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# Non-Enzymatic Glycation with D-Glucose Impairs the Biological Function of Human Serum Albumin: Relevance in Diabetes Mellitus

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Human serum albumin (HSA) non-enzymatic glycation leads to the development of advanced glycation end products (AGEs). HSA was modified with D-glucose for a time span of 40 days at 370C. Post formation of AGEs, the native and glycated-HSA were evaluated for metformin hydrochloride, Cu (II) and oleic acid binding using spectroscopic techniques including UV, fluorescence, and FTIR. The molecular docking with fatty acids and HSA was also performed both in the presence and absence of glucose. The results showed functional impairments of HSA upon non-enzymatic glycation with glucose. The results of the present study clearly showed that hyperglycemia in diabetes mellitus leads to impairments in transport and binding property of HSA.

**Introduction:** Human serum albumin (HSA) is the abundant biomacromolecule in blood plasma and performs vital biological functions. The three-dimensional structure of HSA revealed by X-ray crystallography revealed that HSA has a molecular weight of 66.7 kDa and comprises of the single polypeptide chain of 585 amino acids (1). It is a globular protein with three domains (I- III), each of which consists of two sub-domains A and B stabilize by 17 disulfide bridges (1). Ligands with the aromatic and heterocyclic attributes are found to interact with two hydrophobic pockets present in sub-domains sites IIA and IIIA (1). HSA is known to perform transport and storage functions. HSA is an abundant plasma protein capable of binding with a variety of exogenous and endogenous compounds such as drugs, hormones, fatty acids, metals thereby serving as transporter functions (2). Sulfonylurea drug binding adversely affected by glycation as

shown by several studies (3). These drugs widely prescribed in the treatment of type 2 diabetes that shows effective binding to HSA Sudlow sites I and II. The literature showed that changes in affinity at Sudlow site I and II range from 0.6 fold to 6.0 fold in glycated-HSA and normal HSA for given sulfonylurea drugs (4). Quenching measurement of HSA fluorescence in presence of drug bound to it is an important method to investigate the interactions, as it can reveal the accessibility of quenchers applied to interact with albumin's fluorophore groups, helps to identify the binding mechanisms of HSA with drugs and provide clues for their binding (5). HSA being the transporter for drugs also act as a major contributor for non-esterified fatty acids (FA) for performing vital functions. There are seven binding sites for fatty acids present on HSA as revealed by extensive structure analysis that includes sub-domains IB, IIIA, and IIIB along with subdomain interfaces (6). In one of the previously published study, a 3D model suggested that site for the fatty acid-binding present in the hydrophobic tunnel (7). Human serum albumin including Bovine serum albumin (BSA) consists of N-terminal sequence X+Y+His containing strong, physical interaction sites for endogenous Cu (II) and toxic Ni (II). Another canine or porcine albumin has His-3 substituted with Tyr residue hence unable to bind these metals ions at N-terminus (8).

Non-enzymatic glycation results in the formation of heterogeneous moieties. In the present study, the AGEs formed upon incubation of HSA with glucose may alter the binding properties of the drug, especially metformin hydrochloride, fatty acids such as oleic acid along with metals such as Cu (II). The approach used in the present study clearly showed that even a low glucose concentration initiates the Maillard reaction with HSA that can lead to the alterations of binding properties of HSA and may lead to its impaired biological functions. The clear rationale behind using Cu+2 and oleic acid in the present study is that HSA has the biological ability to bind

reversibly with saturated and unsaturated fatty acids including oleic acid and copper. Moreover, HSA helps in the maintenance of copper homeostasis. It was evident from the literature that 10- 15% copper is HSA bounded that prevents oxidative damage occurring due to Cu+2/Cu+1 in Fenton chemistry.

**Experimental**

Human serum albumin (HSA), metformin hydrochloride (99.5%), Oleic acid and Cu (II) was procured from Sigma chemical company (St. Louis, USA), D-glucose, sodium azide, and Copper(II) chloride anhydrous powder was procured from SRL chemicals, India. All other chemicals and reagents used were of the highest analytical grade available.

