A DISSERTATION ON

N-ACETYL CYSTEINE PROTECTS AGAINST D-RIBOSEINDUCED STRUCTURAL MODIFICATIONS AND AGGREGATION TO SERUM ALBUMIN: INSIGHTS FROM PHYSICOCHEMICAL STUDIES

SUBMITTED TO THE
DEPARTMENT OF BIOSCIENCES
INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILMENT
FOR THE
DEGREE OF MASTER OF SCIENCE
IN BIOTECHNOLOGY

BY

AZKA FATMA
M.Sc. Biotechnology (IV Semester)
Department of Biosciences
INTEGRAL UNIVERSITY, LUCKNOW

UNDER THE SUPERVISION OF

Dr. M. SALMAN KHAN
Associate Professor
Department of Biosciences
Integral University, Lucknow

INTEGRAL UNIVERSITY

Established Under U.P. Act No 09 of 2004 by State Legislation
Approved by University Grants Commission
Phone No.: +91 (0552) 2890812, 2890730,
3296117, 6451039, Fax No.: 0522-2890809

Kursi Road, Lucknow-226026, Uttar Pradesh (INDIA)

TO WHOM IT MAY CONCERN

This is to certify that **Ms. Azka Fatma**, a student of M. Sc. Biotechnology (II Year, IV semester), Integral University has completed her four months dissertation work entitled "***Nacetyl cysteine protects against D-ribose-induced Structural Modifications and Aggregation to Serum Albumin: insights from physicochemical studies***" successfully. She has completed this work from Department of Biosciences, Integral University, under the guidance of **Dr. M. Salman Khan.**

The dissertation was a compulsory part of her M. Sc. degree. I wish her good luck and future endeavours.

Dr. Snober S. Mir
Head,
Department of Biosciences,
Integral University, Lucknow

INTEGRAL UNIVERSITY

Established Under U.P. Act No 09 of 2004 by State Legislation
Approved by University Grants Commission
Phone No.: +91 (0552) 2890812, 2890730,
3296117, 6451039, Fax No.: 0522-2890809

Kursi Road, Lucknow-226026, Uttar Pradesh (INDIA)

TO WHOM IT MAY CONCERN

This is to certify that the study conducted by **Ms. Azka Fatma** during the months Feb–May, 2022 reported in the present thesis was under my guidance and supervision. The results reported by her are genuine and script of the thesis has been written by the candidate herself. The thesis entitled is "***N-acetyl cysteine protects against D-ribose-induced Structural Modifications and Aggregation to Serum Albumin: insights from physicochemical studies***" therefore, being forwarded for the acceptance in partial fulfilment of the requirements for the award of the degree of Master of Science in Biotechnology, Department of Biosciences, Integral University, Lucknow.

Date:

Place: Lucknow

Dr. M. Salman Khan

(Supervisor)
Associate Professor
Department of Biosciences
Integral University, Lucknow

ACKNOWLEDGEMENT

Before I present my work, I would like to gratefully acknowledge the contribution of all those people who have helped in the work described in this Dissertation. I am going to try anyway, and if your name is not listed, it is rest assured that my gratitude is not less than for those listed below.

First, I would like to express my gratitude to **God** for providing me the blessing to complete this work.

Words will hardly help in expressing my sincerest gratitude to my supervisor **Dr. M. Salman Khan, Associate Professor, Department of Biosciences, Integral University,** who not only introduced me the fascinating field of free radical biology and medicine, but also helped me to understand the subject matter in all different possible ways**.** He has always been there for me throughout the work and helped me to overcome all odds. He has always taken a keen interest in my welfare.

It gives great gratification to record my earnest thanks to **Dr. Snober S. Mir, Head of the Department, Department of Biosciences, Integral University,** for providing me all necessary facilities and excellent research climate in pursuing this study.

Special thanks to **Dr. Sahir Sultan Alvi (Young Scientist Fellow, DHR), Mr. Parvej Ahmad, Mr. Mohd. Waiz** and all other laboratory staff members for their relentless help and advices. They generously devoted their valuable time for guidance and without their kind efforts my work would not be possible.

I also thank my group mates. I must write about my family members for their unconditional love, support and encouragement. It is equally important to thank my **parents…**but this acknowledgement will never be complete if their name is not there.

Date: AZKA FATMA

LIST OF ABBREVIATIONS

AGEs	Advanced glycation products
DM	Diabetes Mellitus
T1DM	Type-1 Diabetes Mellitus
T2DM	Type-2 Diabetes Mellitus
IDDM	Insulin dependent diabetes mellitus
NIDDM	Non-insulin dependent diabetes mellitus
HMF	Hydroxy methyl furfural
NBT	Nitro blue tetrazolium
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
CC	Carbonyl content
CR	Congo red
CML	Carboxy methyl lysine
HbA1C	Glycated Hemoglobin
PBS	Phosphate buffered saline
CVD	Cardiovascular disease
NAC	N-acetyl cysteine

CONTENTS

INTRODUCTION

Sweet taste is one of the five fundamental tastes and is associated with pleasurable sensation. Evolutionarily, animals used sweetness to evaluate the energy content of foods. Today our lifestyle has changed but our metabolism has remained the same. And satisfying sweet taste has led us into consuming large quantities of refined sugars. Sugar is an essential metabolite for prokaryotes as well as eukaryotes still high glucose concentration in biological fluids and tissues is involved in chemical modification of reactive amino groups in biological macromolecules (proteins, lipids and DNA) by reactive carbonyls (Sengupta & Swenson, 2005). Diabetes mellitus is a metabolic disorder which is characterized by hyperglycemia (elevated glucose) and insufficiency of secretion or action of endogenous insulin.

The cardiovascular complications of diabetes represent the leading cause of morbidity and mortality in affected subjects. The impact of hyperglycemia may be both direct and indirect: indirect consequences of elevated blood glucose led to generation of advanced glycation endproducts, the products of nonenzymatic glycation/oxidation of proteins/lipids that accumulate in the vessel wall, and are signal transduction ligands for Receptor for AGE (RAGE). Prolonged exposure to hyperglycemia is now recognized as the primary causal factor in the majority of diabetic complications (Pirart J.,1947 and 1973). Indeed, glucose has a wide range of transient and reversible effects on cell function as well as effects that are irreversible and can cause progressive, cumulative dysfunction (Alvi et al., 2021). This suggests that persistent, rather than acute, metabolic alterations are of pivotal importance in the development and progression of diabetic complications. Amongst the irreversible changes which occur as a direct result of hyperglycemia is the formation of advanced glycation endproducts (AGEs) via the Maillard reaction.

Persistent hyperglycemia, without proper management, leads to various secondary complications including diabetic nephropathy, neuropathy and retinopathy in diabetic patients and an ample of evidences suggests that the underlying mechanism is the glycation process (Nabi et al., 2019). Glycation is a non-enzymatic

reaction between reducing sugars and biological macromolecules like proteins, nucleic acids and lipids that results in the formation of respective advanced glycation endproducts (AGEs) (Nabi et al., 2019).

Glycation is the non-specific reaction of sugars with proteins, and proceeds without the need for an enzyme. It happens wherever protein is in contact with sugar. Any protein will in time react with any reducing sugar, but some sugars are more reactive than others and some protein groups are more reactive. Glucose is the least reactive sugar in these uncontrolled pathways and this may have led to its central role in metabolism (Nabi et al., 2019).

The glycation reaction starts as the simple formation of a Schiff base between a sugar in its open chain form and a protein amino group and proceeds to a complex set of reactions to form coloured, fluorescent and crosslinking species called AGEs. Glycation caused conformational change to the proteins (H.T. Beswick & J.J. Harding, 1987) and under harsher conditions, yellowing, aggregation and crosslinking associated with the increased presence of AGEs (Raza et al., 2002). The extent of glycation, and especially of AGE formation, increased with the severity of diabetic complications (Nabi et al., 2020).

The story of glycation reaction was started way back in year 1912 when Louis Camille Maillard first described the glycation reaction after whom the reaction is also known as Maillard reaction. A browning reaction by heating glycine and glucose was first observed in 1912 (Maillard, 1912). Consequently, Maillard response in vivo was named glycation, differentiated from enzymatic glycosylation. Glycation is a nonenzymatic reaction between the carbonyl group of reducing sugar and amino group carrying biomolecules such as proteins, DNA and lipids under in vitro and in vivo conditions. The final products of glycation are a polymorphic gathering of compounds largely alluded to as Advanced Glycation End products (AGEs). The biological function of biomolecules solely depends on their three-dimensional folded structures. There are various conditions, which can alter their folded structure and chemistry; amongst which glycation is the most common. With late advance in science, recognizing and

characterizing these glycation-induced alterations in macromolecules is vital toward understanding their functional significance.

AGEs are heterogeneous molecules derived from the nonenzymatic products of reactions of glucose or other saccharide derivatives with proteins or lipids (Semba et al., 2005). Various environmental factors, including cigarette smoke, high levels of refined and simple carbohydrate diets, hypercaloric diets, high temperature-cooked foods, and sedentary lifestyle, induce AGE production and consequently damage cell lipids and proteins (Peppa et al.,2007). More than 20 different AGEs have been identified in human blood and tissues and in foods. Numerous studies showed that the AGEs and the advanced lipoxidation end products (ALEs) are involved in the development and progression of chronic degenerative diseases, including diabetes (Baynes, 2003).

The Maillard reaction (MR) is characterized by nonenzymatic reactions of reducing sugars with amines. The stable products of this reaction are referred to as AGEs and were initially identified in cooked foods (Sebastian et al., 2005). In these years, numerous other AGEs have been identified in vivo and in vitro. They are classified in different groups based on their chemical structures and ability to emit fluorescence *i.e.*, Fluorescent and cross-linked (fluorescent/crosslinked), Nonfluorescent and non-cross-linked (nonfluorescent/non-cross-linked), Nonfluorescent protein cross-linked, and Fluorescent non-cross-linked. The first isolated and characterized fluorescent crosslinked AGEs are pentosidine. They are obtained from collagen and composed of an arginine and lysine residues crosslinked to a ribose but also from hexoses and ascorbic acid (Sell & Monnier,1989).

AGEs accumulation has been found to be implicated in several chronic diseases including typical diabetic complications, atheroscelerosis, Alzhimer's disease, rheumatoid arthritis and chronic heart failure. Interaction of AGEs with their receptors (RAGEs) causes the oxidative stress and initiation of inflammation cascade. Increased glycation and buildup of tissues AGEs can alter enzymatic activity, decrease ligand binding, modify protein half-life and alter immunogenicity, so they have been considered to be involved in pathogenesis of diabetic conditions. Glycation has been

shown to play an important role in the development of physiological and pathophysiological processes such as aging, diabetes, atherosclerosis, neurodegenerative diseases and chronic renal failure (Nabi et al., 2018).

