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# Fructation Induces Hemin Degradation in Methemoglobin

M. Habibi-Rezaei[a,b,*], M. Bakhti[a,e], A.A. Moosavi-Movahedi[c,d], S.J. Zargar[a] and H. Ghorchian[c]

[a]*School of Biology, College of Science, University of Tehran, Tehran, Iran*

[b]*Nano-Biomedicine Center of Excellence, Nanoscience and Nanotechnology Research Center, University of Tehran, Tehran, Iran*

[c]*Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran*

[d]*Center of Excellence in Biothermodynamics, University of Tehran, Tehran, Iran*

[e]*Institute of Diabetes and Regeneration Research, Helmholtz Zentrum München, Neuherberg, Germany*

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## ABSTRACT

Under prolonged diabetic conditions, $HbA_{1c}$ is produced from normal hemoglobin ($HbA_0$) through a non-enzymatic glycosylation or glycation, a process which enhances the hemoglobin susceptibility to be auto-oxidized to methemoglobin (metHb). Here we are reporting that the non-enzymatic reaction between fructose and metHb (metHb fructation) induces significant changes in the globin structure and degrades the hemin moiety. Moreover, glycation of metHb by fructose brings about the extensive hypochromic effect and a small bathochromic effect in the Soret region. The products of hemin degradation were shown to be dialyzable species. Cathodic peaks of the cyclic voltammogram (CV) of methemoglobin during incubation with fructose resulted positive potential shift and the declined current at the peaks due to a decrease in the number of metHb molecules with the preserved hemin groups. Moreover, we found a significant increase in the hemin oxidation products such as ferryl/oxoferryl-Hb upon fructation of metHb. The production of such species during metHb fructation and hemin degradation might have a key role not only in the induction of hypoxic stress, but also in the reduced protective function of neural hemoproteins in Alzheimer's disease (AD).

**Keywords:** Hemin, Fructation, Reactive oxygen species, Methemoglobin, Cyclic voltammetry, $HbA_{1c}$

## INTRODUCTION

Heme (iron protoporphyrin IX) is a ubiquitous structure in the sorts of hemoproteins with a diverse functionality, including cell respiration/electron transport, oxygenation reactions/oxidative bio-transformations, oxidative stress responses, bistable sensory switches and oxygen-transport/storage [1]. The latest function is carried out by hemoglobin (Hb), myoglobin (Mb), neuroglobin (Ngb) and cytoglobin (Cygb). The distinct functionality of hemoproteins results from both extrinsic (*i.e.* the heme pocket environment and the nature of the heme-ligands) and intrinsic (*i.e.* the substituents on the heme periphery and in some cases, the covalent attachment of the heme to the protein) factors [2]. Hemin ($Fe^{3+}$ protoporphyrin IX) is the oxidized form of the heme ($Fe^{2+}$ protoporphyrin IX) structure, in the oxidized form of Hb, known as methemoglobin (metHb). Under physiologic condition, metHb represents 0-2% of the total Hb within the erythrocytes. However, these cells can tolerate up to 20% of metHb. Symptoms of more profound hypoxia begin when methemoglobin levels exceed 20% of total Hb and at levels above 70% may cause death [3]. However, symptoms depend on the speed of metHb formation. Although, many of patients with lifespan methemoglobinemia are asymptomatic, but the levels of metHb are abruptly increased that may be severely symptomatic including anemia, characterized Heinz bodies in RBC, acute intravascular hemolysis and jaundice [4]. Exposure to the oxidizing substances, including anesthetics (*e.g.* benzocaine, lidocaine, prilocaine), quinines, sulfonamides, *etc.*, is the most common cause of hemin formation and methemo-globinemia. Moreover, under inflammation, the reactive oxygen species (ROSs) such as superoxide anion ($O_2^{\cdot-}$) are produced, which convert Hb to metHb. The formation of these species is increased in diabetic conditions through

*Corresponding author. E-mail: mhabibi@ ut.ac.ir

non-enzymatic glycosylation or glycation of proteins [4]. Glycation is a process in which reducing sugar (s) react spontaneously with amino groups in proteins to produce advanced glycated end products (AGEs) [5]. These reactions introduce considerable alterations in the structure of proteins [6]. The well-known form of the glycated hemoglobin is $HbA_{1c}$, which has attained significant prominence in the modern world of medical biology due to its use as a marker in the long-term control of diabetes mellitus [7]. $HbA_{1c}$ is formed through a non-enzymatic reaction between sugar and N-terminal valine residue of the β-chain of $HbA_0$ [8]. Interestingly, the rate of metHb formation by auto-oxidation is significantly higher in $HbA_{1c}$ than in $HbA_0$ [9]. The advancement of the glycation process, mostly involved by lysyl-ε-amino groups, results in advanced glycation end products of Hb (Hb-AGE) [10]. According the data provided with this paper, although it has been reported that $H_2O_2$ does not cause the degradation of the hemin structure in metHb [11], but hemin can be degraded in diabetic condition upon fructation process. Our data might provide a better understanding of progressive hypoxia involved in diabetic complications.

