

# Studies of human hemoglobin modified with peroxynitrite: A cytotoxic metabolite generated in numerous disorders

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## ABSTRACT

**Objectives:** Peroxynitrite interacts with biomolecules through oxidative reactions or radical-mediated mechanisms leading to oxidative damage and committing cells to necrosis or/and apoptosis. Hemoglobin (Hb) is the oxygen-transporting metalloprotein found in blood that carries oxygen from the lungs to the tissues and subsequently releases it to carry out various metabolic functions. In the present study, we have isolated Hb from human blood and subjected it to modify by peroxynitrite generated *in vitro*. The native and modified Hbs were characterized using various biochemical methods.

**Methods:** The native and modified Hbs were characterized using absorption spectroscopy, thermal melting profile analysis, and other biochemical techniques. We have also tried to ascertain the effect of various scavengers such as uric acid, ascorbic acid, tocopherol, and reduced glutathione as potent peroxynitrite quenchers.

**Results:** The isolated Hb produces distinct peaks while the Hb modified with peroxynitrite showed marked hyperchromicity and the distinct peaks were lost. The chemical denaturation and thermal denaturation studies along with carbonyl content data show that the modified Hb is unstable and shows higher absorbance due to denaturation of the protein.

**Conclusion:** Thus, the formation and effect of peroxynitrite on Hb are deleterious and antioxidant scavengers of the peroxynitrite show that the modification of the Hb can reverse the effect of peroxynitrite modification. The *in vitro* studies presented here show that peroxynitrite is toxic to human Hb and its inhibition by various antioxidants may be helpful in prevention of numerous disorders.

**Keywords:** Biochemical characterization, hemoglobin, peroxynitrite

## Introduction

Hemoglobin (Hb) is the oxygen-transporting iron-containing metalloproteinase found in red blood cells (RBC) of nearly all vertebrate and some invertebrate organisms.<sup>[1]</sup> Hb carries oxygen from the lungs to the tissues releasing it to carry out various metabolic functions. It has a molecular weight of 64 kDaltons. In mammals, Hb makes up about 96% of the RBC with abinding capacity of 1.34 ml/g of Hb, increasing the oxygen binding capacity to many-fold as compared to the dissolved oxygen in blood.<sup>[1]</sup> The mammalian Hb binds to four oxygen molecules to carry them to the site of metabolism. Hb also transports other gases such as carbon dioxide (CO<sub>2</sub>) and nitric oxide (NO). It carries respiratory CO<sub>2</sub> as carbamino Hb (HbCO<sub>2</sub>) and NO bound to the globin moiety of Hb. Hb is also found outside the RBC such as the dopaminergic neurons of

substantia nigra, macrophages, alveolar cells, and mesangial cells of kidney.<sup>[1]</sup> In these cells and tissues, the Hb functions as an antioxidant and a regulator of iron metabolism rather than as an oxygen carrier. The structure of Hb consists of four polypeptide chains (globins) associated with one another which is designated by a Greek letters -  $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\epsilon$ . Adult human Hb (HbA) has two equivalent chains of containing  $\alpha$  and  $\beta$  subunits. Therefore, the biomolecular structure of HbA is  $\alpha_2\beta_2$  since each Hb molecule has two  $\alpha$  and two  $\beta$  subunits.<sup>[2]</sup>

During injury and damage certain reactive oxygen species (ROS) are produced which may cause of modification certain cellular components such as DNA, RNA, proteins, and lipids or may cause inflammation.<sup>[3]</sup> One such species is peroxynitrite (ONOO<sup>-</sup> or ONOOH) formed by the reaction between NO and superoxide anion (O<sub>2</sub><sup>-</sup>). Peroxynitrite is a relatively stable,

but its acidic form, peroxyntrous acid (HOONO), ultimately forms nitrate at pH 7.0 and 37°C with a half-life of 1s.<sup>[4]</sup> The oxidative reactions of HOONO may involve electron transfer reactions in which peroxyntrite is reduced and the substrate oxidized. Peroxyntrite is a powerful oxidant that can attack a wide range of molecular targets.<sup>[5-7]</sup> It interacts with all biomolecules through oxidative reactions or radical-mediated mechanisms triggering cellular responses leading to oxidative injury and committing cells to necrosis or apoptosis.<sup>[8-12]</sup> *In vivo*, peroxyntrite is involved in pathologies such as stroke, myocardial infarction, chronic heart failure, diabetes, shock, inflammatory diseases, cancers, and neurodegenerative disorders. Thus, it is important to control their formation or treatment through appropriate scavengers which may help to mitigate its action.<sup>[13-16]</sup>