Several types of anti-glycating agents have been described (Taha et al., 2014). These can interfere with different potential sites to inhibit glycation and AGE formation. Some may have the ability to compete for the amino groups on the protein. Others can directly bind to the protein or to the glycation intermediates to stop the progression up to the AGE formation stage. Otherwise, they may have the property to eliminate the open chain form of glycating sugars. (Harding et al., 2006) Furthermore, several probable AGE inhibitors have been proposed. Various inhibitors have been developed and some of them are in advanced clinical studies/trials. AGEs and reactive carbonyl compounds are metabolized and eliminated via the kidneys, which are essential organs for AGE filtration (Busch et al., 2010). The kidney accumulation of AGEs causes a function decline, resulting in dysfunction and chronic kidney diseases. In addition, chronic renal failure interrupts the metabolism of AGEs in the glomeruli of the kidneys and leads to an increase in serum AGEs and uremic complications. RAGE is involved in the induction of the reactive oxygen species (ROS) signaling pathway and causes cytosolic and mitochondrial oxidative stress involved in the pathogenesis of diabetic nephropathy (Tan et al.,2007). AGEs–RAGE interaction causes oxidative stress, leading to progressive glomerular damage in kidney glomerular mesangial cells (Yamagishi and Matsui,2010).

REVIEW OF LITERATURE

In 1912, the French chemist Louis-Camille Maillard reported for the first time the formation of brown colored substances during the enzyme-free reaction between reducing sugars and glycine (Maillard,1912). The complex interrelated reactions between the free amino group and reducing sugars are known as the Maillard reaction, which is largely considered as a browning reaction that takes place in the processing of foods and beverages (Zhang et al.,2009). The significance of the Maillard reaction was dormant for a very long time till browning reactions were identified *in vivo* by Monnier and Cerami in 1981. The formation of fluorescent yellow pigments and crosslinks upon incubation of eye lens proteins with reducing sugars was reported, similar to those reported in aging and cataractous lenses (Monnier & Cerami A.,1989).

The classical reaction of NEG (non-enzymatic glycation) occurs through the covalent bonding of reducing sugars (e.g., glucose or fructose) with free amino groups of proteins at N-terminal amino acid residues and/or ε-amino groups of lysine and arginine. NEG preferably occurs at amino groups that are either close (~ 5 Å) to an imidazole residue or a part of lysine doublet (Shilton and Campbell, Walton,1993). The initial reaction between the reactive carbonyl group and a free amino group of a protein results in the formation of a labile Schiff's base. The initial Schiff's base (aldimine) undergoes spontaneous rearrangements to a relatively stable ketoamine (1-amino-1-deoxy-2-ketose), which is known as an Amadori product, after Mario Amadori, an Italian scientist, who attempted to characterize the products of a condensation reaction between glucose and *p*-phenetidine (Amadori M.,1925). Both the Schiff's base and Amadori product can undergo further reactions with accessible lysine/arginine residues of the client proteins and form AGEs (Cerami A, Vlassara H, Brownlee M.,1987). The rate at which the Schiff's base forms takes hours to days, whereas it may take days to weeks and weeks to years to progress to Amadori products and the formation of AGEs, respectively.

Formation of AGEs

The process of AGE formation is complex and is divided into two main steps. First, within a few hours, reducing sugars containing carbonyl groups react reversibly with the free amino groups of proteins and nucleic acids to form unstable Schiff bases. The driving force of this reaction depends on the glucose concentration. Within a few weeks, Schiff base adducts undergo spontaneous intramolecular rearrangements that convert them to relatively stable, covalently bound Amadori products, also known as early glycation products. Second, a small proportion of the Amadori products may be directly transformed into AGEs by irreversible oxidation or hydrolysis through a series of reactions known as the Hodge pathway (C. Ott et al.,2014). The remaining Amadori products may be converted to AGE precursor compounds such as glyoxal (GO), methylglyoxal (MGO) and 3-deoxyglucosone (3-
DG) by dehydration, oxidative cracking or cyclization. Active α-dicarbonyl compounds bind covalently to long-lived proteins and structural components of the connective tissue matrix or basement membrane, such as collagen, to form stable AGE compounds.

Source and classification of AGEs

AGEs may be divided into two main groups according to their origin: endogenous and exogenous. Most endogenous AGEs are formed spontaneously and accumulate in the body under physiological metabolic conditions and during normal aging. Exogenous AGEs are mainly derived from dietary intake, and animal-sourced foods with high fat and protein contents have much higher AGE contents than plantsourced foods, which have high contents of water, antioxidants and vitamins. For example, the AGE levels in heat-treated foods are 10–100 times higher than those in untreated foods. Cooking is another key factor that determines the AGE content of food (Goldberg et al.,2004). investigated the AGE contents of 250 types of food prepared using different cooking methods.

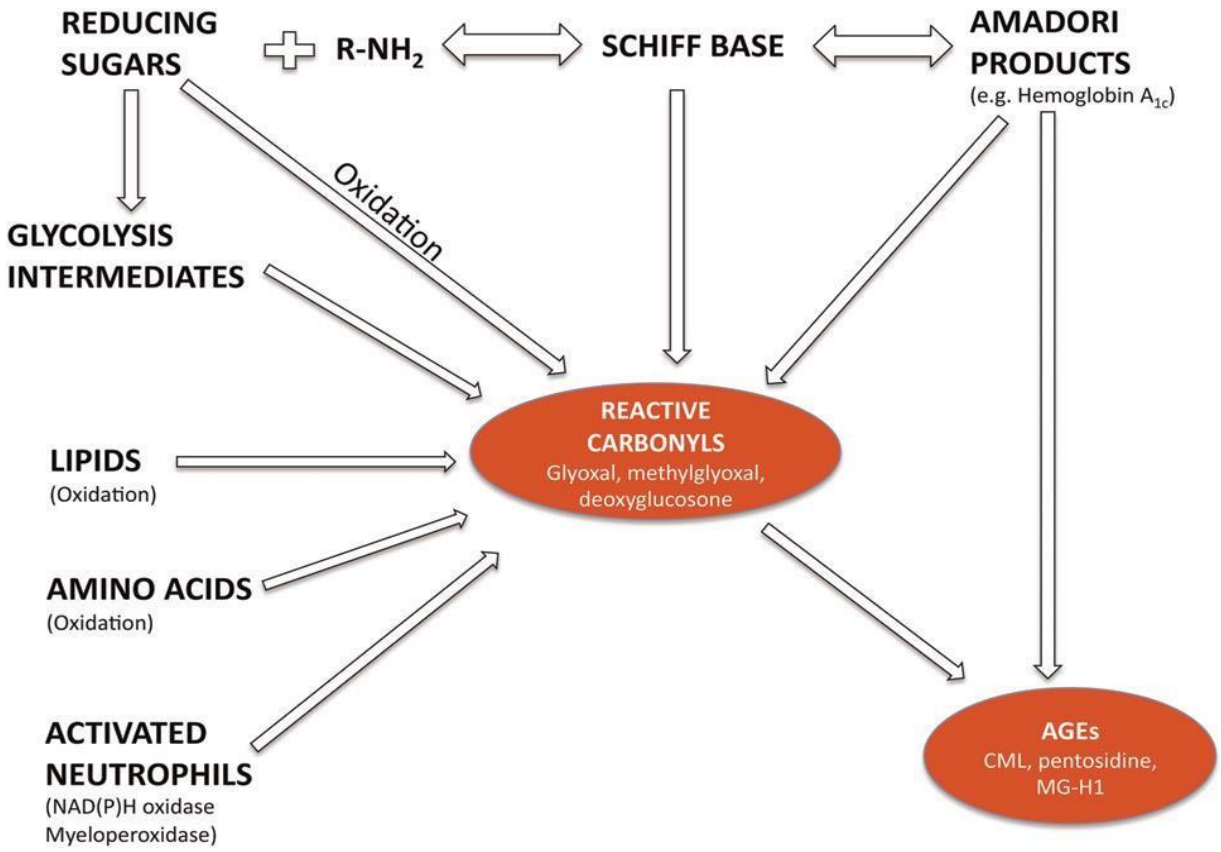

Figure 1: Different pathways of AGE formation.

The highest AGEs levels were found in fried and barbecued foods, followed by baked foods, and the lowest AGE levels were found in boiled foods. To date, more than 20 types of AGEs have been identified in tissue proteins, including carboxymethyl lysine (CML), carboxyethyl lysine (CEL), pyrroline, pentosidine, imidazolines (imidazoline A and imidazoline B) and glucosepane. These AGEs are classified into three groups according to their chemical characteristics (N. Ahmed et al.,2005):

(1) noncrosslinked nonfluorescent products;

(2) crosslinked nonfluorescent products; and

(3) crosslinked fluorescent products

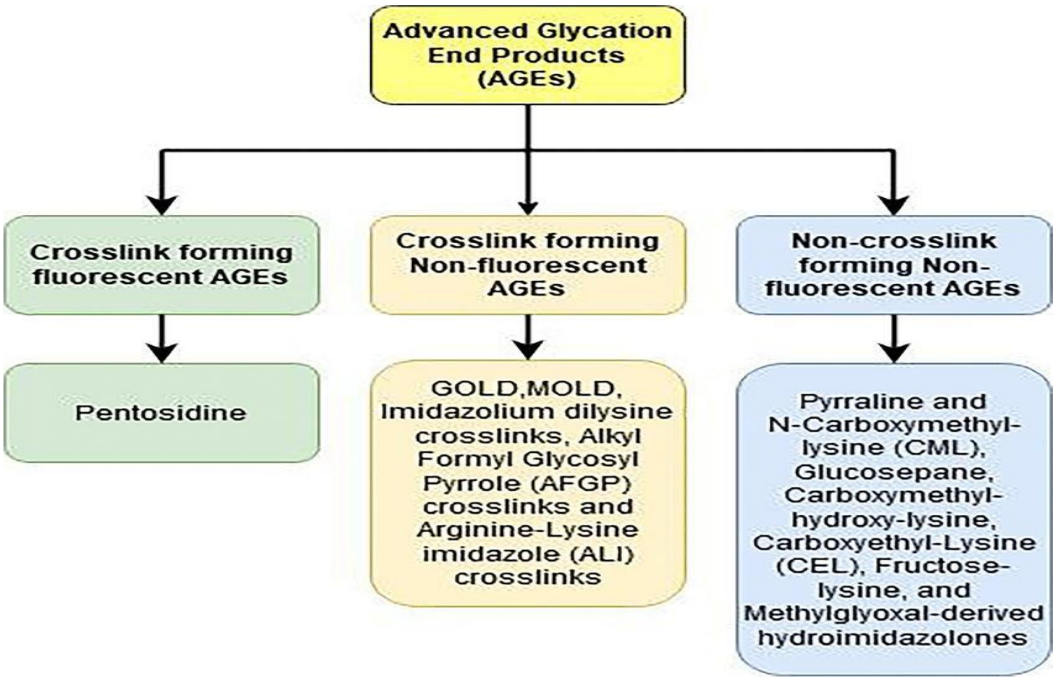

Figure 2. Fluorescent AGEs comprise a large proportion of AGEs

In addition, the oxidative breakdown of Amadori products can lead to the formation of reactive carbonyls such as glyoxal, methyl-glyoxal, and 3-deoxy-glucosone (3DG). These carbonyl compounds can, in turn, react with free amino groups of proteins to form intermediate glycation products. A series of reactions including dehydration, successive β-eliminations, and condensation reactions of both Amadori products and intermediate glycation products eventually result in the formation of irreversible inter- or intra-protein crosslinking AGEs that could persist for the lifetime of the modified substrate.

