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Protective effect of phytochemicals on the triclosan-induced DNA damage

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Abstract

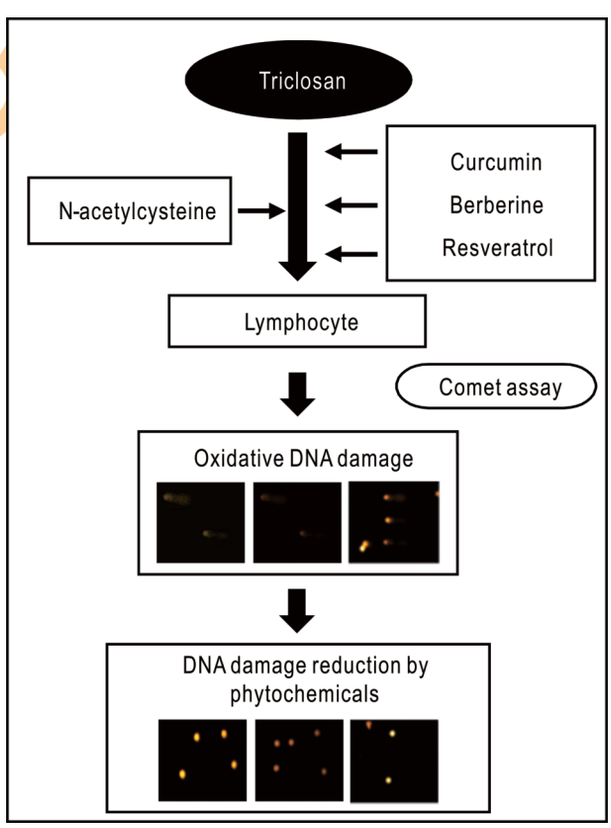
Aim : Triclosan, an antibacterial and antifungal agent, is widely used in several consumer products, including soaps, toothpaste and surgical cleaning treatments. The present study aimed to examine oxidative DNA damage in rat lymphocytes and its protection by phytochemicals *via* comet assay.

Methodology : DNA damage of rat lymphocytes induced by triclosan was measured by the olive tail moment in the comet assay. Following the addition of N-acetylcysteine, curcumin, berberine and resveratrol, the reduction of DNA damage was observed by using comet assay.

Results : The increased olive tail moment induced by triclosan was significantly reduced upon treating N-acetylcysteine and three phytochemicals, such as curcumin, berberine and resveratrol. Notably, the oxidative DNA damage by triclosan was dramatically suppressed by curcumin close to the control value, which means almost complete protection *in vitro*.

Interpretation : These results suggest that *in vitro* suppressive effect of curcumin, berberine and resveratrol against DNA damage by triclosan might be due to their antioxidative properties, and could be utilized for developing a reducing agent for triclosan toxicity.

Key words: Comet assay, DNA damage, Phytochemicals, Protective effect, Triclosan



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Introduction

Triclosan ($C_{12}H_7Cl_3O_2$), an antibacterial and antifungal agent, is widely used in several consumer products, including soaps, toothpaste and surgical cleaning treatments (Ao *et al.,* 2017; Rattan *et al.,* 2017). However, the adverse health effects of triclosan (Yueh *et al.,* 2014; Marshall *et al.,* 2015) includes high risk allergic sensitization, cardiovascular disease, as well as estrogenic and androgenic property (Szychowski *et al.,* 2016; Datta *et al.,* 2017; Teplova *et al.,* 2017). Recently, triclosan is considered as a "contaminant of emerging concern", which means that it is under investigation for public health risk. Triclosan is not commonly monitored in the environment but has potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects (Halden, 2014). Triclosan shows varied toxicity depending on its dose. High dose of triclosan appears as a biocide with multiple cytoplasmic and membrane targets (Ajao *et al.,* 2015; Weatherly *et al.,* 2015; Shim *et al.,* 2016). However, triclosan acts as a bacteriostatic agent in commercial products, inhibiting synthesis of microbial fatty acid at low doses. When low dose triclosan binds to microbial enoyl-acyl carrier protein reductase (ENR), the binding affinity of ENR for nicotinamide adenine dinucleotide increases. Thus, a ternary complex of ENR-NAD^+-triclosan in bacteria might inhibit fatty acid synthesis in bacteria. In human, however, triclosan is suggested to affect with different reaction mechanism as ENR enzyme is absent in humans. The mode of action of triclosan in human has not been elucidated yet (Dann and Hontela, 2011; Verslycke *et al.,* 2016; Ruszkiewicz *et al.,* 2017).