Peroxyntrite (ONOO<sup>-</sup>), a reactive nitrogen species, is a potent oxidant and nitrating species formed from the reaction between NO and O<sub>2</sub><sup>-</sup>. It is a relatively long-lived oxidant that may serve as an important cytotoxic agent. Peroxyntrite may also be produced in the body in response to a variety of toxicologically relevant molecules, environmental toxins, reperfusion injury, and inflammation. Peroxyntrite formation may lead to oxidative stress conditions which may damage biological macromolecules and causes malfunctioning of cellular functions. It is involved in tissue damage in a number of pathophysiological conditions such as neurodegenerative diseases and cardiovascular disorders. Its biological effects are due to its reactivity toward a large number of molecules including lipids, amino acids, and nucleic acids. Peroxyntrite leads to sulfhydryl group oxidation, lipid peroxidation, structural and functional alteration of proteins, and DNA single-strand breakage. Peroxyntrite is a potent trigger of DNA strand breakage and activates nuclear enzyme poly(ADP-ribose) polymerase (PARP) resulting in energy depletion and necrosis of the cells. Therefore, the attenuation of peroxyntrite is a significant therapeutic benefit from environmental toxins as well as in a variety of inflammatory and reperfusion disease conditions.<sup>[17]</sup>

The scavengers are responsible for the complete “absorption” or “quenching” of oxidative stress causing free radicals including peroxyntrite. The direct acting scavengers like reduced glutathione (GSH) donate two electrons to reduce peroxyntrite, while the indirect catalytic scavengers like metalloporphyrins require a two-step process involving cellular antioxidants to quench peroxyntrite. The indirect scavengers do not accelerate the decomposition of peroxyntrite but scavenge the secondary reactive species produced, for example, ascorbate and urate reduce tyrosine nitration by inhibiting the radical intermediates. The various other scavengers are tocopherol (Vitamin E), pyridoxine (Vitamin B<sub>6</sub>), cobalamine (Vitamin B<sub>12</sub>), quercetin (flavonoid), gallocyanin, carmic acid, polyphenols, carotenoids, phytoestrogens, etc.<sup>[18]</sup> In the current study, we have isolated the human Hb and subjected it to modification by peroxyntrite generated *in vitro*. The native

and modified Hbs are then characterized using melting profile spectrometric analysis and other denaturation studies. Later, we have tried to ascertain the effect of various scavengers as potent peroxyntrite quenchers.

## Methods

### Isolation of Hb from the human blood

The present study was approved by the Institutional Ethics Committee of SBSPGI, Dehradun, India, and written consents were taken from all participants. Blood was collected from healthy individuals, and an equimolar volume of Alsever's reagent (name of company with catalog number) was added to it. The supernatant obtained was carefully removed, and the pellet was washed twice with 10% NaCl solution by centrifuging at 2000 rpm at 4°C for 10 min. It was again washed with 10% NaCl for 10 min at 4°C at 3000 rpm, and after discarding the supernatant, the pellet was resuspended in 0.15 M phosphate buffer saline (PBS) (pH 7.2). Hb was then diluted with PBS, and the spectrum was observed from 200 to 700 nm.<sup>[19]</sup>

### Modification of Hb by peroxyntrite

The Hb was diluted in two separate microcentrifuge tubes. In one was added 3 ml of distilled water and to another, an equimolar volume of 0.5 mM sodium nitroprusside, pyrogallol, and EDTA was mixed thoroughly and then incubated at 37°C for 3 h along with blank, i.e., 3 ml of PBS. The samples were analyzed spectrophotometrically.<sup>[11,13]</sup>

### Spectral analysis of modified Hb

The Hb obtained was diluted with PBS. The spectrum was obtained by scanning the sample from 200 to 700 nm. The native and the modified Hb samples were analyzed spectrophotometrically in the range of 200–700 nm with absorbance ranging from 0.00 to 1.50.