The formation of AGEs from autoxidation of Amadori products is known as ‘Hodgepathway’ and cleavage of dicarbonyl compounds from Schiff’s base refers to ‘Namiki pathway’. Alternatively, the formation of dicarbonyls from autoxidation of glucose, ribose, fructose, and glyceraldehyde is known as ‘Wolff pathway’ (Ott C. et al.,2014).

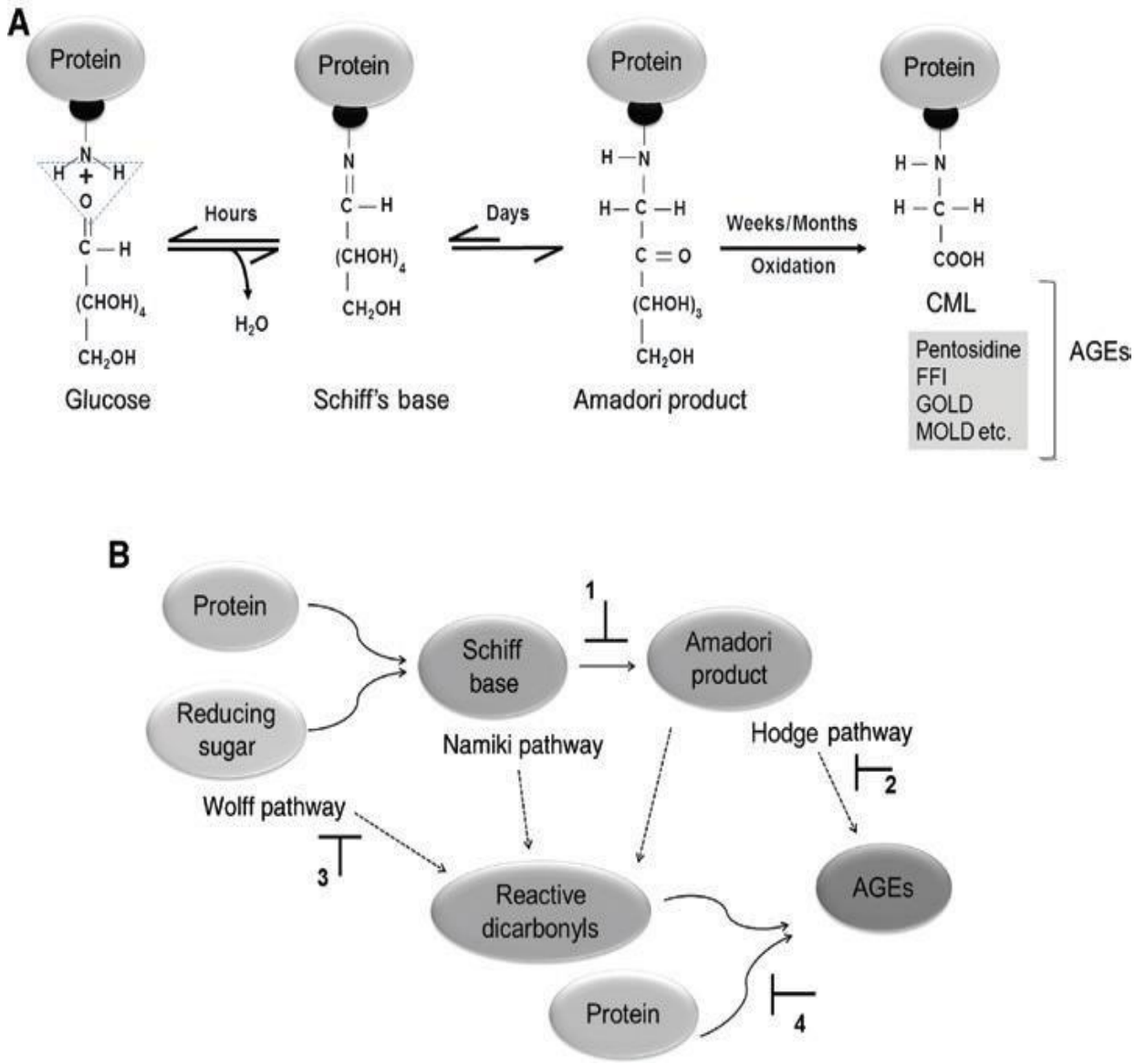

Figure 3: Mechanism and pathways of formation of advanced glycation end products.

(a). Classical reactions in the formation of advanced glycation endproducts (AGEs). (b). A complex network of NEG reactions.

Oxidative stress has a very important role in the mechanism by which AGEs form and accumulate, and has been implicated as a key factor in the progression of various diseases, including chronic diseases such as diabetes, Alzheimer's disease, and aging (Nabi et al., 2020 & 2019). Oxidative stress, more specifically oxidative damage to proteins, is increasingly thought to play a central mechanistic role in this context, as it

is associated with modifications in the activities of biological compounds and cellular processes that may be linked to a pathological environment. Oxidative stress is fueled by the generation of excessive reactive oxygen species (ROS) from glucose autoxidation, and also the nonenzymatic, covalent attachment of glucose molecules to circulating proteins that result in the formation of AGEs.

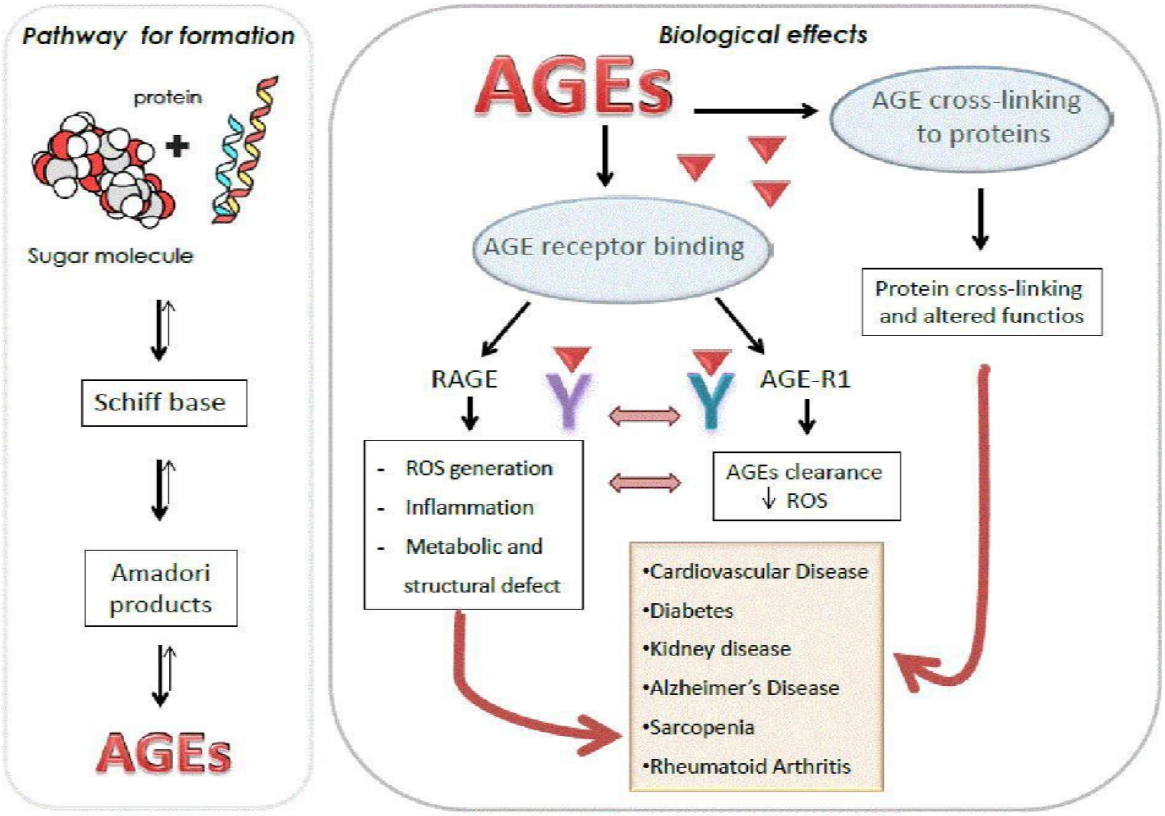

Figure 4. The process of glycation, formation of AGEs and their role in various diseases.

Alternative pathways of AGE formation to the Maillard reaction include thecarbonyl stress pathway, where oxidation of sugars and/or lipids generates a dicarbonyl intermediate, which binds amino acids and forms AGEs (Kurokawa, K. and van Ypersele de Strihou, C.,1998). Another mechanism of AGE formation is the aldose reductase-mediated polyolpathway. Glucose entering the polyol pathway may directly form AGEs via 3-deoxyglucosone AGE intermediates, but this reaction depletes NADPH andglutathione, and the resultant oxidative stress indirectly increases AGE

formation. Since these glycation reactions were slow, it was believed that this process predominantly affected long-lived proteins. However, it was later found that even short-lived compounds such as lipids, nucleic acids, and intracellular growth factors are glycated.

