**RESEARCH ARTICLE**

**In vitro genoprotective and genotoxic effect of nicotine on human leukocytes evaluated by the comet assay**

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**Abstract**

The comet assay was used to measure the DNA damage induced in vitro by nicotine in human leukocytes as the extent of DNA migration in the comet head area, tail length, percent DNA in the tail, and Olive tail moment. Samples of whole blood were collected and blood cells were challenged with acute doses of 0.1, 1 and 10 μM of (−)-nicotine for 60 minutes. We found that nicotine treatment had dose-dependent effects on the level of DNA damage. At 1 and 10 μM of nicotine, both Olive tail moment and percent DNA in the tail significantly increased ($p < 0.001$), compared to the control. In the presence of 10 μM of nicotine, the shortest tail length and the smallest head area were detected. At a concentration of 0.1 μM, surprisingly, DNA damage detected by the comet assay was lower than in the control, which was proved by the observed significantly ($p < 0.001$) lower Olive tail moment and percent DNA in the tail as well as larger head area. The results suggest that nicotine, at a reasonably low concentration (0.1 μM), comparable to those found in the blood of habitual smokers, may have a protective effect, whereas higher doses of nicotine (1 and 10 μM) are genotoxic. The possible participation of reactive oxygen species in the DNA-damaging potential of nicotine is discussed.

**Introduction**

Nicotine is part of the regular human diet, being present in a number of food plants. The content of this alkaloid was estimated as, for example, 5–43 and 16.8 μg/kg of wet weight in tomatoes and cauliflower, respectively, whereas green pepper and eggplant may contain even up to 100 μg/kg (Koleva et al., 2012). The largest amount of nicotine is found in leaves of tobacco plants *Nicotiana tabacum* and *N. rustica*, from 8 to 100 mg/g of dry weight of plants (Idris et al., 1998). However, the main source of nicotine exposure for people is, in general, inhaling tobacco smoke from either active or passive smoking. A cigarette contains approximately 0.3–2.0 mg of nicotine, and plasma nicotine levels in heavy smokers reach up to 100 ng/mL ($\approx 0.6 \mu$M) (Kleinsasser et al., 2005; Matta et al., 2007).

For many smokers, nicotine is delivered in sufficient quantities to produce physiological and behavioral effects that comprise addiction (Swan & Lessov-Schlaggar, 2009). Although it can be treated as addictive, nicotine alone does not fully explain all aspects of symptoms, clinical course or need for treatment that relate to addiction (IARC, 2007). Since the late 1990s, there have been several commercial products containing nicotine offered on the market (Pogocki et al., 2007). Surprisingly, nicotine as medication is widely used in nicotine replacement therapies to assist smoking cessation as transdermal nicotine patches and nicotine-containing gum and, more recently, have been proposed for use concurrently with smoking as part of a risk reduction strategy (Herman & Sofuoglu, 2010). Moreover, despite popular prejudice against this drug, nicotine treatment has been studied as an experimental therapy for Parkinson’s disease, Alzheimer’s disease, ulcerative colitis, and others (Baron, 1996; Jarvik, 1991; Pogocki et al., 2007). Summing up, almost every one of us is exposed to this alkaloid, although the source of nicotine may vary (e.g. food, smoke or medicines).

Nicotine has been identified as a noncarcinogenic component of cigarette smoke (IARC, 2004), and DNA alteration induced by tobacco is not related to nicotine content (Mizusaki et al., 1977). However, its high doses are toxic. The genotoxicity and mutagenicity of drugs in animals were evaluated with the use of several experimental methods, such as alkaline elution (Turner et al., 1982), nucleoid sedimentation (Harris et al., 1986), sister chromatid exchange (SCE) (Doolittle et al., 1995), chromosome aberration (DeMarini et al., 2008), DNA adduct measurements (Hall et al., 1993) and micronuclei counting (Attia, 2007a,b). However, the alkaline comet assay (Singh et al., 1988), increasingly used for in vitro testing of genotoxicity of pharmaceuticals (Hartmann et al., 2001, 2004), is also very useful for...
examination of the DNA-damaging effects of nicotine (Ginzkey et al., 2009; Kleinsasser et al., 2005; Muthukumaran et al., 2008; Sassen et al., 2005; Sudheer et al., 2007a,b). The method has several advantages over other in vitro genotoxicity test methods on leukocytes, especially over those applicable to proliferating cells only (Collins et al., 2008; Lee et al., 2004), whereas almost all leukocytes are in the same phase of the cell cycle (G0) (Collins et al., 2008). The comet assay is also highly sensitive (Leroy et al., 1996; Singh, 2000). Therefore, the method is ideally suited for human investigations because it requires no prelabelling with radioactivity or other harmful procedures and can be applied to easily obtainable cells. Normally, white blood cells are used, because they are obtained in a relatively noninvasive way, do not require tissue disaggregation and behave well in the comet assay (Collins, 2004).

