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Effect of Smoking on Concentration of Carboxyhemoglobin for Persons with β-Thalassemia Minor

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Percentages of carboxyhemoglobin (HbCO) from human venous blood have been determined for four categories of subjects: healthy persons and individuals with β-thalassemia minor, each of these two classes have also been divided into smokers and non-smokers. Identification of β-thalassemic individuals has been realized by performing hemoglobin electrophoresis on agarose gel. Multicomponent analysis based on absorption spectra of hemoglobin has been used in order to obtain HbCO concentrations. Increased values of HbCO have been determined in the blood of smokers, about 6 % for healthy persons while 10 % for those with β-thalassemia minor.

Key Words: Carboxyhemoglobin, β-Thalassemia minor, Electrophoresis, Multicomponent analysis.

INTRODUCTION

Hemoglobin is a macromolecule constituted of four polypeptide chains (two pairs of two identic globin chains), each chain attached to a heme molecule. The two pairs of globin chains differentiate by the number and the sequence of the amino acids forming the chains, confering in this manner specification of various type of known hemoglobins. There have been identified six types of polypeptide chains (ζ, ε, α, γ, δ and β chain) contained in the structure of physiologic hemoglobins.

Heme is a ferroprotoporphyrin, wherein the iron atom is situated at the centre of the porphyrin ring. The iron in the hem is bivalent (Fe^{2+}) and has six coordination sites within a hemoglobin molecule. By four coordinate-covalent bonds the iron atom is coordinated to the four nitrogen atoms of the tetrapyrolic ring. The fifth coordinate-covalent bond makes the contact with the proteic component of hemoglobin by a nitrogen atom from a histidine residue. By the sixth bond, the heme is indirectly linked to the

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histidine residue of the globin chain, by a hydrogen bond realised by an H_2O molecule. This labile bond allows the binding of a molecule of O_2 to the atom of Fe^{2+}, resulting in this manner the oxygenated form of hemoglobin (oxyhemoglobin, HbO_2). Deoxyhemoglobin (Hb) represents the unliganded form of hemoglobin. By its property to reversible binding of O_2, the heme is the active physiologic part of hemoglobin molecule. Hemoglobin can also bind other substances (compounds), resulting in various hemoglobin derivatives. Carboxyhemoglobin (HbCO) is a reversible compound, obtained by binding of a carbon monoxide molecule (CO) to a Fe atom from hemoglobin, at the location normally occupied by O_2. The affinity of hemoglobin for CO is about 200 times higher than that for O_2. Carbaminohemoglobin ($HbCO_2$) results by linking of a CO_2 molecule to the hemoglobin, but not at the iron atom, but at the free aminic groups of the globin chains. Methemoglobin (MetHb) is a ferrihemoglobin wherein the prosthetic group is represented by hematine. In MetHb the iron atom is trivalent (Fe^{3+}) and it cannot binds O_2.

The red cells of a normal adult person contain three types of hemoglobins: HbA_1, HbA_2 and HbF. The HbA_1 has two types of polypeptide chains, the α and β chains, assembled into a tetrameric molecule having the structure $\alpha_2\beta_2$. It is the predominant hemoglobin found in normal adult red cells, its normal concentration being situated in the interval of 96-98 %[1]. HbA_2 and HbF have the subunit composition $\alpha_2\delta_2$ and $\alpha_2\gamma_2$, respectively. Normal values of HbA_2 are placed within the range 2-3.5 %, while those for HbF are less than 2 %.

The α chain is common to these three hemoglobins. The other three globin chains (β, γ and δ) have distinct primary structures that confer different surface charge to the normal human hemoglobins and consequently different electrophoretic mobilities, that allow the separation and quantification of hemoglobins by electrophoresis. Electrophoresis constitutes the basic techniques in diagnosis of quantitative hemoglobinopathies, hereditary diseases that affect the rate of synthesis of one or more polypeptide chains of hemoglobin. β-Thalassemia is a hemoglobinopathy characterized by a deficient or absent synthesis of β-globin chain. In this case, because only synthesis of HbA_1 is affected, the relative concentrations of HbA_2 and HbF are increased with respect to that corresponding of HbA_1.