Biological effects of AGEs

The accumulation of AGEs in our bodies leads to the activation of various signaling pathways through a series of cell membrane receptors. As described above, AGEs comprise a group of molecules with varying chemical characteristics, but their biological differences are not yet fully understood. The biological effects of different AGEs are very similar; AGEs of higher molecular weight have the highest pathogenic potential; crosslinking AGEs have high affinity for proteins and are resistant to degradation, likely making them more toxic to the human body (H. Vlassara et al., 2011). AGEs activate receptors on the cell surface and induce various biological effects. Two main types of AGE receptors have been described: scavenger receptors and the multiligand RAGE receptor.

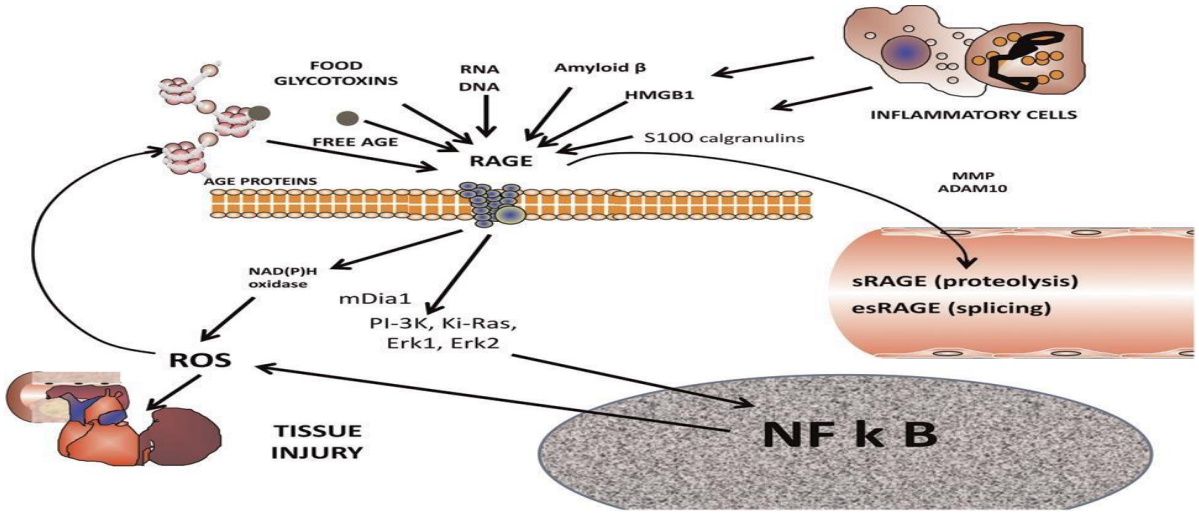

Figure 5. **RAGE, its ligands, and its main signaling pathways enhance oxidative stress.**

These pathways induce the gene expression of monocyte chemoattractant protein-1 (MCP-1), the proinflammatory cytokines IL-6 and TNF-α, vascular cell adhesion molecule-1 (VCAM-1), endothelin-1, and other molecules. Activation of RAGEs also

leads to endoplasmic reticulum (ER) stress, resulting in stress responses that lead to inflammation or apoptosis. Additionally, the accumulation of AGEs in the ER interferes with normal protein folding through its crosslinking effects. AGEs can also cause the crosslinking of mitochondrial proteins in the respiratory chain, reducing the synthesis of ATP and promoting the production of oxidative free radicals (X. Wang et al.,2020). The pathways described above may cause a vicious cycle that leads to intracellular damage, impaired cellular function, and ultimately cell death, resulting in aging and the development of various age-related chronic diseases, such as cancer, Alzheimer's disease, cardiovascular disease, diabetes and other chronic diseases.

Pattern-recognition receptors are involved not only in identifying non self microbial products such as pathogen-associated molecular patterns, but also "danger" signals (dangerassociated molecular patterns—also known as alarmins—from the host that foretell of changes in homeostasis). Of note, many RAGE ligands are now classified as pathogen-associated molecular patterns or danger-associated molecular patterns. Agrowing body of evidence suggests that RAGE has a substantial role in innate immunity (Rojas A et al.,2022).

Natural compounds that affect the formation of AGEs

The main mechanisms that inhibit the formation of AGEs are the reduction of active dicarbonyl compounds, inhibition of ROS formation, protection of the protein structure, and degradation of AGEs. Some chemically synthesized drugs are effective. For example, aminoguanidine, the first drug with clinical therapeutic potential in this regard, inhibits the formation of AGEs by trapping the carbonyl group of Amadori products through nucleophilic addition reactions and inhibiting their further rearrangement. Aminoguanidine can also bind to active α-dicarbonyl intermediates to form triazines, blocking the transformation of Amadori products to AGEs. However, aminoguanidine has severe side effects that limit its clinical applications; these include pernicious anemia, gastrointestinal symptoms, lupus, influenza-like syndrome, vasculitis, and the promotion of oxidation (C.G. Schalkwijk et al.,2020).

“Natural compounds” are chemical substances that are extracted from plants or animals and have distinct pharmacological effects. The natural compounds that potentially inhibit the formation of AGEs are divided into the following six classes based on their structural properties: polyphenols, polysaccharides, terpenoids, vitamins, alkaloids and peptides.

The effects of glycation

Investigation of glycation-induced damage to proteins associated with ageing and complications of diabetes was mostly on long-lived structural proteins, because they are exposed to the sugars for a longer time than enzymes and other non-structural proteins in most tissues. The extent of glycation was approximately doubled in diabetes for a variety of proteins including collagen, myelin basic protein, lens crystallins, lens capsule, LDL, as well as haemoglobin (J.J. Harding,1985). Similar increases were found in ageing. Glycation caused conformational change to the proteins and, under harsher conditions, yellowing, aggregation and crosslinking associated with the increased presence of AGEs (K.M. Biemel et al.,2002). Although the emphasis has been on long-lived proteins even proteins that turn over rapidly like insulin and enzymes are damaged by glycation. In many instances proteins damaged by the inevitable glycation are simply turned over and cause no harm. The extent of glycation, and especially of AGE formation, increased with the severity of diabetic complications (V.M. Monnier et al.,1986). Furthermore, glycation increases in the lens with age and cataract and in other diseases such as Alzheimer's disease.

Primary Sources of Advanced Glycation End Products

Endogenous AGE

Effects of short-lived AGE molecules: Advanced glycation endproduct formation has been known to have significant effects on macromolecular structure and function. Until recently it was thought that AGE formation involves primarily long-lived extracellular proteins and occurs as a function of time, thus representing a form of molecular senescence. It is now clear that AGEs arise on short-lived molecules as well, including

circulating plasma proteins and lipids, and that their levels are significantly elevated in diabetic patients and in patients with impaired renal clearance (Delaney V et al.,1991). It is also recognized that they can form rapidly on cytoplasmic proteins and nucleic acids. Indeed, it has been demonstrated that intracellular AGEs may form at a rate up to 14-fold faster in high (30 mM) glucose conditions (Brownlee M. et al.,1994).

Apart from the high dietary sugar intake that increases the rate of formation of endogenous AGEs, diabetes can accelerate AGE formation due to hyperglycemia.

Elevated glucose level shifts the equilibrium towards the right side facilitating the forward reaction of Schiff's base formation. A variety of diabetic complication such as atherosclerosis, nephropathy, neuropathy and retinopathy have been shown to be exacerbated due to the involvement of AGEs (Vlassara H. et al.,2002).

Exogenous AGEs

Although the formation of AGEs occurs endogenously, they can also be formed during preparation of food. Despite the bioavailability of orally ingested AGEs being as low as 10%, emerging evidence demonstrates the deleterious e_ects of dietary AGEs on health, suggesting a significant proportion of dietary AGEs is absorbed by the gut. As mentioned above, the glycation reaction was first observed when reducing sugar is heated in the presence of amino acids causing non-enzymatic glycation, forming a characteristic golden-brown color. Inducing the Maillard reaction in food preparation is used to obtain the desired food taste, despite its potentially harmful e_ects in health. Ita_ects the properties of the food such as color, aroma, delicate flavors, texture and protein functionality. However, if the Maillard reaction is too pronounced, undesired quality changes can occur, producing bitter and burnt flavors. Temperature and time are two key factors that determine the rate and the extent of exogenous AGE formation. Other factors that influence the rate include, type of reactants, pH and the water activity (Ames J.M.,1990).

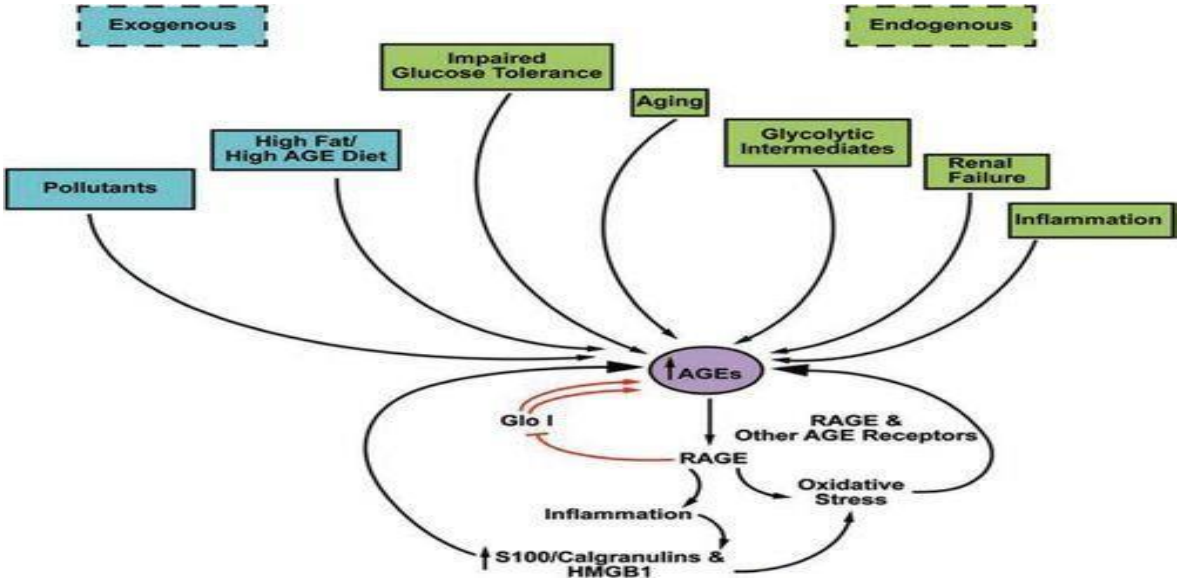

Figure 6. Exogenous and Endogenous Glycation

Glycation is a non-enzymatic reaction that occurs between a carbohydrate and a molecule with a free amino group, such as a protein. This irreversible and cumulative reaction takes place spontaneously in the body. All resulting metabolic intermediates are then reactive. Glycation is a physiological and pathological process that produces so-called glycated proteins and is entirely separate from glycosylation, which represents an enzyme-controlled physiological process that occurs during synthesis of glycoproteins. When it occurs outside of the body, glycation is responsible for the browning process that occurs in food during cooking. Exogenous glycation is the result of a covalent bond between a carbonyl group from a reducing sugar (aldose or ketose) and a free amino group.

