Introduction

Cigarette smoking is a prevalent phenomenon that is linked to many health problems, including heart attack, stroke, chronic obstructive pulmonary disease, cancer, and cardiovascular disease. In South Korea (hereafter Korea), cancer is currently the primary cause of death, while the mortality rate of lung cancer patients is the highest among patients afflicted with major types of cancer [1]. The cigarette consumption rate in Korean adult males reached 75% in 1992, which was the highest rate of smoking prevalence in the world. By 2000, smoking prevalence remained at a relatively high 68% in Korean adult males [2]. Although the rate of smoking prevalence among adult Korean males decreased to 45% in 2007, the smoking prevalence rate in...
Korea remains higher than the average rate of 27.5% in the adult populations of Organization for Economic Cooperation and Development (OECD) countries [3]. According to the Korea Youth Risk Behavior Web-based Survey conducted by the Korea Centers for Disease Control and Prevention, the smoking prevalence rate among male youths in Korea aged 13 to 18 years increased from 14.3% in 2005 to 16.3% in 2012 [4]. These statistics demonstrate that cigarette smoking remains a major public health problem in Korea and reveal the need for toxicological studies of cigarettes sold in the Korean market.

Since carcinogenesis may be induced by the mutagenic and genotoxic effects of cigarette smoke, the aim of this study was to evaluate the mutagenic and genotoxic effects of Korean cigarettes using in vitro assays. The tar and nicotine contents of cigarettes have been reported to be associated with mutagenic and genotoxic effects [5-8]. Packs of TL and TW cigarettes state that each cigarette contains 0.8 mg nicotine and 8 mg tar, which represent the highest level of nicotine and tar contents among around 60 kinds of Korean cigarettes produced in KT&G in 2013. Therefore, we selected 2 types of cigarettes, TL and TW, as benchmark Korean cigarettes. The mutagenic and genotoxic potencies of the TL and TW cigarettes were compared to those of the 3R4F Kentucky reference (3R4F) cigarette. The cigarette smoke condensate (CSC) from each of the 3 tested types of cigarettes was evaluated for mutagenicity and genotoxicity using the Ames test, in vitro comet assay, and in vitro micronucleus (MNvit) assay. Mutagenic potency was expressed as the number of revertants per μg CSC total particulate matter (TPM), whereas genotoxic potency was expressed as a concentration-dependent induction factor (CDI), respectively.

**Materials and Methods**

**Cigarettes and Chemicals**

The 3R4F cigarette was kindly provided by the Korea Institute of Toxicology. The TL and TW were purchased from Korean commercial sources. Dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), 2-aminoanthracene, benzo[a]pyrene and cytochalasin B were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aroclor 1254-induced Sprague Dawley rat liver S9 was obtained from Moltox (Boone, NC, USA). The S9-cofactor, consisting of phosphate buffer, NADP, glucose-6-phosphate, KCl, MgCl₂ and CaCl₂, was purchased from Wako (Tokyo, Japan).

**Preparation of Cigarette Smoke Condensates**

The cigarettes were stored at 22 ± 1°C with 60 ± 2% relative humidity according to International Organization for Standardization (ISO) 3402 [9]. CSCs were generated by a 30-port smoking machine according to the ISO 3308 [10] smoking method (35 mL puff volume, 2 seconds puff duration, 60 seconds between puffs, and no vent blocking). All cigarettes were smoked to 3-mm beyond the end of the filter-tipping paper according to ISO 4387 [11]. Table 1 shows the contents of TPM, nicotine, and tar in the CSC from each type of cigarette. Each CSC was prepared by smoking 3 cigarettes of a particular type onto a Cambridge filter pad (44 mm; Whatman, Maidstone, UK), which was extracted with DMSO for 30 minutes with shaking, such that the final TPM concentration of the CSC was 20 mg/mL. The CSC samples were filtered through 0.44 μm sterile filters and frozen at -80°C.

**Cell Culture**

The CHO-K1 cell line was obtained from the Korea Cell Line Bank. CHO-K1 cells were grown in RPMI 1640 media with 5% fetal bovine serum containing 2 mM L-glutamine, 1% penicillin (100 units/mL), and 100 μg/mL streptomycin at 37°C in an atmosphere of 5% CO₂/95% air with saturated humidity.