A lethal dosage of nicotine for adult humans has been estimated to be 30–60 mg (0.5–1.0 mg/kg or 6 μM) (Gosselin et al., 1988). Many investigators (e.g. Arabi, 2004; Argentin & Cicchetti, 2004; Doolittle et al., 1995; Ginzkey et al., 2009; Kleinsasser et al., 2005; Muthukumaran et al., 2008; Sassen et al., 2005; Sudheer et al., 2007a,b) reported toxic effects of nicotine on different human cells or tissues. However, those investigators have not taken into account the real nicotine blood concentrations in smokers. Peak arterial nicotine concentrations can be as high as 0.6 μM, depending on how the cigarette is smoked, whereas peak venous blood levels are typically 0.06–0.31 μM (Matta et al., 2007). Research on effects of low nicotine concentration in a physiological range with the use of the comet assay is limited (Sobkowiak & Lesicki, 2009, with experiments on Caenorhabditis elegans with nicotine concentrations ranged from 1 to 100 μM; Ginzkey et al., 2012), with experiments on cells of human nasal mucosa and human bronchial epithelial cells with nicotine concentrations ranging from 1 to 4000 μM). Keeping in view the above-cited reports, and the scarcity of the low-concentration in vitro studies on effects of nicotine on DNA damage, we initiated a study on in vitro genotoxic effects of low nicotine concentrations on human leukocytes. To our knowledge, this is the first study demonstrating that nicotine, at concentrations similar to those generally found in the blood of habitual smokers (Benowitz et al., 2009; Hulkkonen et al., 2005), performs a possible genoprotective role in leukocytes.

Methods

Blood collection, preservation and exposure to nicotine

Just before blood collection, (–)-nicotine (CAS no. 54-11-5, free base; purity, >99%; Sigma-Aldrich, St. Louis, MO) was diluted in phosphate buffered saline (PBS; 137 mM of NaCl, 2.7 mM of KCl, 1.4 mM of KH2PO4 and 10 mM of Na2HPO4) to prepare separate tubes with three nicotine stock solutions. To avoid potential effects produced by chronic exposure to nicotine, both volunteers (authors R.S. and J.M.) were nonsmokers. To prevent DNA damage, cell samples were handled in dimmed light. A 100-μL blood sample from each volunteer was collected by a finger prick into a tube. Immediately, the blood was mixed with a one-fourth volume of the anticoagulant (23 mM of citric acid monohydrate, 45 mM of trisodium citrate and 45 mM of glucose) and divided into 24-μL aliquots in four Eppendorf test tubes. To prepare the assay sample, 1 μL of appropriate nicotine stock solution was added – then, the final concentrations of nicotine were reached (0.1, 1 and 10 μM). In the control variant, we added 1 μL of PBS instead of 1 μL of nicotine stock solution. Exposure to nicotine was carried out at 37°C in a water bath for 60 minutes.

We tested nicotine at concentrations simulating physiological blood-nicotine level (or slightly higher) in smokers (Matta et al., 2007). To prove that, in this concentration, the range of nicotine is not cytotoxic, cell viability was examined by the trypan blue test (Sudheer et al., 2007a,b).

These experiments were carried out in accord with the ethics standards as formulated in the Helsinki Declaration of 1975 (revised, 2008).

