Bactericidal Effects and Cytotoxicity of New Aromatic Dialdehyde Disinfectants (Ortho-phthalaldehyde)

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We investigated the bactericidal effects and cytotoxicity of an ortho-phthalaldehyde product in comparison with those of its predecessor glutaraldehyde products. Bactericidal effects were examined on Mycobacterium terrae, a standard organism used for investigating the bactericidal effect of high-level disinfectants. Cytotoxicity as determined by the MTT assay was examined by using four cell lines. The colony forming test, a method to examine residual toxicity, and the evaporation test, a newly developed method to examine the toxicity of the evaporated ingredients, were performed. Test solutions were 2.25% and 3.5% glutaraldehyde (GA) products and a 0.55% ortho-phthalaldehyde (OPA) product, and glutaraldehyde itself. All the disinfectants showed sufficient bactericidal effects on M. terrae. Meanwhile, the OPA product was less toxic than GA products and GA itself to all the cell lines tested. The colony forming test showed that GA products and GA itself exerted residual cytotoxicity more potently than did the OPA product. The evaporation test showed that GA products and GA itself exerted cytotoxicity via evaporation more potently than did the OPA product. In conclusion, OPA appears to be less cytotoxic than GA even though bactericidal effects were comparable. This may be due to the lower concentration of the active ingredient (ortho-phthalaldehyde) in the OPA product.

Key words: Disinfectant / High-level disinfection / Cytotoxicity / Glutaraldehyde / Ortho-phthalaldehyde.

High-level disinfection is recommended for semicritical medical instruments such as gastric and bronchial endoscopes (Akamatsu et al., 2005). Regulatory issues for occupational health and safety include inhalation toxicity, skin sensitivity, and toxic effects on the eyes, requiring proper ventilation, and the wearing of masks, gloves, and protective clothing. Recently ortho-phthalaldehyde (OPA) and peracetic acid have been introduced as new high-level disinfectants as an alternative to conventional glutaraldehyde agents. Glutaral (GA)/OPA with an alkylation effect, and peracetic acid (not approved in Japan), with an oxidation effect, are classified as high-level disinfectants. Previous studies showed rapid bactericidal and virucidal effects of OPA on GA-resistant mycobacterium, HBV, and HCV (Roberts et al., 2008; Shackelford et al., 2006; Walsh et al., 1999; Fraud et al., 2001; Walsh et al., 2001; Cabrera et al., 2002). Their bactericidal mechanisms also have non-specific bio-hazard features. Disinfectants for medical instruments should have strong bactericidal effects as well as minimum adverse effects on tissues.

OPA (Cidex® OPA, Johnson and Johnson KK, Tokyo, Japan) is a new aromatic dialdehyde disinfectant that has been effectively used for cleaning and disinfecting endoscopes (Walsh et al., 1999; Akamatsu et al., 2005). This present study evaluates the cytotoxicity and bactericidal effects of a 0.55% OPA (Cidex® OPA) product in comparison with those

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of conventional 2.25% GA (Cidex®, Johnson and Johnson KK) and 3.5% GA (Cidex® plus, Johnson and Johnson KK) products, and glutaraldehyde itself (Kanto Chemical, CO., INC., Tokyo, Japan).

The following commercially available cell lines were used in this study: SiRc cells (rabbit corneal epithelium, ATCC CCL-60, distributed by American Type Culture Collection, Manassas, VA), Chang conjunctiva cells (human conjunctival cells, ATCC CCL-20.2), FRSK cells (rabbit skin cells, JCRB-0005, distributed by Health Science Research Resource Bank, Osaka, Japan), and HeLa S3 cells (human cervical cancer origin, ATCC CCL2.2, DS pharma Biomedical CO., LTD, Osaka, Japan). Chang conjunctiva cells were cultured in H-199 (Nissui pharmaceutical CO., LTD) with 10% fetal bovine serum (FBS, CELLect®, MP Biomedical LLC, France). These cell lines were cultured in Eagle’s MEM (Nissui pharmaceutical CO., LTD.) with 10% FBS according to the standard protocols provided by the distributors.