**Ames Test**

Mutagenicity was tested based on OECD test guideline (TG) 471 [12]. Among the *Salmonella typhimurium* (*Salmonella enterica* subsp. *enterica*) strains including TA98, TA100, TA102, TA1535, and TA1537 tested in preliminary experiments, frameshift strains TA98 and TA1537 were found to be the most sensitive to CSC and employed in this work. In addition, the number of revertant colonies in most strains was not increased in the absence of S9 mix in preliminary experiments. Therefore, the Ames test was performed in the presence of the S9 mix using a plate incorporation method. Each CSC was combined with 100 μL of an overnight culture (1-2 × 10⁸ cfu/mL) of each strain and the S9 mix, followed by incubation for 30 minutes at 37°C.

| Sample ID | Description                          | TPM (mg/cig) | Nicotine (mg/cig) | Tar (mg/cig) |
|-----------|--------------------------------------|--------------|-------------------|--------------|
| 3R4F      | Kentucky reference cigarette         | 5.92         | 0.43              | 4.65         |
| TL        | Korea benchmark design               | 7.89         | 0.50              | 6.11         |
| TW        | Korea benchmark design               | 7.29         | 0.49              | 5.78         |

TPM, total particulate matter.
The control and CSC-treated strains were mixed with 2 mL of sterile top agar and poured onto minimal glucose agar plates. After the plates were incubated at 37°C for 48 hours, the number of revertant colonies on each plate was counted. The test was carried out with three plates per concentration. Test results were considered positive if the number of revertants was at least double the revertant number of negative control group for 2 consecutive concentrations and a concentration-related increase was observed in the number of revertants [13].

**Cell Viability Assay**

The CHO-K1 cells were seeded onto 96-well plates at a density of 5 × 10³ cells/well. After the cells were cultured for 24 hours, they were treated with the CSC solutions for 24 hours, after which 10 μL of WST-1 reagent (Roche Diagnostic, Montclair, NJ, USA) was added. The absorbance of each test sample was measured at 440 nm and 690 nm using a microplate reader. Cell viability was expressed as a percentage relative to that of the control cells.

**In Vitro Comet Assay**

The comet assay was performed as described by Singh et al. [14]. CHO-K1 cells were seeded on 6-well plates at a density of 4 × 10³ cells/well. After 24 hours of incubation, the cultured cells were exposed to the CSCs for 3 hours in the presence of the S9 mix. The treated cells were resuspended in 0.7% low melting point agar. A 160-μL aliquot of each cell suspension was spread onto a precoated glass slide and covered with a cover glass, after which the slide was incubated for 1 hour at 4°C. In the alkaline comet assay, cells were lysed in pH 10 lysis solution at 4°C for 1 hour. The lysed cells were allowed to unwind for 30 minutes in electrophoresis buffer before electrophoresis for 30 minutes at 25 V on ice. The gels were neutralized with 0.4 M Tris-HCl (pH 7.5) twice for 5 minutes and stained with ethidium bromide (2 μg/mL). DNA migration was assessed using automatic image analysis software. The Olive tail moment (OTM; tail distance × percentage of DNA in the tail) was used to quantify DNA damage based on random scoring of 100 nuclei per slide. In order to compare DNA breakage induced by the 3 tested types of cigarettes, CDI was selected based on the report of Seitz et al. [15]. The CDI was calculated by integrating all concentrations and induction factors for each dose by the following equation:

\[
CDI = \sum_{i=1}^{n} \frac{IF_i}{C_i}
\]

where \( IF_i \) was the induction factor of the concentration, \( C_i \) was the concentration \( i \) (1–4), and \( n \) was 4 (4 concentrations).

**Results**

**Mutagenic Potencies of Cigarette Smoke Condensates**

The mutagenicity of 3 CSCs was evaluated using the TA98 and TA1537 strains with the S9 mix. The revertant number of negative control group in the TA98 and TA1537 strains were 49.9 ± 5.2 and 16.7 ± 1.9 rev/plate, respectively. In both tested strains, CSC exposure dose-dependently increased the number of revertants in comparison with those of the corresponding negative control groups. According to the 2-fold rule, all CSCs showed positive results for mutagenicity in comparison with those of the corresponding negative control groups. The mutagenic potencies of the 3 tested CSCs were expressed as revertants per μg TPM as Mladjenovic et al.’s report (Table 3) [17].