Alkaline single-cell microgel electrophoresis (comet) assay

After incubation with nicotine, without undue delay, the alkaline comet assay was conducted according to the method described by Olive & Banath (2006). In brief, 3 μL of the assay sample were resuspended in 100 μL of 0.5% low-melting-point agarose (Prona; ABO, Gdańsk, Poland) and applied to slides coated with 1% normal-melting-point agarose (Prona, ABO). Slides were covered with a 24 × 60 mm coverslip and left on a cold plate for 3 minutes (to solidify agarose), and next the coverslip was removed. After cell lysis for at least 12 hours at 4°C in alkaline lysis buffer (0.03 M of NaOH, 1 M of NaCl and 0.5% sodium dodecyl sulfate; pH 10), slides were placed in a horizontal gel electrophoresis chamber and covered with alkaline buffer (300 mM of NaOH and 1 mM of Na2 ethylenediaminetetraacetic acid) at pH > 13. After a 50-minute DNA ‘‘unwinding’’ period, the electrophoresis was performed under standard conditions (25 V and 300 mA) for 20 minutes in a chamber cooled on ice. The duration of electrophoresis was designed so that DNA of control cells showed a significant migration. It was necessary to reveal the presence of DNA damages in cells (treated with 0.1 μM of nicotine), which exhibited lower DNA migration, as compared to the control sample. Electrophoresis was followed by neutralization at pH 7.5 (0.4 M of Tris; pH 7.5) and rinsing with water and cold 70% ethanol. Slides were air-dried at room temperature and stored until analysis in a desiccator. Just before image acquisition, DNA was stained with 300 μL of SybrGreen I (dilution, 1:10 000; Molecular Probes, Invitrogen, Eugene, OR) of a sensitivity at least 25 times greater than standard ethidium bromide. Comets were observed at 300× magnification, using a confocal fluorescence microscope (LSM 510; Carl Zeiss, Jena, Germany) equipped with an excitation filter of 500–550 nm and a barrier filter of 590 nm.

Image analysis

Quantification of the DNA strand breaks in the stored images was done using CASP software (CASP image-analysis system; University of Wroclaw, Wroclaw, Poland) (Konca et al., 2003). The head corresponds to the amount of DNA that still remains in the nuclear matrix, whereas the tail
visualizes the fragments of DNA migrating from nuclei. At least 150 cells for every test sample were counted and analyzed for the following parameters to quantify the induced DNA damage: head area; tail length; percent DNA in the tail and Olive tail moment (Olive et al., 1993). Olive tail moment is the product of tail length and percent DNA in the tail. The percent DNA in the tail is a measure of relative fluorescent intensity in the head and tail, measured from the head center to the tail end.

**Statistical analysis**

Data from four experiments were pooled and median values were calculated. Kruskal–Wallis’ analysis of variance test was used to determine statistical differences with nonparametric distribution. Statistical significance was considered at \( p < 0.001 \). Calculations and graphs were done using Statistica software (StatSoft, Inc., Tulsa, OK).

**Results**

After treatment of human leukocytes with nicotine for 60 minutes, even at 10 \( \mu \text{M} \), which was the highest concentration, cell viability (evaluated in the trypan blue test) did not change significantly. Nicotine did not have cytotoxic effects in any of the four experiments, because cell viability was above 95% after exposure to 0.1, 1 and 10 \( \mu \text{M} \) of nicotine (data not presented).

Table 1 shows the individual data sets from each of the four experiments. The DNA alterations in leukocytes are expressed as head area, tail length, percent DNA in the tail and Olive tail moment. Distinct differences were recorded between the experimental variants (Figure 1). At 0.1 \( \mu \text{M} \), we observed lower values of percent DNA in the tail and Olive tail moment than in control experiments (Figure 1; \( p < 0.001 \)). Also, in the presence of 0.1 \( \mu \text{M} \) of nicotine, we observed greater head area than in the control (Figure 1; \( p < 0.001 \)). On the other hand, at 1 and 10 \( \mu \text{M} \), increased DNA damage was detected. For both concentrations, Olive tail moment and percent DNA in the tail were significantly higher than in the control (\( p < 0.001 \)). Besides, in the presence of 10 \( \mu \text{M} \) of nicotine, a significantly lower head area than in the control was observed. At all examined nicotine concentrations, tail length was lower than in the control, but, significantly, this was confirmed only at 10 \( \mu \text{M} \) of nicotine (Figure 1; \( p < 0.001 \)).

**Discussion**

In this study, we used the comet assay to investigate in vitro response of human leukocytes to nicotine at concentrations similar to those found in the blood of habitual smokers (Hukkanen et al., 2005). In this concentration range, nicotine is not cytotoxic for many kinds of cells, including blood cells (Ginzkey et al., 2012; Kleinsasser et al., 2005; Sassen et al., 2005). This is confirmed by the trypan blue test for nicotine-treated lymphocytes. Nicotine did not exert any cytotoxic effects up to the 1000- \( \mu \text{M} \) concentration based on the trypan blue test (Sudheer et al., 2007a,b). Cell viability was between 75 and 85% in the 2000- and 3000- \( \mu \text{M} \) concentrations of nicotine-treated lymphocytes, respectively, but cell viability was dropped to 60% in 4000- \( \mu \text{M} \) nicotine-treated lymphocytes (Sudheer et al., 2007a,b). Cell viability was >95% in all our tested samples. Moreover, nicotine (1, 10 and 100 \( \mu \text{M} \)) did not produce any cytotoxic effects in our previous studies of C. elegans cells (Sobkowiak & Lesicki, 2009). Therefore, we can conclude that the nicotine concentrations used (at the tested duration of treatment) do not have cytotoxic effects.

