technologies Corporation) as the medium.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich Co. LLC., isopropanol from Wako Pure Chemical Industries, Ltd and Non-Essential Amino Acids (NEAA) from Dainippon Sumitomo Pharma Co. Ltd.

Phosphate buffer solution (PBS) was prepared with 8.0g NaCl, 0.2g KCl, 1.15g Na_2HPO_4 , 0.2g KH_2PO_4 in 1L solution (All were purchased from Kanto Chemicals Co. Inc.).

Disinfectant solutions used were 0.3% PAA solution (Saraya Co. Ltd.), 0.55% OPA solution (Johnson & Johnson services Inc.), 2% GA (Maruishi Pharmaceutical Co. Ltd.), 10% povidone-iodine solution (Meiji Seika Pharma Co. Ltd.), and 6% NaClO solution (Saraya Co. Ltd.). The degree of dilution of these concentrations was considered as "1".

As for inactivating agents/Neutralizers, 0.5% $\text{Na}_2\text{S}_2\text{O}_3$ solution (Katayama Chemical Industries Co. Ltd.) & 0.5% catalase (6690u/mg, Funakoshi Co. Ltd.) were used for PAA solution, 0.5% glycine solution (Katayama Chemical Industries Co. Ltd.) for OPA and GA solutions, and 0.5% $\text{Na}_2\text{S}_2\text{O}_3$ solution for povidone-iodine and NaClO solutions.

The MTT assay used HeLa cells. These cells were seeded into 96-well cell culture plates containing EMEM nutrient medium. At 72 hours after incubation, the medium was then replaced with each disinfectant solution containing EMEM nutrient medium. After 48 hours of incubation, the medium was replaced by a fresh EMEM nutrient medium containing 0.5mg/mL MTT. After 3 hours treatment, the formazan was extracted in isopropanol, and absorbance was measured at 570nm.

The cell viability was obtained from the following equation.

$$\text{Cell viability (\%)} = \frac{\text{Absorbance after exposure to disinfectants}}{\text{Absorbance without exposure to disinfectants}} \times 100$$

The cell adhesion assay also used HeLa cells. After trypsin treatment, the HeLa cells were collected and were washed with PBS to prevent any medium effect. After removing PBS, each disinfectant solution (medium-free) was added to the cell pellets and a contact time of 5 minutes was allowed. The disinfectant solutions were then neutralized and diluted with EMEM nutrient medium. The cells were then spread over a culture dish. After one day of incubation, viable cell count was performed.

The survival rate of cells was obtained through the following equation.

$$\text{Cell adhesion rate (\%)} = \frac{\text{Cell count after exposure to disinfection}}{\text{Cell count without exposure to disinfection}} \times 100$$

The direct contact assay similarly used HeLa cells. The cells were spread over a culture dish and allowed one day for incubation. The medium was removed and the cell layer was washed with PBS to prevent any medium effect. After removing PBS, each disinfectant solution (medium-free) was added to the cells and allowed a contact time of 10 minutes. The disinfectant solutions were then neutralized and diluted with EMEM nutrient medium, and viable cell count was performed.

The survival rate of cells was obtained using the equation provided in the cell adhesion assay.

We tested the reaction of disinfectant solutions with the medium content. Since amino acids present in the medium were thought to have a high possibility of reacting with the disinfectant solutions, NEAA was utilized in place of the medium. A ratio of 5:1 (ratio in medium during 1000-fold dilution) disinfectant solution and NEAA was mixed and allowed a contact time of 10 minutes. The residual active components of the disinfectants were then quantified.

PAA was quantified according to the modified method of Sully et al. (1962). After reaction with NEAA, the test solution was measured accurately and 100 mL of 0.1mol/L acetic acid was added into the solution. Ten mL of KI solution (Hayashi Chemical Industry Co., Ltd.) and 1mL of 0.5% starch solution (Wako Pure Chemical Industries, Ltd) were then added. The solution was titrated for its free iodine content with 0.2mol/L $\text{Na}_2\text{S}_2\text{O}_3$ solution until becoming colorless. Titration was continued dropwise as the blue color returned, until 1.5 to 2 minutes elapsed from the addition of KI solution. The stopwatch reading in seconds (t_1) and the buret reading (x_1) were recorded when the blue color returned. Without stopping the stopwatch, titration was continued for another 1.5 to 2 minutes. When the blue color returned, the stopwatch reading (t_2) and buret reading (x_2) were recorded. 0.5 ml of ammonium molybdate solution (solution turns brown/green or blue) was added and the titration continued until reaching a colorless endpoint that remained stable for about one minute. The buret reading in mL (x_i) at the endpoint was noted.

