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Mononuclear leukocyte DNA damage and oxidative stress: The association with smoking of hand-rolled and filter-cigarettes

Abdurrahim Kocyigit[a,*], Sahbettin Selek[a], Hakim Celik[a], Murat Dikilitas[b]

[a] Department of Clinical Biochemistry, Faculty of Medicine, Harran University, 63200 Sanliurfa, Turkey
[b] Department of Plant Protection, Faculty of Agriculture, Harran University, 63200 Sanliurfa, Turkey

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ABSTRACT

Cigarette smoking is a major cause of human cancer at various sites, although its carcinogenic mechanisms still remain unestablished. Based on the use of a filter, cigarette smoke can be divided into a gas phase and a tar phase. Both contain different concentrations of oxidants, free radicals and tobacco-specific carcinogens. To explore the effects of both filtered and non-filtered cigarette smoke on DNA damage and oxidative status, we measured the level of mononuclear leukocyte DNA damage by use of the single-cell gel electrophoresis (Comet) assay. We also determined malondialdehyde (MDA), protein carbonyl content (PC) and total antioxidative capacity (TAC) levels in blood plasma of smokers of manufactured filter-cigarettes and of hand-rolled cigarettes. Cotinine levels were also measured in plasma to estimate the degree of smoking. Mononuclear leukocyte DNA damage, plasma MDA, plasma PC and plasma cotinine levels were found significantly higher, while plasma TAC levels were found significantly lower in smokers of filter-cigarettes and smokers of hand-rolled cigarettes, compared with control subjects. TAC levels in hand-rolled and manufactured filter-cigarette smokers were not significantly different from each other. However, the levels of DNA damage, plasma MDA, plasma cotinine, and plasma protein oxidation were significantly higher in hand-rolled cigarette smokers than in filter-cigarette smokers. There was a significant positive correlation between MDA and DNA damage in both hand-rolled cigarette smokers and manufactured filter-cigarette smokers. This study indicates that smoking of hand-rolled cigarettes has stronger genotoxic and oxidative effects on the metabolism than smoking of manufactured filter-cigarettes. We propose that these harmful effects could be attributed to the higher level of oxidants.

1. Introduction

There is overwhelming evidence that tobacco smoking plays a major role in the epidemiology of lung cancer, cancer at other sites, and a variety of chronic degenerative diseases [1–4]. The mechanisms by which tobacco smoke causes these cancers and their negative effects on health have been studied intensively during the past 20 years. However, the mechanism by which cigarette smoking induces cancer remains un-established since it is probable that more than one mechanism is active in tobacco-related carcinogenesis. It is well known that cigarette smoke contains more than 6000 components emitted in gaseous form and as condensed tar particles, many of which are oxidants and pro-oxidants capable of producing reactive oxygen species (ROS) [4,5]. Besides ROS, tobacco smoke contains major classes of carcinogens that include polycyclic aromatic hydrocarbons, aromatic amines [6] and tobacco-specific nitrosamines [7]. Toxic compounds such as formaldehyde, acetaldehyde, and acrolein may also contribute to the toxic and carcinogenic effects of tobacco smoke [8]. In addition, tobacco smoke contains various compounds that induce damage as assessed by different genetic endpoints [9]. Many studies have demonstrated that cigarette smoke itself can induce DNA strand-breaks in rodents, in mammalian cells in culture, or in DNA *in vitro* [10]. Several studies have confirmed that reactive oxygen or nitrogen species are the primary cause of the DNA strand-breaks [11,12].

Conventionally, tobacco smoke is separated as tar and gas-phase fractions. The gas phase is the material that passes through the filter. Cigarette smoke, passed through the filter, contains only gas phase. However, smoke from cigarettes without filter contains both tar and gas phase [13]. Carcinogenic compounds principally occur in the particulate phase of tobacco smoke, which could induce genetic mutations and chromosomal aberrations as well as the formation of micronuclei in mammalian cells [10]. However, there are carcinogens present in the gas phase as well [14]. The tar

* Corresponding author at: Harran Üniversity, Medical Faculty, Department of Biochemistry, Research Hospital, 63100 Sanliurfa, Turkey. Tel.: +90 414 3183028; fax: +90 414 3151181.
E-mail address: abdurrahimkocyigit@yahoo.com (A. Kocyigit).

