STUDIES ON GLYCATED-HSA: ASSESSMENT OF OPTIMAL CUT OFF VALUE OF GLYCATED-HSA AND ITS CLINICAL RELEVANCE IN DIABETES AND DIABETES ASSOCIATED PATHOLOGIES

ABSTRACT OF THE
THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy
in
Endocrinology

BY

ALOK RAGHAV

Under the Supervision of
PROF. JAMAL AHMAD

RAJIV GANDHI CENTRE FOR DIABETES & ENDOCRINOLOGY,
J.N MEDICAL COLLEGE
ALIGARH MUSLIM UNIVERSITY
ALIGARH-202002, INDIA
2016
**Abstract**

**Background:** In the past 60 years, the entire world population showed an increased incidence of chronic diseases, proving to be a prime concern for developing the World and upcoming generations. Over the past 30 years, diabetes has transformed from its mild version to a major cause of morbidity and mortality affecting middle age and youth population. Diabetes is rapidly emerging as a major threat to public health in India. The pathophysiology of diabetes mellitus reveals that it is not a single disorder, but is a collection of several disorders with different underlying causes and with multiple hormonal abnormalities. It is characterized by disordered carbohydrate metabolism with hyperglycemia resulting from dysfunction of insulin secretion, insulin action or both. The long-term complications of diabetes mellitus include the progressive development of retinopathy, nephropathy, foot ulcers, neuropathy, cardiovascular diseases, and amputation. Albumin is one of the major and largest plasma proteins. Diabetes is characterized by chronic persistent hyperglycemia, due to insulin deficiency or insulin resistance. Hyperglycaemia enables biological macromolecules like proteins, nucleic acids and lipids to participate in the glycation or glucose-induced modifications. Proteins are the best participant for non-enzymatic glycation. Human serum albumin, being the most abundant lysine and arginine-rich plasma protein favors the glycation reaction. The side chains of lysine and arginine forms Amadori adduct via Schiff base reaction, that further undergo chemical rearrangement and forms advanced glycation end products (AGEs). AGEs are the sources of various secondary complications of diabetes mellitus. Diabetic patients with uncontrolled or poor glycemic control show the presence of AGEs in the plasma that initiates the immunological cascades of inflammations upon binding with RAGE receptors. The credit to exacerbating immune response goes to structural alterations also that form neo-epitopes upon glycation during a change in the microenvironment of amino residues within the HSA structure. Impairment in the structure of HSA also impairs its function of binding and transport of essential metals, fatty acids, and life-saving drugs. The aim of this thesis was to establish the relationship between impairment of human serum albumin (HSA) protein structure and functions during glycation, and the establishment of the optimal cut-off value of glycated albumin (GA) as a new diagnostic glycemic marker in diabetic patients with and without complications along with healthy north Indian population. The present work also aims
to establish a correlation between glycated albumin (GA) in stage I, II, III and IV of diabetic nephropathy depending on eGFR values. Association between serum fructosamine content and HbA1c was established in subjects with diabetes and its associated complications.

Methods: The present study is the combo of experimental and clinical approach in glycation. In experimental part HSA (20μM) was incubated with varying D-glucose concentrations of 100, 200, 300 and 400 mg/dL glucose for a period of 40 days to form AGEs at 37°C under sterile conditions followed by overnight dialysis against PBS buffer (10 mM) at 4°C. Confirmation of AGEs was done with UV-spectroscopy at 280 nm, AGEs (pentosidine specific) fluorescence at an excitation wavelength of 370 nm. Intrinsic tryptophan quenching was studied at an excitation wavelength of 285 nm. Biochemical assays such as fructosamine assay, 5-hydroxymethyl furfural assay, free thiol content, carbonyl content, free lysine content, free arginine content were done with glycated and native HSA. Glycation-induced hydrophobicity was demonstrated by i-anilino-8-napthalene sulphonlic acid binding assay. The cross-links produced upon glycation was evaluated with thioflavin-T, congo-red assay. Electrochemical and fluidity paradigm includes specific electrical conductance, hydrodynamic radius, stokes radius and intrinsic viscosity measurement was done for native and glycated-HSA. Gel electrophoresis of all protein samples were done to confer glycation induced changes.