In β-thalassemia, anemia is the result of two mechanisms *i.e.*, ineffective erythropoiesis and decreasing of time of life for red blood cells. An increased rate of destruction of red blood cells implies an increasing in the heme catabolism that leads to an increased production of carbon monoxide. The catabolism of the porphyrin ring of the heme involves multiple steps *viz.*, from a mole of heme there results a mole of Fe, a mole of CO and a mole of bilirubin.

Degradation of red blood cells under pathological conditions such as thalassemia, anemias and other hematological diseases accelerates carbon monoxide production[2]. For patients with hemolytic anemia the carboxyhemoglobin levels can be as high as 4 %[3].

Concentration of HbCO in blood is also influenced by the quantity of inhaled CO. Healthy non-smokers have a percentage carboxyhemoglobin of about 1 %, while for the healthy smokers a mean value for HbCO of 6.2 % has been reported by Sagone *et al.*[4].

In order to analyze the influence of smoking on HbCO concentration for individuals with β-thalassemia, we studied four group of persons: healthy persons (separated in smokers and non-smokers) and persons with β-thalassemia minor (also divided in smokers and non-smokers).

Hemoglobin electrophoresis on alkaline agarose gel has been used for quantification of HbA_2 and identification of healthy persons and individuals with β-thalassemia minor.

Percentage of HbCO from venous blood has been determined by multicomponent analysis of hemoglobin derivatives based on absorption spectra of hemoglobin.

EXPERIMENTAL

Identification of healthy persons and of those with β-thalassemia was realized by hemoglobin electrophoresis on alkaline agarose gels by using a Sebia electrophoresis system and Hydragel 7 Hemoglobin(e) K20 kits[5].

Venous blood for analysis was collected from adult persons and was anticoagulated with EDTA. The first step for sample preparation was the centrifugation of anticoagulated blood at 5000 rpm for 5 min. The plasma was removed and the red blood cells were washed 2 times with 10 volumes of saline solution (9 g/L NaCl solution in distilled water). 10 μL of red cells were diluted (hemolyzed) with 130 μL of hemolyzing solution. After vortex for 10 s, the samples were incubated 5 min at room temperature. 10 μL of each hemolyzed samples have been applied on agarose gel by using an applicator that has been placed into a position corresponding to the migration start point for hemoglobins. The gel was placed into an electrophoresis chamber with the gel side facing down, according to the polarity indicated on the gel, namely the samples on the cathodic side. At alkaline buffer, normal hemoglobins have different negative electric charges and under the action of an applied electric field they migrate to the anodic side of gel with distinct electrophoretic mobilities, being separated in this manner. At pH 8.5, HbA_1 is the normal adult hemoglobin with the highest electrophoretic mobility, thus, after electrophoretic separation, it is the fraction with the longest migration space. HbF has an electrophoretic mobility less than that of HbA_1, while HbA_2 is the hemoglobin with the lowest electro-

phoretic mobility. The power supply was programmed to generate a constant voltage of 165 V for a migration time of 15 min. We used *tris*-Barbital buffer pH 8.5.

After migration, the hemoglobin denaturation has been realized by immersion for 15 min of gel in a fixative solution containing 60 % ethanol, 10 % acetic acid and 30 % distilled water. The gel has been dried for 10 min with hot 80 ºC air flow in a Sebia IS 80 Incubator-Dryer. The staining of the hemoglobin fractions has been performed by immersing the dried and cooled gel in an Amidoblack solution for 5 min. In order to eliminate the excess of dye and also to obtain a background completely colourless and clear, the gel has been destained in three succesive baths of destaining solution (0.05 g/dL citric acid solution in distilled water). The gel has been again dried with hot 80 ºC air and then scanned at 570 nm. By densitometry, the relative concentrations of hemoglobin fractions were obtained.