Endogenous physiological glycation involves glucose, the body's most prevalent reducing sugar, and the functions of free amino groups present in the body as well, especially amino acids in proteins like lysine and arginine. It has also been shown to play a role in numerous pathologies in diabetic and non-diabetic patients alike. These intermediaries are very reactive and cause carbonyl stress, which in turn can aggravate inflammation and oxidative stress. The reactive intermediaries described above are located at the "crossroads" of various metabolic pathways.

RAGE and cell signalling

The receptor for advanced glycation endproducts (RAGE) was first described as a signal transduction receptor for advanced glycation endproducts (AGEs), the products of non-enzymatic glycation and oxidation of proteins and lipids that accumulate in diabetes and in inflammatory foci. The discovery that RAGE was a receptor for inflammatory S100/calgranulins and high mobility group box 1 (HMGB1) set the stage for linking RAGE to both the consequences and causes of types 1 and 2 diabetes receptor for advanced glycation endproducts (RAGE), an immunoglobulin superfamily molecule whose multiple ligands have been shown to accumulate in diabetic tissues. RAGE was discovered as a receptor for advanced glycation endproducts (AGEs), such as carboxymethyl lysine (CML). AGEs, the products of nonenzymatic glycation and oxidation of proteins, form to an accelerated degree in hyperglycemia. AGEs, largely via RAGE, activate signaling mechanisms that cause cell stress, contribute to cellular dysfunction, and damage target organs, leading to complications.

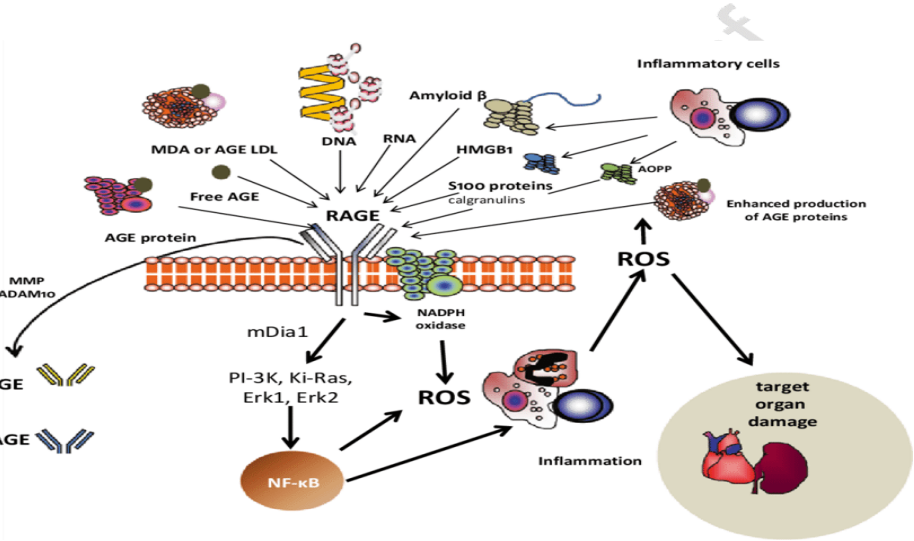

Figure 7. The RAGE, its ligands and main signaling pathways enhance oxidative stress.

The receptor for advanced glycation end-products (receptor for AGEs, RAGE) is a multi-ligand protein that integrates the immunoglobulin superfamily of receptors. RAGE recognizes a variety of ligands including high mobility group box 1 protein (HMGB1), the leukocyte integrin Mac-1, S100/calgranulins, modified LDL, DNA, RNA and amyloid fibrils.

RAGE Expression

RAGE can be constitutively or inducibly expressed in different cells, depending on the cell type and developmental stage. During embryonic development, RAGE is highly expressed in a constitutive manner (Shi Du Yan et al.,1993). Compared to embryonic cells, there is relatively low expression of RAGE in a wide range of differentiated adult cells such as cardiomyocytes, neurons, neutrophils, monocytes/macrophages, lymphocytes, dendritic cells (DCs), and vascular endothelial cells. Unlike constitutive RAGE expression during embryonic development, RAGE is expressed in a regulated manner in adult life. This means that RAGE expression can be induced in situations when there is an accumulation of ligands and inflammatory mediators.

RAGE and Inflammation

First, RAGE has been found on numerous immune cells that play key roles in perpetuating the immune response. These cells include neutrophils, T and B lymphocytes, monocytes, macrophages, and also dendritic cells (S. S. Saleh et al.,2002). Second, many of the extracellular ligands that trigger RAGE signaling have been determined to be involved in acute and chronic immune responses (Al-Fakhri et al.,2003). Third, RAGE expression has been found on endothelial cells, and this expressed RAGE can physically interact with the leukocyte β2 integrin Mac- 1. The RAGE-Mac-1 interaction enables RAGE to function as an adhesion receptor for leukocytes (Gebhardt et al.,2006).

Fourth, pro-inflammatory transcription factor nuclear factor kappa B (NF-κB) and its downstream target genes are activated following engagement of RAGE. Among these NF-κB regulated target genes, some of them are regulators of the adaptive and innate immune systems (Morcos et al.,2005). Interestingly, RAGE itself is also an NF-κB regulated target gene, exhibiting a functional binding site for NF-κB in its proximal promoter. Fifth, accumulation of RAGE ligands at sites of tissue injury and inflammation has been found to induce intracellular activation of NF-κB. RAGEligand interactions also lead to sustained NF-κB signalling via *de novo* RelA (p65) mRNA

synthesis because the *de novo* synthesis produces a constantly growing pool of proinflammatory transcriptionally active NF-κB (Schwaninger et al.,2001).

The nonenzymatic glycation of proteins in vivo may alter their secondary structure, affect cell biological activity and function, and cause cell damage and cell death. AGEs regulate various signaling pathways, including the mitogen-activated protein kinase (MAPK), nuclear transcription factor (NF-κB) and signal transducer and transcriptional activator (STAT) pathways. AGEs play a pathological role in the development of some metabolic diseases, including diabetes, atherosclerosis and Alzheimer's disease (K. Byun et al. & V. Gill et al.,2019). Reducing their endogenous formation and accumulation in vivo has become an important consideration to prevent and control diabetes. Thus, many strategies targeting the formation of AGEs have been developed. For example, metformin reduces blood glucose levels and induces glyoxalase I (GLO1) activity, thereby reducing the level of methylglyoxal (MG), an important precursor of AGEs. Aminoguanidine more specifically prevents the formation of AGEs by eliminating reductive carbonyl groups and acting as a scavenger of α-dicarbonyl compounds. However, the administration of aminoguanidine is accompanied by inevitable side effects that limit its clinical application.

AGEs in Health and Diseases

Interest in the role of AGEs in health and disease was sparked by initial reports of progressive rise of in vivo AGEs with normal aging, by their ability to cross-link proteins in an irreversible fashion, and by their modulation of extracellular- kinases (ERK) signaling. A series of reports demonstrating rise in circulating AGEs in people with diabetes and chronic kidney disease stimulated further interest in health implications of AGEs.

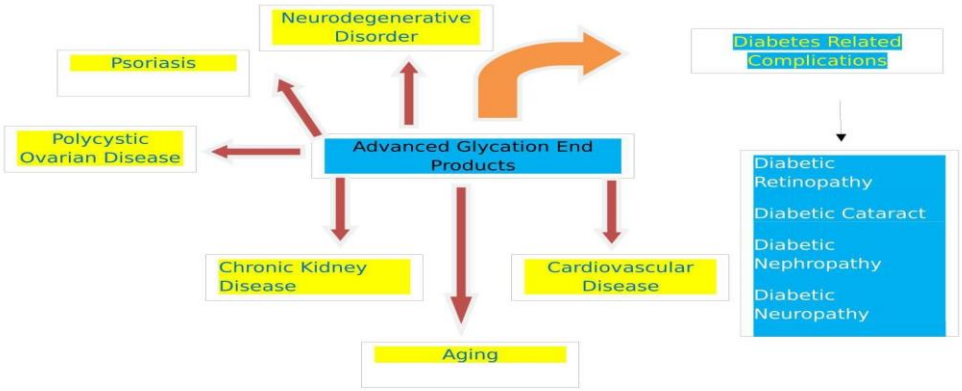

Figure 8. Advanced Glycation End Products and the diseases

Diabetic retinopathy

The formation of AGE compounds and their role in the progression of diabetic retinopathy could be due to the death of various lens proteins and retinal cells of the eye as a result of AGEs accumulation (Stitt. 2011). The AGEs bind to its receptor RAGE in lens, which leads to the activation of a signaling pathway that causes oxidative stress, and release local hormones, cytokines and adhesive molecules. The AGE–RAGE interaction causes vascular changes in pericytes (contractile cells of endothelium) leading to apoptosis of pericytes, which is an early sign of retinopathy (Ogata M. et al.,2003).

Diabetic nephropathy

It is the major cause of End Stage Renal Disease (ESRD). Even though genetic susceptibility is the risk factor, hyperglycemia is also linked with the pathogenesis of diabetic nephropathy. The pathological link between hyperglycemia and development of diabetic nephropathy has been attributed to the formation of AGEs. These AGEs and related products form cross-links with collagen, which could lead to structural and functional changes in the kidneys (Kruse et al.,1998) AGEs also induce production of inflammatory cytokines, chemokines, adhesive molecules, and growth factors which could be involved in the pathogenesis of diabetic nephropathy. In vitro and in vivo

studies indicates that AGEs via RAGE leads to overproduction of matrix proteins and inhibits its breakdown, initiating an oxidative stress pathway further damaging the organs involved.

Diabetic neuropathy

Polyneuropathy (damage of peripheral nerves) or mononeuropathy (damage of a nerve due to injury) is a common diabetic neuropathic complication. The glycation of cytoskeletal proteins, structural or functional changes of nerve fibers leads to diabetic neuropathy. Experimental studies on diabetic rats showed decreased sensory motor conduction velocity, decreased nerve potential and also functions of sciatic nerve due to accumulated glycated proteins. AGE accumulation may cause the loss of myelin sheath around neuron, further leading to vascular abnormalities (Yagihashi et al.,1997).

