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Evaluation of the Effect of Hydrogen Peroxide (H_2O_2) on Haemoglobin and the Protective Effect of Glycine

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Abstract:
Oxidative stress has focused the attention of world wide researchers for its deleterious effects on the human body, reactive oxygen species (ROS) are often called radicals. These radicals if present in smaller amounts pose no threat to our body, but instead they provide defense against various pathogens. When the concentration of these radicals exceeds beyond a limit then the antioxidants provided can not perform the scavenging of all the radicals resulting in oxidative stress. The targets of oxidative stress are DNA, lipids and proteins. In organisms including humans ROS are produced during metabolic and immune system functions. Oxidative stress is now recognized to be an important factor in the development or enhancement of human diseases. Among ROS, H_2O_2 is relatively stable in the presence of ferrous iron H_2O_2 forms hydroxyl radical (·OH) via the Fenton reaction.

$$Fe^{+2} + H_2O_2 \longrightarrow {}^{\cdot}OH + OH^{-} + Fe^{+3}$$

Key words: *Hydrogen peroxide, Glycine,Spectro-photometer,Circular-Dichroism*

1. Introduction

Human body is provided with a balanced system for the maintenance of a proper state. This proper state is maintained by the interaction of various biomolecules which include DNA, Proteins and lipids. The disturbance to these molecules results in the impairment in the proper functioning and ultimately the disturbance in the metabolic state and growth and development of the cell. Various agents and chemicals are known to cause the disturbance in the cell development and growth. One of the damage to cell is oxidative stress[7].It is the phenomenon in which there is a considerable elevation in the concentration of reactive oxygen species (ROS) and reactive nitrogen species (RNS) to the ratio of the antioxidant defense provided to our body[3].

Among various radical producing agents, hydrogen peroxide is noteworthy because it readily permeates membranes and it is therefore not compartmentalized in the cell[4]. Numerous enzymes (peroxidases) use hydrogen peroxide as a substrate in oxidation reactions involving the synthesis of complex organic molecules. The well-known reactivity of hydrogen peroxide is not due to its reactivity per se, but requires the presence of a metal reductant to form the highly reactive hydroxyl radical which is the strongest oxidizing agent known and reacts with organic molecules at diffusion-limited rates[8].In the presence of Fe^{+2}, H_2O_2 decomposes to form hydroxyl radicals that are extremely reactive[6].When the endogenous oxidant H_2O_2 reacts with Hb transient radicals are generated during the peroxidative consumption of H_2O_2,If not neutralized, these radicals can lead to tissue toxicity[10].

Interactions of hemoglobin(Hb) with hydrogen peroxide alters thiol levels and course of endothelial cell death.The oxidation of Hb generates potentially cytotoxic products such as the ferryl heme intermediate (Fe^{+4}), methemoglobin (Fe^{+3}), hemichromes, and free heme or iron[5]. In addition, the auto oxidation of oxyhemoglobin to methemoglobin releases reactive oxygen species that in turn may lead to tissue damage[2].

when haemoglobin is dissociated into free subunits such as alfa and beta chains, they are more susceptible to haemichrome formation than in tetrameric state,superoxide and peroxide exert an influence on haemichrome formation, based on the studies of the autoxidation of alfa-chains and beta-chains of haemoglobin. It became clear that haemoglobin, when dissociated into its subunits, is easily transformed into haemichrome by a small amount of H_2O_2. Furthermore Haemoglobin subunits were transformed directly to Haemichrome by oxidation with H_2O_2 and this process did not involve methaemoglobin formation[1].

Therefore the objective of present study included evaluation of the effect of H_2O_2 on haemoglobin and its protection by glycine.

2. Experimental

2.1. Experimental Sample
Packed cell volume of blood (RBC's) were procured from Rotary Blood Bank, near Batra Hospital, New Delhi.

2.2. Isolation of Haemoglobin
10 ml of packed cell volume blood(RBCs) were diluted in 30 ml of water for hemolysis, in a 50 ml centrifuge tube. After making the sample to stand for 10 minutes, it was centrifuged at 10000 rpm for 15 min. The supernatant containing the hemoglobin was collected in another tube and the pellet containing the stroma of RBCs was discarded.