The study has been continued with a group of 25 adult persons constituted by eleven healthy individuals (five non-smokers and six smokers) and fourteen persons with β-thalassemia minor (7 non-smokers and 7 smokers).

Relative concentrations of HbA_2 for the healthy persons were between 2 and 3 % while for individuals with β-thalassemia minor the values of HbA_2 are included in the range of 4-5 %. Each smoker that participated in this study smoked between one and two packs of cigarettes per day.

For the determination of percentages of HbCO, for each participant 2 mL of its venous blood was anticoagulated with EDTA. The total hemoglobin concentration of each sample was determined by using an ABX Pentra 120 hematology analyzer.

Samples preparation for obtaining absorption spectra began by separation of erythrocytes and plasma. The blood was centrifugated at 5000 rpm for 5 min, then the plasma was discarded by using a transfer pipette. The red blood cells were washed 2 times with 10 volumes of physiologic serum, each time the separation of red blood cells has been realized by centrifugation and then elimination of used physiologic serum. After washing, the red blood cells were diluted up to 2 mL with distilled water, obtaining in this manner the initial hemoglobin concentration.

In order to obtain with better accuracy the absorption spectra of samples with an Ocean Optics S2000 spectrophotometer, by using cuvettes with lightpath length (l) of 1 cm, we diluted the samples such that the concentration of total hemoglobin in each diluted sample (spectral analyzed) has a value between 2 and 3.2 g/L.

For the samples with an initial total hemoglobin concentration greater than 100 g/L we diluted 20 μL of aqueous solution of red blood cells with 980 μL of distilled water. In the case of samples with initial total hemoglo-

bin concentration less than 100 g/L, 25 μL of aqueous solution of erythrocytes were diluted up to 1000 μL with distilled water.

The absorption spectra of diluted samples have been obtained immediately after preparation, in order to diminuate the effect of autoxidation of HbO_2 ($HbO_2 \rightarrow$ MetHb), effect that can modifies the concentrations of hemoglobin derivatives. Since HbCO cannot autoxidize, its concentration is not influenced by this phenomenon[6].

Simultaneous determination of concentrations of Hb, HbO_2, HbCO and MetHb in diluted samples has been realized by multicomponent method[7]. Using the absorption spectra of all diluted samples, we measured the absorbances of each sample at 542, 554, 576 and 630 nm. These wavelengths have been choosed by taking into account the principal peaks of the absorption spectra of these four hemoglobin derivatives investigated and also the values of the wavelengths corresponding to the linear silicon CCD array of the spectrophotometer that we used.

The wavelength 542 nm is near the maximum of absorption of HbCO (at 540 nm) and the wavelength 554 nm corresponds to the peak of absorption spectrum of Hb, situated at 544 nm. Absorption spectrum of HbO_2 has a peak at 576 nm and MetHb has an important absorption band[8] at 632 nm.

In order to consider a possible increase of absorbance with a certain value (denoted by f) due to an eventual presence in diluted samples of a little quantity of physiologic serum, remained from the washing of erythrocytes, we noted the absorbance at 700 nm, where the effect of this correction is more important because the hemoglobin derivatives have low absorptivities in the vicinity of 700 nm.

We obtained a system with five equations and five unknowns (the concentration of the four hemoglobin derivatives and the value f).
Each equation has the form:

$$A(\lambda) = [\varepsilon_{Hb}(\lambda) \cdot c_{Hb} + \varepsilon_{HbO_2}(\lambda) \cdot c_{HbO_2} + \varepsilon_{HbCO}(\lambda) \cdot c_{HbCO} + \varepsilon_{MetHb}(\lambda) \cdot c_{MetHb}] \cdot \frac{l}{16.1145} + f \quad (1)$$

where $A(\lambda)$ is the absorbance of the diluted sample at wavelength λ, $\varepsilon_{Hb}(\lambda)$, $\varepsilon_{HbO_2}(\lambda)$, $\varepsilon_{HbCO}(\lambda)$ and $\varepsilon_{MetHb}(\lambda)$ are the millimolar absorptivities of Hb, HbO_2, HbCO and MetHb, respectively, at wavelength λ, c_{Hb}, c_{HbO_2}, c_{HbCO} and c_{MetHb} are the concentrations of hemoglobin derivatives and l is the lightpath length.