Neurodegenerative diseases

The AGEs accumulation in the brain has been noted to increase with the advance in age. The recent data has suggested that the accumulated AGEs in brain and other organs of the Central Nervous System (CNS) may be responsible for neurodegenerative disorders like the Alzheimers, Parkinson, Prionopathies and lateral amylotrophic sclerosis (Swamy S, et al.,2014). The accumulation of AGEs varies according to the pathology of the disease. Increased AGEs were noted in hippocampal region in Alzheimers, substantia nigra in Parkinsons, ventral spinal cord in amylotrophic lateral sclerosis patients. The measurement of AGEs, the methyl glyoxal derivative can be done by immunological, and chemical methods and further confirmed by proteomic analyses.

Cardiovascular diseases

AGEs accumulation has been observed both during diabetes and also in nondiabetic conditions. Smoking, consuming more amounts of deep fried and fatty foods and food cooked at high temperatures etc. could lead to increased AGEs in blood. The adverse effects of AGEs and related product is due to their cross-linking nature with matrix proteins, which decreases the flexibility and causes sequential dysfunction of the protein. The cross linking of AGEs with collagen, elastin, laminin in the myocardium leads to the rigidity and diastolic dysfunction of the heart. Similarly, formation of AGE–RAGE complex in the myocardium induces fibrosis by enhancing the activity of Transformation Growth Factor β (TGF-β) (a super family of cytokines involved in cell functions). AGEs also may cause reduction in calcium concentrations by delaying the calcium re-uptake.

Endogenous glycation and aging

Aging is an overall change in a collection of physiological functions and an increased susceptibility to various diseases. Aging is also associated with chronic, low-level inflammation evidenced by increased blood levels of inflammatory mediators that contribute to functional disruptions, the development of chronic disease, and the state of fragility that occurs throughout aging. Moreover, the accumulation of AGEs has been found in many parts of the body, including the blood, blood vessel walls, retina, lens, kidney, brain, peripheral nerves, joints, and skin. The build-up of these products results in significant changes in the metabolism, appearance, and biomechanical properties of these organs (Wang et al 2015).

A build-up of glycation products is correlated with increased rigidity in the arteries, tendons, and skin. AGEs play adverse proinflammatory roles in osteoporosis and the serum level of sRAGE could therefore have a potential diagnostic role in the monitoring of osteoporosis progression (Galliera et al 2017). AGEs also play a role in the aging of skeletal muscle. Muscle mass and strength decrease during the aging process, which can increase the fragility and dependence of the elderly (Van et al.

2014). Glycation and oxidation, especially with respect to lipids, also affect the pathophysiological process of age-related macular degeneration and formation of cataracts, thereby disrupting the quality of vision and the visual field (Roehlecke et al 2016).

Glycation and skin aging

In aging, the skin becomes dryer, thinner, and less elastic and dark spots and wrinkles also appear. The skin's sensory abilities also change. Undoubtedly, skin aging is strongly influenced by exterior factors such as tobacco use, exposure to ultra-violet (UV) rays, pollution, and lifestyle (Rabbani & Thornalley 2015). The skin of elderly patients is characterized by a thinner epidermis, a lack of dermal papillae, and an atrophied and disorganized dermal extracellular matrix (including the presence of fragmented collagen bundles and thickened, denatured elastic fibers). UV rays accelerate skin aging through a variety of mechanisms (induction of oxidative stress, increased production of metalloproteases, etc..Aged skin that is exposed to the sun is characterized by a more significant accumulation of denatured elastic fibers (solar elastosis). Glycation is a process that contributes to skin aging in a variety of ways AGEs accumulate with age in the extracellular matrix of the dermis and epidermis. They bind with long-lived components of the extracellular matrix like collagen and elastin fibers and play a role in intermolecular connections. Glycation modifies the skin’s physical properties, rendering it more rigid and less elastic.

Glycation and cerebral aging

Alzheimer's disease is a neurodegenerative disease, characterized by neuronal death that most often affects people over the age of sixty-five. It manifests as a progressive loss of memory, spatial and temporal orientation, and reasoning leading to a concomitant and considerable reduction in the patient's autonomy. Alzheimer's disease is the result of two pathological processes that first develop in the hippocampus and then spread to the cerebral cortex: 1) the aggregation of hyperphosphorylated tau proteins in neurons and 2) the accumulation of amyloid

peptides on the surface. Current treatments have only a moderate effect on symptoms and progression of the disease (Arbor et al. 2016). Studies have shown that diabetes increases a patient's risk of developing Alzheimer's disease. The Rotterdam study examined over 6,000 patients without any signs of dementia, around 11% of whom were diagnosed with diabetes mellitus. Subjects were then monitored for one year. The study showed that diabetes nearly doubled a patient's risk of developing dementia and Alzheimer's disease.

Antiglycation Strategies

Recently, antiglycation has been considered as an effective strategy to slow down human aging and disease development. The inhibition of glycation can suppress inflammasome activation to reduce the development of inflammatory reactions. The antioxidant and anti-inflammation abilities of polyphenol substances have been extensively studied, and their antiglycation functions have been screened in many in vitro experimental platforms. The results show that polyphenols can inhibit the biosynthesis of AGEs through their antioxidant properties, metal-chelating ability, protein interaction, MG trapping, and/or blocking the receptor for advanced glycation end products (RAGE) (Tan D. 2008) Polyphenols were classified into four large groups in this article, phenolic acids, stilbenes, lignans, and flavonoids, and different antiglycation functions of polyphenols found in recent years were examined to evaluate the antiglycation potential of polyphenols.

Role of Aminoguanidine (Pimagedine) to prevent the formation of AGES:

Aminoguanidine (AG) is a prototype therapeutic agent for the prevention of formation of advanced glycation endproducts. It reacts rapidly with alpha,betadicarbonylcompounds such as methylglyoxal, glyoxal, and 3-deoxyglucosone to prevent the formation of advanced glycation endproducts (AGEs).Aminoguanidine (Pimagedine, AG)1 is a prototype a,b-dicarbonyl scavenging agent that prevents the formation of advanced glycation endproducts (AGEs) from a,b-dicarbonyl precursors. Since then, use of AG to prevent AGE formation in vitro and in

vivo has given evidence of the involvement of advanced glycation in many disease proesses and abnormal physiological state.

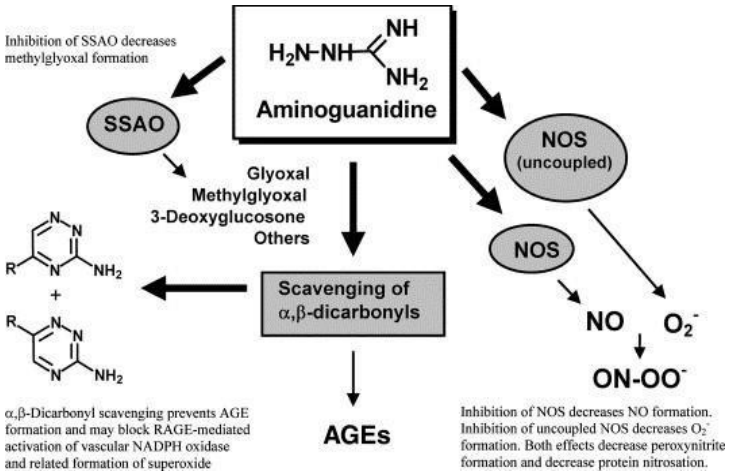

Figure 9: Role of aminoguanidine to prevent the formation of AGEs.

NAC(N- acetyl cysteine)

NAC is most notably found in plants of the Allium species, especially the onion (Allium cepa, 45 mg NAC/kg). NAC is well known for its substantial antioxidant, anticancer, antimicrobial and other pharmacological properties. N-acetylcysteine

SPECIFIC OBJECTIVES

1. **Anti-glycation studies of N-acetyl-cysteine (NAC) against D-ribose mediated BSA glycation.**
2. **Characterization of native, D-ribose glycated BSA, and NAC treated glycated BSA via various Biochemical techniques.**
3. **Characterization of native, D-ribose glycated BSA, and NAC treated glycated BSA via various Biophysical techniques.**
4. **To delineate the protective effects of NAC against D-ribose-induced protein aggregation using non-fluorescent and fluorescent probes.**

Materials and methods

D-Ribose, BSA, thiobarbituric acid (TBA), guanidine hydrochloride and 2,4dinitrophenyl-hydrazine (DNPH)were procured from HiMedia Laboratories, Mumbai, India. NAC was purchased from Sigma Aldrich Co. USA.

D-Ribose mediated in-vitro glycation and NAC-mediated antiglycation assay of BSA

The glycation of BSA with D-ribose was performed using a previously standardized protocol in which we established the concentrations of both D-ribose and BSA was selected on the basis of a recent report with slight optimizations for appropriate absorbance on UV-VIS spectroscopy (Nabi et al., 2020). Briefly, appropriate amount of BSA (0.5 mg/mL) was modified with 80mM D-ribose in 0.1M phosphate buffer saline (PBS), pH 7.4 containing 0.05% sodium azide. The reaction mixture was incubated either in presence or absence of different concentrations of NAC (100, 200, and 300 μM) at 37 °C for 30 days followed by extensive dialysis against PBS to remove unbound constituents. Native BSA was used as control.

Physicochemical characterizations

Characterization via UV-VIS spectral analysis

The UV-VIS spectral analysis of native, glycated and inhibitors (NAC) BSA samples were recorded in a wavelength range of 200–800 nm in order to depict any alterations in the absorption patterns of BSA on daily basis for 18 days using Eppendorf Biospectrometer. The anti-glycation potential of NAC was calculated by using absorbance at 280 nm via the below mentioned equation and the results were represented as percent hyperchromicity by respective inhibitors.

% Hyperchromicity = [(Absorbance of glycated sample−Absorbance of native or

Inhibitor treated sample/Absorbance of glycated sample] X 100

Determination of ketoamine content through NBT reduction assay

The glycation of a protein leads to the formation of Amadori products or ketoamine content that undergo further rearrangement and cyclization to form the AGEs (Nabi et al., 2020; Alvi et al., 2021). These Amadori products are also called as the early glycation products (EGPs). Therefore, the formation of EGPs in the unmodified BSA and Gly-BSA (in presence or absence of NAC was assessed by NBT reduction assay on daily basis using Eppendorf Biospectrometer (Nabi et al., 2020). Briefly, 20 µL samples from unmodified BSA, Gly-BSA and NAC treated Gly-BSA were mixed with 180 µL of sodium carbonate-bicarbonate buffer (100 mM; pH 10.8) containing 0.25 mM NBT and kept for incubation at 37 °C for 10 min. The absorbance was read at 525 nm on daily basis until we observed a decline in the absorbance which signifies the transformation of EGPs into AGEs that do not reduce the NBT. Sodium carbonate-bicarbonate buffer was used as blank.