**Preparation of glycated-HSA**

HSA (20 µM) was incubated under sterile conditions with varying concentration of D-glucose (5.55, 11.10, 16.65 and 22.20 mmol/L) in phosphate buffer saline (20 mM, pH=7.5) in the presence of 0.01 % sodium azide solution at 370C under sterile conditions in capped sterile tubes for 40 days. At the termination of incubation, the samples were extensively dialyzed against sterile PBS buffer (10mM, pH=7.4) with consecutively two changes of PBS buffer overnight at 40C to remove excess glucose (9-12).

***In- vitro* Drug Binding**

Fluorometric measurements for drug interaction methods were carried out with Shimadzu (RF- 5301- PC) spectrofluorimeter. The fluorescence spectra were recorded with an excitation wavelength of 370 nm and emission in the range of 400-700 nm with a slit width of monochromator was taken 2.5 nm both in excitation and emission. Native (unmodified) and

glycated- HSA (modified with 5.55, 11.10, 16.65 and 22.20 mmol/L glucose) (3 mL) was titrated against the concentrations of metformin hydrochloride and fluorescence intensity was taken after an equilibrium time of 1 min. The concentration of HSA protein (20 µM) chosen for titration with drug concentrations ranging from 20 to 500 µM.

***In- vitro* Fatty acid Binding**

Native and glycated-HSA (20 µM) are mixed with ethanolic solutions of unlabeled oleic acid (100 mM). The ethanolic solution (one vol.) was added to HSA samples (50 vol.). The fatty acids bound HSA samples were lyophilized in a vacuum and dried in presence of silica gel at 40C followed by dissolution in PBS buffer (20 mM) pH =7.4 forming clear solutions for analysis (13).

In-vitro metal binding

Native and glycated-HSA (20 µM) was mixed with 3 mM Cu (II) to adjust the final concentration ratio of 1:10. HSA was adsorbed onto a negative ion exchange resin (DEAE). The excess metal was removed from the HSA sample. The supernatant was discarded followed by the addition of 50mM HEPES buffer (pH=7.4) and centrifugation from the resin at 15000 rpm for 1 min. The supernatant was removed followed by washing. The HSA protein was eluted by 1 M NaCl solution, centrifugation at 15000 rpm for 1 min for the recovery of the supernatant. The supernatant was diluted 10-15 times with 50 mM HEPES buffer (pH 7.4) and HSA samples were centrifuged at 3000 rpm. The spin-column was used to concentrate on the metal-bound protein samples (14).

**Absorption spectroscopy analysis**

UV absorption spectra of native and glycated samples bound with ligands i.e. metformin hydrochloride, oleic acid and Cu (II) were recorded in the wavelength range of 250-400 nm on Shimadzu UV-1700 spectrophotometer with quartz cuvette having 1 cm path length (9-12).

**Fluorescence spectroscopy analysis**

Fluorescence spectra to detect structural changes of native and glycated samples bound with ligands i.e. metformin hydrochloride, oleic acid and Cu (II) were performed on Shimadzu (RF- 5301-PC) spectrofluorometer at 25+0.20C using 1.00 cm cuvettes at an excitation wavelength of 370 nm and emission spectra range of 400-700 nm (9-12).

**Attenuated total reflectance-Fourier transform infrared spectroscopy**

To demonstrate the change in secondary structure upon glycation Fourier transform- infrared spectroscopy analysis of native and HSA modified with glucose bound with ligands i.e. metformin hydrochloride, oleic acid and Cu (II) was recorded. Briefly, 10 µl (20 µM) of the protein sample was placed on the ATR accessory on the FT-IR spectrophotometer (8201 PC) with a resolution of 4 cm-1. FT-IR measurements of native and glycated samples were carried on Shimadzu FT- IR spectrophotometer (8201-PC) in the spectral range of 1200- 2000 cm-1 (9-12).

**Molecular docking**

The structural coordinates of fatty acids; Oleic Acid [PubChem CID: 445639], Linoleic Acid [Pubchem: 5280450] and Arachidonic Acid [PubChem CID: 444899] were retrieved from NCBI PubChem compound database (https://pubchem.ncbi.nlm.nih.gov/) in three dimensional (3D) SDF-file format and then were converted to PDB format using Open Babel (15). The crystallographic structure of HSA with glucose (PDB ID: 4IW2) is downloaded from the RCSB

PDB database (http://www.rcsb.org/) (16) and binding pockets and sub-pockets are predicted using DoGSiteScorer (18). The protein is then prepared for two series of docking experiments;

(i) by deleting all the heteroatoms present in the macromolecule and (ii) by deleting all the heteroatoms except Glucose present in them. These two series of experiments were carried to see the effect of presence and absence of Glucose in fatty acid-binding.

