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Assessment of Genotoxicity of Waterpipe and Cigarette Smoking in Lymphocytes Using the Sister-Chromatid Exchange Assay: A Comparative Study

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Tobacco smoking is a major world health problem. Recently, waterpipe smoking has become more popular in many countries. Although the genotoxicity associated with cigarette smoking has been extensively investigated, studies evaluating such toxicity in waterpipe users are still lacking. In this study, we examined the genotoxicity of waterpipe smoking in lymphocytes compared with the genotoxicity of cigarette smoking. Genotoxicity was evaluated using the sister chromatid exchanges (SCEs) assay. Fifty waterpipe smokers and 18 healthy nonsmokers participated in this study. Additionally, 18 heavy cigarette smokers (CS) were recruited for comparison. The results show that waterpipe smoking and cigarette smoking significantly increase the frequencies of SCEs ($P < 0.01$) compared with those of nonsmokers, indicating the genotoxic effect of tobacco smoking. In addition, frequencies of SCEs were significantly higher among waterpipe smokers compared with CS ($P < 0.01$), indicating that waterpipe smoking is more genotoxic than cigarette smoking. Moreover, the frequency of SCEs increased with the extent of waterpipe use. In conclusion, waterpipe smoking is genotoxic to lymphocytes and the magnitude of its genotoxicity is higher than that induced by regular cigarette smoking. Environ. Mol. Mutagen. 00:000–000, 2010.

Key words: waterpipe; smoking; cigarette; genotoxicity

INTRODUCTION

Smoking is a major world health problem. Globally, 5–6 million deaths each year are attributed to tobacco use and this annual toll may increase to 10 million within the next 20–30 years [WHO, 2009]. Tobacco smoke contains over 4,000 chemical compounds, including more than 50 known carcinogens, such as polycyclic aromatic hydrocarbon (PAHs), N-nitrosamines, aromatic amines, and trace metals [IARC, 2004; Rogers, 2008]. Tobacco smoking increases the risk of lung, cavity, esophagus, stomach, pancreas, liver, kidney, and bone marrow cancers [IARC, 2004; Sasco et al., 2004; Jha, 2009; Lee and Hamling, 2009].

Tobacco is commonly consumed in different ways including cigarette, pipe, cigar, and waterpipe smoking. The popularity of waterpipe smoking is growing in the eastern Mediterranean and throughout the world including the USA and other western countries, especially among youth [Eissenberg et al., 2008; Maziak, 2008; Primack et al., 2008; Warren et al., 2009]. Smoking using this method includes the use of a heavily flavored and...
hydrated, shredded tobacco known as “moassel” and it relies on burning charcoal placed on top of the tobacco to provide the heat needed to produce the aerosol, since unlike cigarette tobacco, the “moassel” is incapable of self sustained combustion [Shihadeh, 2003]. Similar to cigarettes, waterpipe smoke contains abundant toxicants that are thought to render smokers more prone to cancer [Shihadeh and Saleh, 2005].

The genotoxicity of cigarette smoking has been extensively studied [DeMarini, 2004]. Most reports indicate that cigarette smoking is genotoxic as assessed by several tests including sister chromatid exchanges (SCEs) and chromosomal aberrations (CAs) [Rowland and Harding, 1999; Tawn and Whitehouse, 2001; Karaoguz et al., 2005; DeMarini et al., 2008; Lu and Morimoto, 2008; de Assis et al., 2009]. A recent study by Milic et al. [2008] showed that even occupational exposure to tobacco dust in the cigarette manufacturing industry increases CA and SCE frequencies. However, only two studies have examined the genotoxicity of waterpipe smoking. The first report showed a significant increase in chromosomal damage in lymphocytes of waterpipe users [Yadav and Thakur, 2000] whereas the second one showed high levels of micronuclei in buccal mucosa cells of users [El-Setouhy et al., 2009]. In addition, the genotoxicity of tobacco smoking using cigarette has not been compared with that of waterpipe smoking. In this investigation, SCE analysis was used to compare the genotoxicity of waterpipe smoking in heavy, medium, and light smokers to the genotoxicity of cigarette smoking.

MATERIALS AND METHODS

Subjects

The study was performed on 50 waterpipe smoker and 18 heavy smoker subjects recruited from different places in Irbid city/Jordan such as coffee shops and student dorms. As a control, 18 nonsmoking subjects were selected to match smokers for age and geographical area. Selection criteria: waterpipe smokers were those who use only waterpipe to smoke tobacco and cigarette smokers (CS) were those who use only cigarettes to smoke tobacco with at least 30 or more cigarettes per day. Waterpipe smokers (50 subjects) were divided into three groups: (a) heavy group (Wh, use waterpipe daily), (b) medium group (Wm, use waterpipe in 4–5 days/week), and (c) light group (Wl, use waterpipe in <3 days/week). All subjects were healthy adult males and did not use alcohol or drugs. This study was approved by the Institutional Review Boards of Jordan University of Science and Technology. Prior to the start of the study, written informed consents were obtained from all subjects. A 5-ml blood sample was collected from each subject via peripheral vein using coded heparinized vacuum tubes and was cultured within 2 hr of sampling.