Bactericidal effects were examined on M. terrae (JCM12143), a standard organism used to investigate bactericidal effects of high-level disinfectants. The organism was cultured in Middlebrook 7H10 medium (BD, BectonDickinson and Company, Le Pont De Claix, France) with glycerol (Wako Pure Chemicals, Osaka, Japan) and Middlebrook OADC enrichment (BD, BectonDickinson and Company). Following incubation for 10 to 14 days in the broth, a bacterial suspension was prepared in the medium to contain approximately $1 \times 10^6$ cells/ml. Nine hundred μl of the test disinfectant was added to 100 μl of the bacterial suspension and incubated for 1, 5, 10, 30, and 60 minutes at 4, 10, 15, and 20°C. The number of colony forming units (CFU) in a ten-fold dilution was determined. Furthermore, CFU of M. terrae in a solution containing 800 μl of the test disinfectant, 100 μl of the bacterial suspension, and 100 μl of FBS was counted after being washed at 10°C to simulate the organic load. Solutions to which saline, instead of the test disinfectant, was added as a control were then incubated at 10°C for 5, 10, 30, or 60 minutes.

In the cytotoxicity assay, an aliquot (100 μl) of medium containing approximately $1 \times 10^6$ cells (Chang, SiRc, FRSk, or HeLa S3) was dispersed into each well of a 96-well culture plate (NUNC167008, Thermo Fisher Scientific, Denmark) and incubated for two days at 37°C under an atmosphere of 5% CO2/95% air. The culture medium was then replaced with 100 μl of diluted test solution and the cells were further incubated for 48 h. Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann, 1983). Cell viability in test solutions was calculated as a percentage of the control cell viability. The experiments were repeated 8-16 times and results are presented as the average +/- standard deviation. The colony forming test was performed using SiRc cells and HeLa S3 cells. Five ml of each disinfectant product (GA2.25%, GA3.5%, and OPA0.5%) was incubated in cell culture dishes (Falcon353003, BD Bioscience, USA) for one minute and the disinfectant product was removed. Then the dish was washed with PBS without FBS once, three, or five times, and culture medium with 500 cells was added. The number of colonies was counted under Gimza staining after 14 days of incubation.

To determine the cytotoxic effect by the evaporation of the disinfectant from adjacent wells, 104 cells /100 μl of Chang conjunctiva was cultured in all of 96 wells except 12 wells in the bottom row. After the wells were incubated for two days under an atmosphere of 5% CO2/95% air, 100 μl of the test disinfectant product (GA2.25%, GA3.5%, and OPA0.5%) was added to each well of the bottom row. Following further incubation for two days, the MTT assay was performed. The cytotoxic effect of the evaporation of the disinfectants was determined by the cell viability in each well of the rows above the bottom row.

Cell viability obtained from the MTT assay was subjected to statistical analysis using analysis of variance (ANOVA) to determine whether factors such as disinfectant product, cell line, and dilution rate of the products affected significantly the variance of the results.

The bactericidal effect of each disinfectant was augmented with an increase in temperature (Figure 1) although almost no bactericidal effect was observed at 4°C for up to 60 minutes. At 10°C, the number of CFU was decreased from 107 to 103 by GA3.5% and from 104 to 102 by GA2.25% and OPA0.55%. At 15°C, the number of CFU was decreased to an undetectable level by GA2.25% and GA2.0% after 60 minutes, whereas OPA0.3%, OPA0.55% and GA3.5% decreased the number of CFU to an undetectable level in 5 minutes. At 20°C, it took 10 minutes to reach an undetectable level for GA2.0%-treated bacteria, 5 minutes for GA2.25%-treated bacteria, and one minute for the others. The solutions with FBS exhibited no difference in the number of CFU at 10°C as compared with the solutions without FBS (Figure 2).

The minimum concentrations to kill most (>75%) of the cells of any of the four cell lines were 0.035% (100-fold dilution) for GA3.5%, 0.0225% (100-fold dilution) for GA2.25%, and 0.55% to 0.11% (undiluted to 5-fold dilution) for OPA0.55% (Figure 3). Glutaraldehyde as an active ingredient of the GA products exhibited remarkable cytotoxicity up to
FIG. 1. Bactericidal effect of disinfectants at various temperatures. CFU of *Mycobacterium terrae* were counted after incubation at 4, 10, 15, and 20°C. Bactericidal effect was low at 4 and 10°C. The OPA product was more effective than the GA products with dose dependency.