Comet assay, also referred to as single cell gel electrophoresis, has been extensively used for the detection of DNA damages and repair at individual cell levels. Comet assay is a simple, sensitive and fast technique for assessing DNA strand breaks, alkali-labile sites, DNA cross-links and base-pair damages (Nandhakumar *et al.,* 2011). The applications of comet assay range from DNA damage in specific genomic sequences within eukaryotic cells to massive human biomonitoring.

A wide variety of phytochemicals, named polyphenols and alkaloids, have been known to provide beneficial effects for human through notable antioxidant activity. Phytochemicals have been reported to diminish oxidative stress and the relevant risk of chronic diseases (Sreelatha *et al.,* 2012). Information on the toxic effect of triclosan on the lymphocyte DNA and the ameliorative capability of phytochemicals against triclosan toxicity has not been fully addressed yet. In light of the above, the ameliorative effect of N-acetylcysteine and phytochemicals such as curcumin, berberine and resveratrol on the oxidative DNA damage by triclosan was examined in this study.

Materials and Methods

Preparation of lymphocytes: Male Sprague-Dawley rats (Orient Bio Inc. Seongnam, Korea), weighing approximately 250 g, were maintained in an experimental room under controlled conditions of temperature (22 ± 2°C) humidity (50 ± 10%), and a 12-hour light/dark cycle. Five rats were anesthetized and whole blood was collected into EDTA-containing tubes. A 200 µl of fresh whole blood was added to 800 µl of phosphate-buffered saline (PBS) and layered onto 200 µl of Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). After centrifugation at 1,450 rpm for 5 min at room temperature, the lymphocytes were collected from the buffy-coat layer and washed with PBS. Comet assay using rat blood was conducted with the approval of the Animal Research Ethics Committee at Soonchunhyang University (approval number: SCH15-0007).

Lymphocyte treatment : Triclosan and stock solution was made by dissolving triclosan in DMSO. N-acetylcysteine (NAC) and three phytochemicals such as curcumin, resveratrol and berberine were dissolved in DMSO and then diluted with PBS. They were directly added to the lymphocytes which were treated for 30 min at 37°C before triclosan treatment. Phosphate buffered saline was treated to lymphocytes as a control.

Determination of DNA damage by comet assay : The alkaline comet assay was performed following the method of Singh *et al.* (1988) with slight modification (Kim *et al.,* 2017) to evaluate triclosan-induced DNA damage and determine the ameliorative effect of selected phytochemicals. The lymphocytes were mixed with 75 µl of 0.7% low-melting-point agarose and placed onto slides pre-coated with 1% normal-melting-point agarose, which was then allowed to solidify over 30 min at 4°C. Once solidified, the slides were further covered with 100 µl of 0.7% low-melting-point agarose. After the last applied agarose had been solidified, the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris,1% Triton X-100 and 10% DMSO) for more than one hour at 4°C in dark. To unwind the DNA, the slides were placed in an electrophoresis tank containing electrophoresis buffer [300 mM NaOH and 10 mM Na_2EDTA (pH 13.0)] for 20 min at 4°C. Electrophoresis was performed at 25 V/300 mA for 20 min at 4°C. The slides were washed with neutralizing buffer (0.4 M Tris•HCl pH 7.5) three times for 5 min at 4°C and fixed with crude ethanol for 5 min. Slides were air dried for 15 min and stored in a slide box at 4°C.

