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Risk Assessment of Triclosan Using Animal Cell Lines

Kalyanee Jirasripongpun*, Thanate Wongarethornkul and Sunisa Mulliganavin

ABSTRACT

Triclosan is an antimicrobial ingredient used in many health-care products and household items including plastics, textiles and food packaging materials. However, the safety of triclosan has been questioned recently. In this study, cytotoxicity of triclosan to BHK-21, Vero and KB cells were performed by incubating these cells in culture medium containing various concentrations of triclosan for 3 days. Vero and KB cells were more sensitive and suitable as models to evaluate triclosan toxicity. The 50% inhibition concentration (IC_{50}) of triclosan to Vero and KB were at 0.036 and 0.034 mM, respectively, while that of BHK-21 was at higher concentration (0.26 mM). Analyzing for triclosan content in popular use of personal health-care products revealed the maximum triclosan content at 0.27 % (w/w) or 0.023 mM in liquid facial soap. Genotoxicity analysis based on comet assay demonstrated the DNA breakage by triclosan. Its genotoxic potency was significantly increased as the triclosan concentration and the exposure time increased. Exclusion of cell death or cytotoxicity, the genotoxic effect of triclosan in this study could be observed noticeably at the IC_{20-30} of triclosan after 5 days treatment. Therefore, animal cell lines could be used for primary evaluation on the risk assessment of triclosan.

Key words: triclosan, cytotoxicity, genotoxicity, personal care products

INTRODUCTION

Triclosan (5-chloro-2-(2, 4-dichloro-phenoxy) phenol) is a crystalline powder with molecular weight of 289.55. It is soluble in many organic solvents with very low solubility in water (10 mg/l) but high lipophilicity. Broad spectrum on antibacterial, antifungal and antiviral activity (Bhargava and Leonard, 1996; John *et al*., 2000) has made triclosan a popular ingredient in many professional health-care products and household items including plastics, textiles and food packaging materials (Schweizer, 2001). Recently, triclosan safety has been questioned with respect to environmental impact and human health risk (Kepner, 2005). The United States Environmental Protection Agency (EPA) has registered triclosan as a pesticide (EPA Registration Number: 100-502). Furthermore, triclosan photoconversion to 2, 8-dibenzodichloro-ρ-dioxin at pH >8 under UV light of the wavelength at 280 nm (Latch and Packer, 2003) and the presence of dioxin as synthesis impurities from the manufacturing process alert the consumers on their risk in daily use of triclosan products.

There is a paucity of data on the toxicity of triclosan. Tatarazako *et al*. (2004) reported the acute toxicity of triclosan on the tested organism

Department of Biotechnology, Faculty of Engineering and Industrial Technology, Silpakorn University, Nakornpathom 73000, Thailand.

* Corresponding author, e-mail: kalyanee@su.ac.th

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of bacterium (*Vibrio fischeri*), microalga (*Selenastrum capricornutum*), crustacean (*Ceriodaphnia dubia*) and fish (*Danio rerio* and *Oryzias latipes*) showing microalga to be the most sensitive at IC_{25} of 0.0034 mg/l while those of other organisms were in the range of 0.07 to 0.29 mg/l. Therefore, algae were determined to be a susceptible organism for assessing toxicity of triclosan in the environment (Orvos *et al*., 2002). Toxicity of triclosan to mammalian cells was also performed to test for the safety of daily use of triclosan products. The 24 h midpoint cytotoxicology of triclosan on Smulow-Glickman (S-G) human gingival epithelial cell line was in the range of 0.14-0.17 μg/l as assessed by the neutral red (NR) assay (Zuckerbraun, 1998). In this study, a susceptible cell model was chosen among BHK-21, KB and Vero cells for cytotoxicity testing of triclosan. The maximum triclosan concentration determined from personal health-care products and the 50% inhibition concentration of triclosan to the cell lines were also analyzed for its genotoxicity using comet assay and Vybrant Apoptosis Assay Kit 2.