Genotoxic in vitro effects of nicotine have been reported in human gingival fibroblast (Argentin & Cicchetti, 2004), tonsillar cells (Kleinsasser et al., 2005), lymphocytes

<table>
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<tr>
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<th>Mean</th>
<th>Median</th>
<th>SD</th>
<th>SE</th>
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<tr>
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HA, head area; TL, tail length; TD%, tail DNA %; OTM, Olive tail moment; N, number of comets; SD, standard deviation; SE, standard error.
(Sudheer et al., 2007a,b), cells of human nasal epithelia (Ginzkey et al., 2012; Sassen et al., 2005), tumor-free human parotid glands (Ginzkey et al., 2009), oral epidermal carcinoma cells (Wu et al., 2005) and spermatozoids (Arabi, 2004), as well as in animal cells: Chinese hamster ovary (CHO) cells (Doolittle et al., 1995); mouse bone marrow cells (Attia, 2007a,b) and culture of *C. elegans* cells (Sobkowiak & Lesicki, 2009). In most experiments, nicotine tested at concentrations from 1 to 4000 μM induced a dose-dependent increase in DNA migration, with statistical differences from the control, starting at 1 μM (Argentin & Cicchetti, 2004; Wu et al., 2005). However, taking into account a realistic dispersion of nicotine after usage, for example, after cigarette smoking (Matta et al., 2007), a 1000-μM nicotine concentration or higher chosen for experiments is unrealistically high.

In this study of nicotine effects on human leukocytes, we observed dual effects of this alkaloid. Similarly to results of other researchers (Argentin & Cicchetti, 2004; Ginzkey et al., 2012; Kleinsasser et al., 2005; Sassen et al., 2005; Sudheer et al., 2007a,b; Wu et al., 2005), we observed genotoxic effects in the presence 1 and 10 μM of nicotine (Figure 1C and D). In the comet assay, the tail length increases at relatively low damage levels and therefore is treated as the most informative (Collins et al., 2008). In some cases, significant dose-dependent DNA damages may appear, which is manifested by an increased amount of DNA in the tail, with no tail elongation and sometimes even tail contraction. We observed this situation in the presence of 10 μM of nicotine, and the best description of DNA damage level in such cases was provided by percent DNA in the tail and Olive tail moment parameters. Generally, percent DNA in the tail covers the widest range of damage, and it is linearly related to break frequency over most of this range (Collins et al., 2008; Lovell & Omori, 2008). However, in our study, we also found that the percent DNA in the tail, together with the head area and Olive tail moment, were significantly different in cells treated with 0.1 μM than in control cells. These results suggest that DNA in leukocytes was not only undamaged, but also even protected against natural damage detected in control cells. We suppose that relatively high Olive tail moment values in control cells were a result of spontaneous DNA damages (Kohn & Bohr, 2002) caused by, for example, oxidative damage from sudden exposure to the
high concentration of O₂ in the atmosphere, compared with blood (Collins et al., 2008) or damages generated in every experimental manipulation. However, these types of damages appeared in control, as well in nicotine-treated, samples. Nicotine, at concentrations ranging up to 6000 μM, was not genotoxic to CHO cells tested by SCE assay (Doolittle et al., 1995). DNA damages at nicotine concentrations of 1 and 10 μM were lower than in the control in our study of in vitro response of cell culture of C. elegans to nicotine (Sobkowiak & Lesicki, 2009). This was reflected in significantly lower Olive tail moment and tail length in comet assays for both concentrations. Similarly, Ginzkey et al. (2012), in the comet assay, observed lower DNA damage in isolated single cells of human nasal mucosa of some patients in the presence of 1 μM of nicotine than in the control, although this difference is not significant. It should be stressed that our results indicate a significantly lower value of DNA damage in cells treated with 0.1 μM of nicotine than control cells (Figure 1). Leukocytes are known to express the nicotinic acetylcholine receptor (nAChR) as a possible target for nicotine, and these results underscore the fact that nAChR plays an important role in nicotine-induced genotoxicity (Arias et al., 2009; Sobkowiak & Lesicki, 2011). Nicotinic receptors mediate responses in PC12 cells and probably in C. elegans cell culture, as in most in vitro systems utilizing transformed cells, but these responses require substantially higher concentrations than in vivo (Abreu-Villaca et al., 2005 and references cited therein). Abreu-Villaca et al. (2005) and Sobkowiak & Lesicki (2009) reported on significant effects elicited at nicotine concentrations approximately 10-fold higher than those in venous blood of smokers.