## MATERIALS AND METHODS

### Chemicals

Bovine methemoglobin (metHb) was obtained from Sigma Chemical Co. as a lyophilized powder and confirmed by the absorptivity [12]. All other chemicals were purchased from Merck.

### *In Vitro* Preparation of Glycated metHb

Bovine methemoglobin (15 µM) and fructose (30 mM) were dissolved in a 50 mM phosphate buffer (pH 7.4). The solution was sterilized by filtering through low protein binding filter (Millex®-GV 0.22. µm filter unit, Millipore) and then incubated under sterile conditions at 37 °C in the dark. The control solution was prepared in the same way, but without fructose. At the intervals, 5 ml samples were collected and the protein concentrations were estimated by the Bradford method in which bovine methemoglobin was used as the standard. The samples were immediately frozen and stored at -70 °C for further analysis.

### UV-Vis Spectroscopy

The UV-Vis absorption spectra were obtained in a 1 cm quartz cuvette at 25 °C (Camspect model M550). Spectral changes of the fructated hemoglobin were monitored by recording the UV-Vis absorption in the range of 250-500 nm. Phosphate buffer 50 mM (pH 7.4) was used to adjust the protein concentration at 100 µg $ml^{-1}$.

### Electrochemical Analysis

For electrochemical analysis, the samples were dialyzed extensively against 100 mM $KNO_3$ at 4 °C, to remove the interference of phosphate in the cyclic voltammetry CV spectrum. Electrochemical analysis measurements were carried out with a Potentiostat/Galvanostat (Model 263A, EG&G, USA) using a single-compartment voltammetric cell equipped with a platinum rod auxiliary electrode, an Ag/AgCl reference electrode (purchased from Metrohm Co.) and an iodide modified working electrode of silver with a disk diameter of 1 mm (from Azar Electrode Co., Iran).

### Monitoring the Heme Degradation Products

Fluorescent heme degradation products during glycation of hemoglobin were detected using a Hitachi F-4500 spectrofluorometer. Sets of fluorescent products were analyzed against fructation time in which the emission spectra were scanned from 340-500 nm with an excitation wavelength of 321 nm and from 480-600 nm after the excitation at 460 nm at 10 nm band. The spectra were corrected with appropriate protein and buffer blanks. These experiments were performed at 25 °C.

### Detection of Ferrylhemoglobin Formation

Ferryl/Oxoferryl-hemoglobin (oxoferrylHb) was detected by its reaction with sodium sulfide ($Na_2S$) as it was formed during the incubation of hemoglobin with fructose through an irreversible production of sulfhemoglobin [13]. $Na_2S$ (500 µM) was added to the samples (7.5 µM) and incubated at 25 °C for 30 min and sulfhemoglobin was detected at 620 nm.