2.3. Protein Estimation
Blood protein in blood sample was estimated by lowery method.

3. Spectral Analysis
The spectral analysis was done in UV-Vis spectrophotometer in a range of 200-600nm (Spectro DV-80). The instrument operates by passing a beam of light through a sample and measuring the intensity of light reaching a detector. The number of peaks and the absorption was recorded. 50 µl sample was diluted in 1ml of water [1/20 dilution] to make the stock solution. This stock solution contains haemoglobin in a concentration of 100µg/1000µl. 1000µl of Hb sample was taken for the spectral analysis. The sample was scanned from 200-600 nm with the help of spectrophotometer. 500 µl sample (Hb Stock) was added to 500 µl H_2O_2 (25mM). Then the spectra were taken as a function of time at 0-min, 1-min, and 5-min to evaluate the effect of H_2O_2 on Hemoglobin. 500 µl sample(1/20) was mixed with 500 µl glycine (0.2M). The spectra were recorded. To analyse the effect of glycine against the H_2O_2 induced damage. A combined sample of Hb + Glycine + H_2O_2 was prepared by taking 200µl from Hb stock, 200µl of 25mM H_2O_2 and 600µl of 0.2M Glycine. The effect of glycine was monitored at 0-min,1-min and 5-min.

4. Circular Dichroism (CD) Spectroscopy
Circular dichroism is the difference in the absorption of left-handed circularly polarised light (L-CPL) and right-handed circularly polarised light (R-CPL) and occurs when a molecule contains one or more chiral chromophores (light-absorbing groups). Circular dichroism = $\Delta A(\lambda) = A(\lambda)_{LCPL} - A(\lambda)_{RCPL}$, where λ is the wavelength. CD spectroscopy is used extensively to study chiral molecules of all types and sizes, but it is in the study of large biological molecules where it finds its most important applications. The far-UV (ultraviolet) CD spectrum of proteins can reveal important characteristics of their secondary structure. CD spectra can be readily used to estimate the fraction of a molecule that is in the alpha-helix conformation, the beta-sheet conformation, the beta-turn conformation, or some other (e.g. random coil).

5. Results and Discussion
Spectral analysis: The figures below show the spectra of Human Hb in presence and absence of H_2O_2 and glycine (antioxidant).

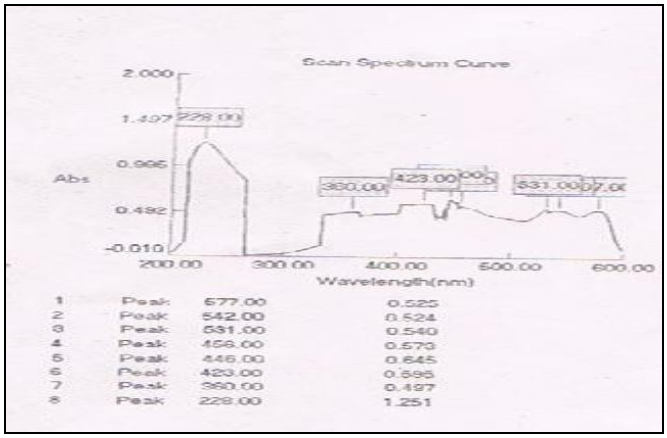

Figure 1: Spectra showing normal Haemoglobin peaks (λ 200-600nm)