**In Vitro Micronucleus Assay**

MNvit assays were conducted in compliance with OECD TG 487 [16]. The treatment concentration of each CSC was determined by measuring the cytokinesis-block proliferation index. CHO-K1 cells were seeded onto 8-well chamber slides at a density of 1.5 × 10⁴ cells/well for 24 hours. The cells were treated with the 4 doses of CSCs in the presence of the S9 mix for 3 hours, followed by a 21-hour recovery period under exposure to 0.75 μg/mL cytochalasin B. After washing the cells twice with PBS, 1% trisodium citrate was added for 5 minutes at 4°C, after which the slides were placed in fixative solution at 4°C. Ribonuclease A was added to each slide for 5 minutes at 30°C, after which the slides were rinsed in 2 × saline sodium citrate. After the slides were dried thoroughly, they were stained overnight with 5% Giemsa solution with shaking. Micronuclei (MN) 1000 binucleated cells per duplicate culture (total 2000 binucleated cells) were scored by 2 scorers who were blind to the treatments. The CDI was calculated to allow comparison of the genotoxic potency of the 3 tested types of cigarettes using the MNvit assay.

**Statistical Analysis**

Sigma Plot (Jandel Scientific, San Rafael, CA, USA), Excel (Microsoft, Redmond, WA, USA), and SPSS version 21.0 (IBM Corp., Armonk, NY, USA) were used to analyze the data. The results of each assay are expressed as mean ± standard deviation. Differences between groups were assessed by one-way ANOVA followed by Duncan’s post-hoc test. Statistical significance was accepted at \( p < 0.05 \) or 0.01.
Table 2. Mutagenicity of 3 CSCs in Salmonella typhimurium

| Dose (μg TPM/plate) | TA98  | TA1537 |
|---------------------|-------|--------|
|                     | 3R4F  | TL     | TW     | 3R4F  | TL     | TW     |
| 0                   | 49.9±5.2 | 40.6±0.4 | 41.8±4.3 | 16.7±1.9 | 13.8±1.5 | 14.8±2.3 |
| 25                  | 84.0±9.9* | 61.0±10.5 | 48.2±1.1 | 24.8±5.3 | 13.8±1.5 | 16.8±2.0 |
| 50                  | 185.0±17.0** | 115.2±8.6** | 114.7±11.9** | 34.5±1.3** | 23.5±1.4** | 16.8±2.0 |
| 100                 | 231.3±18.0** | 224.3±14.6** | 204.8±13.3** | 39.8±1.1** | 29.5±2.6** | 22.8±2.5** |
| 200                 | 272.7±21.2** | 293.4±12.2** | 321.7±4.0** | 51.0±5.7** | 52.8±2.4** | 34.0±0.7** |
| 300                 | 346.0±19.7** | 368.4±17.3** | 340.6±4.4** | 58.8±5.3** | 51.7±5.1** | 55.0±7.8** |
| 400                 | 335.0±22.6** | 60.5±7.1** | 51.3±2.5** | 56.2±1.6** |        |        |

Values are presented as mean±standard deviation. Values significantly different from control (0 μg/plate). The inhibitory effect of CSCs on cell growth was observed in the tested strains at concentrations of 500 μg/plate and higher. CSC, cigarette smoke condensate; TPM, total particulate matter; 3R4F, 3R4F Kentucky reference.

*The concentration of 2-aminoanthracene was 1 μg/plate.

The concentration of 2-aminoanthracene was 10 μg/plate.

*p<0.05, **p<0.01.

Table 3. Mutagenic and genotoxic potencies (rankings) of 3 CSCs

| CSC    | Ames test | Comet assay | MNvit assay |
|--------|-----------|-------------|-------------|
|        | TA98 rev/μg TPM | TA1537 rev/μg TPM | Control b(a)p μg/plate | CDI/μg TPM/mL | CDI/μg TPM/mL |
| 3R4F   | 1.99 1 0.39 1 1.41 1 1.11 1 | 3R4F TL TW | 3R4F TL TW | 3R4F TL TW |
| TL     | 1.15 2 0.32 2 1.32 2 1.02 2 | 3R4F TL TW | 3R4F TL TW | 3R4F TL TW |
| TW     | 1.13 3 0.29 3 0.69 3 0.93 3 | 3R4F TL TW | 3R4F TL TW | 3R4F TL TW |

CSC, cigarette smoke condensate; MNvit assay, in vitro micronucleus assay; rev, revertants; TPM, total particulate matter; CDI, concentration-dependent induction factor; 3R4F, 3R4F Kentucky reference.