SCE assay

Lymphocyte cultures were established by adding 1 ml of fresh heparinized whole blood to 9 ml of PB max complete karyotyping medium (Gibco-Invitrogen, UK) containing 5'- Bromodeoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO) solution at a final concentration of 0.1 µg/ml. All cultures were incubated in the dark at 37°C for 72 hr in a CO₂ incubator. Tow hours prior to harvesting, colcemid (final concentration of 0.075 M KCl) for 15–20 min at 37°C. The swollen lymphocytes were collected by centrifugation and fixed in freshly prepared fixative (absolute ethanol:glacial acetic acid 3:1, v:v) at room temperature for 15 min. The cell suspension was centrifuged, washed with fixative three times, and then resuspended in 1 ml of the fixative before dropping on prechilled microscopic slides to obtain metaphase spreads. Slides were then allowed to air dry, aged for 24 hr in the dark and stained with Fluorescence-plus-Giemsa as previously described [Sadig et al., 2006; Azab et al., 2009]. To score SCEs, twenty-five clearly differentiated second metaphases that contained only 44–48 chromosomes were examined for each donor.

Cell Kinetics Analysis

The mitotic index was calculated by analyzing 1,000 cells from each subject and scoring the cells that were in metaphase [M’Bemba-Meka et al., 2007]. For the cell proliferation index, 100 metaphase cells from each donor were scored. The proliferation index was calculated using the following formula = (1 × M1 + 2 × M2 + 3 × ≥M3)/100, where M1, M2 and M3 are the number of cells at the first, second and third metaphase, respectively [Ivett and Tice, 1982]. Depending on the proliferation index, the average generation time was calculated as the number of hours for the cells in BrdU, divided by proliferation index [Kaya and Topaktas, 2007]. The changes in the above parameters were used as indicators that reflect the cytotoxicity of smoking in blood lymphocytes.

Statistical Analysis

Statistical analysis was performed using Prism statistical software (version 4.0). Data were expressed as mean ± standard deviation (SD). The comparisons of parameters were performed with ANOVA multiple comparison test, followed by Newman - Keuls Post-hoc test. A Pearson test was used to analyze the correlation between the extent of waterpipe use and mean SCEs. High frequency SCE cell (HFCs) analysis was calculated according to Moore and Carrano [1984]. HFCs were defined as cells that display a number of SCEs/cell that exceeds the 95th percentile of the distribution of SCEs/cell in the controls. The frequencies of HFCs in the different groups were compared using Chi square test. All differences were regarded as significant at P < 0.05.

RESULTS

Table I shows the characteristics of all participants. The mean age was similar between groups (ANOVA, F = 0.63, df = 85, P = 0.59) and the sample sizes were equivalent in the different groups. However, the number of smoking sessions/week in the three waterpipe groups was significantly different (ANOVA, F = 77.3, df = 49, P < 0.001).

SCEs were observed in differentially stained M2 metaphase cells that have 44–48 chromosomes. There were significant differences in SCE frequencies between the control (C) group and the heavy CS as well as the waterpipe heavy (Wh) users (ANOVA, F= 81.57, df = 53, P < 0.001, Figure 1, post-hoc results, C vs. CS: P < 0.01, C vs. Wh: P < 0.01). In addition, significant differences in SCE frequency was observed between the Wh group and the CS group (P < 0.01).
To investigate whether SCEs increase with increasing waterpipe use, the frequencies of exchanges were investigated in lymphocytes of the medium and light waterpipe users. As shown in Figure 2, there were significant differences in the frequencies of SCEs between the control group and all waterpipe user groups (ANOVA, $F = 65.2$, df = 67, $P < 0.01$). In addition, the level of SCEs in Wh was higher than the Wm group ($P < 0.01$) and higher in the Wm than the Wl group ($P < 0.05$). More importantly, the mean SCEs in waterpipe users was positively correlated with increasing waterpipe use ($r = 0.53$, $P < 0.01$, Fig. 3).

SCE data were further analyzed using the HFCs measure proposed by Moore and Carrano [1984]. The threshold of HFCs with a percentile of 95 was 8 SCE/cell. The percentage of HFCs in the control was 3.8%. Approximately, 34.8%, 17.3% and 7.1% of M2 cells from Wh, Wm, and Wl smokers were HFCs respectively, compared to 11.5% in CS group. Smoking using cigarette and waterpipe (Wh and Wm) caused a significant increase the percentage of HFCs ($P < 0.01$). In addition, a significant difference in HFCs was observed between the Wh group and CS group ($P < 0.01$), and between the Wh group and both the Wm and Wl groups ($P < 0.01$). Thus, the HFCs results were similar to those of the mean SCEs.