Spectroscopic and biochemical analysis confirm that non-enzymatic glycation forms AGEs with altered physiology of human serum albumin. Fluorescent AGEs were characterized by pentosidine specific fluorescence at 370 nm excitation wavelength. The occurrence of non-fluorescent AGEs such as carboxymethyl-lysine (CML) in glycated-HSA samples was estimated with liquid chromatography mass spectroscopy (LCMS) scan with a range of 60 to 400 m/z values. Fourier transform infrared spectroscopy (FTIR) spectra of native and glycated-HSA (400 mg/dL) was recorded from 1200 to 2000 cm⁻¹ range and result in transmittance were obtained to demonstrate the impairment in secondary structure upon glycation. Far (200-250 nm) and near UV (250-320 nm) circular dichroism (CD) spectroscopy of native and glycated-HSA was done to confirm the damage to secondary structures of HSA upon glycation. High-performance liquid chromatography (HPLC) was done for acid hydrolysed native and glycated (400 mg dL glycated-HSA) samples along with CML
standard alone. Non-enzymatic glycation increases the intact mass of HSA due to the addition of sugars to the lysine and arginine of the proteins. The content of % carbon and hydrogen was quantified with the LECO-CHNS-932 elemental analyzer in native and glycated-HSA.

Structural alterations in HSA upon glycation possess the formation of neo-epitopes that contribute to the immunogenicity potential of HSA in experimental animals and human subjects. Well characterized AGEs (100 µg) complexed with complete Freund's adjuvant followed by six weekly booster dose of Freund's incomplete adjuvant were induced in New Zealand white rabbits. Sera of immunized and preimmunized rabbits were taken after completion of immunization and direct binding ELISA along with Inhibition ELISA was performed. Rectal temperature measurements along with hematological and biochemical analysis were done. To confer the specificity of epitopes against IgG immunoglobulin, further the rabbit serum was subjected to IgG isolation by affinity column. Direct binding and inhibition ELISA was performed with affinity purified IgG along with band shift assay. The antibody-antigen complex was subjected to UV analysis at 280 nm, fluorescence emission profile, and X-ray diffraction to study the binding of neo-epitopes present on IgG to glycated and native HSA.

In-situ Congo Red staining also demonstrates the formation of aggregates in the glycated albumin. The monoformazonc formation in fructosamine assay was evaluated for aggregation formation in glycated HSA at 25,000 X by transmission electron microscopy images. Furthermore, scanning electron microscopy images of native and glycated-HSA (modified with 400 mg/dL glucose) was obtained to decipher the aggregation formation. The In-situ antioxidant capacity of human serum albumin during the course of non-enzymatic glycation was assessed by AAPH assay.

In order to access the possible role of glycated-HSA in the etiology of diabetes mellitus subjects, sera from patients (T2DM (n= 50), T1DM (n= 50), GDM (n= 50) and T2DM with Chronic kidney disease (CKD) (n= 50) was assessed for their binding to native and glycated-HSA (modified with 400 mg/dL glucose) with direct binding and inhibition ELISA. The sera were subjected to affinity purification to isolate IgG from the subjects showing a high titre of binding and direct binding and Inhibition ELISA
was performed to determine the specificity of native and glycated-HSA with IgG class of immunoglobulins.

Albumin besides contributing to the maintenance of osmolality also possesses the transport of Cu(II), metformin hydrochloride, and oleic acid. Oleic acid (100 mM each) was added to an ethanolic solution of native and glycated-HSA in the ratio 1:50 (FA:HSA). Native and glycated-HSA bound fatty acid complex was evaluated for UV absorption profile at 280 nm to demonstrate the binding effects. Pentosidine specific fluorescence at an excitation of 370 nm was taken for native and glycated-HSA complexed with fatty acids to evaluate the changes during binding. FTIR spectra of native and glycated-HSA complexed with fatty acids were recorded between 1200 to 2000 cm⁻¹.