### Chemical denaturation studies of the native and modified Hb

Chemical denaturation was done to analyze the extent of modification of the Hb through guanidine HCl. To denature Hb, the native and modified Hb were treated with 50 mM guanidine HCl (pH 4, 4.5, 5, and 6) and incubated for 60 min on ice. The sample was centrifuged for 10 min at 4°C at 4000 rpm. The protein content in the supernatant was estimated by extrapolation method on the standard curve of BSA plotted by the Lowry's method.<sup>[20]</sup>

### Estimation of carbonyl content in native and modified Hb

Carbonyl content was estimated using the standard method stated by Schmorak and Lewin.<sup>[21]</sup> Native and modified Hb

were taken to which 2.5M HCl was added. The sample was mixed thoroughly and 20% TCA was added to it. It was then centrifuged for 10 min at 4000 rpm. The pellet was collected and washed thrice with a 1:1 solution of ethanol: ethyl acetate and was dissolved in 6M Guanidine HCl and finally incubated at 37°C for 15 min. It was centrifuged at 4°C to collect the supernatant, and then the absorbance was recorded at 600 nm. The carbonyl contents are indicative of the amount of carbon associated with oxygen atom through a double bond.

### Thermal denaturation studies of native and modified Hb

Heat treatment was given to both the Hb samples, i.e., native and the modified to estimate the degree of denaturation in the samples. The samples were exposed to temperatures ranging from 30 to 100°C at an interval of 10°C between each reading. The absorbance was recorded at 280 nm using a spectrophotometer and a graph plotted.

### Treatment of native Hb with the peroxynitrite scavengers

The Hb samples were treated with the scavengers, i.e., chemical species which bind to peroxynitrite and thereby protect the protein from modification. The scavengers used are chemicals (uric acid and sodium azide), vitamins (ascorbic acid and tocopherol), and enzyme (reduced GSH). The native Hb was added with equal volumes of five scavengers in different test tubes and incubated at 37°C for 2 h. The samples were subjected to a mixture of pyrogallol, sodium nitroprusside, and EDTA in the ratio 1:1:1. The samples were then incubated at a temperature of 37°C for 3 h. The spectrophotometric readings of the samples were taken at 280 nm.

## Results

### Spectral analysis of modified Hb

The modification of Hb was carried out using the *in vitro* generation of peroxynitrite ion as determined by Wani *et al.*<sup>[22]</sup> and Habib *et al.*<sup>[23]</sup> using NO donor, i.e., sodium nitroprusside (5 mM) and pyrogallol (5 mM), in the presence of EDTA (5 mM) in equimolar concentration. The spectrophotometric spectrum analysis along with thermal and chemical denaturation studies confirm the formation and modification of the Hb by the generation of the peroxynitrite ion. The isolated Hb was dense red precipitate which was dissolved in PBS and was diluted with the same for further work. It was stored at -20°C. Hb on spectral analysis from 200 to 700 nm showed three distinct peaks at 415 nm, 543 nm, and 577 nm and a shoulder at 369 nm [Table 1]. Peroxynitrite was generated *in vitro* and the human blood Hb was treated with it. Spectrometric analysis was further done to observe the effects. The spectral analysis showed that the absorbance of modified Hb increased, i.e., hyperchromicity was observed throughout the spectrum from 200 to 700 nm as compared to the native Hb. The distinct peaks at 577 and 543 were lost

with large increase in absorbance at the 415 nm peak. The hyperchromicity indicates that the protein is denatured and thus the absorbance of the modified product is increased. Hyperchromicity of the modified protein varied from 1.3% at 542 peak to 52.2% at 349 nm peak. Hyperchromicity is calculated using the formula:

$$\% \text{ Hyperchromicity} = (\text{O.D modified} - \text{O.D native} / \text{O.D native}) * 100$$