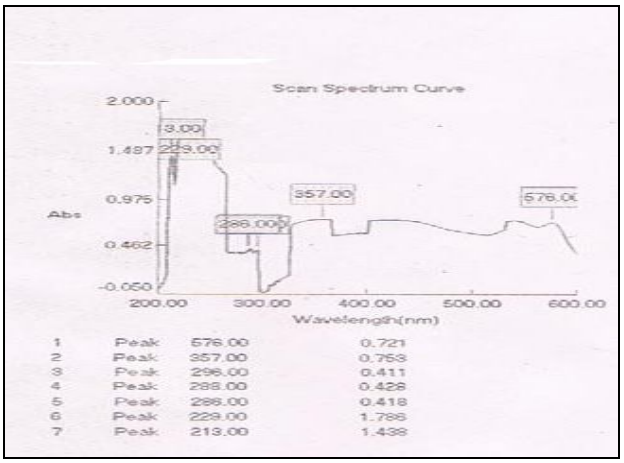

Figure 2: spectra showing Hb peaks treated with H_2O_2 (25mM) at 0-min

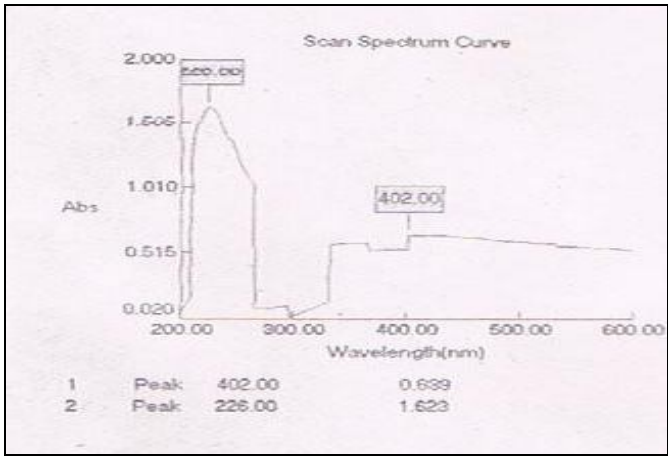

Figure 3: spectra showing Hb peaks treated with H_2O_2 (25mM) after 1-min

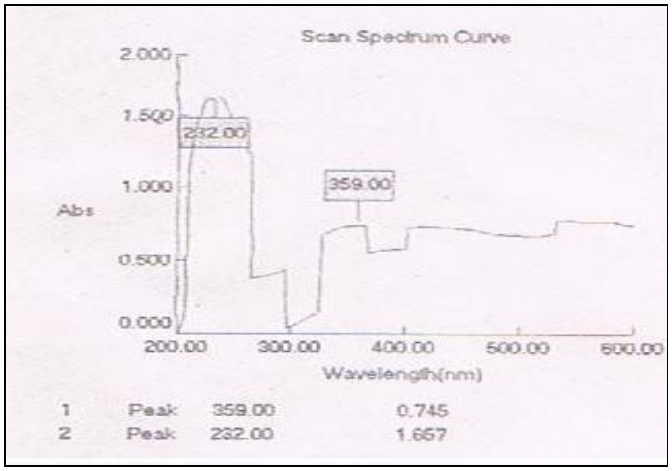

Figure 4: spectra showing Hb peaks treated with H_2O_2 (25mM) after 5-min

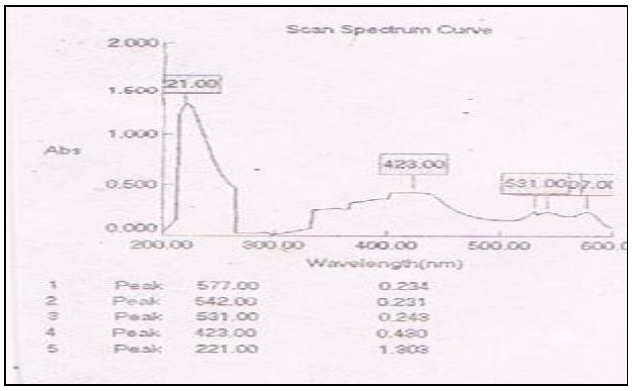

Figure 5: Spectra showing Hb peaks in presence of glycine (0.2M) as an antioxidant

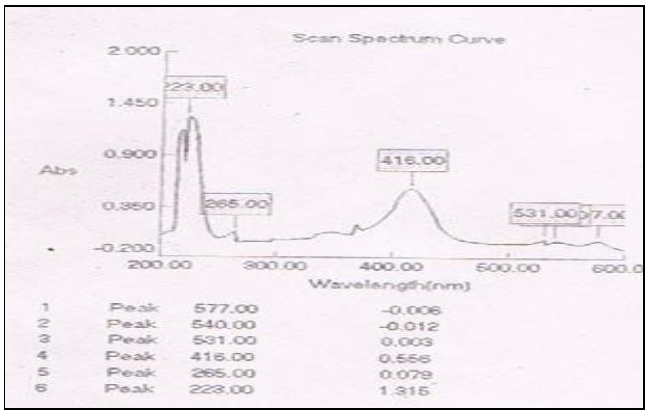

Figure 6: spectra showing Hb peaks treated with H_2O_2 (25mM) in presence of glycine(0.2M) as an antioxidant after 1 min

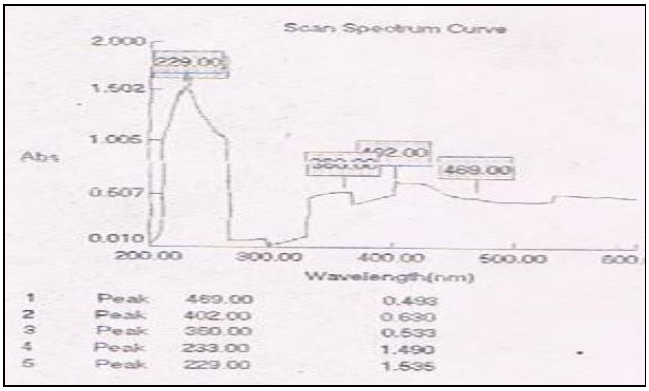

Figure 7: Spectra showing Hb peaks treated with H_2O_2(25mM) in presence of glycine(0.2M) as an antioxidant after 5-min