Native and glycated-HSA was evaluated \textit{In-silico} binding with metformin hydrochloride using dockingserver.com to obtain the lig plots and binding energies. Native and glycated-HSA (1 mg/mL) was titrated fluorometric against the concentration of metformin hydrochloride from 20 to 500 µM to obtain equilibrium in fluorescence intensity. Native and glycated-HSA bound drugs complex was evaluated for UV absorption profile at 280 nm to demonstrate the binding effects. Pentosidine specific fluorescence at an excitation of 370 nm was taken for native and glycated-HSA complexed with drugs to evaluate the changes during binding. FTIR spectra of native and glycated-HSA complexed with drugs were recorded between 1200 to 2000 cm⁻¹.

Native and glycated-HSA (20 mg/mL) was mixed with 3 mM Cu(II) to adjust final concentration ratio of 1:10. HSA was adsorbed onto a negative ion exchange resin (DEAE-650C). Excess metal was removed from the HSA sample. Native and glycated-HSA bound Cu(II) complex was evaluated for UV absorption profile at 280 nm to demonstrate the binding effects. Pentosidine specific fluorescence at an excitation of 370 nm was taken for native and glycated-HSA complexed with metals to evaluate the changes during binding. FTIR spectra of native and glycated-HSA complexed with metals were recorded between 1200 to 2000 cm⁻¹.

The clinical regime of this thesis includes the quantification of glycated-HSA (GA) with ELISA method in healthy (n=100) and T2DM subject with eGFR≥90mL min⁻¹ (n=167), eGFR 60-89 mL min⁻¹ (n=91), eGFR 30-59 mL min⁻¹ (n=68) and eGFR ≤29
mL/min (n=21) to establish the optimal cut-off value. Association between serum fructosamine content and HbA1c was established in subjects with diabetes and its associated complications. GA/HbA1c ratio was estimated to determine the glycemic status of in the diabetes mellitus. Other anthropometric and demographic data are recorded to know the glycemic status of the subjects.