Characterization of intrinsic fluorescence via fluorescence emission spectral studies

Native, glycated BSA in presence or absence of NAC were excited at 370nm and their respective fluorescence emission intensities (FI) were recorded in the wavelength range of 360–600 nm on Agilent Cary Eclipse Spectro-fluorimeter at 25 ± 0.1 °C. Percent increase/decrease in FI for all the samples was calculated from the following equation

%Increase/Decrease in FI = [(FI of glycated sample−FI of native or inhibitor treated sample)/FI of glycated sample]X 100.

Intrinsic fluorescence measurements have been widely used to notice the structural and conformational changes in proteins upon ligand binding. To explore the changes induced in protein upon incubation with sugars, aliquots at different time intervals were withdrawn and checked for protein intrinsic fluorescence. It can be observed that there was an initial increase in fluorescence intensity reaching maximum followed by a concomitant decrease for D-ribose incubated samples. Depicting the formation of a

structural intermediate which may be somewhat close to the molten globular state of the protein the decrease in fluorescent intensity after day 18 for the sugar incubated samples can be attributed to the transition of chromophores towards polar solvent.

Determination of protein-bound carbonyl groups

Total protein bound carbonyl contents (CC) of native, glycated BSA in presence or absence of NAC were determined using 2,4-dinitrophenylhydrazine (DNPH) method by Levine et al. In brief, 400µL of DNPH solution was added to 100µL of each sample and incubated for 60 min in dark at room temperature. The reaction mixture of each sample was precipitated with 500µL (20%) of trichloroacetic acid (TCA) solution and incubated on ice for 5 min. After the reaction, the samples were centrifuged at 10,000 rpm for 10 min at 4 °C. The precipitates were washed three times with ethanol-ethyl acetate in the ratio of (1:1) and the final precipitates were dissolved in 250µL of 6M guanidine hydrochloride (GH), which was used as a blank. The absorbance was read at 360 nm and the carbonyl content was determined using extinction coefficient of 22,000 $M^{-1}cm^{-1}$. The results were expressed as the ratio of nmol of DNPH reacted mg^{-1} of protein.

Thiobarbituric acid assay

Hydroxy methyl furfural (HMF) content of native, glycated BSA in presence or absence of NAC was estimated by thiobarbituric acid (TBA) assay. 100µL of each of the above samples were added to 400µL of oxalic acid. The reaction mixture was boiled for 1 h. After boiling the reaction mixture was cooled to room temperature for
10 min. The reaction mixture of each sample was precipitated by adding 500µL (40%) of TCA and centrifuged at 8000 rpm for 10 min at 24 °C. After centrifugation, th pellets were discarded and 500µL (0.05 M) of TBA was added to the supernatant. The supernatant was incubated at 40 °C for ½ hour. TBA was used as a blank. The absorbance was read at 443 nm and the HMF content was determined using extinction coefficient of 40,000 M^{-1} cm^{-1}.

Determination of free arginine residues

Like free lysine residues, free arginine are also the potent sites of glycation and are well reckoned to produce fluorescence on reacting with phenanthrenequinone. Free arginine content in the unmodified and Gly-BSA in presence or absence of NAC (100, 200 and 300 µM) was assessed by previously described standard method (Nabi *et al*., 2020). In brief, 500 µL of phenanthrenequinone of (200 µM) (dissolved in ethanol) was added to all samples and the reaction mixture was mixed properly followed by addition of 200 µL of 2 N NaOH. This reaction mixture was incubated for 60 min at 30 °C and subsequently 200 µL of 1.2 M HCl was added to all samples. FI of all the above-mentioned samples was recorded in the wavelength range of 350–450 nm using an excitation wavelength of 312 nm (Nabi *et al.*, 2020).

Estimation of free lysine residues

Free lysine residues are the potent sites of glycation and are well reckoned to react with 2,4,6-trinitrobenzene-1-sulphonic acid (TNBS) and the level of free lysine residues tends to decline upon glycation (Nabi et al., 2020). Free lysine content in unmodified and Gly-BSA in presence or absence of ID and AG (1, 5, 25 and 50 µM) was estimated by using TNBS method. 500 µL of samples was added to 500 µL of 4% (w/v) sodium bicarbonate buffer (pH 8.5) and 500 µL of 0.1% aqueous TNBS and mixed thoroughly. The reaction mixtures were incubated for 2 h at 40 °C followed by addition of 2.25 ml of concentrated HCl and incubated again at 110 °C for 90 min. The reaction mixture was then cooled to room temperature and centrifuged at 3000 rpm for 10 min 5 mL of ether was then added to the supernatant to eliminate the αTNP amino complex and the resulting solution was kept in hot water to evaporate any residual ether. The absorbance of the solution was recorded at 346 nm on Eppendorf Biospectrometer. The % Free lysine residue content was determined by using following equation:

% Free lysine residues = [(Abs. of Gly-BSA – Abs. of unmodified or inhibitor treated BSA) / Abs. of Gly-BSA] X 100.

Aggregation studies

Congo red (CR) binding assay

Aggregation in native and glycated BSA sample in presence or absence of inhibitor (NAC) was measured using amyloid specific Congo red dye according to the method described previously by (Tupe et al. 2013). Congo red (100µM), was prepared in PBS (pH 7.4) containing ethanol (10%, v/v). 500 µL of each of the above sample was incubated with 100µL Congo red solution for 20 minutes. The absorbance was measured after incubation at 530 nm.

Detection of thioflavin T-specific extrinsic fluorescence

Thioflavin T (ThT), a benzothiazole dye used to detect protein fibrils, exhibits elevated fluorescence while interacting with amyloid fibrils (Alvi et al., 2021; Nabi et al., 2020). Thus, the formation of amyloid fibrils in the current study was measured in unmodified and Gly-BSA in presence or absence of NAC (0, 100, 20, and, 300 µM) using a recently described ThT method. The samples were excited at 440 nm and the emission was recorded in wavelength range of 460–600 nm on Agilent Cary Eclipse Spectro-fluorimeter. The percentage increase/decrease in FI was measured using following equation for all samples at 490 nm and the results were represented as arbitraty units (a.u.).

% Increase or Decrease in ThT FI = [(FI of Gly-BSA − FI of unmodified or inhibitor treated Gly-BSA) / FI of Gly-BSA] X 100

ANS fluorescence measurements

Extrinsic fluorescence dyes like ANS are well known to emit significantly greater fluorescent when exposed to more hydrophobic environments including protein's interior apolar surfaces due to structural binding with unmodified and Gly-BSA in presence or absence of NAC (0, 100, 200, and 50 µM) was assessed following the standard protocol (Alvi et al., 2021). The samples were excited at 380 nm and the emission spectra were recorded in the wavelength range of 400–600 nm on Agilent

Cary Eclipse Spectro-fluorimeter. The concentration of ANS used in the assay was 10 μM. ANS fluorescence was normalized by using following equation at 525 nm and the results have been represented as arbitrary units (a.u.), as recorded on the Spectro-fluorimeter.

% Increase or decrease in ANS FI = [(FI of Gly-BSA − FI of unmodified or inhibitor treated Gly-BSA) / FI of Gly-BSA] X 100

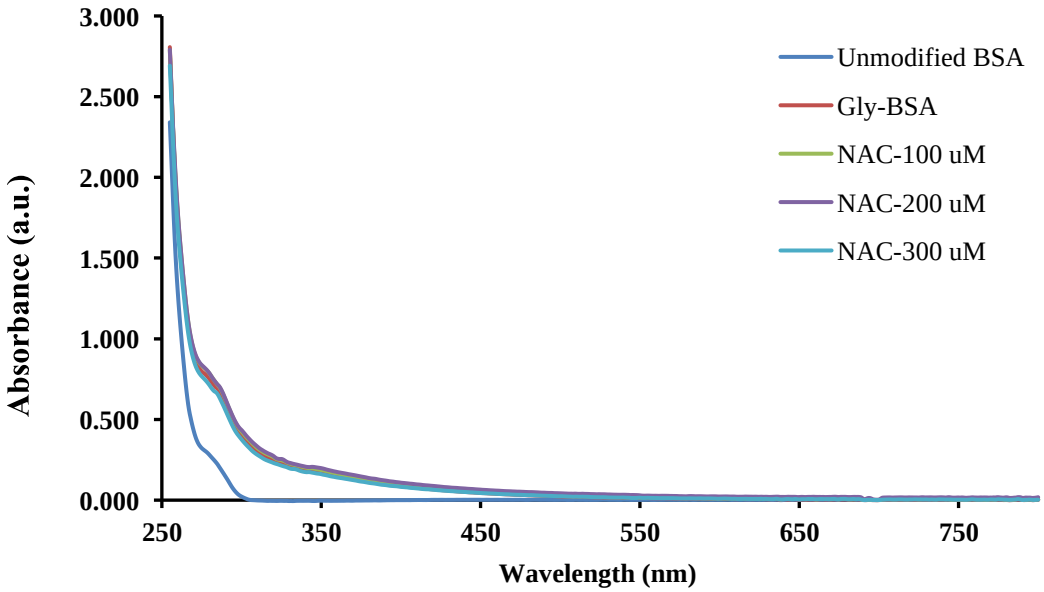

Figure 10. Hyperchromicity in unmodified BSA, Gly-BSA and N-acetyl cysteine (NAC) treated samples after 24 h of incubation (Day 1).