Image analysis : The slides were stained with ethidium bromide (50 µM) and the fluorescence was measured using a fluorescence microscope (Leica, Wetzlar, Germany) in dark condition and viewed with a CCD camera (Hitachi, Japan). The captured image was analyzed using Komet 5.5 software (Kinetic Imaging, UK). DNA damage was quantified *via* comet assay, wherein the olive tail moment was calculated by the following formula: (Tail. mean-Head.mean) x Tail% DNA / 100. A total of 100 lymphocytes were randomly captured and the comets were examined from each slide. All measurements were made in duplicate and performed from three independent experiments.

Statistical analysis: All comet data were analyzed using the SPSS package for Windows version 13 (SPSS Inc., Chicago, IL). The olive tail moments, values of DNA damage, from each treatment were compared with each other by one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. $p< 0.05$ was considered significant.

Results and Discussion

The triclosan-induced DNA damage occurred in a dose-dependent manner. All the concentrations of triclosan (6, 9, 12 and 15 µg ml^{-1}) induced statistically significant DNA damage ($p<0.05$). The olive tail moment at 6 µg ml^{-1} triclosan was about 19.2 ± 4.77, whereas that of PBS-treated control was about 1.3 ± 0.32, indicating approximately a 14-fold increase in DNA damage with 6 µg ml^{-1} triclosan assessed by olive tail moment (Fig. 1). The popular antioxidants, N-acetylcysteine (NAC) suppressed DNA damage caused by triclosan in lymphocytes as shown in Fig. 2 (A). NAC has been clinically used for diseases including cardio-vascular disease, sickle cell anemia and inflammation. The efficacy of NAC is due to its nucleophilicity and antioxidant property (De Flora *et al.,* 2001).

Lymphocytes were pre-treated with N-acetylcysteine at three concentrations (1, 2 and 3 µg ml^{-1}), and DNA damage was induced by exposing the lymphocytes to 6 µg ml^{-1} triclosan. The DNA damage in the NAC pre-treated group was notably inhibited in a concentration-dependent manner. NAC, derived from reduced glutathione, seems to act as a direct ROS scavenger (Sadowska *et al.,* 2007). The results show that triclosan induced oxidative DNA breakage in lymphocytes, but the oxidative DNA damage was blunted upon application of NAC, probably due to the antioxidative capability of NAC.

Next, the protective effect of curcumin, resveratrol and berberine on triclosan-induced oxidative DNA damage was studied. The herbal phytochemicals showed significant suppressive effects on triclosan toxicity, probably due to their function as scavengers and quenchers against free radical (Gao *et al.,* 2017). Phytochemicals have been utilized as complementary and alternative agents to conventional pharmacological therapeutics (Grabacka *et al.,* 2014; Park and Jang, 2017). They are involved in a range of efficacies from inhibiting cancer cell proliferation to protecting against oxidative damage that prevents chronic diseases such as heart disease, hypertension, diabetes and multiple cancers. Moreover, they act as reducer of cytochrome P_{450} enzyme system in the liver and anti-inflammatory chemical, carbohydrate metabolism promoter and immune system modulator (Ko *et al.,* 2016; Dhupal *et al.,* 2017; Nilnumkhum *et al.,* 2017).

Curcumin is the principal curcuminoid of turmeric, and possess anti-inflammatory, antioxidant, anti-cancer and neuro protective properties (Derochette *et al.,* 2013). Curcumin has been reported to be an effective scavenger of reactive oxygen species and reactive nitrogen species *in vitro.* Berberine is an isoquinoline alkaloid present in *Berberis*. It is known for its anti-bacterial, anti-aging and anticancer activity (Kim *et al.,* 2016). Resveratrol,3,5,4'-trihydroxy-*trans*-stilbene, is one of the phytoalexin induced by external stimuli including pathogen in several plants including grape. There is no conclusive human evidence for an effect of resveratrol on heart disease, cancer and metabolism. However, resveratrol has been reported to have numerous positive health effects including the anti-inflammatory, anti-oxidative and anti-cancer effect in various cancer cells, mice and rats (Rauf *et al.,* 2017).