People are continuously exposed to a variety of harmful (e.g. genotoxic) and beneficial (e.g. antioxidant) chemicals. The basal level of DNA damage in primary human (diploid) lymphocytes has been estimated to contain approximately 5000 different DNA damages detected by the simple alkaline comet assay (Møller et al., 2000). Probably immediately after isolation, leukocytes suffer oxidative damage from sudden exposure to the high concentration of O₂ in the atmosphere, compared with blood (Collins et al., 2008). This process increases the damage to the DNA and emphasizes the protective effects of nicotine. We suppose that nicotine at low concentrations (approximately 0.1 μM) protects DNA from damage, which is proved by higher comet head area and lower value of percent DNA in the tail and Olive tail moment than in the control (Figure 1).

The results suggesting opposite – genotoxic versus genoprotective – roles of nicotine are controversial. The differences between results obtained in different test systems can be attributed to different drug concentrations and treatment duration, as well as different endpoints of genotoxicity, and the repair capacities of the various cell types used can explain the discrepancies.

Nicotine may act as an oxidative stressor or as an antioxidant. This suggests biphasic effects of nicotine, with the shift somewhere between 10 and 100 μM (Abreu-Villaca et al., 2005; Guan et al., 2003; Sobkowiak & Lesicki, 2009). Villablanca (1998) described bimodal, dose-dependent activities of nicotine in calf pulmonary endothelial cells (ECs). Lower concentrations (0.1–10 nM; i.e. lower than the concentrations found in the blood of smokers, but at the level of exposure to nicotine from food) stimulated proliferation of ECs (up to 180%), whereas a higher concentration (100 μM) led to a decrease in DNA synthesis and apoptosis (Villablanca, 1998). It is plausible that nicotine treatment may play dual effects on oxidative stress (OS) and cell protection, in which the effects are dependent on differences in dosage of the drug used and its mechanism of action (Guan et al., 2003). Generally, high doses of nicotine may induce toxicity and stimulate OS, whereas reasonably low concentrations may act as an antioxidant and play an important protective role (Guan et al., 2003). The fact that only high doses of nicotine (1000 μM and over), but not a low dose (100 μM), can induce the decrease in glutathione content and increase in the level of lipid peroxidation markers in CHO cells and lymphocytes also provides evidence to show dose-differentiated effects of nicotine (Sudheer et al., 2007a,b; Yildiz et al., 1998).

Although the exact mechanism of nicotine influence on DNA is still unknown, OS induced by nicotine seems to play an important role. It has been reported that nicotine disrupts the mitochondrial respiratory chain, leading to increased generation of superoxide anions and hydrogen peroxide (Gvozdjakova et al., 1992). High doses of nicotine (over 1000 μM) stimulate OS and increase the activity of cytochrome P 450 enzymes during intracellular metabolism of nicotine (Yildiz et al., 1998). Interestingly, nicotine in vitro has been found to have radical scavenging properties (Ferger et al., 1998). It has been suggested that nicotine may have a capacity to chelate Fe²⁺ to block the Fenton reaction (Soto-Otero et al., 2002).

Overall, smoking harms health and this statement contains the assumption that smoking leads to DNA damage resulting from the presence of a large number of carcinogens in tobacco smoke. However, Hoffmann & Speit (2005), under the experimental conditions of the comet assay used in their study, did not reveal – as it could be expected – any effect of cigarette smoking on the amount of DNA damage in peripheral blood cells. This negative finding is in agreement with their previous study (Speit et al., 2003) and the majority of reviewed human biomonitoring studies (Faust et al., 2004), which also failed to show an effect of smoking on DNA migration in the comet assay (Møller et al., 2000). Several other articles dealing with smoking effects in the comet assay reported discrepant results (for a review, see Faust et al., 2004).

Conclusions

Many factors can contribute to the generation of conflicting results, but, in our opinion, a bimodal and partly protective effect of nicotine should be taken into account. The role of nicotine in reducing DNA damage by other components of tobacco smoke in human cells needs to be confirmed in future experiments.

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Declaration of interest

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