FIG. 2. Bactericidal effects of disinfectants with or without fetal bovine serum. CFU of *Mycobacterium terrae* were counted after incubation at 10°C. The bactericidal effect of neither the GA products nor the OPA product was affected by fetal bovine serum.

0.05%. The critical concentrations not to exert cytotoxicity were 0.0007% (5,000-fold dilution) for GA3.5%, 0.00045% (5,000-fold dilution) for GA2.25%, 0.0055% (100-fold dilution) for OPA0.55%, and 0.001% for glutaraldehyde. Regarding the vulnerabilities of cell lines to the test disinfectants, the cell viability of Chang conjunctiva tended to be lower than that of other three cell lines and it was most evident in the case of GA3.5% and GA2.25%. Table 1 summarizes the results of ANOVA of the MTT assay indicating that each of the factors (disinfectant product, cell line, and dilution rate) significantly affected the cell viability. Of these factors the dilution rate showed the highest F value followed by F values of the disinfectant product, indicating that the dilution rate was the factor that most affected the variance of cell viability and that the cell line affected it the least. Furthermore, significant interaction effects among the factors were found. Especially the interaction between disinfectant product and dilution rate showed the highest F values.

In the colony forming test, no colony was observed even after five washings following the GA3.5%- and
FIG. 3. Cytotoxicity of disinfectants assayed by MTT.
Cell viability was examined by the MTT assay after incubation with a disinfectant for two days. Cell viability in test solutions was calculated as a percentage of the control. The critical concentrations not to exert cytotoxicity were 0.0007% (5,000-fold dilution) for GA3.5%, 0.00045% (5,000-fold dilution) for GA2.25%, 0.0055% (100-fold dilution) for OPA0.55%, and 0.001% for glutaraldehyde. Regarding the susceptibility of cell lines to the tested disinfectants, Chang conjunctiva tended to be more susceptible than the other three cell lines.

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<td>65460.314</td>
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<tr>
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GA2.25%-treatment (Figure 4). In contrast, colony formation was observed after three washings for OPA0.55%, and slight growth inhibition was observed after five washings as compared with the control cell growth.

The evaporation test revealed that all disinfectants exhibited cytotoxicity (Figure 5). The borders of distinct cytotoxicity observed were wells of the first row above the bottom row for OPA0.55% and wells of the third row for GA3.5% and GA2.25%. GA3.5% showed toxic effect in wells of all rows above the bottom row.

Practice Guideline Endoscope Disinfection has been proposed by WGO—OMGE／OMED (Rey et al., 2005) and three academic societies including the Japanese Society of Environmental Infections, Japanese Society of Gastrointestinal Endoscope, and
FIG. 4. Colony forming test.
After one minute incubation of each well of the culture plate with disinfectant followed by washing once, three, or five times, SIRC cells or HeLa S3 cells were added to the wells and further incubated for 14 days. Then the number of colonies was counted under Gimza staining. No colony was observed in GA3.5%- and GA2.25%-treated wells even after five washings, whereas colony formation was observed in the wells treated with OPA0.55% after three washings.

FIG. 5. Cytotoxic effect of the evaporation of disinfectants. The cell viability of Chang conjunctiva in the wells of discrete rows from the bottom row in which wells were treated with disinfectant was determined after incubation for two days in 96 wells. All disinfectants exhibited cytotoxicity by evaporation. The border of distinct cytotoxicity was observed at one row for OPA0.55% and three rows for GA3.5% and GA2.25%.

Japanese Society of Technicians for Gastrointestinal Endoscopy made multi-society guidelines for the disinfection and washing of endoscopes (Japanese Society of Environmental Infections; 2008). The rationale includes: 1) every human tissue is considered to be a potential source of infection, 2) compliance for infection control should be shared by all staff involved, 3) disinfection should be done after sufficient washing, 4) occupational health and environmental control are the first priority, and 5) local manuals for compliance should be determined according to the guidelines.