Docking simulations were performed using AutoDock 4.2-MGL Tools version 1.5.6, a molecular docking software package that implements the Lamarckian genetic algorithm. All fatty acids were converted to PDBQT file format which stores the atomic coordinates, partial charges, and AutoDock atom types. Torsion angles were calculated to assign the fixable and non-bonded rotations of the molecule (18). In this study, we used a flexible docking procedure, in which protein is treated as a rigid molecule whereas the fatty acids, as flexible. Polar hydrogen atoms were added to the protein and non-polar hydrogen is merged. Gasteiger charge of the macromolecule was added. The grid size was set to 100, 100 and 100 along the X-, Y- and Z-axis and spacing set to 0.636 Å with the intention of covering all predicted binding pockets

**For finding out best possible binding site both on the surface and inside of the domains.**

During the docking procedure, 100 conformations were considered for each fatty acid inhibitor for Lamarckian genetic algorithm search, other assorted parameters were set to the default values of the AUTODOCK 4.2 program. The best-docked conformations of fatty acids were selected based on binding energy, inhibition constant and intermolecular energy. The docking results and predicted interactions were investigated using Discovery Studio Visualizer and LIGPLOT (19).

**Results and Discussion**

Non-enzymatic glycation induced structural alterations were reported by ultraviolet absorption spectroscopy. Native HSA (unmodified) gives characteristics peak at λ280 nm (Fig. 1a), while the glycated-HSA (glucose modified) bound with metformin hydrochloride, Cu(II), and oleic acid showed hypochromic as clearly demonstrated in Fig. 1b - 1d respectively. HSA (20 µM) modified with varying D-glucose for the formation of advanced glycation end products. Metformin hydrochloride, Cu (II), and oleic acid were added to post modified HSA. Glucose modified HSA showed 65.82%, 71.98%, 73.62% and 76.63% hyperchromicity without ligandswith 5.55, 11.10, 16.65 and 22.20 mmol/L glucose respectively {Fig 1a (10)}. Whereas, glycated-HSA bounded ligands showed less UV absorption (i.e hypochromic effect) compared to unbound glycated-HSA.

Fluorescence measurement of the fluorogenic AGEs was measured for glycated- HSA both in the presence and in the absence of ligands. Fig 2a, clearly demonstrated that native HSA with negligible fluorescence, while glycated-HSA with increased fluorescence intensity of 23.82%, 35.69%, 42.87%, and 68.22% {Fig 2a (10)}. Similarly, AGEs specific fluorescence of native and glycated-HSA recorded at an excitation wavelength of 370 nm and with an emission range of 400- 600 nm showed a decrease in fluorescence intensity in glycated-HSA bound with ligands (Fig. 2b-2d) compared to HSA with unbound ligands.

FTIR spectra of both native and glycated-HSA bound with ligands (i.e. metformin hydrochloride, Cu (II) and oleic acid) were recorded as shown in Fig 3a and 3b-d respectively to demonstrate the alterations in the secondary structure on the basis of frequency and shapes of amide I and amide II bands. The figure depicts that the native amide I peak appears at 1656 cm- 1, while the glycated HSA showed shifting in the amide I peak at 1659 cm-1. The ligands bound to the glycated-HSA also showed shifting in the amide I peak compared to the native one.

Similarly, amide II peak (N-H bend vibrations of peptide bonds) in glycated-HSA bound with ligands showed a shifting of 1554 cm-1 compared to native unmodified HSA, which appeared at 1564 cm-1.