## RESULTS

Glycation of metHb by 30 mM fructose caused profound

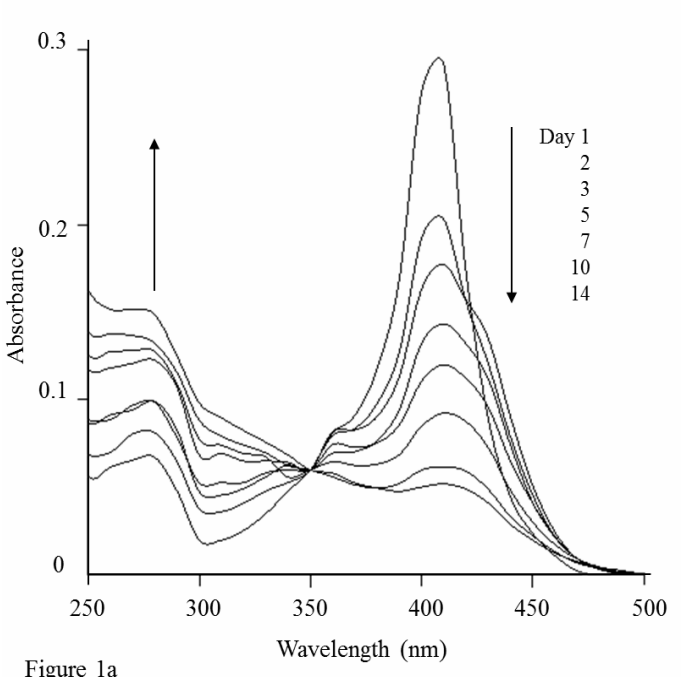


Figure 1a

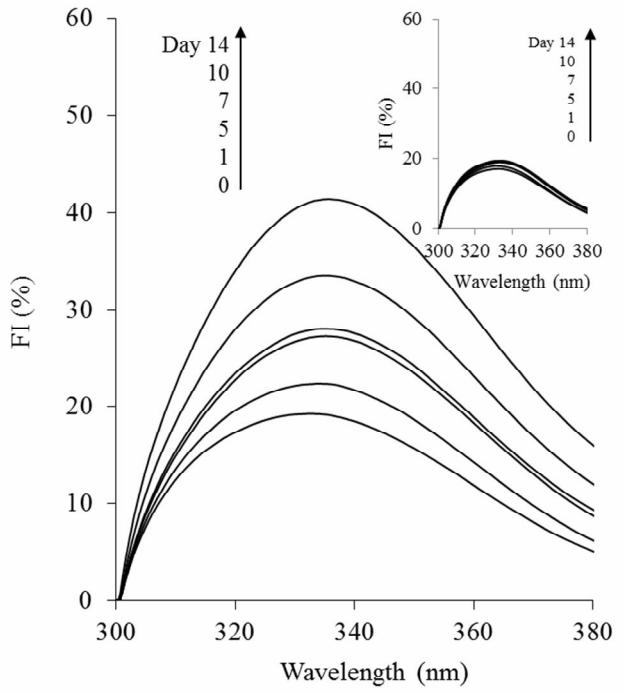


**Fig. 1.** a) UV-Vis spectra of methemoglobin during 14-days incubation with fructose. b) Intrinsic fluorescence emission spectra (ex wavelength 280 nm) of Hb-AGE during 14 days of incubation of metHb with fructose. Inset to figure presents the intrinsic fluorescence emission of control sample incubated in fructose-free condition.

effects on the UV-Vis spectrum of the protein during 14 days of incubation time (Fig. 1a). We observed the extensive hypochromic effect and a small bathochromic effect in the Soret region (410 nm) upon glycation of metHb by fructose. The hyperchromic effect was also detected in the UV region due to the structural alteration in metHb. The sugar-free control samples however, exhibited no changes in the UV-Vis spectrum throughout the time course of the experiment. Interestingly, the fructation-induced alterations of metHb structure progressed through the formation of intermediate products with the well-defined isobestic point at 350 nm (Fig. 1a). Next, we analyzed the intrinsic fluorescence spectra of fructated metHb. Excitation at 280 nm of glycated metHb revealed an increase in emission intensities along with a slight shift (3 nm) towards the red region of the wavelengths (Fig. 1b), meanwhile as depicted in the inset to Fig. 1b, the intrinsic fluorescence of the control sample has not been remarkably changed.

Next, we performed electrochemical analysis of fructated hemoglobin using cyclic voltammetric approach. Figure 2 presents the cathodic peaks of cyclic voltammogram (CV) of methemoglobin during 7-days incubation with fructose. We found positive shifts in the cathodic potential of methemoglobin incubated with fructose throughout the time course of the experiment. Concurrently, the current also declined along with the progression of the fructation process.

Fluorescence spectroscopy analysis revealed the formation of two hemin degradation products during fructation of metHb. These fluorescence species are characterized by emission at 417 and 525 nm after excitation at 321 and 460 nm, respectively (Fig. 3). We found an increase in the emission intensity for both species during fructation of metHb. Control samples exhibited no remarkable changes.

To explore whether the fructation-induced hemin degradation products are disassociated from the metHb structure, samples were dialyzed. We observed a significant decrease in the emission intensity of fluorescence species (321/417 nm and 460/525 nm) after dialysis procedure (Fig. 4).