Figure 1. Viability of CHO-K1 cells exposed to 3 CSCs. The cells were incubated with 3R4F (green bar), TL (sky blue bar), and TW (red bar) CSCs for 24 hours, after which the WST-1 assay was performed. Cell viability was expressed as a percentage of that of the control cells (0.0 μg TPM per mL). Each value represents the mean±standard deviation of 5 separate experiments. CSCs, cigarette smoke condensates; 3R4F, 3R4F Kentucky reference; TPM, total particulate matter. *p<0.05, **p<0.01 for values significantly different from those of the control group.

response to the CSCs, but the CSCs were less potent than they were in the TA98 strain. The mutagenic potencies of the CSCs from 3R4F, TL, and TW cigarettes were 0.39±0.30, 0.32±0.15, and 0.29±0.17 rev/μg TPM, respectively, in the TA1537 strain.

In the TA98 and TA1537 strains, the 3R4F CSC had the greatest mutagenic potency, followed by the TL CSC, whereas the TW CSC had the lowest mutagenic potency.

Figure 2. DNA breakage in CHO-K1 cells exposed to 3 CSCs. The cells were treated with 3R4F (green bar), TL (sky blue bar), and TW (red bar) CSCs for 3 hours in the presence of the S9 mix. The positive control cells were exposed to 10 μM b(a)p. DNA breakage was expressed as Olive tail moment (tail distance × %DNA in the tail), which was expressed as a fold-induction relative to the control group (0.0 μg TPM per mL). Each value represents the mean±standard deviation of 5 separate experiments. CSCs, cigarette smoke condensates; 3R4F, 3R4F Kentucky reference; b(a)p, benzo[a]pyrene; TPM, total particulate matter. *p<0.05, **p<0.01 for values significantly different from those of the control group.
**Genotoxic Potencies of Cigarette Smoke Condensates**

Cytotoxicity tests were performed prior to DNA breakage evaluation to avoid false-positive results owing to interference with the genotoxicity assay by acute cell toxicity. Significant cytotoxicity was observed in cells treated with all CSCs at concentrations greater than 50 μg TPM/mL (Figure 1). Therefore, the concentrations of CSCs used in the genotoxicity tests were 25 μg TPM/mL or less. Figure 2 shows the dose-response curve of each CSC for DNA breakage. All CSCs dose-dependently increased DNA breakage. The CSCs from 3R4F and TL cigarettes significantly increased OTM at all tested concentrations, whereas the CSC from the TW cigarettes significantly increased OTM at concentrations of 12.5 and 25.0 μg TPM/mL. The TL CSC produced more DNA damage (3.83 ± 0.24-fold) than the 3R4F (3.68 ± 0.25-fold) and TW (1.93 ± 0.54-fold) CSCs at a concentration of 25 μg TPM/mL. However, the CDI values produced by the 3R4F, TL, and TW CSCs were 1.41, 1.32, and 0.69/μg TPM/mL, respectively (Table 3). In the MNVit assay, all tested CSC concentrations dose-dependently and significantly increased MN formation (Figure 3). Similar to the results of the comet assay, the TL CSC induced the highest frequency of MN formation (2.62 ± 0.19-fold), followed by the 3R4F (2.59 ± 0.05-fold) and TW (2.49 ± 0.13-fold). However, the CDI values were 1.11, 1.02, and 0.93/μg TPM/mL in 3R4F, TL, and TW, respectively.

**Discussion**

Cigarette smoke is a deleterious and complex mixture of more than 7000 gaseous and particulate compounds, including at least 70 carcinogens [18]. The cytotoxicity, genotoxicity, and mutagenicity of commercial brands of cigarettes sold in Japan, the US, and Canada have been reported [19-21]. However, to the best of our knowledge, this study is the first report to evaluate the mutagenic and genotoxic effects of CSCs derived from Korean cigarettes and compare them with those of the 3R4F cigarette.