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Table 1
Demographic characteristics of smokers and non-smokers.

	Hand-rolled cigarette group ($n=26$) $X \pm SD$	Manufactured cigarette group ($n=37$) $X \pm SD$	Control group ($n=36$) $X \pm SD$	p
Mean age (year)	36.30 ± 8.11	34.18 ± 8.42	36.05 ± 5.05	>0.05
Body mass index (kg/m2)	23 ± 2	22 ± 1	24 ± 2	>0.05
Number of smoking (per day)	15.12 ± 9.36	14.96 ± 7.72		>0.05
Smoking period (year)	18.04 ± 9.3	16.75 ± 7.7		>0.05

phase of tobacco smoke contains about ten-fold more free radicals than the gas phase. A puff of smoke contains $>10^{15}$ free radicals in the tar phase and $>10^{14}$ free radicals in the gas phase, besides many more carcinogenic chemicals [15]. Therefore, people who smoke cigarettes with a different design and pattern according to their habits and economic condition (hand-rolled tobacco is much cheaper) are exposed to different components [16,17]. People generally use filter-cigarettes, considering that hand-rolled cigarettes may be worse than the manufactured filter-cigarettes because tobacco smoke from hand-rolled cigarettes has higher tar content due to the absence of a filter [18]. There are quite a few studies on the effects of cigarette smoke on DNA damage and oxidative-stress markers [19–22]. To the best of our knowledge only a few studies have been carried out so far on hand-rolled cigarettes, but there are no studies comparing the effects of smoke from hand-rolled and manufactured filter-cigarettes on DNA damage and oxidative-stress markers. In this study, we measured mononuclear leukocyte DNA damage, and malondialdhyde (MDA), protein carbonyl content (PC) and total anti-oxidative capacity (TAC) levels in blood plasma of smokers, in order to evaluate the effect of smoke from both hand-rolled and manufactured filter-cigarettes on DNA damage and oxidative status.

The comet assay is a reliable *in vitro* assay to detect the genotoxic effect of cigarette smoke [23]. The assay method is sensitive and straightforward for measuring DNA damage in individual cells [24–26], and has been previously applied for the monitoring of DNA damage in connection to smoking habits [9]. In the last 20 years, since its introduction by Östling and Johanson in 1984 [24], a great number of reports have been published. This technique allows the detection and quantitation of DNA damage in single cells and is simpler and faster than other conventional genotoxicity techniques. The comet assay has also shown high sensitivity for detecting carcinogens – although non-carcinogens have not been tested extensively – [27] and therefore, it is a useful tool for human bio-monitoring studies [28].

2. Materials and methods

2.1. Subjects

Ninety-nine men were enrolled in this study, 36 of whom (mean age ± SD, 36.05 ± 5.05 years) never smoked, 26 (age, 36.30 ± 8.11 years) smoked hand-rolled cigarettes, and 37 (age, 34.18 ± 8.42 years) smoked manufactured filter-cigarettes. The smokers consumed at least 14.96 ± 7.72 manufactured filter-cigarettes or 15.12 ± 9.36 hand-rolled cigarettes daily during the last year. None of the subjects had any history of cardiovascular, endocrine or gastrointestinal disorders, and none had received any medication or had been given any nutritional supplements. In the smoker groups, the average age when smoking started was 15 ± 7 years and the subjects had been active smokers since. In the non-smoker group, the subjects had never smoked, not even occasionally. All the subjects had similar socio-cultural background as they belonged to the same social group, i.e., all came from the rural areas. Demographic features of the subjects are presented in Table 1. Each person was interviewed about their history of cancer, previous radio- or chemotherapy, use of therapeutic drugs, exposure to diagnostic X-rays during the last six months, severe infection during the last six months, high alcohol consumption, and intake of vitamins or intensive sports activities during the last week. If so, these subjects were excluded from the experimental trial.