### Chemical denaturation studies of the native and modified Hb

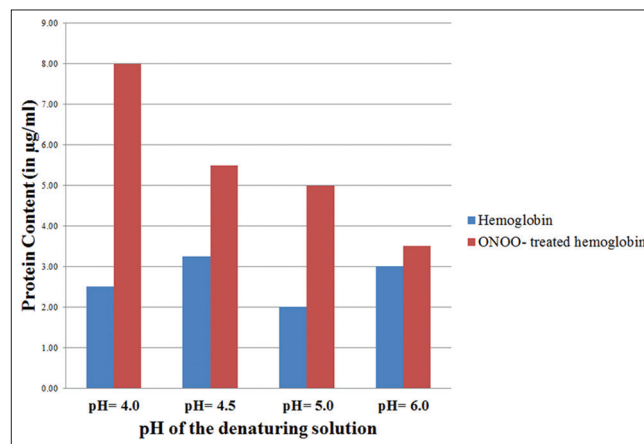
The native and modified Hb were denatured using guanidine HCl at different pH (pH = 4.0, 4.5, 5.0, and 6.0). The results showed that the protein content of modified Hb was higher than the native Hb at all the pH studied [Figure 1]. Maximum variation of 5.5 µg/ml of protein content was observed at the pH 4.0 while the minimum variation of 0.5 µg/ml of protein content was observed at the pH 6.0.

### Estimation of carbonyl content in native and modified Hb

Protein-bound carbonyl is regarded as a biomarker of protein oxidation as it is generated by free radicals in blood, tissues, and cells. The carbonyls modified proteins represent an irreversible form of modification are found to be relatively stable. These carbonyls are formed during overall oxidative conditions not due to specific oxidant and thus they can be called a marker of general protein oxidation. The carbonyl content of the native and modified Hb varied considerably as shown in Figure 2.

**Table 1:** Wavelengths peaks of native and peroxynitrite-modified hemoglobin

Native hemoglobin wavelengths	Modified hemoglobin peaks
369, 543, 577, 415	541, 577, 415



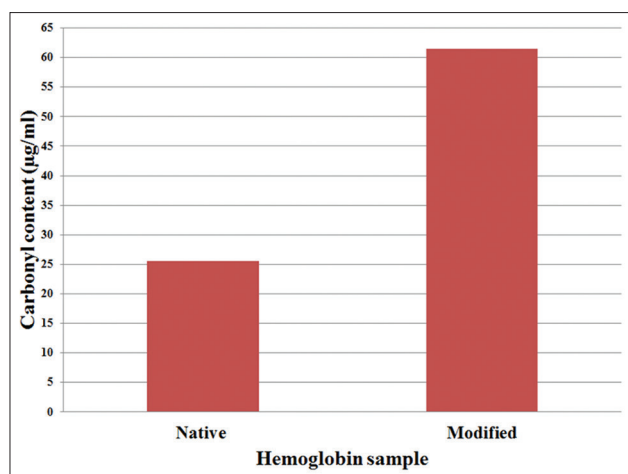
**Figure 1:** The protein content of native and modified hemoglobin after exposure to denaturing buffer at different pH

## Thermal denaturation studies of native and modified Hb

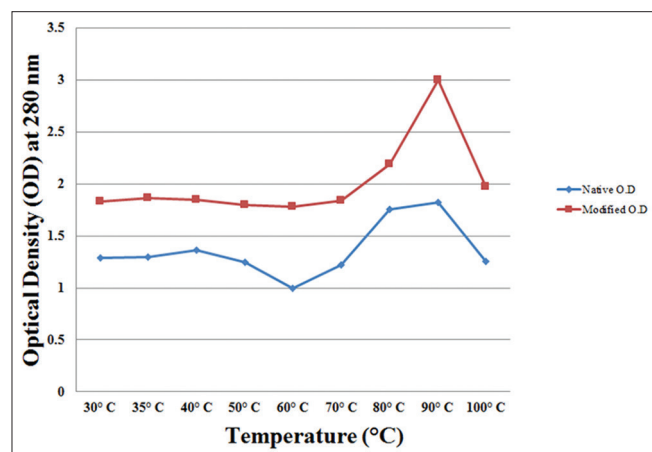
Thermal denaturation studies show that the modified Hb shows more denaturation and the absorbance of the modified Hb is higher than the native Hb since the beginning until the end [Figure 3]. This suggests that the modified Hb melts or denatures more rapidly than the native Hb.