MATERIALS AND METHODS

Animal cell lines and cell cultures

BHK-21 and Vero cell lines were provided from The Center for Vaccine Development, Mahidol University, Thailand. KB cell line was from The National Cancer Institute, Thailand. They were cultured in minimum modified Eagle medium (MEM) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 humidified incubator. Cultures were dissociated with trypsin-EDTA and cells with a typical viability of 87-95% were used as seed at a cell density of 1×10^4 cells/ml in all experiments.

Triclosan preparation and determination in personal care products

Triclosan (industrial grade; Irgasan DP-300) was obtained from the Department of Materials Science and Engineering, Silpakorn University. Stock solution at 1 mM was prepared in dimethylformamide (DMF) and filtered to sterilize. It was diluted with medium when used and the final concentration of DMF in the culture medium was less than 1% in all culture tests.

Toothpaste, bar soap and liquid facial soap were analyzed for triclosan concentration according to the method of Honghirunroung and Hirunthanakitjakul (1993).

Cytotoxicity assay

The BHK-21, Vero and KB cell lines were seeded in 24-well plate in MEM supplemented with 10% fetal bovine serum to provide 70-80% confluence after 4 days incubation. Thereafter, the spent medium was removed and replaced with a new medium containing triclosan at 0, 0.025, 0.050, 0.10 and 1.00 mM. The cultures were further incubated for 3 more days and triplicate of samples were counted for cell viability each day using Trypan blue exclusion method and haemacytometer. Results from averaged percent cell viability were plotted with concentrations of triclosan. The 50% inhibition concentration (IC_{50}) was defined as a triclosan concentration causing a 50% decrease in cell viability.

Genotoxicity assay

The comet assay and apoptosis assay were used to determine genotoxicity of triclosan. The alkaline comet assay was used according to Ostling and Johnson (1984) with slight modification. Briefy, the selected cell lines of KB and Vero cells after 3 and 5 days treatment of triclosan (at the IC_{50} and the maximum triclosan concentration found in personal care products) were trypsinized and centrifuged to wash out growth medium. Cells (10^5 cells in 0.5 ml phosphate buffer saline) were suspended in 0.8% low melting point agarose and dispensed onto a

1% normal melting point agarose precoated slides. After the gel settle, 1% normal melting point agarose was layered on top. The cells were lysed in pH 10 lysis solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 1% Trion X-100 and 10% DMSO for 20 h at 4°C. Slides were then incubated in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min at 4°C followed by electrophoresis in the same buffer at 25 V, 300mA for 1 h. The slides were rinsed twice with neutralization buffer (400 mM Trizma-base, pH 7.5) for 5 min and left to dry. The slides were then stained with ethidium bromide for 5 min before washing with cold distilled water. Images were analysed by epi-fluorescent microscope (Olympus) equipped with a digital camera. Cells with comet image were counted based on the total cells.

Triclosan-induced apoptotic death of the selected cell lines was determined and monitored by Vybrant Apoptosis Assay Kit 2 (Molecular Probes, Inc., Eugene, OR) and epi-fluorescent microscope. Briefly, after the treatment of cells with triclosan at the maximum triclosan content of personal health-care product and IC_{50} for 3 and 5 days, cells were collected and subjected to annexin V and propidium iodide staining using the kit by following the step-by-step protocol provided by the manufacturer. Annexin V binds to phospatidylserine, which was translocated from inner to outer leaflet of the plasma membrane in only apoptotic cells and stained green. While propidium iodide stained necrotic cells and became red. The green and red cells were then counted to determine the percent cell death via apoptosis and necrosis based on the total cells.