2.2. Blood-sample collection and preparation of cells

After overnight fasting, peripheral blood samples (total 6 ml) were collected from an antecubital vein into heparinised tubes, stored at 2–4 °C in the dark to prevent further DNA damage, and processed within 2 h. Mononuclear leukocyte isolation for the comet assay was performed by centrifugation on Histopaque 1077 (Sigma). 1 ml heparinised blood was carefully layered over 1 ml Histopaque and centrifuged for 35 min at 500 × *g* at 25 °C. The interface band containing mononuclear leukocytes was washed with phosphate-buffered saline (PBS) and then collected by a 15-min centrifugation at 400 × *g*. The resulting pellets were re-suspended in PBS and the cells were counted with an automatic cell counter (Abbott 3700, USA). Membrane integrity was assessed by means of the Trypan-Blue exclusion method. The remaining blood was centrifuged at 1500 × *g* for 10 min to obtain the plasma. The separated plasma was then stored at −80 °C until further analysis of MDA, PC, and TAC.

2.3. Comet assay

The comet assay, also known as single-cell gel electrophoresis (SCGE) assay, was performed as described by Singh et al. [25] with the following modifications: 10 μL of fresh mononuclear leukocyte cell suspension (roughly 20,000 cells) were mixed with 80 μL of 0.7% low-melting agarose in PBS at 37 °C. Subsequently, 80 μl of the mixture was layered onto a slide pre-coated with thin layers of 1% normal melting point agarose (NMA), and immediately covered with a cover slip. Slides were left for 5 min at 4 °C to allow the agarose to solidify. After removing the cover slips the slides were submersed in freshly prepared cold (4 °C) lysing solution (2.5 M NaCl, 100 mM EDTA-2Na, 10 mM Tris–HCl, pH 10–10.5, 1% Triton X-100 and 10% DMSO, added just before use) for at least 1 h. Slides were then immersed in freshly prepared alkaline electrophoresis buffer (0.3 mol/L NaOH, and 1 mmol/L Na_2EDTA, pH > 13) at 4 °C for unwinding (40 min) and then electrophoresed (25 V/300 mA, 25 min). All steps were carried out under minimal illumination. After electrophoresis, the slides were neutralized (0.4 M Tris–HCl, pH 7.5) for 5 min. The dried microscope slides were stained with ethidium bromide (2 μg/ml in distilled H_2O; 70 μL/slide), covered with a cover slip and analyzed with a fluorescence microscope (Olympus BX51, Japan) at 400× magnification provided with epi-fluorescence and equipped with a rhodamine filter (excitation wavelength 546 nm; barrier 580 nm). Fifty cells were randomly scored by eye in each sample, on a scale of 0–4, based on fluorescence outside the nucleus as previously described by Kobayashi et al. [29]. The scale used was as follows: 0, no tail; 1, comet tail < half the width of the nucleus; 2, comet tail equal to the width of the nucleus; 3, comet tail longer than the nucleus; 4, comet > twice the width of the nucleus. Scoring cells in this manner has been shown to be as accurate and precise as using computerized image analysis. The individual scoring of the slides was blind, using coded slides. The visual score for each class was calculated by multiplying the percentage of cells in the appropriate comet class by the value of the class. The total visual comet score characterizing the degree of DNA damage in the entire study groups was the sum of the scores in the five comet classes. Thus, the total visual score could range from 0 (all undamaged) to 400 (all maximally damaged) arbitrary units (AU), as reported by Collins et al. [30]. The comets were scored independently by two investigators.

2.4. Measurement of lipid peroxidation in plasma

Plasma lipid peroxidation was evaluated by a fluorometric method based on the reaction between MDA and thiobarbutiric acid (TBA) [31,32]. Briefly, 50 μL of plasma was added to 1 mL of 10 mmol/L diethylthiobarbutiric acid (DETBA) reagent in phosphate buffer (0.1 mol/L, pH 3). The mixture was mixed for 5 s and incubated for 60 min at 95 °C. Samples were placed on ice for 5 min and then 5 mL of butanol was added. The mixture was then shaken for 1 min to extract the DETBA-MDA adduct, and finally centrifuged at 1500 × *g* for 10 min at 4 °C. Fluorescence of the butanol extract was measured with excitation wavelength 539 nm and emission wavelength 553 nm. For these measurements, 1,1,3,3, tetraethoxypropane (Sigma) was used as a standard solution and the values were expressed as μmol/L.

