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Human hemoglobin structural and functional alterations and heme degradation upon interaction with benzene: A spectroscopic study

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ABSTRACT

Here, the effect of benzene on hemoglobin structure, stability and heme prosthetic group integrity was studied by different methods. These included UV–vis absorption spectrophotometry, normal and synchronous fluorescence techniques, and differential scanning calorimetry (DSC). Our results indicated that benzene has high hemolytic potential even at low concentrations. The UV–vis spectroscopic results demonstrated that benzene altered both the globin chain and the heme prosthetic group of hemoglobin increasing met- and deoxy-Hb, while decreasing oxy-Hb. However, with increasing benzene the concentration of all species decreased due to heme destruction. The spectrophotometric results show that benzene has a high potential for penetrating the hydrophobic pocket of hemoglobin. These results were consistent with the molecular docking simulation results of benzene-hHb. Aggregation and thermal denaturation studies show that the increased benzene concentration induced hemoglobin aggregation with a decrease in stability, which is consistent with the DSC results. Conventional fluorescence spectroscopy revealed that the heme degradation species were produced in the presence of benzene. The results of constant wavelength synchronous fluorescence spectroscopy (CWSFS) indicated that at least five heme-degraded species were produced. Together, our results indicated that benzene has adverse effects on hemoglobin structure and function, and heme degradation.

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1. Introduction

Hemoglobin constitutes approximately one third of the mass of a mammalian red blood cells. Its major function is to carry oxygen from the lungs through the arteries to the tissues and help to carry carbon dioxide through the veins back to the lungs. The process whereby hemoglobin performs this essential physiological role is characterized by a cooperative interaction among its constituent subunits [1,2]. This macromolecule is composed of four chains which are held together through non-covalent interactions [3]. Each chain contains one heme prosthetic group, thus providing four binding sites for oxygen on the hemoglobin. In the alpha chain the 87th residue is histidine, while in the beta chain the 92nd residue is histidine. A heme group is attached to each of the four histidines in the pocket. Also the heme consists of an organic part containing nitrogen atoms for complexation with the iron atom as a metallic core. The iron atom in the heme binds to the four nitrogens in the center of the protoporphyrin ring [3–5]. The regulated complex structure of hemoglobin is important for oxygen transferring from long to the body and carbon dioxide from body to long. Many factors can impact the normal physiological functions of hemoglobin. Hemoglobin is affected by various endogenous physiological conditions and exogenous agents or environmental pollutants such as drugs, heavy metals, herbicides (alachlor, aminopielik D), paraquat and insecticides (chlorpyrifos and cypermethrin), and organic pollutants [6]. These could have adverse effects on protein structure and function [6]. In addition, the oxidation state of heme iron center is very important for its true action. If the iron is in the ferric state (met-hemoglobin) the hemoglobin cannot bind to the oxygen molecules and consequently loss common physiological function. Normally, met-hemoglobin levels are <1%. In oxidative stress, hemoglobin acts as oxidase resulting in increased level of ferric state hemoglobin and met-hemoglobinemia [7–10]. Hemoglobin lifetime is 120 days due to the limited life span of the RBCs. As the heme is not recycled, degradation of heme is the only way of removal. In most cells, heme degradation is done with heme oxygenase. However, due to the absence of heme oxygenase enzyme in the mature red blood cells and in blood serum, the released heme has to be transported to other body systems in order to be degraded by heme oxygenase enzyme. The redox ability of oxy-hemoglobin leads to producing superoxide and hydrogen peroxide reagents which is responsible for nonenzymatic heme degradation. In other words, these reagents are initiators in producing various reactive oxygen species that resulted in nonenzymatic heme degradation and heme turnover in red blood cells. Also it

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should be mentioned that met-hemoglobin shows catalytic properties, such as peroxidase-like and catalase-like activities, so the redox chemistry of hemoglobin plays an important role in living systems and in specific conditions. Peroxidase activity of hemoglobin makes great interest for application in environmental processes, where the metHb system can be used as biocatalyst to remove and oxidate organic pollutants [11,12].

Various environmental pollutants, hazard materials and toxins can increase oxidative stress in blood. Small chain alkyl benzenes such as BTEX (benzene, toluene, ethyl-benzene and xylene) are a class of toxic, volatile organic compounds. These compounds represent some of the most hazardous materials in gasoline. These chemicals belong to an important class of volatile environmental contaminants and are frequently analyzed in the environment and drinking water. Chronic effects of BTEX include damage to the liver and harmful effects on kidneys, heart, lungs and nervous system. Aromatic hydrocarbons have also been associated with the induction of non-immune hemolytic anemia or met-hemoglobinemia. Currently in the U.S. there are significant concentrations of benzene in ambient air, due in large part to vehicle emissions. Benzene concentration in indoor air is also significant contributors to children's exposure particularly in homes where people smoke [13–16].

Chronic exposure of benzene is associated with various health effects for human. Benzene (water solubility at 15 °C ≈ 1.8 g/L [17]) is known as clastogenic and carcinogenic chemical agent that induces primarily hematopoietic cancers in humans [18,19]. The most important blood system disorders can be divided to met-hemoglobinemia, aplastic anemia, pancytopenia, myelodysplasia and acute myeloid leukemia. 1 ppm is the accepted concentration of benzene as exposure standard limit in the USA by OSHA (Occupational Safety and Health Administration) in occupational conditions. However, nowadays, during the progress of the both developing and developed countries the exposure to high levels of benzene is unavoidable [18–21]. Moreover, the toxicity to blood system and carcinogenicity of benzene at or below 1 ppm is unclear and an ongoing concern [20,21].