Cell kinetic indices were used as an indicator reflecting the cytotoxicity of smoking to blood lymphocytes. The mitotic indices in waterpipe and CS were higher than those of the controls (mean ± S.E, C: 8.1 ± 0.51, CS: 9.2 ± 1.2, Wh: 10.4 ± 0.82, Wm: 9.1 ± 0.3, and Wl: 9.2 ± 0.38), but this difference was not statistically significant ($P > 0.05$). Similarly, no significant differences were obtained from the proliferative index measurement between groups (control: 2.2 ± 0.2, CS: 2.12 ± 0.18, Wh: 2.14 ± 0.2, Wm: 2.3 ± 0.32, Wl: 2.21 ± 0.17, $P > 0.05$).

**DISCUSSION**

In this study, the genotoxic effect of waterpipe smoking on lymphocytes was examined using the SCE assay. SCEs provide a sensitive indicator of genotoxicity that is widely used, especially in the assessment of genotoxicity in human subjects [Kao-Shan et al., 1987]. We found that SCE frequencies in waterpipe smokers were significantly higher than those in healthy controls. In addition, the genotoxicity of waterpipe smoking was positively correlated with the extent of waterpipe use. These data are in agreement with the study of Yadav and Thakur [2000], demonstrating a significant increase in SCEs and CAs in the lymphocytes of waterpipe users in India. In addition, waterpipe use has been shown to cause an increase in the level of micronuclei in buccal mucosa cells of smokers.
Thus, our results and those of others [Yadav and Thakur, 2000; Boulos et al., 2009] indicate that waterpipe smoking is strongly genotoxic to users, regardless of the ways in which the waterpipe is smoked and the genetic background of the population.

Previous studies have shown that cigarette smoking is highly genotoxic. Prabhavathi et al. [2000] reported a significant increase in the frequency of CAs in smokers compared with nonsmokers, while Milic et al. [2008] showed that occupational exposure to tobacco increases the frequency CAs. Also, smoking has been shown to increase the frequencies of micronuclei [Larramendy and Knuutila, 1991], SCEs [Rowland and Harding, 1999; Akbas et al., 2003], and DNA damage [Akbas et al., 2003] in peripheral lymphocytes. In accordance, the results of the present study demonstrate that waterpipe smoking causes an increase in the frequencies of SCEs in lymphocytes, indicating the general genotoxic effect of tobacco smoking.

The mechanism for the potentiated genotoxicity of waterpipe smoking compared with that of regular cigarettes is unknown. In general, tobacco and charcoal might contribute to the genotoxicity observed in lymphocytes of waterpipe users, since waterpipe smoke has been shown to contain more concentrated and widely diverse toxic compounds compared with cigarette smoke [Shihadeh et al., 2004; Shihadeh and Saleh, 2005]. Studies on the mainstream smoke aerosol of the waterpipe showed that the “tar” (volatile aldehydes) of a single smoking session is startlingly high, typically two order of magnitude greater than that produced from smoking a single cigarette [Al Rashidi et al., 2008]. In addition, CO exposure is greater in waterpipe smoking compared with cigarette smoking [Bacha et al., 2007; Eissenberg and Shihadeh, 2009; Maziak et al., 2009]. Furthermore, the quantities of 3- or 4- ring compounds of PAH in waterpipe smoke are many times more than that of cigarette smoke [Shihadeh et al., 2004; Shihadeh and Saleh, 2005; Monzer et al., 2008; Sepetdjian et al., 2008]. Recently, it has been reported that the levels of carboxyhemoglobin after waterpipe smoking are approximately triple when compared with those obtained after cigarette smoking [Eissenberg and Shihadeh, 2009]. Also, the style of waterpipe smoking results in a dramatically higher exposure volume to smoke, more tobacco consumption per smoking event, and a longer smoke inhalation period [Bacha et al., 2007; Eissenberg and Shihadeh, 2009; Cobb et al., 2010]. Waterpipe smoke has been shown to increase the amount of free radicals in the bodies of smokers [Sharma et al., 1997]. Free radicals can cause oxidative DNA damage, including single strand breaks, formation of apurinic/apyrimidic lesions, base modification, and chromosomal damage [Salmon et al., 2004]. Therefore, waterpipe smoking seems to induce more genotoxic effects than cigarette smoking. The results of the current study highlight the fact that smoking via waterpipe is not a safer alternative to smoking cigarettes.

The present results demonstrate that the frequency of SCEs is related to waterpipe use. However, direct measurement of exposure to tobacco (by assessing the plasma cotinine level) and how it might correlate to genotoxicity in white lymphocytes was not investigated in this study. Other variables that might affect the extent of genotoxicity in waterpipe users include the length of the smoking session, the type of charcoal, the type of “moassel”, the reuse of water in the bowl, and fruit flavors. The current study also did not investigate the effects on passive waterpipe smokers. The contribution of these variables to waterpipe genotoxicity, including an analysis of passive waterpipe smokers, will be the subject of future research.

Collectively, waterpipe smoking is genotoxic and the extent of waterpipe induced genotoxicity is higher than that of regular cigarette smoking. In addition the genotoxicity of waterpipe smoking increases with use.

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