2.5. Measurement of protein carbonyl content in plasma

Plasma protein carbonyl (PC) content was measured according to Reznick and Parker [33]. Plasma (15 μL) was treated with 500 μL of 10 mmol/L 2,4-dinitrophenylhydrazine (DNPH), dissolved in 2 M HCl. Samples were then incubated for 1 h at room temperature in the dark, precipitated with 10% trichloracetic acid (TCA), and centrifuged at 13,000 × *g* for 5 min. The pellet was washed three times with 1 ml of ethanol/ethyl acetate (1:1, v/v) and redissolved in 1 ml of 6 mol/guanidine in 10 mM phosphate buffer/trifluoroacetic acid (pH 2.3). The dif-

Table 2
Mononuclear leukocyte DNA damage and oxidative stress parameters.

	Hand-rolled cigarette group ($n=26$) $X \pm SD$	Manufactured cigarette group ($n=37$) $X \pm SD$	Control group ($n=36$) $X \pm SD$	Significance p
Mononuclear leukocyte DNA damage (arbitrary units)	25.23 ± 8.92[a,c]	18.81 ± 8.27[b]	8.29 ± 25.23	0.001
Plasma protein oxidation level (nmol/mg prot)	5.04 ± 3.55[a]	4.09 ± 2.99[b]	3.28 ± 1.66	0.006
Plasma malondialdehyde level (μmol/L)	46.22 ± 23.24[a,c]	34.31 ± 25.62[b]	25.86 ± 13.45	0.005
Plasma total antioxidative capacity (meq troloks/L)	1.09 ± 0.34[a]	1.11 ± 0.23[a]	1.33 ± 0.17	0.010
Plasma cotinine level (ng/mL)	382.84 ± 171.54[a,c]	361.59 ± 260.58[b]	12.93 ± 7.25[a]	0.001

Significance was defined as $p < 0.05$.
[a] Statistically significant, compares controls and hand-rolled cigarette group.
[b] Statistically significant, compares controls and filter-cigarette group.
[c] Statistically significant, compares hand-rolled and filter-cigarette group.

ference in absorbance between the DNPH-treated and the HCl-treated samples was determined at 366 nm. The results were expressed as nmol of carbonyl groups per mg protein, using the extinction coefficient of 22,000 M^{-1} cm^{-1} for aliphatic hydrazones.

2.6. Measurement of total antioxidant capacity in plasma

TAC in plasma was determined by use of a novel automated measuring method, developed by Erel [34]. In this assay, a standardized solution of Fe_2^+–ortho-dianisidine complex reacts with a standardized solution of hydrogen peroxide in a Fenton-type reaction, producing hydroxyl radicals. These potent ROS oxidize the reduced colorless o-dianisidine molecules to yellow–brown colored dianisidyl radicals at low pH. The oxidation reactions progress among dianisidyl radicals and further oxidation reactions develop. The colour formation is increased with further oxidation reactions. Antioxidants in the sample suppress the oxidation reactions and colour formation. The results are expressed as mmol Trolox equivalent/L.

2.7. Statistical methods

The values were expressed as mean ± SD for the smokers and the control group, separately. The comparisons of parameters were performed with the one-way ANOVA test and correlation analyses were performed with Pearson's correlation test. The relationship between plasma MDA contents and DNA-damage values are illustrated in a scatter graph, and the rest of the parameters such TAC, plasma protein oxidation level, plasma cotinine levels, etc. is presented in Table. A p-value less than 0.05 was considered as significant. Data were analyzed by use of the SPSS® for Windows computing program (Version 11.0).