**Results:** Glycation of HSA protein with varying D-glucose concentrations (100, 200, 300 and 400 mg/dL) damages its structure, that impairs its functions and biological properties. The primary investigations done with native and glycated-HSA showed fructosamine content in 100, 200, 300 and 400 mg/dL glycated-HSA samples as 25.32±1.35, 29.78±1.06, 41.49±0.50 and 73.16±0.97 (nmol/mg protein) respectively that was much higher than native HSA 1.44±0.05 (nmol/mg protein) at day 21s1. HMF content in nmol/mg protein of the same day samples was found much higher in glycated-HSA modified with 100 mg/dL (11.6±0.97), 200 mg/dL (21.24±1.07), 300 mg/dL (29.56±1.22) and 400 mg/dL (38.26±1.19) glucose. Native HSA showed significantly negligible HMF content of 0.92±0.01 nmol/mg. Free thiol content decreases significantly in glycated-HSA modified with 100 (84.00±5.65 nmol/mg), 200 (77.00±15.55 nmol/mg), 300 (55.00±15.55 nmol/mg) and 400 (32.50±4.94 nmol/mg) mg/dL glucose compared to native HSA (124.5±20.50 nmol/mg). Glycation lead to the formation of carbonyl adducts in glycated-HSA modified with 100 (2.82±0.03nmol/mg), 200 (3.48±0.09 nmol/mg), 300 (4.1±0.04 nmol/mg) and 400 (6.09±0.44 nmol/mg) mg/dL glucose compared to native HSA (2.37±0.03 nmol/mg). Free lysine content in 100 mg/dL glucose+HSA (70.36±0.09 nmol/mg), 200 mg/dL glucose +HSA (65.28±1.19 nmol/mg), 300 mg/dL glucose +HSA (54.00±0.18 nmol/mg) and 400 mg/dL glucose +HSA (41.25±1.06 nmol/mg) was found significantly less than native HSA (78.62±1.84 nmol/mg). Similarly content of free arginine was found to be less in glycated-HSA modified with 100 (29.77±0.09 nmol/mg), 200 (18.36±1.16 nmol/mg), 300 (11.00±0.87 nmol/mg) and 400 (5.29±0.06 nmol/mg) mg dL glucose compared to native HSA (32.56±1.08 nmol/mg). 1-anilino-8-napthalene sulphonlic acid binding assay showed percent increase of fluorescence intensity of 17.21%, 21.56%, 28.70% and 35.47% in 100 mg dL glucose +HSA, 200 mg dL glucose +HSA, 300 mg dL glucose +HSA and 400 mg dL glucose +HSA respectively compared to native. Thioflavin T binding assay showed percent increase of fluorescence intensity of 11.68%, 21.41%, 37.52% and 48.11% in 100...
mg/dL glucose +HSA, 200 mg/dL glucose +HSA, 300 mg/dL glucose +HSA and 400 mg/dL glucose +HSA respectively compared to native. The cross-links formation in glycated-HSA was demonstrated by congo red dye that showed a red shift of 490 to 540 nm compared to native HSA. Spectroscopic studies demonstrate the damage to native conformation of HSA structure. UV absorption profile at 280 nm of glycated-HSA modified with 100, 200, 300 and 400 mg/dL glucose showed percent hyperchromicity of 65.82%, 71.98%, 73.62% and 76.63% compared to native HSA. Similarly pentosidine specific fluorescence in glycated-HSA modified with 100, 200, 300 and 400 mg/dL glucose showed increase in percent fluorescence intensity of 23.82%, 35.69%, 42.87% and 68.22% compared to native HSA thereby confirming the presence of pentosidine in glycated HSA. Non fluorescent AGEs i.e carboxymethyl lysine (CML) was confirmed with LCMS analysis with a peak of 279.1 in acid hydrolyzed glycated-HSA along with CML standard alone. Native HSA devoid of such peak. The FTIR spectroscopy of glycated-HSA (modified with 400 mg/dL glucose) showed shifting of amide I and amide II peaks compared to native HSA. Glycated-HSA also showed appearance of C=O bond stretching peak, whereas native HSA devoid of such peak. Circular dichroism (CD) spectroscopy analysis in both far UV and near UV showed a marked increase of β-pleated sheets structures in glycated-HSA compared to native HSA at mean residual ellipticity (MRE) 208nm, thereby showing damage to secondary structure of HSA upon glycation due to disruption of non-covalent interactions. HPLC chromatograms of acid hydrolyzed glycated-HSA (modified with 400 mg/dL glucose) showed more than one retention time (4.851, 6.348, 6.852, 6.892, 8.352, 8.417 mins) probable due to formation of cross-links or aggregates due to glycation. Standard CML also showed a retention time of 4.851 min that is identical with one of the retention time of glycated-HSA. Acid hydrolyzed native HSA showed a retention time of 3.469 mins. The % carbon in 100mg/dL glucose +HSA (0.287±0.003), 200 mg/dL glucose +HSA (0.364±0.004), 300 mg/dL glucose +HSA (0.541±0.003) and 400 mg/dL glucose +HSA (0.894±0.019) comparative higher than native HSA (0.156±0.003). Similarly the % hydrogen in 100mg/dL glucose +HSA (1.677±0.004), 200 mg/dL glucose +HSA (1.714±0.002), 300 mg/dL glucose +HSA (1.812±0.004) and 400 mg/dL glucose +HSA (3.482±0.006) comparative higher than native HSA (1.471±0.012). The data showed the addition of glucose carbon and hydrogen atoms to the HSA protein. Glycation
occurred on charged lysine and arginine residues of the HSA thereby altering its electrochemical behaviour. The specific electrical conductance (ohm$^{-1}\cdot$cm$^{-1}\times10^5$) of 100 mg/dL glucose + HSA (30.07±2.18), 200 mg/dL glucose + HSA (23.99±2.22), 300 mg/dL glucose + HSA (20.30±1.95) and 400 mg/dL glucose + HSA (4.92±0.41) was found lowered compared to native HSA (38.14±1.61) thereby showed occupancy of charged residues in glycation with glucose. Native HSA showed the hydrodynamic diameter of 4.11±0.15 nm. In the case of glycated-HSA, (modified with 400 mg/dL glucose) we observed four values of hydrodynamic radius as 7.99±0.22 nm. Stokes radius (Rs) showed greater value in glycated-HSA modified with 100 (81.29 nm), 200 (97.52 nm), 300 (112.47 nm), 400 (127.85 nm) mg/dL glucose compared to native HSA (65.26 nm). Addition of glucose to HSA non-enzymatically impaired the intrinsic viscosity (\(\theta\)) characteristics of glycated-HSA modified with 100 (4.99±0.02 mL/gm), 200 (5.74±0.11 mL/gm), 300 (6.41±0.13 mL/gm), 400 (7.25±0.12 mL/gm) mg/dL glucose compared to native HSA (4.16±0.06 mL/gm). There was decrease in surface contact angle (\(\theta\)) in glycated-HSA modified with 100 (57.65±1.01°), 200 (55.59±0.10°), 300 (54.80±0.00°), 400 (53.73±0.25°) mg/dL glucose compared to native HSA (66.83±1.73°).

