Effects of Cigarette Smoke, Nicotine and Cotinine on Red Blood Cells Hemolysis and their -SH Capacity

S. Asgary, Gh Naderi, A. Ghannady and M. Soghraty

1Assistant Professor of Pharmacognosy, Head of Basic Department
2Assistant Professor of Biochemistry, Head of laboratories
3Associate Professor of Pharmacognosy, Isfahan University
4General Practitioner, Isfahan Cardiovascular Research Center, Isfahan, IRAN

INTRODUCTION

Smoking is one of the greatest and most important causes of mortality, which is fortunately preventable.

Prevalence of some diseases such as, pulmonary, cardiovascular, cataract and cancers, in smokers is higher than non-smokers (1–5). There are many active molecules like aldehyde, heavy metals, hydrogen cyanide, low molecular weight phenols, nitrosamines and aromatic polycyclic hydrocarbons in cigarette smoke. Cigarette smoke is a source of metallic ions, kinones and hydroxyl radicals. High volume of oxidants increases oxidative stresses level in smokers (6). Free radicals in cigarette smoke start and develop lipids peroxidation that causes low density lipoprotein (LDL) oxidation and atherosclerosis (7–9).

As the RBC membrane lipids are rich of polyunsaturated fatty acids, oxidative effects of oxygen on their membrane is more than other tissues. In addition, RBCs contain hemoglobin that is one of the most potent catalyzors for lipid peroxidation start.

Peroxidants invasion to RBC membrane due to hemoglobinopathies, radioactive rays, oxidative drugs, increase of some metals and decrease the function of antioxidant systems, can lead to cell hemolysis (10). Oxidants can cause -SH groups oxidation in proteins and RBC membrane besides lipids peroxidation.

-SH groups are very active and can be a target in oxidative stress and decrease after glutathione reduction (11).

Glutathione plays a direct protective role against of membrane proteins and reserves their stability. When glutathione level is decreased, membrane -SH groups are oxidized and membrane will loss its stability (12).

In this study cigarette effect as a free radical producer, on RBC hemolysis has been investigated.
For this reason, RBC sensitively against 2, 2′ Azobis (2-amidinopropane) dihydro chloride (AAPH) in smokers and non-smokers, and direct effects of cigarette smoke, nicotine and cotinine (the main metabolite of nicotine) on RBC hemolysis have been studied.

MATERIAL AND METHODS

This study was an analytic sectional study. 25 smoker volunteers (20 cigarettes per day for at least 2 years) and 25 non-smoker volunteers were chosen.

All of the volunteers were 20–50 year-old healthy men without history of cardiovascular, endocrine and gastrointestinal diseases.

They were on no special medication, diet or nutritional supplement.

Preparation of Globular Suspension

Blood samples were obtained from smoker and non-smoker volunteers and collected in heparinized tubes separately and then RBC were isolated by centrifugation and washed with Normal Saline (NaCl 0.9%) 3 times.

At the last washing, they were centrifuged with 12000g for 10 minutes for obtaining packed cells.

Globular suspension from healthy individuals for study of cigarette smoke, nicotine and cotinine effect was prepared too. For this reason blood samples (from healthy volunteer) were collected in citrated tubes and after isolation of RBC from plasma and Buffy Coat by centrifugation, RBC were washed by Normal Saline for 3 times and at the last stage, upper layer was separated and then 20% globular suspension in phosphate buffer saline (PBS) was prepared.

RBC Hemolysis

For the study of direct cigarette smoke effect on RBC hemolysis, exhalation of 3 cigarettes by a healthy volunteer, was entered into the test tube (0.900 globular suspension) and after 15 minutes incubation, 1 ml AAPH was added and incubated for 2 hours.

Tests on nicotine (Merck-Germany) in 3 concentrations (1, 1.5, 2.5) µg/ml and cotinine (Sigma Chemical Co.) in 3 concentration (1.25, 2.5, 5) µg/ml were done.

For the study of nicotine and cotinine effects, 0.100 of cotinine or nicotine stock solutions in Normal Saline, was added in test tubes separately and 1ml of Normal Saline added in control tubes.

After that 0.900 of PBS and 0.100 of globular suspension were added to each control and test tube and incubated for 15 minutes.

Then, 1ml of AAPH (25µM) was added into all of the tubes and tubes were placed on the shaker for 2 hours and finally they were centrifuged for 10 minutes with 3000 rpm and upper solution was used for hemolysis measurement in 415nm.

For comparison of RBC sensitivity against AAPH in smokers and non-smokers, the method mentioned above was done.

-SH Groups Measurement per mg of Protein

At first RBC membrane was prepared according to ref.14. Then 0.1ml of prepared RBC membrane was solved in 1ml of sodium dodecyl sulfate 10% and 0.8 ml of phosphate buffer (pH = 8), 5.0 in 412nm for Reduced measurement Effects of Statistical results from t-test.

All of the

RESULTS

The mean is significant 21.6% more

<table>
<thead>
<tr>
<th></th>
<th>Non-sm</th>
<th>n =</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.204 ±</td>
<td></td>
</tr>
<tr>
<td>A 20% susp was determ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>There is sig</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Study Exposure (Table 2).

<table>
<thead>
<tr>
<th></th>
<th>Cot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.180</td>
</tr>
</tbody>
</table>

At first R induced 1

There is s

Nicc

concenb

On t

effect or

In at up to 5l
(pH = 8), 5.0 mM and 5.5 Dithiobis (2 Nitrobenzoic Acid) were added and photo absorption in 412 nm for 1 hour in 37°C was measured.

Reduced glutathione (GSH) was selected as reference for -SH group content (mg/protein) measurement and protein was determined according to Lowry et al. method.