The corrected titre at zero time (x_0) was calculated from the following equation:

$$x_0 = x_1 - \{t_1 \times (x_2 - x_1) / (t_2 - t_1)\}$$

The peracetic acid (PAA) content can be calculated as follows:

$$\text{PAA (\%)} = x_0 \times 0.2 \times f_1 \times 38.03 / (10 \times W_1)$$

$$\text{H}_2\text{O}_2 \text{ (\%)} = (x_i - x_0) \times 0.2 \times f_1 \times 17.01 / (10 \times W_1)$$

Where: W_1 = weight of the product used as the test sample (g)

f_1 = factor of the 0.2N $\text{Na}_2\text{S}_2\text{O}_3$ solution

OPA and GA were quantified according to the Standard Methods of Analysis for Hygienic Chemists. A few drops of hydrogen peroxide solution (Wako Pure Chemical Industries, Ltd) were added to the test solution after it had reacted with NEAA. The addition of 4-amino-3-hydrazine-5-mercapto-1,2,4-triazole (Wako Pure Chemical Industries, Ltd) followed. When the color of the resulting mixture changed from colorless to red violet, absorbance was measured at 550nm.

Povidone-iodine was quantified according to The Japanese Pharmacopoeia. One gram of DPD (N,N-diethyl-p-phenyldiammonium sulfate, Tokyo Chemical Industry Co. Ltd.) was ground on a mortar and mixed well with Na_2SO_4 (Wako Pure Chemical Industries, Ltd), serving as the diluted DPD powder mixture. After the disinfectant solution had reacted with NEAA, 0.1g of the diluted DPD powder mixture was added and mixed well. Within one minute, the change in color was observed from the side and compared with the reference solutions to obtain the suitable concentration of the residual free iodine. Then, 0.5g of KI was added to the mixture. After the mixture was allowed to stand for about 2 minutes, the change in color was again compared to the reference solutions to obtain the chlorine content. This also served as the amount of total residual iodine.

NaClO was quantified according to The Japanese Pharmacopoeia too. The determination of residual free chlorine and total chlorine content was the same as that for the quantity of povidone-iodine.

The cytotoxicity of disinfectants was investigated using the most general cytotoxicity assay, the MTT assay. The results are shown in Fig.1. The vertical axis indicates the cell survival rates. The horizontal axis provides the rate of dilution of the test solution and the concentrated solution having 1 as its value. At a 100-fold dilution, the cytotoxicity of NaClO and PAA could be observed, while at a 1000-fold dilution, no toxicity was observed for all disinfectants.

The cytotoxicity of disinfectants was evaluated after direct contact with the disinfectants through the measured cell adhesion rate (Fig.2). The adhesion rate of the cells was low as the dilution rate decreased, making the cytotoxicity easily understood. With different results from the MTT assay, the increasing order of cytotoxicity of disinfectants was observed to be as follows: PAA, povidone-iodine, GA, OPA and NaClO .

From the cell viability rate obtained after treatment with disinfectants, the trend in the cytotoxicity of the disinfectants was similar to that of the cell adhesion assay (Fig.3). However, in comparison to the cell adhe-

FIG. 1. Cytotoxicity of disinfectants analyzed by the MTT assay. The vertical axis indicates cell viability. The horizontal axis provides the rate of dilution of the test solution and the concentrated solution having 1 as its value. The MTT assay was used to analyze the effects of disinfectant solutions diluted from 100 to 10000-fold on the cell viability of HeLa cells after a 48-hour exposure. Bars indicate standard error ($n=3$). At a 100-fold dilution, the cytotoxicity of NaClO and PAA could be observed, while at a 1000-fold dilution, no toxicity was observed for all disinfectants.

FIG. 2. Cytotoxicity of disinfectants analyzed by the cell adhesion assay. The vertical axis indicates the cell adhesion ratio. The horizontal axis provides the rate of dilution of the test solution and the concentrated solution having 1 as its value. The cell adhesion assay was used to analyze the effects of disinfectant solutions diluted from 100 to 100000-fold on the cell viability of HeLa cells after a 10-minute exposure. Bars indicate standard error ($n=3$). The adhesion rate of the cells became low as the dilution rate decreased. With different results from the MTT assay, the increasing order of cytotoxicity of disinfectants was observed to be as follows: PAA, povidone-iodine, GA, OPA and NaClO .

sion assay, the cell viability shifted slightly to the lower dilution rate.