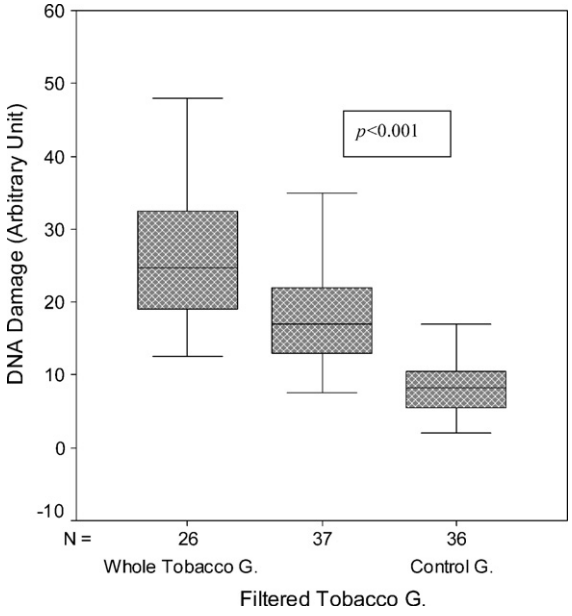

Fig. 1. DNA damage in peripheral mononuclear leukocytes, measured by use of the comet assay.

3. Results

Demographic characteristics of participants, smokers and nonsmokers, are summarized in Table 1. The levels of plasma cotinine were 382.8 ± 171.5 ng/mL, and 361.6 ± 260.5 ng/mL for smokers of hand-rolled cigarettes and filter-cigarettes, respectively, while 12.9 ± 7.2 ng/mL was the value for the control subjects. Plasma levels of cotinine were found to be markedly higher in both hand-rolled and filter-cigarette smokers than in nonsmokers.

Mononuclear leukocyte DNA damage (Fig. 1), plasma MDA, PC and cotinine levels were found to be significantly higher in both hand-rolled and manufactured filter-cigarette smokers than in the control group ($p < 0.001$). The levels of DNA damage and MDA, on the other hand, were significantly higher in the hand-rolled cigarette smokers than in the filter-cigarette smokers. Plasma TAC was also found significantly lower in both hand-rolled cigarette and filter-cigarette smokers than in the control group. The levels of TAC were lower in the hand-rolled cigarette smokers than in the filter-cigarette smokers, although this result was not statistically significant (Table 2). There was a slightly positive correlation between MDA and DNA damage in smokers of hand-rolled cigarette ($r = 0.518$, $p < 0.01$) and smokers of manufactured filter-cigarettes ($r = 0.537$, $p < 0.001$) (Figs. 2 and 3, respectively). These results indicate that the negative effect of cigarette smoking resulted in a higher MDA content along with a higher level of DNA damage compared with the nonsmokers (Table 2).

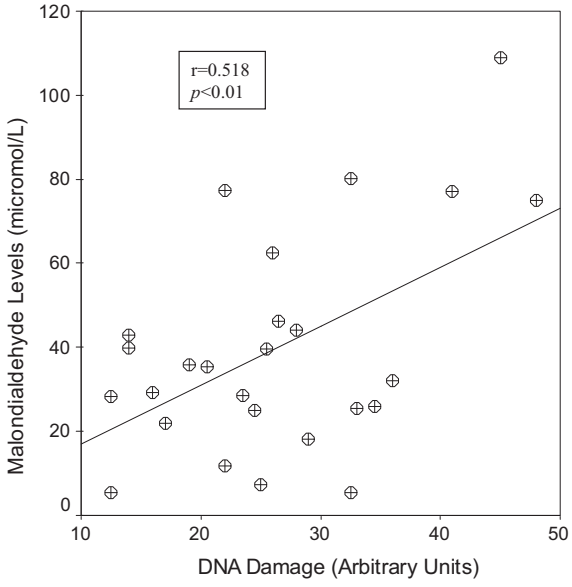

Fig. 2. Relationship between mononuclear leukocyte DNA-damage and plasma malondialdhyde-levels in smokers of hand-rolled cigarettes.