The protective effect of curcumin on triclosan-induced oxidative DNA damage is shown in Fig. 2 B. The olive tail moment from all curcumin-treated group significantly decreased close to the level of the control group. The olive tail moment with 1, 5 and 7 µg ml^{-1} curcumin treatment was about 6 ± 0.3, 4.4 ± 1.84 and 4.2 ± 0.41, respectively, while that at 6 µg ml^{-1} triclosan was 41.8 ± 5.55. Fig. 2 C indicates the inhibitory effect of berberine on triclosan-induced oxidative DNA damage. The olive tail moment of the berberine-treated group significantly reduced down to the level of control group. The olive tail moment with 1, 5 and 7 µg ml^{-1} berberine application was about 11.9 ± 1.32, 16.2 ± 4.05 and 11.5 ± 4.31, respectively, while that at 6 µg ml^{-1} triclosan was 41.8 ± 5.75 (Fig. 2C). This result suggests the protective capacity of berberine against oxidative stress in lymphocytes. Berberine showed ABTS•+free radical scavenging activity of 43.87% at 125 µg ml^{-1} in our previous study (Ryu *et al.,* 2016). The result suggests that the inhibitory effect of berberine on oxidative DNA damage by triclosan might be due to its radical scavenging activity. Fig. 2 D shows the inhibitory effect of resveratrol on triclosan-induced oxidative DNA damage. The olive tail moment with 1, 3, 5 and 7 µg ml^{-1} resveratrol treatment was approximately 36.7 ± 4.17, 19.3 ± 2.41, 8.2 ±0.34 and 6.8 ± 0.34, respectively, while that at 6 µg ml^{-1} triclosan was 56.5 ± 5.8. The olive tail moment of 5 and 7 µg ml^{-1} resveratrol-treated group was diminished close to the control value with a statistical significance.

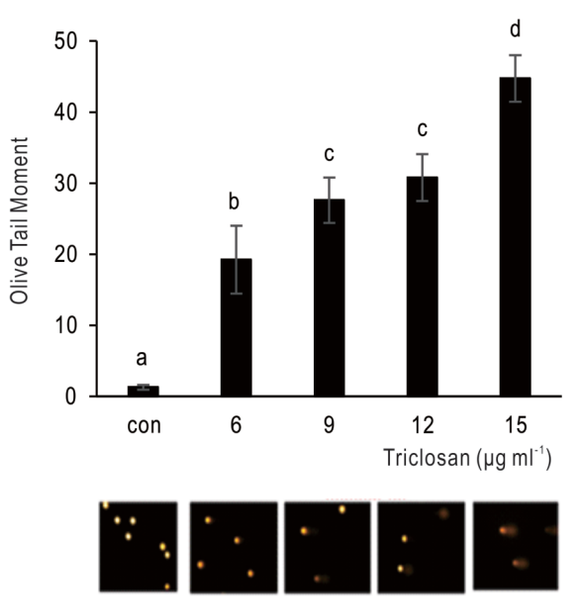

Fig. 1 : Triclosan-induced oxidative DNA damage measured by comet assay. Values not sharing the same letter are significantly different from one another ($p<0.05$) according to Duncan's Multiple Range Test.