Finally, we investigated the formation of ferryl and oxoferryl metHb by detecting sulfhemoglobin, which is produced by reaction of these molecules with $Na_2S$. Figure 5 shows a significant increase in ferrylHb/oxoferrylHb formation during metHb fructation. The formation of these structures attained its maximum level at day 3 and then decreased to an almost steady level. We found no significant changes in the control samples.

## DISCUSSIONS

In this study we investigated the fructation-induced hemin degradation of metHb, based on the following facts; 1) the amount of fructose has been shown to be increased more effectively than glucose in the erythrocytes of diabetic patients [14]; 2) the rate of metHb formation by auto-oxidation is significantly higher in $HbA_{1c}$ than in $HbA_0$ [9]; 3) although, $H_2O_2$ induces the degradation of heme moiety in Hb, it does not stimulate degradation of the hemin structure in metHb [11]. However, we are reporting that fructose induces the oxidative degradation of hemin structure during glycation of metHb. We showed structural changes of fructated metHb using near-UV-Vis. spectroscopy and intrinsic fluorescence properties. Observed extensive hypochromic effect in the Soret region, indicates the hemin loss/degradation during the fructation process. Interestingly, the presence of a well-defined isobestic point at 350 nm suggests that the fructation-induced alterations succeeded through defined intermediate products.

These observations were supported with cyclic voltammetery (CV) analysis by monitoring the cathodic current as the function of the electrochemical potential. Observed positive potential shift and the declined current at the peaks might be due to a decrease in the number of metHb molecules with the preserved hemin groups during the fructation of this protein. The reversible nature of the heme-globin binding in hemoglobin [15] and a weaker heme-globin linkage in $HbA_{1c}$ than $HbA_0$ [9] have been reported. The iron-protoporphyrin leakage makes it a preferential target to be attacked by reactive oxygen species (ROS) generated during the glycation process [11]. This interpretation is strongly supported by high susceptibility of hemoglobin and myoglobin damage by glucose which may leads to the separation and/or degradation of heme groups [16]. In the diabetic conditions, the loosening of the heme and its degradation not only alters the normal function of

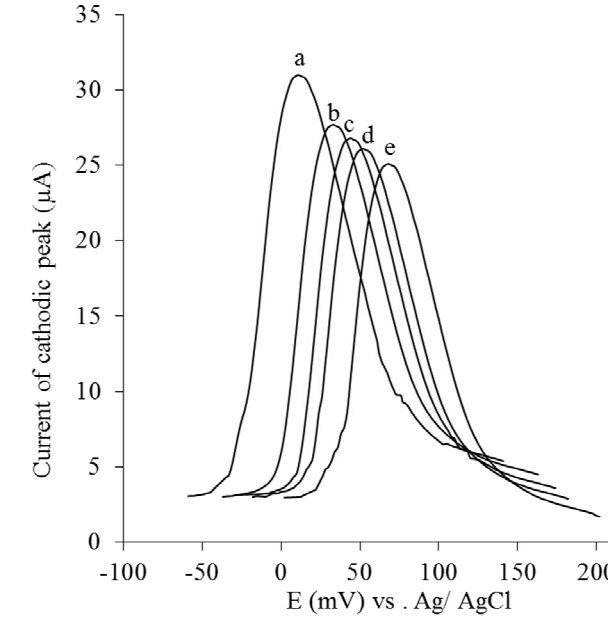


**Fig. 2.** Cathodic peak of methemoglobin by iodide modified silver electrode during different days of incubation with fructose.