## Treatment of native Hb with the peroxynitrite scavengers

The Hb when treated with scavengers (concentration) such that uric acid, sodium azide, ascorbate (Vitamin C), tocopherol (Vitamin E), and reduced GSH showed considerable decrease in the absorbance as compared to Hb not treated with the scavengers [Figure 4]. Thus, this shows that the scavengers are successful in quenching (maximum and minimum amount of quenching) the peroxynitrite species where the best results are shown by the reduced GSH followed by uric acid, ascorbate, sodium azide, and tocopherol.



**Figure 2:** Carbonyl content of native and modified hemoglobin after treatment with guanidine HCl



**Figure 3:** Thermal melting profile of the native and modified hemoglobin

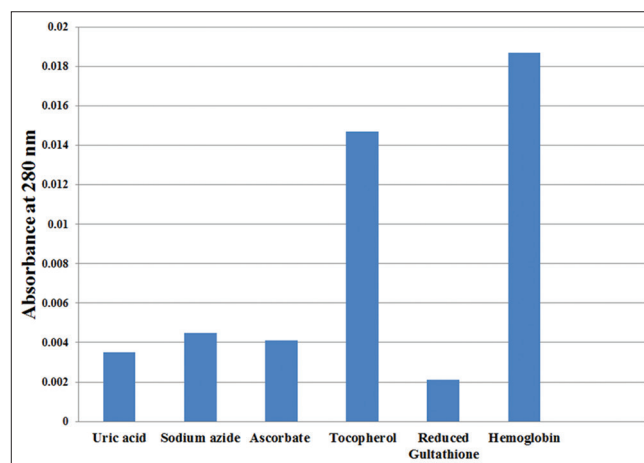
## Statistical analysis

All the experiments were repeated at least 3 times or otherwise stated. The data presented here represents one such typical experimental observation.

## Discussion

Peroxyntirite is an important biological oxidant produced by the reaction between NO and O<sub>2</sub><sup>-</sup> and reacts with a number of biomolecular targets including amines, lipids, and proteins.<sup>[24]</sup> It's increased production is involved in various pathophysiological conditions in cardiovascular, neurodegenerative, and inflammatory diseases.<sup>[25-27]</sup> Peroxyntirite induces cell death, influence signal-transduction processes, mitochondrial function, and signaling of apoptosis. The products of peroxyntirite reactions have been detected in several pathophysiological conditions including ischemia-reperfusion injury, circulatory shock, inflammation, and neurodegenerative disorders. Reaction of peroxyntirite or peroxyntirite-derived radicals (carbonate and nitrogen dioxide radicals) with targets results in one- and two-electron oxidations and nitration. The diffusion of peroxyntirite through plasma membranes can cause oxidative damage. In these conditions, the pharmacological inhibition of peroxyntirite was shown to be beneficial. The pharmacological strategies to attenuate the toxic effects of peroxyntirite involves its rapid catalytic reduction to nitrite (NO<sub>2</sub>) or its isomerization to nitrate (NO<sub>3</sub>) by metalloporphyrins. Manganese and iron metalloporphyrinic compounds have been shown to rapidly react with peroxyntirite and promote its decomposition in a catalytic manner. These compounds attenuate peroxyntirite-dependent toxicity *in vitro* and *in vivo* and are potential candidates for drug development in cardiovascular, inflammatory, and neurodegenerative diseases.<sup>[28]</sup>

A majority of ONOO<sup>-</sup> mediated oxidations results in redox-signaling, cell damage, and/or apoptosis in organisms.<sup>[29]</sup> Reaction of peroxyntirite with intracellular oxyHb is much faster than with CO<sub>2</sub> at 37°C since 80% of the peroxyntirite



**Figure 4:** Effect of various scavengers on modified hemoglobin



inside RBC will react with Hb. The ferrylHb intermediate is formed during the reaction of peroxynitrite with oxyHb<sup>[30]</sup> and will be reduced to metHb by peroxynitrite, nitrite, or amino acid residues adjacent to the heme moiety in globin. Antioxidant scavengers of peroxynitrite protect organisms because they quench ROS before they damage biological molecules or prevent oxidative stress, for example, by interfering the radical chain reaction of lipid peroxidation.<sup>[31]</sup> Various antioxidants including nutrient and synthetically synthesized could be used as a quencher for oxygen species.