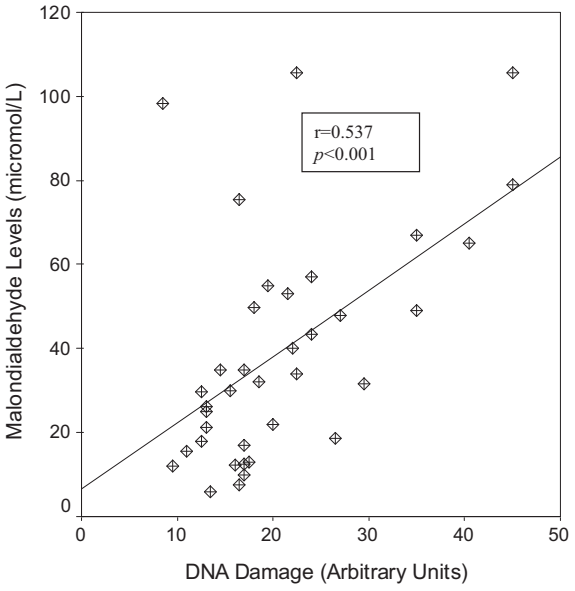

Fig. 3. Relationship between mononuclear leukocyte DNA-damage and plasma malondialdhyde-levels in smokers of filter-cigarettes.

4. Discussion

Our findings show that the DNA damage in mononuclear leukocytes, plasma concentrations of cotinine, MDA and PO levels were significantly higher and that TAC levels were significantly lower in both hand-rolled and filter-cigarette smokers compared with control subjects (nonsmokers). However, the levels of DNA damage and cotinine concentrations were significantly higher in hand-rolled cigarette smokers than in filter-cigarette smokers. Scoring of cells after the comet assay via the 0–4 scale and assessment of the DNA damage by eye has been shown by previous investigators to be as accurate, sensitive and fast as computerized image analysis. Therefore, this method was suitable for testing the genetic integrity of the mononuclear leukocytes. Nicotine is the major addictive substance in cigarette smoke and the cotinine level in body fluids serves as an indicator of the nicotine dose and is an important marker of smoking behaviour [35]. Therefore, we assessed plasma cotinine levels to determine smoking behaviour.

It is well known that cigarette smoke contains a large number of toxic components, including a variety of tobacco-specific carcinogens, co-carcinogens and tumour promoters. Most of the components are present in the gas phase and more than one thousand components are present in the particulate and tar phase, both of which contain high concentrations of oxidants and free radicals [13]. At least 200 of these components are toxic to humans and/or experimental animals, and over 50 have been identified as potential human carcinogens [14,36]. It is not exactly known which agent (tar or gas phase) causes more damage [37], but it is evident that both the use of hand-rolled cigarettes and manufactured filter-cigarettes cause extensive DNA damage and oxidative stress in the metabolism of the smokers (Fig. 1 and Table 2).

It has been demonstrated that cigarette smoke can induce DNA strand-breaks either in rodents, mammalian cells in culture, or in DNA *in vitro* [38,39]. Several of these studies indicate that reactive oxygen and nitrogen species are the primary cause of the DNA damage [21,40,41]. All these studies show that the association between smoking and genotoxicity is well-established. However, some major questions still remain unanswered, such as the relative importance of the kind of tobacco and the way of smoke inhalation. A few studies have been published, though with limited conclusions. Several epidemiological studies have demonstrated that smokers of hand-rolled cigarettes showed an increased risk for cancer compared with smokers of manufactured filter-cigarettes [42–44]. However, they have not exactly explained the underlying mechanism and there is no study implicating any relationship between the type of smoking and DNA damage. Rather, studies on total time of smoking or age of the smoker received quite a remarkable attention [45]. The relationship between the level of DNA strand breaks and the number of cigarettes smoked per day draw interest although the correlation between them was reported as not significant [46].