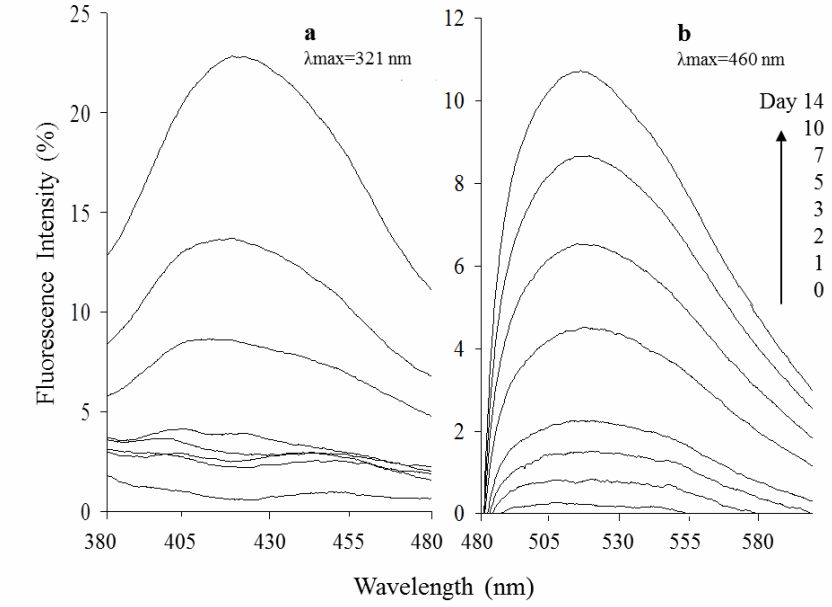


**Fig. 3.** Time course experiment of hemin degradation, monitored through the formation of two fluorescence species during the reaction of metHb with fructose. (a) The emission spectra from 340-500 nm at excitation wavelength of 321 nm and (b) the emission spectra from 480-600 nm at excitation wavelength of 460 nm.

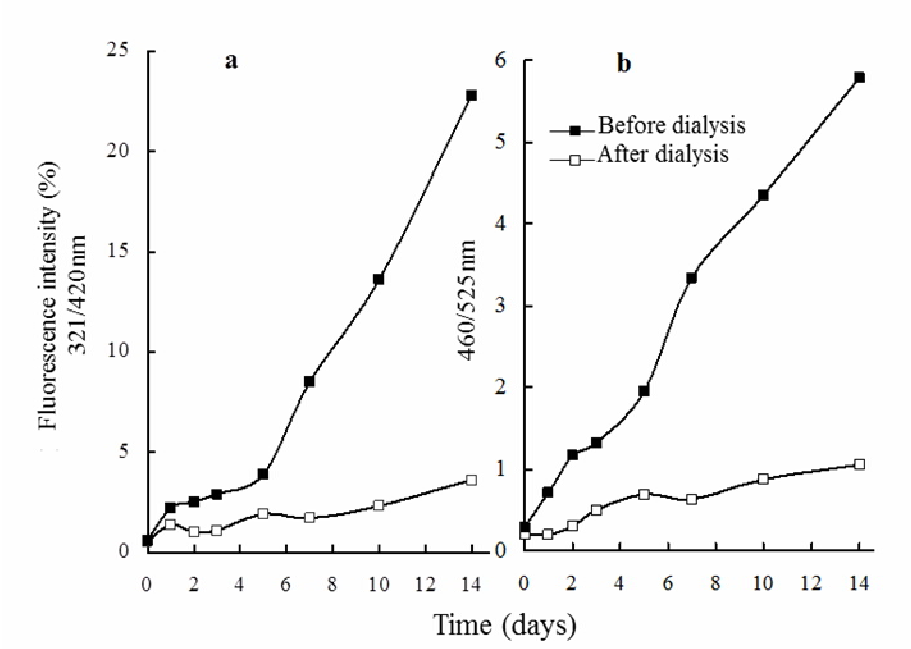


**Fig. 4.** The hemin degradation products of metHb with and without dialysis process. (a) Progressive intensification of the fluorescence emission (a) at 417 nm after the excitation at 321 nm and (b) at 525 nm after the excitation at 460 nm.