RESULTS AND DISCUSSION

Cytotoxicity of triclosan

Three animal cell lines of one rodent and two primates used for cytotoxicity test of triclosan in this experiment were baby hamster kidney cell (BHK-21), human epithelial carcinoma cell (KB) and African green monkey kidney cell (Vero). The concentration-response cytotoxicity curve for 3 days exposure of triclosan to BHK-21, KB and Vero cells, as determined by Trypan blue exclusion method are shown in Figure 1. Triclosan exhibited toxicity to BHK-21, KB and Vero cells similarly; viability of each cell type was decreased as the concentration of triclosan increased. The mechanism of toxic triclosan to animal cells may possibly be the same as the bactericidal activity of triclosan (Regos *et al*., 1979); it may solubilize lipid in plasma membrane of animal cell which caused cell membrane disruption and then finally led to cell death. However, the different amounts of percent cell viability remaining in each cell type, after the treatment with triclosan, revealed that BHK-21 seemed to be able to tolerate triclosan better than Vero and KB cells. These could be the characteristic of cell type that rodent cells may be more resistant to toxic chemical than the primate cells.

In this study, the 50% inhibition concentration (IC_{50}) after 3 days exposure of triclosan to BHK-21, KB and Vero cells, determined from the curve, were at 0.26, 0.034 and 0.036 mM, respectively. The lower IC_{50} of KB and Vero cells implied that they were more susceptible to triclosan than the BHK-21 cells, and that they were more suitable models for cytotoxicity assay of triclosan. These two IC_{50} concentrations of KB and Vero cells in our study were slightly lower than that obtained by Zuckerbraun *et al.* (1998); the IC_{50} of triclosan for 24 h treatment to Smulow-Glickman (S-G) human gingival epithelial cell line was at 0.05-0.06 mM. Although a little higher value of the IC_{50} by Zuckerbraun *et al.* (1998) may suggest that S-G human cell line was more tolerant to triclosan than the cells used in our study, but several factors affecting cytotoxicity, i.e., chemicals, concentrations and durations of exposure should be closely considered. In our

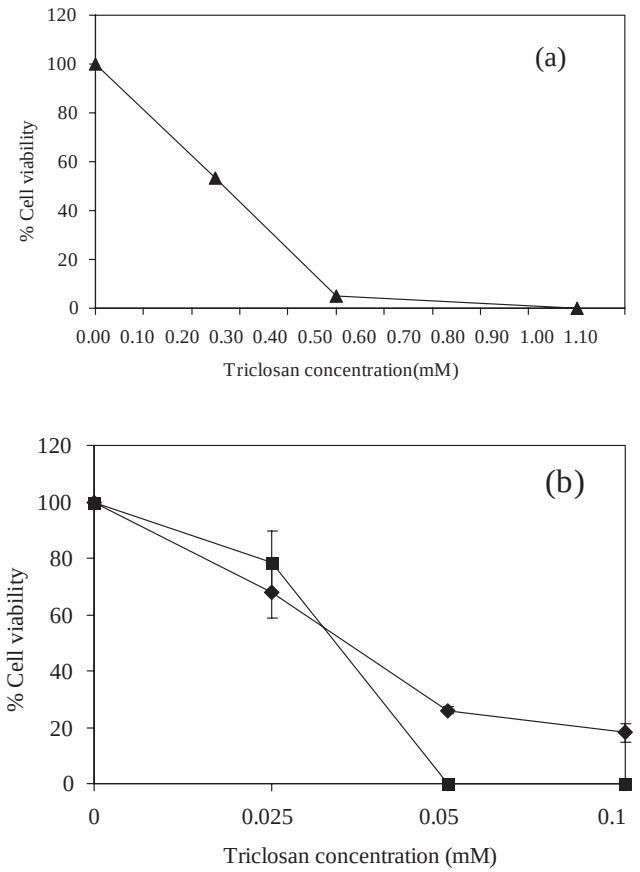

Figure 1 Cell viability of (a) BHK-21 (—▲—) and (b) Vero (—◆—) and KB cells (—■—) after 3-day exposure to triclosan at concentrations ranging from 0-0.1mM (for Vero and KB cells) and 0-1 mM (for BHK-21 cells). The control was exposed to 1 % dimethyformamide without triclosan.

study, the triclosan treatment was done for a longer period (3 days), while that of Zuckerbraun *et al*. (1998) was only for 1 day. This reasonably explained the low values of IC_{50} in 3 days exposure time of our study. Therefore, the IC_{50} obtained from this study was practically correlated with the result of Zuckerbraun *et al*. (1998). The duration of 3 days treatment was set in the study because there is a possibility that lipophilic triclosan may be accumulated in cells or fatty tissue (Glaser, 2004). This may lead to long term exposure of triclosan to cells. Therefore, we chose KB and Vero cells for the further study in long term treatment on genotoxicity of triclosan.