Effects of nicotine and cotinine were studied in highest concentration on -SH amount (16).

Statistical Analysis

Results from all of the tests have been calculated as Mean ± SD and studied through student t-test.

All of the tests have been repeated 4 times and the mean of them have been calculated.

RESULTS

The mean age of smokers was 35 ± 7 years and non-smokers 34 ± 6 years that there was no significant difference between them. Results demonstrate that RBC hemolysis in smokers is 21.6% more than non-smokers (p < 0.05) (Table 1).

Table 1. Mean of RBC hemolysis in smokers and non-smokers.

<table>
<thead>
<tr>
<th>Absorbance rate in 415 nm</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers</td>
<td>Smokers</td>
</tr>
<tr>
<td>n = 25</td>
<td>n = 25</td>
</tr>
<tr>
<td>0.204 ± 0.069</td>
<td>0.248 ± 0.083</td>
</tr>
</tbody>
</table>

A 20% suspension of smokers and non-smokers incubated against AAPH for 2 hours. Then hemolysis was determined in smokers and non-smokers. There is significant statistical difference compared with control group (p < 0.05).

Study of direct cigarette smoke effect on RBC hemolysis has showed remarkable results. Exposure to cigarette smoke has increased RBC hemolysis against oxidant up to 281.7% (Table 2).

Table 2. Direct cigarette smoke effect on AAPH-induced RBC hemolysis.

<table>
<thead>
<tr>
<th>Absorbance rate in 415 nm</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Test</td>
</tr>
<tr>
<td>0.180 ± 0.023</td>
<td>0.687 ± 0.068</td>
</tr>
</tbody>
</table>

At first RBC were exposed to cigarette smoke and after adding AAPH and incubating for 2 hours, induced hemolysis was measured and increased percentage was calculated. There is significant statistical difference compared with control group (p < 0.05).

Nicotine has inhibited hemolysis up to 36.7% in highest concentration but in lower concentration has increased hemolysis.

On the contrary, cotinine has increased RBC hemolysis up to 13.8% with antioxidant effect on the highest concentration.

In addition, nicotine and cotinine in the highest concentration have decreased -SH groups up to 50% (Table 3 and 4).
Table 3. Effect of different concentration of nicotine and cotinine on AAPH-induced RBC hemolysis.

<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></td>
<td>1.5</td>
<td>19.7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>Cotinine</td>
<td>5</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>

RBC were exposed to different concentrations of nicotine (1, 1.5, 2.5) µg/ml and cotinine (1.25, 2.5, 5) µg/ml and after adding AAPH and incubating for 2 hours induced hemolysis have been measured.

Table 4. Effect of nicotine (2.5 µg/ml) and cotinine (5 µg/ml) on -SH groups of RBC.

<table>
<thead>
<tr>
<th>Material</th>
<th>µ mol/ mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (without tetrathionate)</td>
<td>507</td>
</tr>
<tr>
<td>tetrathionate</td>
<td>296</td>
</tr>
<tr>
<td>tetrathionate + nicotine</td>
<td>268</td>
</tr>
<tr>
<td>tetrathionate + cotinine</td>
<td>261</td>
</tr>
</tbody>
</table>

DISCUSSION

Cigarette smoke contains more than thousands chemical compounds and high volume of oxidants and it seems, many of cigarette smoking effects are due to oxidative damages (3, 4). Whereas cigarette smoke hasn’t any access to plasma lipoproteins directly in vivo, in vitro results may not show in vivo events.

For example there are contradictory results in different studies of cigarette effect on low density lipoprotein (LDL) oxidation (17-21). In some of the studies, effects of cigarette smoke on LDL oxidation have been protective or nicotine has inhibited LDL oxidation (22). Also, this study showed in direct effect of cigarette smoke, hemolysis is more, compared with smokers' blood exposure to oxidant. In this study has been shown that nicotine in 2.5 µg/ml concentration, has inhibited RBC hemolysis up to 36.7% but in lower concentration hemolysis has increased gradually, whereas cotinine in this concentration has caused dose dependent RBC hemolysis. Discussion about cigarette effects and its compounds is very complex because cigarette contains many compounds that some of them may have protective effect on one mechanism but destructive effects on other mechanisms. -SH groups of proteins (either in the cell membrane or in the hemoglobin and especially glutathione, intracellularly) are very important factors in cell and cell membrane stability. In oxidative stresses -SH groups protect other cellular structures against free radicals by oxidation and disulfide bands formation(23). Results show that nicotine and cotinine decrease -SH groups. Previous studies has demonstrated that cigarette
smoking not only causes oxidative damage in lungs but also have destructive effects on all of the biologic systems that these effects are due to free radicals in the cigarette smoke(6), that increase peroxidation products in smokers' blood and vascular tissues. Increase of cigarette smoke has decreased plasma antioxidants in vitro and Vit C has decreased in smokers' plasma and leukocytes, too(5-24).

Previous studies have shown that folate level in smokers' serum and RBC is significantly lower than non-smokers and this reduction depends on amount of cigarette smoking(25) and RBC lipid peroxidation in smokers is higher than non-smokers, too(26). On the other hand, it has confirmed that biological tissues lose their glutathione due to exposure to cigarette smoke, cigarette smoke increases free radicals production from t-BH by Iron and Scorbate chelating and increases cells sensitivity to free radicals damages through iron(27). Our study confirms the results of previous studies based on destructive and oxidative effects of cigarette smoke that is dangerous for both smokers and non-smokers.

REFERENCES


27. Mehlihorn RJ. Increased vulnerability of human erythrocytes to hydroperoxide damage after exposure to cigarette smoke or 1-chloro-2,4-dinitrobenzen in vitro Nicotine Tob Res 2000, 2(2), 141-8.