Effects of cigarette smoke, nicotine and cotinine on red blood cell hemolysis and their -SH capacity

S Asgary PhD¹, GH Naderi PhD¹, A Ghannady PhD²

BACKGROUND: Smoking is a leading cause of premature death. Red blood cell (RBC) membrane lipids are rich in polyunsaturated fatty acids; therefore, the effect of oxygen on RBC membranes is more prominent than on other body tissues. The attachment of peroxidants to RBC membranes can result in hemolysis.

OBJECTIVES: The present study was conducted to assess the sensitivity of RBCs to 2,2'-azo-bis-(2-amidinopropane) dihydrochloride in smokers and nonsmokers. The effect of cigarette smoke, nicotine (1 µg/mL, 1.5 µg/mL and 2.5 µg/mL) and cotinine (1.25 µg/mL, 2.5 µg/mL and 5 µg/mL) on RBC hemolysis was also examined.

RESULTS: RBC hemolysis in smokers was 21.6% higher than in nonsmokers (P<0.05). Cigarette smoke increased 2,2'-azo-bis-(2-amidinopropane) dihydrochloride-induced RBC hemolysis by 281.7%. Nicotine inhibited RBC hemolysis by 36.7% at the highest concentration used, but increased RBC hemolysis at the lower concentrations. Cotinine caused a 13.8% increase in RBC membrane peroxidation at the highest concentration used and its effects were dose-dependent. At their highest concentrations, nicotine and cotinine decreased -SH groups by 50%.

CONCLUSIONS: The present study confirms the results from previous studies of the oxidative and destructive effects of cigarette smoke, which are detrimental to the health of both active and passive smokers.

Key Words: Cigarette; Cotinine; Nicotine; Red blood cell; Smoking

Smoking is an important preventable cause of mortality worldwide. The prevalence of pulmonary and cardiovascular disease, cataracts and some cancers is higher in smokers than in nonsmokers (1-5).

Active molecules, such as aldehydes, heavy metals, hydrogen cyanide, low molecular weight phenols, nitrosamines and aromatic polycyclic hydrocarbons, are abundant in cigarette smoke. Cigarette smoke is also a source of metallic ions, quinones and hydroxyl radicals. Large volumes of oxidants increase the level of oxidative stress in smokers (6). Free radicals in cigarette smoke trigger and augment lipid peroxidation, which causes low-density lipoprotein (LDL) oxidation and atherosclerosis (7-9).

Red blood cell (RBC) membrane lipids are rich in polyunsaturated fatty acids; therefore, the oxidative effects of oxygen on RBC membranes are greater than on other tissues. Moreover, RBCs contain hemoglobin, which is one of the most potent catalysts of lipid peroxidation.

The invasion of the RBC membrane by peroxidants, which occurs with hemoglobinopathies, radioactive radiation, the consumption of oxidative drugs, increased levels of certain metals and the decreased function of antioxidant systems, can lead to RBC hemolysis (10). In addition to lipid peroxidation, peroxidants can cause the oxidation of -SH groups in proteins and RBC membranes. The -SH groups are highly reactive and can be a target during oxidative stress.

Glutathione directly protects membrane proteins and preserves their stability. Decreased levels of glutathione lead to a decrease in -SH groups (11) and can result in the oxidation of membrane -SH groups and loss of membrane stability (12).

The present study examined the effect of cigarette smoke as a source of free radicals on RBC hemolysis. The sensitivity of RBCs from smokers and nonsmokers to 2,2'-azo-bis-(2-amidinopropane) dihydrochloride (AAPH) and the direct effects of cigarette smoke, nicotine and cotinine (a major nicotine metabolite) on RBC hemolysis were studied.

PARTICIPANTS AND METHODS

Subjects

The present analytical, cross-sectional study involved 25 smokers (with a history of smoking 20 cigarettes per day for at least two years) and 25 nonsmokers who volunteered for participation. The volunteers were healthy men who were between 20 and 50 years of age, with no history of cardiovascular, endocrine or gastrointestinal disease.

The subjects received no special medications or nutritional supplements and were not on special diets.