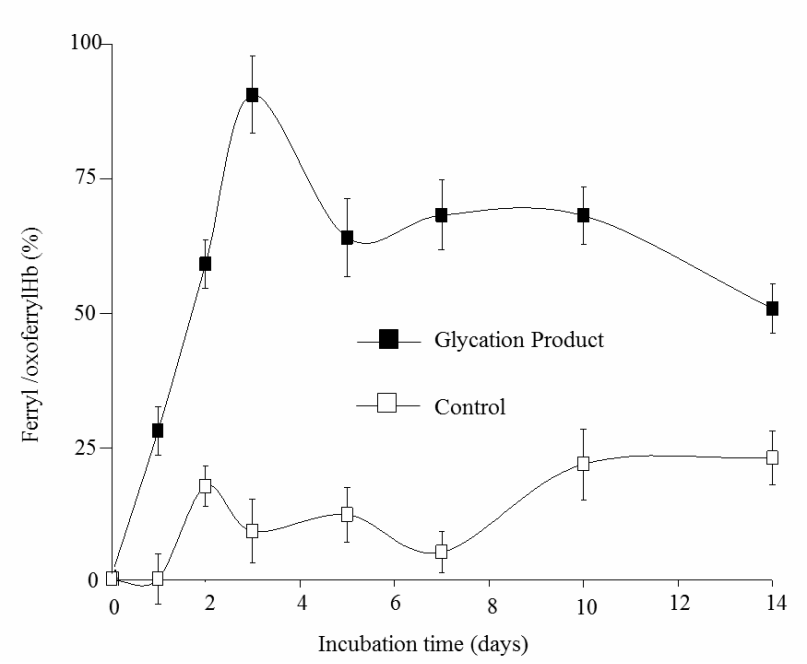


**Fig. 5.** Ferryl/OxoferrylHb formation during glycation of metHb by fructose. Glycated metHb and control samples were incubated with sodium sulfide ($Na_2S$) and the formation of sulfhemoglobin was detected at 620 nm.

Hb but affects the erythrocytes membrane [17].

Although the heme or hemin enzymatic degradation products by heme oxygenases (HO) have been well characterized, the non-enzymatic degradation products of these molecules have not been identified [18]. Intact heme, by itself, is known to be non-fluorescent, whereas heme degradation products have been detected *via* their featured fluorescence excitation/emission ($\lambda_{ex}/\lambda_{em}$) pair properties [6,18,19]. The reaction of hemoglobin with $H_2O_2$ results in the production of dityrosine structure with fluorescent properties of excitation at 320 nm and emission at 410 nm [20]. Morover, non-enzymatic degradation of heme by $H_2O_2$ [21] or auto-oxidation of oxyhemoglobin [22], produce two fluorescent species at 321/465 and 460/525 ($\lambda_{ex}/\lambda_{em}$) nm. We found two fluorescent species, with fluorescence properties at 321/417 and 460/525 nm ($\lambda_{ex}/\lambda_{em}$) during glycation of metHb by fructose. Interestingely, excitation at 321 nm of hemin-degradation products induced emission neither at 410 nor at 465 nm but at 417 nm. To explore whether the observed emission at 417 nm is due to the dityrosine-related fluorescence, the fructated samples were extensively dialyzed against buffer. The observed fluorescent species were significantly reduced upon dialysis, indicating the different characteristics of hemin degradation products with the non-dialysable di-tyrosine moieties formed during the oxidation of hemoglobin by $H_2O_2$ [20].

It has been shown that the degradation of heme or hemin by free radicals, occur through the formation of intermediate molecules ferrylhemoglobin (ferrylHb) and oxoferryl-hemoglobin (oxoferrylHb), respectively [19].

$$\text{Hb (FeII)} + H_2O_2 \rightarrow \text{ferrylHb (FeIV)} + H_2O + O_2$$

$$\text{metHb (FeIII)} + H_2O_2 \rightarrow \text{oxoferrylHb (FeV)} + H_2O$$

Our data showed that the early production of these unstable species is increased until day 3, then decreased to almost steady level during fructation-induced hemin degradation. The same pattern of ferryl formation during interaction of Hb with $H_2O_2$ has been previously reported [11]. Accordingly, a major fraction of the heme degradation occurs after the utmost ferrylHb formation, indicating that the degradation is not a direct result of ferrylHb formation, but involves a subsequent reaction of ferrylHb [11].

Development of Alzheimer's disease (AD) in diabetics not only occurs by possible glycation involved processes [23-25] but also the heme deficiency may contributes to AD pathogenesis [6,18,26]. If true, factors that contribute to the intracellular heme deficiency could potentially alter the course of AD [27]. Neuronal hemoprotein, neuroglobin (Ngb) is a recently discovered member of the globin family with protective effects in animal models of AD but the relevance of these effects to Alzheimer's disease in humans is unknown. Interestingly the level of Ngb is significantly reduced in mouse model of AD and negatively correlated with tau phosphorylation [28]. Moreover the level of this protein is increased in early and moderately advanced AD, but declined in the severe stages [29]. Therefore our data might explain the possible contribution of glycation-induced hemin degradation to the evelopment of Alzheimer's disease (AD) in diabetic conditions.

In conclusion, reactive oxygen species are assumed to be involved in hemin degradation upon metHb fructation. Our results supports that the glycation-induced hemin destruction may involved in long term diabetic conditions in which heme of the $HbA_0$ is significantly auto-oxidized to hemin in $HbA_{1c}$. Our data might provide a better understanding of progressive hypoxia involved in diabetic complications that may explain a possible mechanism involved in the reduced or loss of protective role of neural hemoprotein (*i.e.* Ngb) in AD that remains to be explored.

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