The mutagenic potencies of various commercial cigarettes have been evaluated in TA98 strain, because TA98 showed greater susceptibility to CSCs than other strains, including TA100 and TA1537 [22,23]. Mladjenovic et al. [17] reported that the mutagenic potencies of CSCs from 3 types of cigarettes chosen as benchmarks of Canadian commercial cigarettes were 0.6 to 0.7 rev/μg TPM, which corresponded to about half of the mutagenic potency of the 3R4F CSC. The mutagenic potencies of kretek cigarettes, a type of commercial cigarette originating from Indonesia, were approximately 1.1 rev/μg TPM in the TA98 strain with the S9 mix [24]. In addition, Japanese cigarettes with nicotine and tar contents similar to those in our samples showed mutagenic potencies of 0.73 to 1.19 rev/μg TPM in the TA98 strain with the S9 mix [19]. Therefore, based on calculated mutagenic potency, the mutagenic effects of TL (1.15 rev/μg TPM) and TW (1.13 rev/μg TPM) cigarettes would be expected to be similar to those of previously tested foreign commercial cigarettes. In contrast, US commercial cigarettes had stronger mutagenic potency than Korean cigarettes. Virginia Slims are a brand of commercial cigarettes sold in the US that have similar nicotine and tar contents to the TL and TW cigarettes tested in our study. The mutagenic potencies of Virginia Slims cigarettes ranged from 3.37 to 4.23 rev/μg TPM in the TA98 strain with the S9 mix [20].

The genotoxic potencies of CSCs were expressed as CDIs, which were calculated from the induction factors at all concentrations. The CDI provides information that is adequate for straightforward, precise, and realistic assessment of genotoxic potential by integrating responses to compounds across wide concentration ranges [15]. However, the CDI may overestimate effects at low concentrations, because substances with minor genotoxic effects at low concentrations tend to result in higher CDIs than substances with very strong effects at high concentra-
tions [25]. The CDI value of 3R4F CSC was the greater than that of TL and TW CSCs in both comet and MNvit assays. This result was consistent with the relative mutagenicity potencies of the 3 tested CSCs. After all, the CSC from the 3R4F cigarette produced the most severe mutagenic and genotoxic potencies, followed by the CSC from the TL cigarette, whereas the CSC from the TW cigarette produced the least severe mutagenic and genotoxic potencies (Table 3). However, our results do not indicate that domestic cigarettes typically have weaker mutagenicity and genotoxicity than foreign cigarettes, since the 3R4F cigarette is not represented cigarettes on sale in entire US cigarette market.

The tar and nicotine contents of cigarettes have been reported to be associated with smoking-related diseases such as lung cancer. The level of daily exposure to cigarette tar was a positive significant predictor of genotoxicity [5]. In addition, smokers of lower tar cigarettes had a risk of lung cancer 23% lower than that of smokers of higher tar cigarettes [6]. Although nicotine itself is not classified as a carcinogen, nicotine may contribute to the carcinogenic effects of cigarette owing to its genotoxic properties [7]. However, a few studies reported that the mutagenic activities of so-called low-tar brands are not always lower than that of the other brands [19,26]. These findings are supported by epidemiologic studies that demonstrate no difference in lung cancer risk among smokers of cigarettes having tar levels of regular, light and ultralight [27]. Some studies also reported that nicotine and its major metabolites are not genotoxic [28] and that mutagenic effects induced by cigarettes are not related to nicotine content [29]. The role of tar in mutagenic and genotoxic effects induced by CSCs is harder to explain in this study, because CSCs derived from 3 types of cigarettes have similar toxic effects induced by CSCs have similar. However, the genotoxicity of CSCs cannot usually be compared directly, because there is no standardized or commonly used method for quantifying the genotoxic effects of CSCs. In this study, we evaluated the mutagenic and genotoxic effects of Korean cigarettes and compared them to those of 3R4F cigarettes. The mutagenic and genotoxic potencies of the tested CSCs were calculated as the number of revertants per μg TPM in the Ames test and CDI values in comet and MNvit assays, respectively. The mutagenic and genotoxic potencies of the 3R4F CSC were greater than those of the TL CSC, which were greater than those of the TW CSC. Further study on standardized concepts of toxic equivalents for cigarettes needs to be conducted for more extensive use of in vitro tests.

**Conflict of Interest**

The authors have no conflicts of interest associated with material presented in this paper.

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