Because we used the values of millimolar absorptivities reported by Zijlstra *et al.*[9], that are expressed in $L \cdot mmol^{-1} \cdot cm^{-1}$ and we expressed the concentration of hemoglobin derivatives in $g \cdot L^{-1}$ and the lightpath length in cm, we inserted in eqn. 1 the factor 16.1145 that represents the mass (in g) of one millimole of hemoglobin monomer[10].

By solving, for each sample, the system of five equations of the type (1) for the chosen wavelengths, where the values of millimolar absorptivities were deduced by Zijlstra *et al.*[9] and the lightpath length has a value of

1 cm, we obtained the concentration of hemoglobin derivatives from diluted samples and the correction factor f. By using the values of total hemoglobin concentrations in the diluted samples, we computed the percentages of hemoglobin derivatives in these samples.

Because HbCO is a hemoglobin derivative that cannot autoxidize, its percentages determined for diluted samples can be considered very close to those from venous blood.

RESULTS AND DISCUSSION

In the Fig. 1 we present the absorption spectra in the spectral range of 450-700 nm for two subjects with β-thalassemia minor: one non-smoker and one smoker.

Total hemoglobin concentrations for the diluted samples (with absorption spectra presented in Fig. 1) were 2.90 g/L for non-smoker and 2.72 g/L for smoker, while percentages of HbCO deduced for non-smoker and smoker were 6.14 and 12.48 %, respectively. Although these two total hemoglobin concentrations are close one to the other, we remark some differences between the two absorption spectra. Near the wavelength $\lambda = 500$ nm the absorbance for the smoker is greater than that for the non-smoker. This result can be explained by the fact that in the blood of the smoker person there exists a higher concentration of HbCO than in the case of the non-smoker person and in this spectral range the absorptivity of HbCO exceeds the absorptivities of Hb and HbO_2. A remarkable difference between the shapes of these spectra consists in the fact that for the smoker, the absorbance at 542 nm is higher than the absorbance at 576 nm, while for the non-smoker the absorbance at 576 nm is higher as compared to that for 542 nm. The explanation is based on the existence of an absorption band of HbCO situated at 540 nm, whose presence becomes important at high concentrations of HbCO (the case of smoker persons). The absorption bands of HbCO at 540 and 570 nm justify why the absorbances are higher for the smoker in the spectral range of 535-580 nm. We observe that near the wavelength 630 nm the absorption spectrum for the smoker is over that for the non-smoker. This result is due to the higher concentration of HbCO and also to the existence of a concentration of 8.02 % MetHb for the smoker, as for the non-smoker the percentage of MetHb was 3.61 %.

The values of percentages of HbCO obtained for all individuals included in present study are presented in Fig. 2.

The HbCO levels for healthy non-smoker persons were between 0.93 and 1.85 %, the mean value being 1.32 %, in a good agreement with the value of 1.00 % reported by Sagone *et al.*[4]. For healthy smoker persons the values of HbCO were situated in the range of 5.18-6.70 %, with a mean value of 6.15 %, close to that of 6.20 % resulted by the Sagone study.