¹Isfahan Cardiovascular Research Center; ²Faculty of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran

Correspondence: Dr S Asgary, Isfahan Cardiovascular Research Center, PO Box 81465—1148, Isfahan, Iran. Telephone 98-311-335-9090 or 98-311-335-9696, fax 98-311-337-3435, e-mail s_asgary@hotmail.com or crc@mui.ac.ir
The UV-3100 spectrophotometer. (0.1 mL) and absorption at 412 nm was measured at 37°C for 1 h on (5.0 mM, pH 8). 5,5-

in 1 mL of 10% sodium dodecyl sulphate and 0.8 mL phosphate buffer by Dodge et al (14), in which 0.1 mL of RBC membrane was dissolved

RBC membranes were prepared according to the method described

-SH group measurement per milligram of protein

RBC membranes were prepared according to the method described by Dodge et al (14), in which 0.1 mL of RBC membrane was dissolved in 1 mL of 10% sodium dodecyl sulphate and 0.8 mL phosphate buffer (5.0 mM, pH 8). 5,5'-dithiobis-(2-nitrobenzoic acid) was then added (0.1 mL) and absorption at 412 nm was measured at 37°C for 1 h on the UV-3100 spectrophotometer.

Reduced glutathione was selected as reference for -SH group content per milligram of protein measurement and protein was quantified according to the method described by Lowry et al (15).

\[
\text{TABLE 1}
\]

Mean red blood cell hemolysis in smokers and nonsmokers

<table>
<thead>
<tr>
<th>Increase in hemolysis* (%)</th>
<th>Absorbance at 415 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Smokers (n=25)</td>
</tr>
<tr>
<td>21.6</td>
<td>0.248±0.083</td>
</tr>
</tbody>
</table>

*There was a significant difference compared with the control group (P<0.05)

Preparation of globular suspension

Blood samples were obtained from the smokers and nonsmokers and kept in heparinized tubes. The RBC content was isolated by centrifugation (3000 rpm for 10 min) and then washed three times using normal saline (0.9% NaCl). The tubes were then centrifuged at 12,000 g for 10 min to obtain packed cells.

A globular suspension was also prepared using blood samples from healthy individuals to study the effects of cigarette smoke, nicotine and cotinine. The blood samples from healthy volunteers were collected in citrated tubes. The RBC content was isolated from the plasma and buffy coat by centrifugation and was then washed three times using normal saline. The upper layer was separated and a 20% globular suspension in phosphate buffered saline was prepared.

RBC hemolysis

To study the direct effect of cigarette smoke on RBC hemolysis, the exhalations of three cigarettes by a healthy volunteer were entered into a test tube containing 0.9 mL globular suspension. After a 15 min incubation, 1 mL of AAPH was added and the test tube was incubated for 2 h.

Tests were conducted with nicotine (Merck Inc, Germany) and cotinine (Sigma, Germany) using three concentrations (1 µg/mL, 1.5 µg/mL and 2.5 µg/mL, and 1.25 µg/mL, 2.5 µg/mL and 5 µg/mL, respectively).

To study the effect of nicotine and cotinine, 0.1 mL solutions of cotinine and nicotine prepared in normal saline were added to the test tubes separately. Normal saline (1 mL) was added to the control tubes. Phosphate buffered saline (0.9 mL) and globular suspension (0.1 mL) were then added to the control and test tubes. Following a 15 min incubation, 1 mL of AAPH (25 µM) was added to each of the tubes. The tubes were placed onto a shaker and after 2 h were centrifuged for 10 min at 3000 rpm. The upper solution was used for the measurement of hemolysis at 415 nm on a spectrophotometer (UV-3100, Shimadzu, Japan) (13).

The above method was used to compare RBC sensitivity with AAPH in smokers and nonsmokers.

The effect of nicotine and cotinine on -SH group content was studied at their highest concentrations (2.5 µL/mL and 5 µL/mL, respectively) (16).

Statistical analysis

All tests were repeated four times. Mean results were calculated and expressed as mean ± SD. Significance was determined using Student's t test.

**RESULTS**

Smokers and nonsmokers had mean ages of 35±7 years and 34±6 years, respectively, which were not significantly different. The results showed that RBC hemolysis in smokers was 21.6% higher than in nonsmokers (P<0.05) (Table 1).