Spectrophotometric spectral analysis showed maximum absorbance at 369, 543, 577, and 415 nm against the suggested 541, 577, and 415 nm. The spectrophotometric spectrum analysis along with thermal and chemical denaturation studies confirm the formation and modification of the Hb by the generation of the peroxynitrite ion. The chemical denaturation studies indicate that the maximum difference in the denatured state of the native and modified Hb was observed at the pH of 4.0 while minimum difference was observed at the pH of 6.0 [Figure 2]. The higher carbonyl content of the modified Hb compared to the native Hb is the indicative of the denatured state of modified Hb. The thermal denaturation studies clearly indicate that the denaturation state of modified Hb is higher than the native Hb and increases more rapidly as compared to the native hemoglobin.

Various antioxidants have been indicated as potential scavengers for the peroxynitrite species such as Vitamins C, E, A, flavonoids, uric acid, and quercetin.<sup>[32]</sup> Among these two chemicals (uric acid and sodium azide), two vitamins (ascorbate and tocopherol), and one enzyme (reduced GSH) were used during the study. The results clearly indicate that these are effective in scavenging the peroxynitrite species generated and thus could possibly reverse the damage caused by peroxynitrite. The biological activity of peroxynitrite is modulated by various antioxidant mechanisms and neutralized by synthetic peroxynitrite-scavenging compounds. A large number of these compounds have been identified with pharmacological potential to reduce the harmful effects of peroxynitrite and peroxynitrite-derived free radicals.<sup>[33]</sup> Several neutralizers and scavengers of peroxynitrite are metalloporphyrins, thiol-based antioxidants (mercaptoalkylguanidines, N-acetylcysteine, and dihydrolipoic acid), selenium-containing proteins (selenoprotein P and GSH peroxidase), tempol, cabergoline, acetaminophen, drugs (hydralazine, pindolol, zileuton, penicillamine, simvastatin, edaravone, propofol, deprenyl, and rasagiline), and molecules present in natural or dietary products (carotenoids, polyphenols, and epicatechin). However, their *in vivo* therapeutic potential as peroxynitrite neutralizing agent was found to be relatively low.<sup>[34]</sup> A potentially strong candidate against peroxynitrite-mediated toxicity both *in vitro* and *in vivo* is uric acid, an antioxidant and the end product of purine metabolism in humans. The reaction of uric acid with peroxynitrite may be due to the scavenging of free radicals and inhibition of tyrosine nitration products.<sup>[33]</sup> Previous

studies showed that when peroxynitrite was added to intact erythrocytes it leads to generate met Hb from intracellular oxy Hb by oxidation. Furthermore, the peroxynitrite causes nitration of intracellular Hb in a process that was enhanced in thiol-depleted erythrocytes.<sup>[35]</sup> In views of the our results and the findings discussed above, it is now clear that peroxynitrite is harmful and has potential to damage our circulating proteins, which are directly or indirectly have linked with numerous disorders particularly cancer and autoimmune disorders.

## Conclusion

Endogenous peroxynitrite leads to sulfhydryl oxidation, lipid peroxidation, structural and functional alteration of proteins and DNA, and DNA single-strand breakage. Hence, these modified biomolecules could have important implications in several pathophysiological conditions. The data presented here clearly demonstrates that the modified Hb is unstable and shows a higher absorbance due to denaturation of the protein molecule. Thus, it can also be confirmed that the effect of peroxynitrite on the Hb molecule is deleterious. The antioxidant quenching of the peroxynitrite by various scavengers shows that the damage incurred on Hb could be reversed using these substances. Future research in free radical interventions might suggest some strategies to remove peroxynitrite therapeutically. A possible mechanism to prevent peroxynitrite-mediated damage with potential pharmacological applications is the use of cell-permeable tyrosine-containing peptides. Tyrosine peptides interfere with tyrosine nitration reactions but spare critical protein tyrosine residues. Although their precise mechanism of action is elusive, the intracellular delivery of tyrosine-containing peptides can protect cells from peroxynitrite mediated damage.

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