The molecular docking analysis was done and the output file was generated from the docking study and as shown in Fig 4. During the analysis, the

binding energy, inhibition constant (Ki), intermolecular energy, total internal energy and the no. of hydrogen bonds were considered for the analysis. All fatty acids were found to dock on the area between 2 HSA domains. The results are shown in table 1 showing details of the docking procedure. Figure 5 clearly showed docking poses (i.e. Position of fatty acids and glucose in HSA after docking). The 2D lig plots between HSA and fatty acids were also obtained both in the presence and absence of glucose and shown in Fig 6.

Glycation enabled structural perturbations in glycated-HSA samples were determined by UV absorption spectroscopy. Native HSA gave a characteristic peak at 280 nm, whereas in glucose modified samples of HSA showed an increasing % hyperchromicity. Furthermore, binding of native HSA and glycated-HSA with metformin hydrochloride, oleic acid and Cu (II) causes decreases in the absorbance. This decrease in absorbance contributed mainly by masking and binding of aromatic amino acids with or by the ligands in the structure of HSA. Masking of bonding electrons also contributes to this characteristic. There is an abundance of positively charged amino acids located in fatty acid-binding sites that contribute to the static relation with the anionic carboxylate of fatty acid and ε-amino group of lysine also of quite an importance in this interaction. The interaction between these chemical moieties leads to a decrease in absorbance (20). Glycation primarily occurs at Lys-525 residue of HSA that contributes to about 30 % of glycation mechanism (21). X-ray crystallography study by Bhattacharya et. al. revealed

HSA interact with six molecules of fatty acids such as myristic acid, palmitic acid and stearic acid proving that Lys-525 in sub-domain IIIb interacted with carbonyl myristic acid (22), this interaction with glycated-HSA become loosen thereby contributing to reduction of UV absorption due to steric hindrance.

The formation of AGEs was measured from the emission fluorescence intensity recorded at an excitation wavelength of 370 nm. Native HSA at this excitation wavelength showed negligible changes in fluorescence. Similarly, there is a decrease in fluorescence intensity with glycated- HSA and native HSA bounded with metformin hydrochloride, Cu(II) and oleic acid were found in the present study. Previously published literature demonstrated the effects of glycation on drug and fatty acids binding with the major binding regions of HSA. For instance, drug warfarin and its enantiomeric analog have been widely used as site-selective probes for Sudlow site I of HSA (23).

The alterations that have been observed for binding of ligands to native and glycated- HSA using fluorescence spectroscopy or equilibrium dialysis at 20-250 C noted a six-fold decrease in the intensity (20). Glycated-HSA showed a comparatively lower affinity for fatty acids, drugs, and metals compared to native HSA. Sub-domains IB and IIA of HSA form the drug interacting site I which contributes mainly in the binding pocket for anionic heterocyclic molecules, whereas site II can accommodate drugs and fatty acids with aromatic rings and extended conformations. The reduced binding of drugs, fatty acids, and metals with glycated- HSA can mainly attribute to the modification of arginine residues (Arg- 194, Arg-196, Arg-198, Arg-217, and Arg-409), which are the major ligand-interacting residues. Lys- Lys-204, Lys- 411, Lys-431 of the site I and II also involved in drug binding. The formation of AGEs HSA also reduces the accessibility of these pockets to the drugs that contribute to decreasing fluorescence intensity. HSA has an intrinsic fluorescence because of the Trp and Tyr amino acids in its structure which using

excitation wavelength at 295nm and the emission of Trp can be detected. The quenching process can be usually induced by a collision process or a formation of a complex between quencher and fluorophore. The former is referred to as a dynamic quenching mechanism and the latter a static quenching mechanism.

Binding of ligand metformin hydrochloride, oleic acid and Cu (II) with native and glycated- HSA induces conformational changes in the secondary structure of the HSA that leads to the shifting of amide I and amide II bonds in HSA. The microenvironment of the native HSA upon glycation gets altered due to a decrease in the compactness of the globules and reduction in the interaction between domains I and II of HSA. Being a protein of importance, HSA bears 18 tyrosine residues that reshuffle due to change in microenvironment upon the addition of glucose.

**Conclusions**

In conclusion, the results of the present work reveal that the non-enzymatic glycation of human serum albumin with glucose leads to the formation of advanced glycation end products that furthermore impairs it's binding and transport function. During a state of uncontrolled hyperglycemia in diabetes mellitus, human serum albumin loses its function leading to diabetes- associated complications.