Triclosan content in personal care products

The popular use of selected toothpaste, bar soap and liquid facial soap were analyzed for their triclosan contents. The triclosan content of toothpaste was 0.025% (w/w), while those of soap and liquid facial soap were at 0.0675 and 0.27% (w/w) respectively. Triclosan contents in all selected samples were less than 0.3% (w/w) set by FDA as the maximum content for consumer products (www.fda.moph.go.th/ fda-net/html/ product/cosmetic/cosmetic/dat/article/ Triclosan.pdf). The maximum triclosan content in the liquid facial soap was at 0.27% (w/w) or 0.023 mM. This concentration of triclosan was

still lower than the IC_{50} obtained in our study. Therefore, the amounts of triclosan present in the health care products were not toxic to consumer. However, people may have taken triclosan unintentionally. Chung *et al*. (2003) reported the releasing of triclosan from triclosan coated food packaging at 1.2% in 10% aqueous ethanol (the treatment simulated to aqueous/acidic foods) and at 65% in n-heptane (the treatment simulated to fatty foods). The lipophilic triclosan may gradually be accumulated in cells and may lead to toxicity to cells. Therefore, the genotoxicity of triclosan at the maximum concentration of triclosan in products (0.023 mM) and the concentration at IC_{50} were analyzed based on comet assay and Vybrant Apoptosis Assay Kit 2, after the cell treatment for 3 and 5 days period.

Genotoxicity of triclosan

The DNA damage by triclosan was analyzed by comet assay or single cell electrophoresis to determine its activity on genotoxicty in individual cells. If the image of a cell after the assay appears red round head without tail, it represents the cell as non-DNA damage. But if it appears comet-like, it is considered a DNA breakage cell where the tail contains the damaged DNA. In our study, cell treated at IC_{50} (0.034 and 0.036 mM) and IC_{20} or IC_{30} (0.023 mM) of both cell lines showed that comet cells increased as concentrations and exposure times increased (Figure 2). However, the percent comet cells were rapidly decreased when the potency of the toxic triclosan increased. Apoptotic cell death is also resulted to DNA fragmentations. Therefore,