Since all the disinfectants react with amino acids, it was speculated that disinfectants were being neutral-

FIG. 3. Cytotoxicity of disinfectants analyzed by the direct contact assay. The vertical axis indicates cell viability. The horizontal axis provides the rate of dilution of the test solution and the concentrated solution having 1 as its value. The direct contact assay was used to analyze the effects of disinfectant solutions diluted from 100 to 100000-fold on the cell viability of HeLa cells after a 10-minute exposure. Bars indicate the standard error ($n=3$). From the cell viability rates obtained after treatment with disinfectants, the trend in the cytotoxicity of the disinfectants was similar to that of the cell adhesion assay. However, in comparison to the cell adhesion assay, the cell viability shifted slightly to a lower dilution rate.

FIG. 4. Residual disinfectants and the influence of the culture medium. The disinfectant solution and NEAA were mixed and allowed a contact time of 10 minutes. The residual active components of the disinfectants were then quantified. The vertical axis indicates the disinfectant residual ratio. The horizontal axis provides the concentration of amino acids. When PAA was allowed to come in contact with pure amino acids for 10 minutes, about 10% reactivity was observed, whereas for OPA, as high as 60% was observed.

ized due to the amino acids they contained (Fig.4). In addition, when PAA was allowed to come in contact with pure amino acids for 10 minutes, about 10% reactivity was observed, whereas for OPA, as high as 60% was observed.

After the disinfection of medical devices, the cytotox-

icity of the residual disinfectants was evaluated after rinsing the devices. In this study, to avoid any medium effect, the cells and disinfectants were allowed to come in direct contact. The cell viability and cell adhesive rate were obtained and the cytotoxicity of the disinfectants was assessed. The results from these assays were clearly different from those of the MTT assay. The disinfectants that did not show cytotoxicity in the MTT assay had displayed cytotoxicity in the direct contact assay. Observing the NEC, the MTT assay provided 1 to 50x higher values than that of the direct contact method, specifically 1x for PAA, 1 to 5x for povidone-iodine, 5x for GA, 10x for OPA and 50x for NaClO.

The amino acids including glycine are usually being used to neutralize GA, OPA and other dialdehyde-containing groups (Cheung and Brown, 1982; Gelinas and Goulet, 1983). Since the culture medium also contained amino acids, this was thought to be the reason behind the difference in the results of the two assays. Reaction measurements between disinfectants and the medium were performed to verify this phenomenon. As expected, the dialdehydes reacted with the culture medium, as the obtained concentration decreased. Furthermore, halogens were also thought to be reacting with the medium as halamine would be formed. While the active halogen content did not decrease, there was a remarkable decrease in the active free halogen content. In the investigation of the reaction, the contact time of 10 minutes was rather short, whereas in the MTT assay, the contact time of the disinfectants with the media was 48 hours long. This can allow the reaction to progress further.

From the obtained cytotoxicity values, the direct contact assay is recommended compared to MTT assay in terms of verifying the presence of residual disinfectants after rinsing medical devices like endoscopes due to its lower concentration and larger margin of safety. In the actual disinfection process of medical devices, two points should be taken into consideration when it comes to the residual cytotoxicity of the disinfectants: mere poor rinsing, and the liberation of the disinfectant after its adsorption and sorption on medical device surfaces. In this investigation, the residual disinfectant after rinsing can be assumed and its concentration after rinsing as well, with the highest NEC as base. In terms of automated processing, the number of necessary rinse cycles can also be established.

When disinfectants and cells come in contact, the cytotoxicity can be assessed in two ways. One is through the cell adhesion assay, and the other is through the cell viability assay. The latter is more sensitive to the disinfectants than the former. Through the cell viability assay, the obtained NEC and post-rinsing potential toxic index of each disinfectant at the concen-

tration at which it is used can be understood.

Lastly, the obtained cytotoxicity of high-level disinfectants from the direct contact assay was clearly different from that obtained from the MTT Assay, especially in terms of aldehyde- and halogen-containing disinfectants. With the MTT assay, the evaluation of cytotoxicity could be underestimated. When evaluating the cytotoxicity of residual disinfectants after disinfecting endoscopes, with the margin of safety in mind, evaluating the cell viability through the direct contact with cells and disinfectants is recommended.

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