Overall characterization of all attributes of glycated and native HSA showed the formation of AGEs in HSA incubated for 40 days with D-glucose. The AGEs formed showed impairment in the structure of native structure upon non-enzymatic glycation. Glycated-HSA showed defective or less efficient binding with drugs, fatty acids, and metals.

Immunogenicity of native and glycated-HSA was probed by inducing antibodies in rabbits. Native HSA and glycated-HSA modified with 400 mg/dL glucose showed a titre of <1:6400 and >1:12,800 respectively. Under identical conditions pre-immune serum of animals immunized with both native and glucose modified antigen showed negligible binding titre. Inhibition ELISA of Native HSA and glycated-HSA modified with 400 mg/dL glucose showed percent inhibition of 66.6% and 84.65% at 20 \(\mu\)g/mL. Under identical conditions, pre-immune serum of animals immunized with both native and glucose modified antigen showed negligible inhibition. Specificity of immunogen was evaluated with isolated and affinity purified IgG. The saturation of native and glycated-HSA (+400 mg/dL) was obtained at 40 and 60 \(\mu\)g/mL of IgG concentration respectively. However, preimmune IgG showed negligible binding
under identical conditions. Similarly, a value of 91.23% inhibition was achieved upon binding with glycated-HSA (400 mg/dL). Inhibition of 65.23% in antibody binding at 20μg/mL was achieved with native HSA. Hematological analysis showed a significant increase in blood count in post-immune rabbits immunized with glycated-HSA modified with 400 mg/dL glucose compared to preimmune animals. Rectal temperature also showed a rise in post-immune animals compared to native rabbits.

The possible involvement of glycated-HSA in diabetes mellitus patients has been probed. Sera from T2DM, T1DM, GDM and T2DM+CKD patients were screened for the presence of antibodies reactive to native and glycated-HSA. Direct binding ELISA showed greater recognition of glycated-HSA (modified with 400 mg/dL glucose) as compared to native HSA in all groups. Similarly, percent inhibition ELISA in all group against glycated-HSA (modified with 400 mg/dL glucose) showed a maximum percent inhibition in all groups compared to native form. The affinity purified IgG from sera of patients showed appreciable high binding towards glycated-HSA (modified with 400 mg/dL glucose) in direct binding ELISA. Inhibition ELISA with affinity purified IgG showed maximum percent inhibition with glycated-HSA (400 mg/dL glucose) compared to native HSA.