The aim of this study was to investigate the effect of hand-rolled or manufactured filter-cigarettes on DNA damage and oxidative status in smokers. We have found that smoking of both types of cigarette caused extensive mononuclear leukocyte DNA damage in smokers compared with the control subjects. The damage was approximately two-fold higher in the filter-cigarette smokers and three-fold higher in the hand-rolled cigarette smokers when compared with the control group. These findings seem to demonstrate that the tar content of cigarette smoke results in a much higher genotoxic effect than the gas phase. To best of our knowledge, no previous report was available to compare hand-rolled cigarette smokers and manufactured filter-cigarette smokers with respect to DNA damage and oxidative status. Several mechanisms may be operating here. For example, the half-life of most free radicals in the gas phase is too short (seconds) to reach the cell nucleus, while some free radicals in the particulate or tar phase are stable and long-lived and can easily pass through the cell membrane. This causes increased production of ROS and produces a condition of oxidative stress that can result in the oxidation of DNA [46–48]. The exposure to cigarette tar in addition to gas phase components, especially in smokers of hand-rolled cigarettes has resulted in more DNA strand-breaks as assessed by the alkaline comet assay when compared with the filter-cigarette smokers. We, therefore, suggest that the smoke from hand-rolled cigarettes itself is responsible for the higher DNA damage. We also propose that the deposition of higher tar contents in the lungs of hand-rolled cigarette smokers, due to the lack of a filter, may be responsible for DNA damage.

In this study, we found that the plasma MDA level, as an indicator of lipid peroxidation, was significantly higher in smokers of both types of cigarette that in the control subjects. However, MDA content was found significantly higher in the hand-rolled cigarette smokers than in the filter-cigarette smokers. There are studies that report increased levels of lipid peroxidation in cigarette smokers [49,50]. Our findings also support their conclusions. There is no report available that compares the situation in both groups with respect to DNA damage and MDA levels.

Cigarette smoke also causes acute inflammatory reactions in the lung, characterized by the accumulation and activation of leukocytes, producing reactive oxygen and nitrogen species in high concentrations [51]. Polyunsaturated fats and fatty acids are a major target for free-radical attack resulting in lipid peroxidation, which is a process that may continue as a chain reaction to generate peroxides and aldehydes [52]. Our findings demonstrate that smoke from both types of cigarette are serious risk factors for lipid peroxidation, but the hand-rolled cigarette poses a higher risk than the filter-cigarette.

PC content was also found significantly higher in the two groups of smokers than in control subjects. PC content was higher, on the other hand, in hand-rolled cigarette smokers than in filter-cigarette smokers, but the difference was not statistically significant. In the case of proteins, almost all amino acids can be oxidized by reactive oxygen species, but the sulphur-containing (cysteine and methionine) amino acids are among the most susceptible. Metal-bound amino acids are also specific targets for metal-catalyzed oxidative damage [53]. Several studies have demonstrated that smoking causes oxidation of protein thiols, and alterations in PC content in

plasma [54], and the potency of smoking is revealed by its capacity to oxidize proteins. Our findings also show that filter-cigarette smoke resulted in protein oxidation, as was also observed for smoke from hand-rolled cigarettes.

Antioxidant molecules that may prevent or inhibit these harmful reactions could be reduced under stress conditions. The measurement of antioxidants such as albumin, uric acid, bilurubin and ascorbic acid is time-consuming, labor-intensive, and costly; their effects are additive [55,56]. Instead of those parameters, we have measured TAC, which was found significantly lower in both groups of smokers compared with the control group. However, there was no significant difference between the two groups of smokers. There is more evidence that smoking is related to increased free-radical production and antioxidant depletion [57,58], i.e., the level of oxidants would generally increase and that of the antioxidants decrease. As a consequence, the anti-oxidative mechanisms may be exiguous to prevent oxidative damage completely in smoking conditions. The low TAC observed in both groups of smokers indicates that the smokers and even the smokers of filter-cigarettes are subject to the same oxidative effect. This finding demonstrates that smoking filter-cigarettes is not much less dangerous than smoking of hand-rolled cigarettes.

In conclusion, smoking of filter-cigarettes and hand-rolled cigarettes both strongly increase DNA damage and oxidative stress in humans. However, both DNA and lipids are more negatively affected by the smoke from hand-rolled cigarettes. People with smoking habits generally use manufactured filter-cigarettes thinking that they avoid the harmful effects. However, the data presented here clearly show that both filter-cigarettes and hand-rolled cigarettes have genotoxic effects and cause extensive oxidative damage, although hand-rolled cigarettes produce significantly more DNA damage and lipid peroxidation than do the filter-cigarettes.

Conflict of interest

The authors have no conflicts of interest to declare.

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