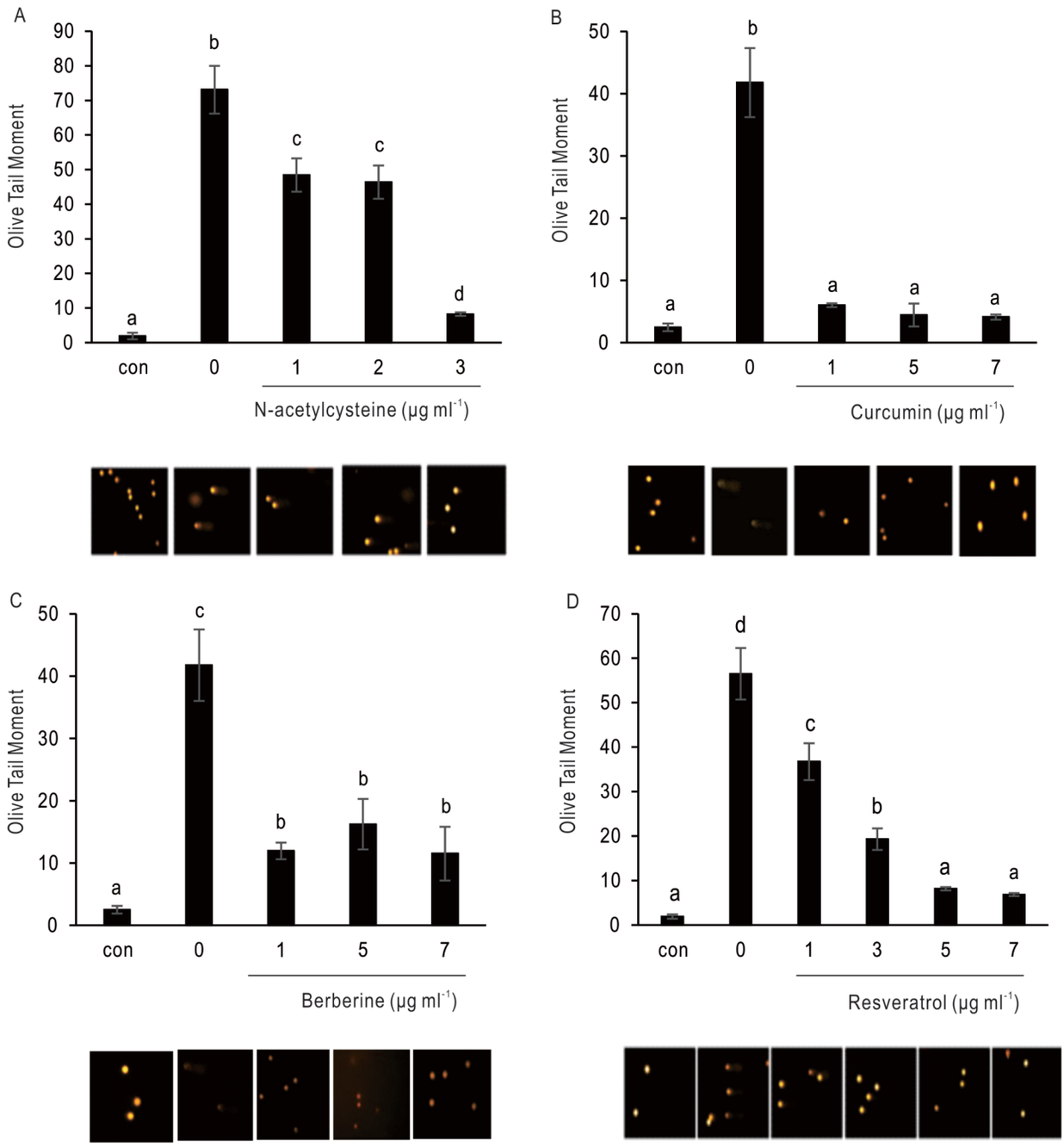

Fig. 2 : Suppressive effect of N-acetylcysteine (A) curcumin (B) berberine (C) and resveratrol (D) on triclosan-induced oxidative DNA damage. Values not sharing the same letter are significantly different from one another ($p<0.05$) according to Duncan's Multiple Range Test period.

Above results demonstrate the ameliorative effect of phytochemicals, such as curcumin, berberine and resveratrol on triclosan-induced oxidative DNA damage. Recently, comet assay has been applied not only for measuring DNA damage, but also in DNA repair studies. Most toxic compounds exert their toxicity *via* producing ROS, and it is generally accepted that the genotoxicity of toxic compounds can be reversed by antioxidants *in vitro*. Eukaryotic cells possess several repair systems for DNA damage before genome alterations occur, including base excision repair and nucleotide excision repair (Azqueta *et al.,* 2014). Despite the mechanism underlying the mode of action of antioxidants implicated with cellular repair systems remains unclear, these

phytochemicals could be developed as therapeutics for preventing DNA damage caused by triclosan *via* notable antioxidant properties.

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