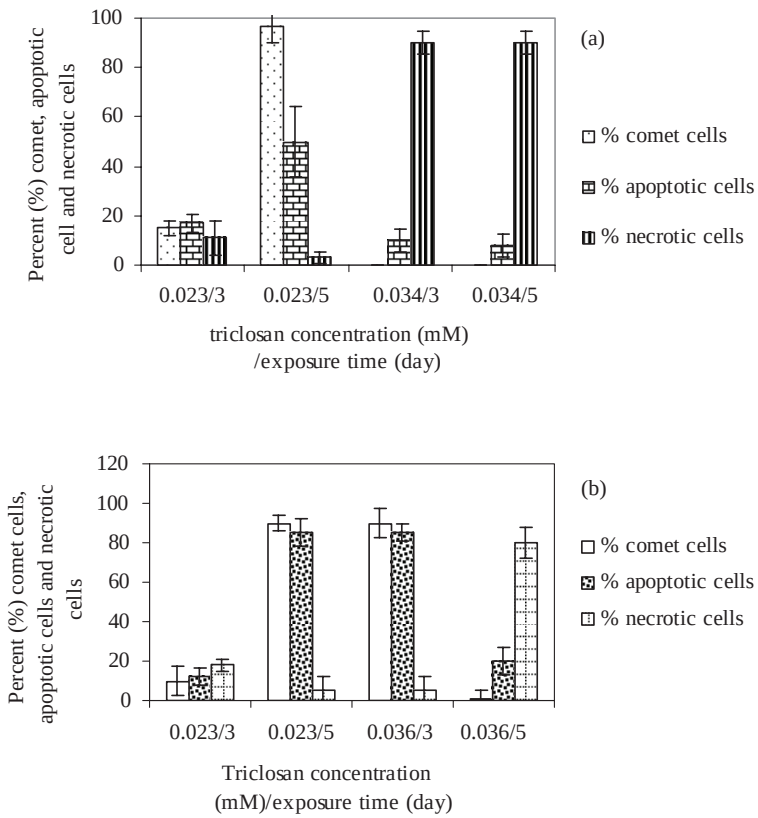

Figure 2 Genotoxic effect of triclosan at maximum triclosan concentration in products (0.023 mM) and the 50 % inhibition concentration (IC_{50}) of triclosan for 3 and 5 days exposure time to KB (a) and Vero cells (b) using comet assay and Vybrant Apoptosis Assay Kit 2.

Vybrant Apoptosis Assay Kit was performed concurrently in order to exclude cytotoxicity effect of triclosan.

The higher toxic of triclosan which resulted in decreasing comet cells to only 1 or zero percent could be explained; low toxicity of triclosan (0.023 mM) may break DNA and lead to apoptotic cell death which was increased as exposure time increased. The amount of comet cells was correlated with the percent apoptotic level at the condition studied. However, when cells were treated with a higher toxic concentration (at the IC_{50}), necrotic cell death was dominant. The DNA breakage in cells may be too severe and lead cells to necrotic death. The necrotic dead cells presented no tail of comet cells when analyzed on gel electrophoresis. This was similar to the finding of Collins (2004) that necrotic cells displayed a characteristic view with large nuclear remnants and almost invisible tails while apoptotic cells clearly formed typical comet-like structures with large tails and small heads (Figure 3 a). Therefore, triclosan caused DNA breakage and resulted in comet cells including apoptotic cells, and if the DNA damage was severe then the cell would die by necrosis.

As genotoxicity should not occur concurrently with cytotoxicity, therefore, only the results from using 0.023 mM triclosan were considered. The short term exposure of triclosan (3 days) to both KB and Vero cells showed comet cells at only 10-15%. The levels of apoptotic cells at this condition were at 12-17% which was slightly higher than that of the untreated condition (where apoptotic cells were determined to be at 10-12%). As a result, triclosan may exhibit slightly or almost non-genotoxic by the treatment at 0.023 mM for 3 days to KB cells and Vero cells. This genotoxic would be pronounced after 5 days treatment and led to more comet cells. Therefore, DNA breakage from triclosan was time and dose dependent at the cellular level. This research information may not directly be extrapolated to the complex animal model or human being, where the DNA damage of cells would be repaired by the body mechanisms. However, the basic information derived with cell culture on the health risk agent of triclosan would recommend us to expose to triclosan as little as possible. Furthermore, triclosan was also classified as an agent that may cause adverse environmental effects and hence the use should be restricted also from an ecotoxicological standpoint.

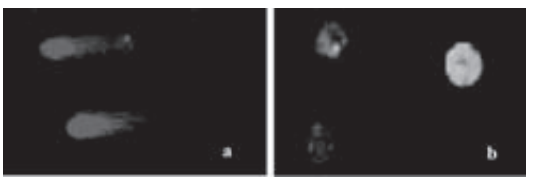

Figure 3 Comet appearance with long tail of cell from triclosan treatment after electrophoresis (a) and appearance of apoptotic cell (green) and necrotic cell (red) using Vybrant Apoptosis Assay Kit 2.

CONCLUSION

The results of this study demonstrated that triclosan was more toxic to primate cells than the rodent cells. KB and Vero cells would be good models to determine risk assessment of triclosan in personal health-care products. The 50% inhibition concentration (IC_{50}) of triclosan to KB and Vero cells were at 0.034 and 0.036 mM, respectively. Triclosn contents of samples from personal health-care products were not cytotoxic and genotoxic to human. However, human exposure to lipophilic triclosan should be as less as possible to avoid an accumulated triclosan in the body to the genotoxic level.

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