The examination of the direct effect of cigarette smoke on RBC hemolysis yielded remarkable results. Exposure to cigarette smoke increased RBC hemolysis by 281.7% with exposure to peroxidants (Table 2).

Nicotine inhibited RBC hemolysis by 36.7% at its highest concentration; however, RBC hemolysis increased at the two lower concentrations (Table 3). In contrast, cotinine increased RBC hemolysis by 13.8%, showing its peroxidant effect at the highest concentration. Furthermore, at their highest concentrations, nicotine and cotinine decreased the -SH groups by more than 50% (Table 4).

**DISCUSSION**

Cigarette smoke contains numerous chemicals and large quantities of oxidants. Many of the harmful effects of cigarette smoke are due to oxidative damage (3,4). Because cigarette smoke does not come into direct contact with plasma lipoproteins in vivo, the in vitro scenario may not entirely reflect the in vivo events. For example, different studies (17-21) examining the effect of cigarette smoke on LDL oxidation have produced contradictory results. In some studies (17-21), the effects of cigarette smoke on LDL oxidation have been protective and nicotine has been shown to inhibit LDL oxidation (22). It has also been observed that RBC hemolysis resulting from the direct exposure of blood samples from smokers to cigarette smoke was higher than that resulting from exposure to oxidants (Table 2).

In the present study, nicotine inhibited RBC hemolysis by 36.7% at a concentration of 2.5 µg/mL, but hemolysis increased at the lower concentrations. In contrast, cotinine caused an increase in RBC hemolysis at this concentration. Cotinine also caused dose-dependent increases in RBC hemolysis. The analysis of the effects of cigarette smoke is highly complex because it contains a vast array of compounds, each
with their own protective or destructive effects. The -SH groups of proteins residing in the cell membrane or existing intracellularly, especially those found in glutathione, are essential to cell membrane stability. Under oxidative stress, the -SH groups protect other cellular structures against free radical-induced oxidation and disulfide band formation. Results have shown that nicotine and cotinine decrease -SH groups. Previous studies have demonstrated that cigarette smoke not only causes oxidative damage in the lungs, but also exerts destructive effects on all biological systems. The free radicals in cigarette smoke, which increase peroxidation products in smokers' blood and vascular tissues, are chiefly responsible for these deleterious effects. Increased exposure to cigarette smoke was shown to decrease plasma antioxidants in vitro and to lower vitamin C levels in the plasma and leukocytes of smokers.

### REFERENCES

6. Pryor WA, Stone K. Oxidants in cigarette smoke. Radicals, chelating iron and ascorbate, making the cell more vulnerable to damage by free radicals mediated by iron ions.

### TABLE 4

<table>
<thead>
<tr>
<th>Chemical Combination</th>
<th>Percentage of -SH groups (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetrathionate + nicotine</td>
<td>44.2</td>
</tr>
<tr>
<td>Tetrathionate + cotinine</td>
<td>52.8</td>
</tr>
</tbody>
</table>

Previous studies (25) have shown that serum folate levels and RBCs of smokers were significantly lower than those of nonsmokers, and that these reductions were dependent on the intensity of smoking. RBC lipid peroxidation in smokers was also higher than in nonsmokers (26). In addition, it has been confirmed that glutathione levels decrease in biological tissues due to exposure to cigarette smoke. Cigarette smoke increases the production of free radicals from tert-butylhydroperoxide by chelating iron and ascorbate, making the cell more vulnerable to damage by free radicals mediated by iron ions (27).

### CONCLUSIONS

The present study confirms the results from previous studies, which also highlighted the destructive and oxidative effects of cigarette smoke and its harmful effects on smokers and non-smokers alike.

---

**TABLE 3**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration (µg/mL)</th>
<th>Increase hemolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>2.5</td>
<td>-36.7</td>
</tr>
<tr>
<td>Cotinine</td>
<td>5.0</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>4.1</td>
</tr>
</tbody>
</table>

---

Asgary et al

---

Exp Clin Cardiol Vol 10 No 2 2005