Very limited and few studies are available on BTEX effects on proteins, particularly hemoglobin [22,23], and there are no reports regarding structural effects, stability, heme destruction effects, and heme degradation species produced in interaction of benzene with hemoglobin. In this study, we used spectrophotometric techniques focusing on the effects of benzene on structure, stability, function, aggregation and also effects on heme destruction and hemolysis of red blood cells (RBCs).

2. Experimental

2.1. Materials

Benzene, all the buffer salts, and other chemicals were obtained from E. Merck (Germany). Nitrogen and oxygen gases (99.999% purity) were from Air Products (UK). All the solutions were prepared using double distilled water.

2.1.1. Human adult hemoglobin extraction

Human hemoglobin was extracted in our laboratory using a previously described method [24]. In brief, new fresh and heparinized blood was centrifuged at 3000 rpm to remove plasma components. The upper yellowish solution was decanted and the packed red cells were washed three times in an isotonic saline solution (0.9% NaCl) at a ratio of 1:10 for 5 min and subsequently centrifuged at 10,000 rpm. Red cells were osmetically lysed using cold double distilled water. Membrane components were removed by centrifugation (10,000 rpm). The soluble Hb was centrifuged at high speed (18,000 rpm) at least two additional times to remove any insoluble materials. The hemoglobin solution was then brought to 20% saturation with ammonium sulfate, left standing for 20 min and centrifuged at 20,000 rpm. The resulting Hb solution was dialyzed 3 times against 0.2 M phosphate buffer solution (pH 7.4) for 24 h. The purity of the extracted hemoglobin was determined using SDS-PAGE (15%) and catalase test [25].

2.2. Methods

2.2.1. Hemolytic studies

Blood samples (6 mL) were collected from healthy and non-smoker volunteers of HbA genotype in 50 mL sample tubes containing 1 mL of ascorbic acid (4%). The samples were centrifuged at 2000 rpm for 15 min at 15 °C to remove plasma and washed with saline buffer solution (0.9% NaCl solution) at ambient temperature. The upper yellowish solution was decanted and the packed red cells were washed again for three times using an isotonic saline buffer solution at a ratio of 1:10 for 5 min and subsequently centrifuged at 1600 rpm until upper phase color changed from yellow to clear solution. Upper clear solution was removed and the red cells were diluted to 5% with deionized water at room temperature. The prepared sample (1.5 mL) was placed in 2 mL vials and the analytes were added to the vials at desired concentration. The control (0%) was considered without any analyte addition, and the control (100%) was considered by adding triton X-100. The vials were incubated at 37 °C for 45 min in water bath. The samples were centrifuged at 1600 rpm and the upper solution was used for lysed hemoglobin concentration determination using a UV–vis spectrophotometer at 540 nm [26].

2.2.2. Aggregation potential studies

The hemoglobin solution (50 mL) was prepared (2 μmol·L^{-1}), and 1 mL was placed in a quartz spectrophotometer cell and quickly sealed using parafilm. Various concentrations of benzene were injected into the cell using a Hamiltonian syringe. To consider the aggregation induction potential of analytes and blank solutions the increasing absorption at 365 nm at 60 °C was recorded for 600 s.

2.2.3. Thermal denaturation and Tm determination

The hemoglobin solution (1 mL of 2 μmol·L^{-1}) was placed in a spectrophotometer cell and sealed quickly with parafilm. Appropriate amounts of benzene were injected into the cells and temperature was raised from 25 °C to 90 °C at a rate of 1 °C/min and the absorption was recorded at 280 nm. Tm was obtained by calculating the midpoint of the spectra.

2.2.4. Differential scanning calorimetry (DSC) measurements

Differential scanning calorimetry studies were carried out using a nano-DSC differential scanning calorimeter (Setaram, France) equipped with 0.348 mL cells. The sample cell was filled with hemoglobin and hemoglobin–benzene solutions and the reference cell was filled with a buffer solution that contained all of the sample constituents except the protein. The cells were carefully filled to avoid air bubbles. DSC scans were performed in the temperature range of 20–90 °C. The hemoglobin concentration in all of the experiments was 1 mg/mL. The heating rate was fixed at 1 °C/min.

2.2.5. Normal fluorescence measurements of heme degradation species

Simple fluorescence measurements were carried out using a Cary fluorescence spectrophotometer at 25 °C and 2 μM hemoglobin. Benzene was added to the sample solution and emission fluorescence spectra were recorded at 330–650 nm using 321 nm as the excitation wavelength and from 450 to 650 nm at the excitation wavelength of 460 nm. Excitation and emission slits were fixed at 10 nm with a scan speed of 5 nm/s.

2.2.6. Synchronous fluorescence spectroscopic studies

Synchronous fluorescence spectra of hemoglobin were recorded in a Cary Eclipse spectrofluorometer (Varian Co., Australia) and analyzed to determine the changes around tryptophan and tyrosine residues as a

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