Biochemical and spectroscopic characterization confirms the presence of AGEs in the glycated-HSA upon incubation with 40 days time period. Glycation alters the structural properties of albumin and caused severe damage to its secondary and tertiary structures. HSA perform the transport function of mineral, drugs, and metals targeted to tissue delivery for action. Native HSA showed efficient and effective binding for the same. Wet lab experiments after In-silico studies for testing binding of the oleic acid was performed with glycated-HSA modified with 100, 200, 300 and 400 mg/dL glucose showed less efficient binding compared to native. Similarly pentosidine specific fluorescence of glycated-HSA modified with 100, 200, 300 and 400 mg/dL glucose complexed with the oleic acid showed a decrease in fluorescence intensities compared to native HSA. FTIR spectra of native and glycated-HSA complexed with oleic acid was recorded between 1200 to 2000 cm⁻¹ and showed the further shifting of amide I and amide II peaks.

Glycated-HSA and native HSA was In-silico tested for binding for metformin hydrochloride. Results obtained from In-silico studies confirmed inefficient binding
of metformin hydrochloride to glycated-HSA. Native HSA showed efficient and effective binding for the same. Wet lab experiments after In-silico studies for testing binding of metformin hydrochloride was performed with glycated-HSA modified with 100, 200, 300 and 400 mg/dL glucose complexed with the drug showed less efficient binding compared to native. Similarly pentosidine specific fluorescence of glycated-HSA modified with 100, 200, 300 and 400 mg/dL glucose complexed with the metformin hydrochloride showed a decrease in fluorescence intensities compared to native HSA. FTIR spectra of native and glycated-HSA complexed with metformin hydrochloride were recorded between 1200 to 2000 cm⁻¹ and showed the further shifting of amide I and amide II peaks.

Glycated-HSA and native HSA was evaluated for Cu (II) binding. UV absorption profile showed less effective binding of glycated-HSA modified with 100, 200, 300 and 400 mg/dL glucose compared to native HSA. Similarly pentosidine specific fluorescence of glycated-HSA modified with 100, 200, 300 and 400 mg/dL glucose complexed Cu (II) showed a decrease in fluorescence intensities compared to native HSA. FTIR spectra of native and glycated-HSA complexed with Cu (II) were recorded between 1200 to 2000 cm⁻¹ and showed the further shifting of amide I and amide II peaks.

The optimal cutoff value of glycated albumin (GA %) in north Indian healthy population was established with a value of 14.2 ± 2.25. Glycated albumin (GA %) in T2DM subject with eGFR>90mL/min, eGFR 60-89 mL/min, eGFR 30-59 mL/min and eGFR ≤29 mL/min was found to be 24.53 ± 9.00, 28.23 ± 8.51, 29.66 ± 7.23 and 21.65±6.52 respectively. Serum fructoseamine content was found to be higher in diabetes patients with and without complications compared to healthy subjects. Similarly, HbA1c, fasting and postprandial plasma glucose was found higher in diabetes patients with and without complications compared to healthy subjects. A positive correlation among fructoseamine and HbA1c in patients with diabetes and its associated complications compared to healthy subjects.

**Conclusions:** In view of the above studies, it could be concluded that glucose-induced modification of HSA resulted in the formation of covalent adduct causing structural perturbation as revealed by biochemical, biophysical, electrochemical, spectroscopic analysis. Combinatorial results obtained from this work clearly
demonstrate the HSA glycation within low sugar range can confer the formation of AGEs that initiate the complications in diabetes-associated complications due to its impaired structure and functions. Glucose-induced modifications appear to have generated highly immunogenic epitopes on HSA. Structurally altered HSA upon glycation preferably showed higher recognition of epitopes in patients with diabetes with and without complications. Glycated albumin (GA) in healthy and diabetic nephropathy patients showed a promising short-term glycemic index compared to long-term HbA1c.

A positive correlation was found between HbA1c and fructosamine. A promising novel diagnostic marker other than HbA1c was discovered in the form of glycated albumin (GA) in North Indian population establishing its optimal cutoff value. Overall, the results of this thesis implicate that credit for diabetes associated complications development goes to somehow HSA glycation induced AGEs formation keeping in mind GA as an alternate marker for diagnosis of diabetes mellitus. The present study also suggests that glucose modified epitopes may be used as the antigenic probe for detecting specific auto-antibodies that can serve as reliable biomarkers for the evaluation of the disease severity in diabetes patients with and without complications.