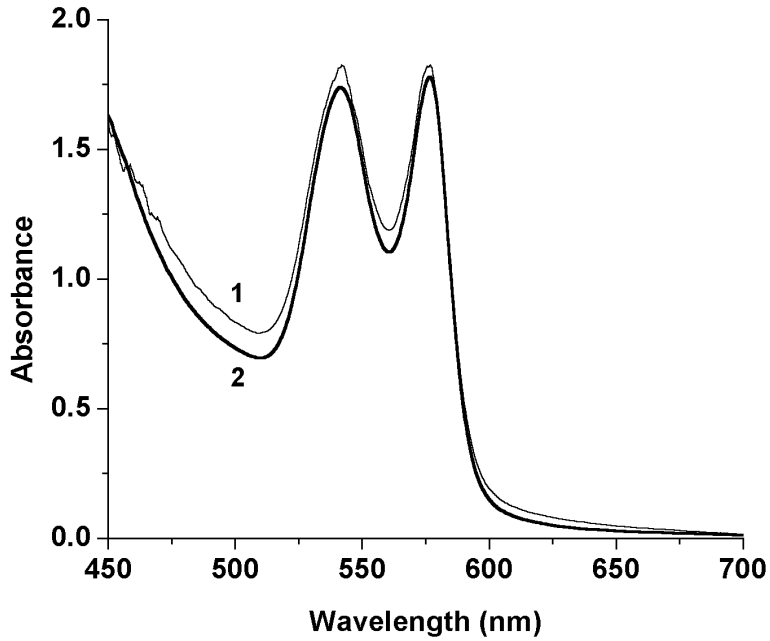

Fig. 1. Absorption spectra for a smoker (1) and a non-smoker (2), both with β-thalassemia minor

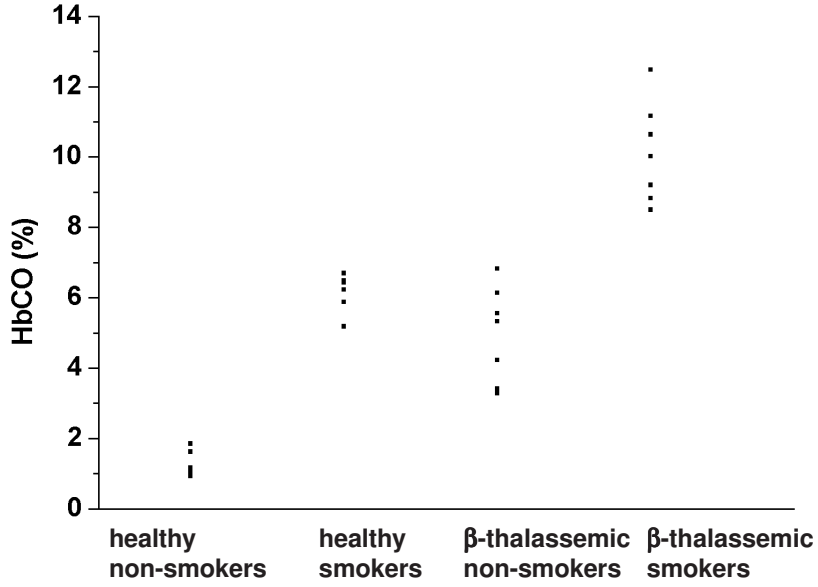

Fig. 2. Percentage carboxyhemoglobin for the four groups of persons

In the case of non-smoker individuals with β-thalassemia minor there were obtained percentages of HbCO between 3.29 and 6.83 % and a mean value of 4.97 %, characteristic value for persons with hemolytic anemia[3]. We observe in this case a large variation in the HbCO levels as compared to the two groups of healthy persons, that can be due to different forms of affectation produced by β-thalassemia minor and the effect of this genetic disease on the rate of heme catabolism. The values of HbCO obtained for smoker persons having β-thalassemia minor were between 8.50 and 12.48 %, the mean value was 10.12 %. We remark that for this group we obtained the highest concentrations of HbCO, which that can be explained by addition of endogenous CO resulted by heme catabolism to the CO inhaled by smoking. If such elevated levels of HbCO in blood persist long time, then the chronic hypoxia appears, that increases the risk of appearing of atherosclerosis. We observed an increased mean value of HbCO for healthy smokers is higher than the mean value obtained for non-smokers with β-thalassemia minor. This result supports the idea that the effect of CO inhaled by smoking surpasses that of endogenous CO.

The obtained results suggests that the elimination of smoking will conduce to a decreasing in the HbCO percentage in blood of about 6 % both for healthy smokers and smokers with β-thalassemia minor.

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