Spectral analysis data shows that untreated Hb gives absorbance at 577, 542, 531, 458, 446, 423, 260 and 228nm respectively and peaks were observed at these wavelengths which confirmed the presence of Hb in our sample. After treatment with hydrogen peroxide the spectra showed considerable change in both the absorbance and number of peaks. The hydrogen peroxide induced damage was studied at different time intervals viz 0, 5 and 10 mins. It was observed that hydrogen peroxide induces severe damage to Hb structure and stability. It was seen that at 0 min after Hydrogen peroxide treatment the spectra showed a small change. At 5-min spectra showed disappearance of peaks and absorbance also changed. Same results were observed after 10 min. Glycine used as a protective agent against hydrogen peroxide induced damage showed its effect on Hb. After treatment with both H_2O_2 and glycine the Hb showed no change in the sprctra. Therefore it can be concluded that glycine acts as a protective agent against H_2O_2 induced oxidative damage.

6. Circular Dichroism Analysis

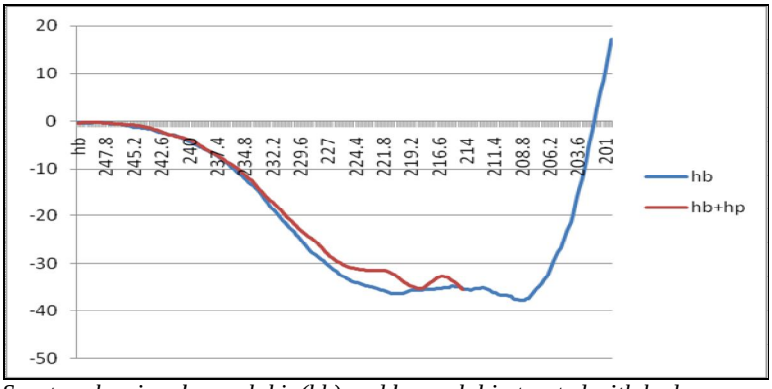

Figure 8: Spectra showing hemoglobin(hb) and hemoglobin treated with hydrogen peroxide(hp)

Circular dichroism was performed to check the structural changes in biomolecules. When performed circular-dichroism to check any structural changes in the hemoglobin on the treatment with hydrogen peroxide, It was observed that hydrogen peroxide induced secondary structural changes in the hemoglobin which confirms the denaturing ability of hydrogen peroxide as an oxidant. There was a gradual decline in the helical content of the hemoglobin upon being oxidized. Therefore we conclude that hydrogen peroxide affects the structure of hemoglobin thereby also alters functional properties of hemoglobin because of which the cell or the organism will undergo deleterious effect.

7. Summary and Conclusion

Proteins are amongst the most abundant and extremely versatile biological molecules of life, which are crucial for the cell function. The structure of a protein molecule plays an important role in its function, and any change or loss in the 3-dimentional structure of the protein results in the loss or lowering of the activity.

Various kinds of stresses such as the oxidative stress, heat stress, and other denaturing stresses perturb the structure of protein molecules. Oxidative stress which has been implicated in large number of pathological conditions, often results from the excessive production of partially reduced oxygen moieties or the ROS that include super oxide anion radical, hydroxyl radical, hydrogen peroxide and singlet oxygen. The ROS are produced from both the exogenous and endogenous sources as inescapable by-products of normal aerobic metabolism. Being the partially reduced oxygen moieties, ROS are highly reactive and are capable of damaging almost any type of bimolecules such as proteins, sugars, DNA and lipids.

ROS can attack proteins in a variety of ways such as the modification of amino acid residues in proteins. In pathological conditions and with age, these modifications increase possibly due to disruption of antioxidant protective mechanisms. Thus the antioxidants property of glycine against oxidative stress condition was analyzed using techniques like absorption spectroscopy, the studies of absorption spectroscopy suggested that the effect of H_2O_2 on Hb was an irreversible modification.

The results of absorption spectroscopy indicate that the haem proteins are more susceptible to oxidative damage, a possible explanation : the oxidative degradation of protein is enhanced in the presence of metal cofactors, which are capable of redox cycling, such is the major constituents of heme proteins, the become susceptible to damage .

When performed circular dichroism to check any structural changes in the hemoglobin on the treatment with hydrogen peroxide. It was seen that hydrogen peroxide induced secondary structural changes in the hemoglobin which confirms the denaturing ability of hydrogen peroxide as an oxidant. There was a gradual decline in the helical content of the hemoglobin on being oxidized. Therefore it was conclude that hydrogen peroxide affected the three dimentional of hemoglobin thereby also altered functional properties of hemoglobin.

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