Infection control has been improved after guidelines had been set in Japan. Other contributory factors include the introduction of equipment for automatic washing that led to the replacement of manual procedures, and preventive strategies involving ventilation, masks, goggles, and gloves to protect medical personnel from inhalation toxicity, skin problems, and eye injuries. Special attention should be paid to the seasonal and regional fluctuation of tap water temperature and room temperature due to unique Japanese conditions. Considering these issues, we made an experimental design simulating actual hospital settings to explore the effect of temperature and organic loads, and the evaporation effect of disinfectants as a biohazard.

Our results indicate that all of the tested products had sufficient disinfection effect at 20°C as shown in Figure 1. The disinfection effect decreased by lowering the temperature especially below 15°C. We should be careful that GA may be ineffective if a GA product is used just before the expiration date in winter because there might be an insufficient disinfection effect due to the low temperature. We examined the effect of FBS, a simulated organic load after insufficient washing, and the results showed that the disinfection effect was not affected.

We further conducted a cytotoxicity assay according to the protocol in our previous study for corneal cells (Ayaki et al., 2007). The present results indicate that 100-fold diluted GA and 10-fold diluted OPA exhibited severe toxicity as shown in Figure 3. According to ANOVA as shown in Table 1, it is suggested that any of the factors including the disinfectant product, cell line, and dilution rate, can significantly affect the variance of cell viability in the MTT assay. In addition, significant interaction effects that represent the combined effects of factors on the cell viability were found. When an interaction effect is present, the impact of one factor depends on the level of the other factor. In the present study, the major interacting factors were the disinfectant product and dilution rate, suggesting that effect of each disinfectant product on the cell viability is prominently affected by the dilution rate but not by the cell line. The origin of toxicity in GA products may be the active ingredient (glutaraldehyde) itself since 0.05%-0.01% glutaraldehyde showed similar toxicity. Although we did not examine toxicity of the active ingredient (ortho-phthalaldehyde) of OPA, we speculate that the reason for the lower toxicity of the OPA product in comparison with that of GA products is due to the lower concentration of the active ingredient, and only ten to 50-fold dilutions were enough to exclude completely the toxicity of OPA.

Although our results indicated that the cytotoxicity of OPA product was less than that of GA products, we have to be aware of adverse events of OPA including anaphylaxis (Spool et al., 2004) and
chemical burn (Venticinque et al., 2003). Basic experiments also suggested that OPA might induce allergic or inflammatory reactions (Hasegawa et al., 2009; Morinaga et al., 2010; Anderson et al., 2010).

Endoscopes are immersed in a container with a lid in manual disinfection. Immersion in a closed circuit is also performed in automatic disinfection. In both cases, high-level disinfection is required, and such disinfectants are generally highly cytotoxic. Nevertheless, the harmful effects of the evaporated disinfectant during immersion and the effects of the residual disinfectant after washing have not been fully evaluated. The present study revealed that more washing was required to remove GA products than to remove OPA since considerable residual toxicity of GA products was observed in the colony forming test even after five washings. The evaporation test is a new assay system that we developed to evaluate the cytotoxicity of evaporated ingredients. According to the results of the cytotoxicity assay as shown in Fig. 3, the minimum concentrations required to kill most (>75%) of the cells of Chang conjunctiva were 0.007% for GA3.5%, 0.0225% for GA2.25%, and 0.55%~0.11% for OPA 0.55%. Thus, it is speculated that GA evaporated from the GA products in the bottom rows and in turn reached the walls of at least the third row from the original source of GA, and was dissolved in the medium to the concentrations as described above (0.007% for GA3.5%, and 0.0225% for GA2.25%). Similarly, it is supposed that the OPA that evaporated from OPA 0.55% in the bottom row reached the first row and was dissolved in the medium to the OPA concentration of 0.55%~0.11%.

Taken together, the present study revealed that the toxic effects of OPA are smaller than those of GA although their disinfection effects are similar to each other. The results of the cytotoxicity test are consistent with the results for human corneal endothelial cells (Ayaki et al., 2007). The reason for the lower toxicity of the OPA product compared to that of the GA products is probably related to the lower concentration of the active ingredient, ortho-phthalaldehyde.

REFERENCES