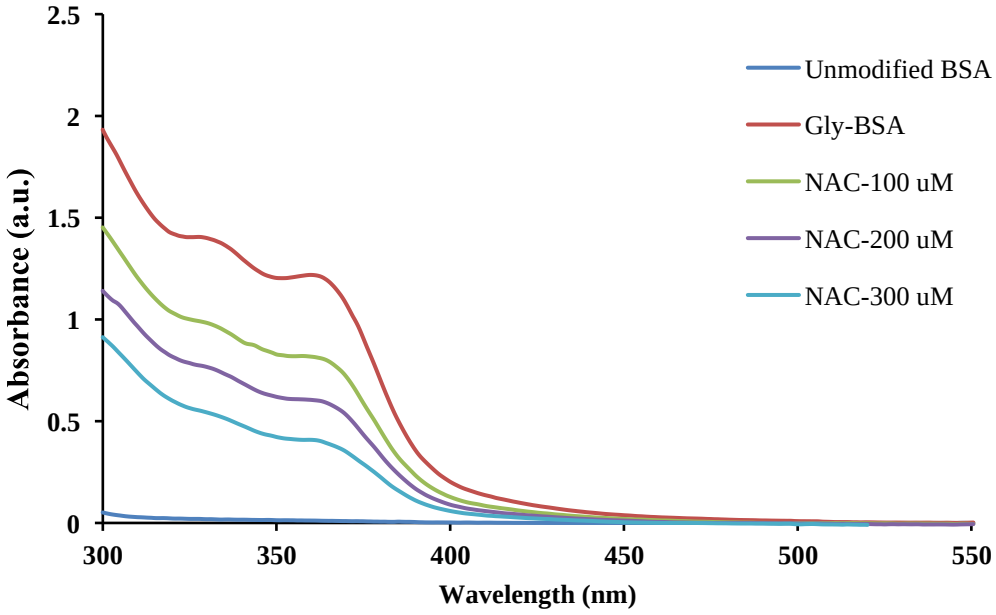

Figure 11. Hyperchromicity in unmodified BSA, Gly-BSA and N-acetyl cysteine (NAC) treated samples (Day 19).

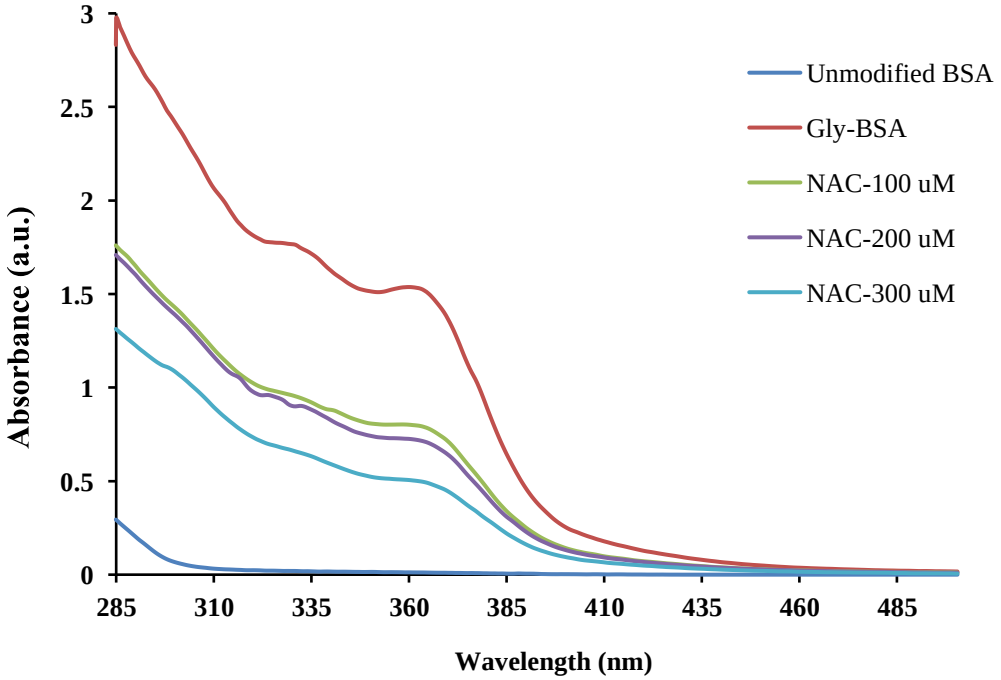

Figure 12. Hyperchromicity in unmodified BSA, Gly-BSA and N-acetyl cysteine (NAC) treated samples (Day 30).

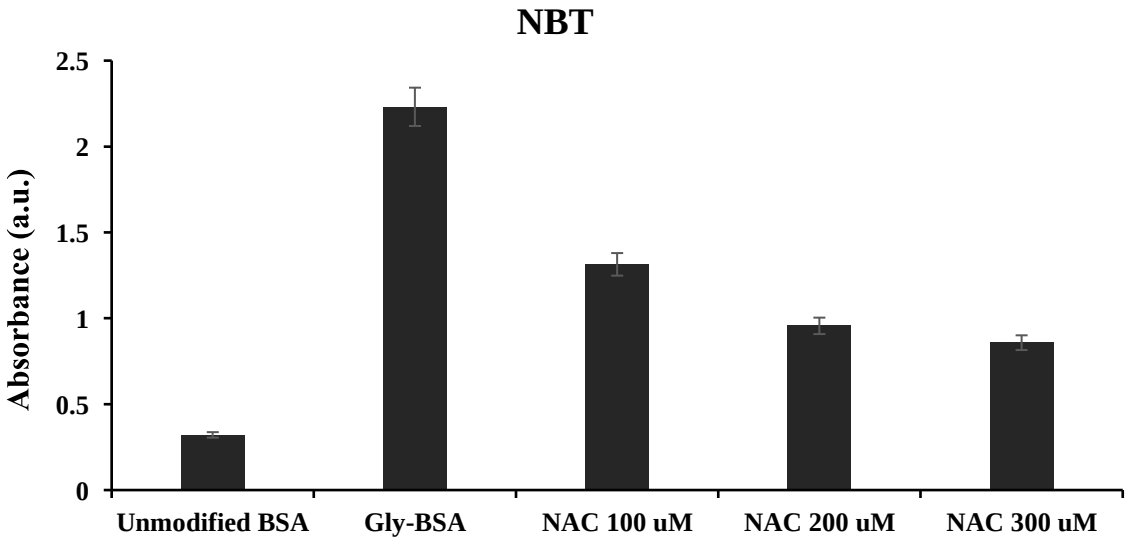

Figure 13. NBT reduction potential in unmodified BSA, Gly-BSA and N-acetyl cysteine (NAC) treated samples (Day 11). NBT reduction in each sample was

measured at 525 nm. The data represented are the mean ± SD of three independent experiments.

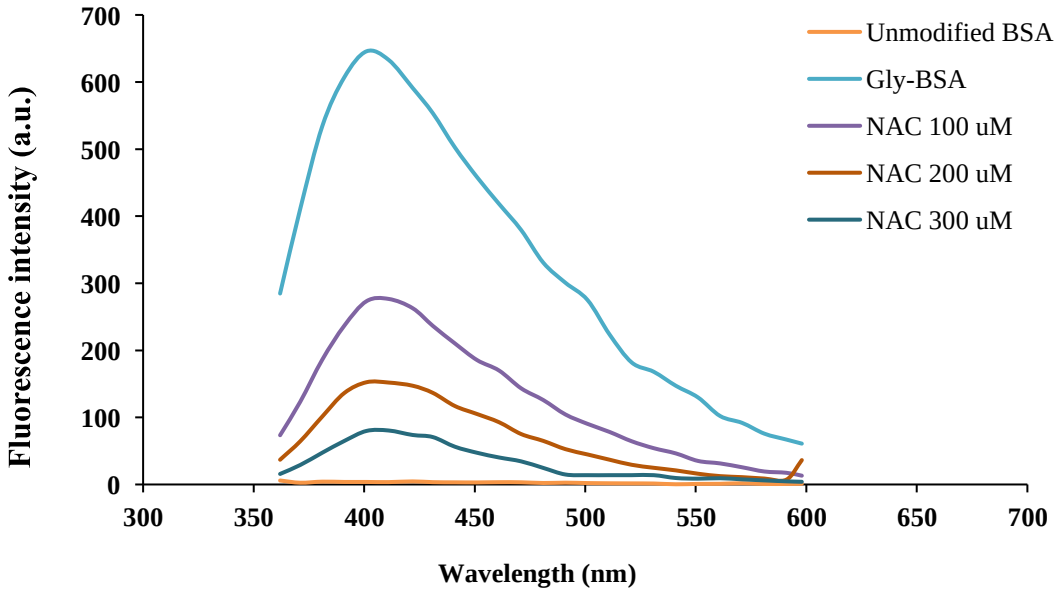

Figure 14. Fluorescence emission spectra of unmodified BSA, Gly-BSA and NAC (100, 200 and 300 µM) treated Gly-BSA on 19th day. The data shown in graph is a representative of three independent assays. Samples were excited at 370 nm and their emission was recorded in the wavelength range of 330–600 nm.

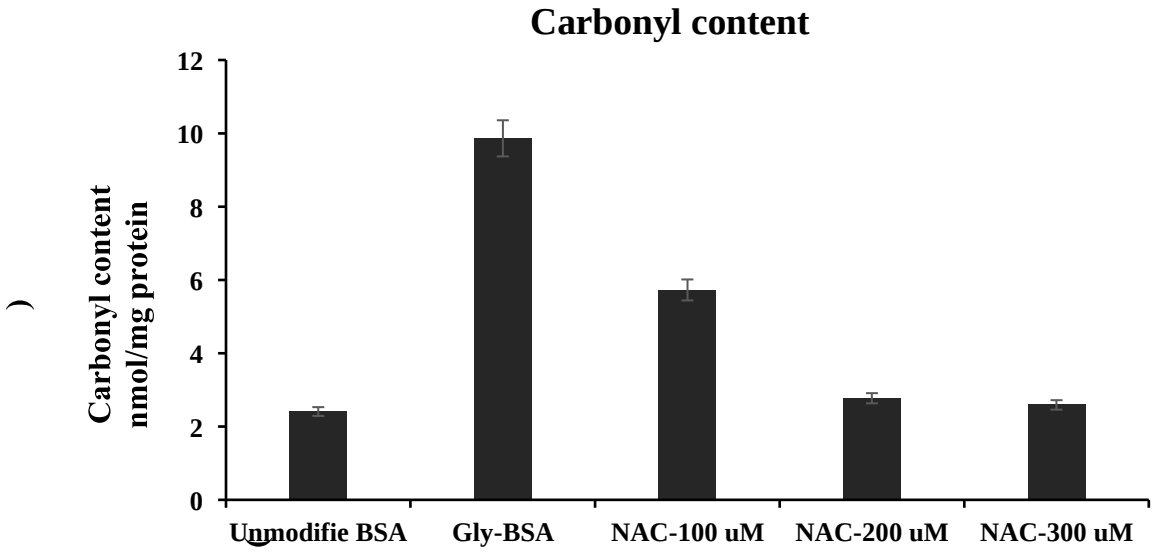

Figure 15. Carbonyl content of unmodified BSA, Gly-BSA and NAC treated GlyBSA. The data represented are the mean ± SD of three independent experiments.

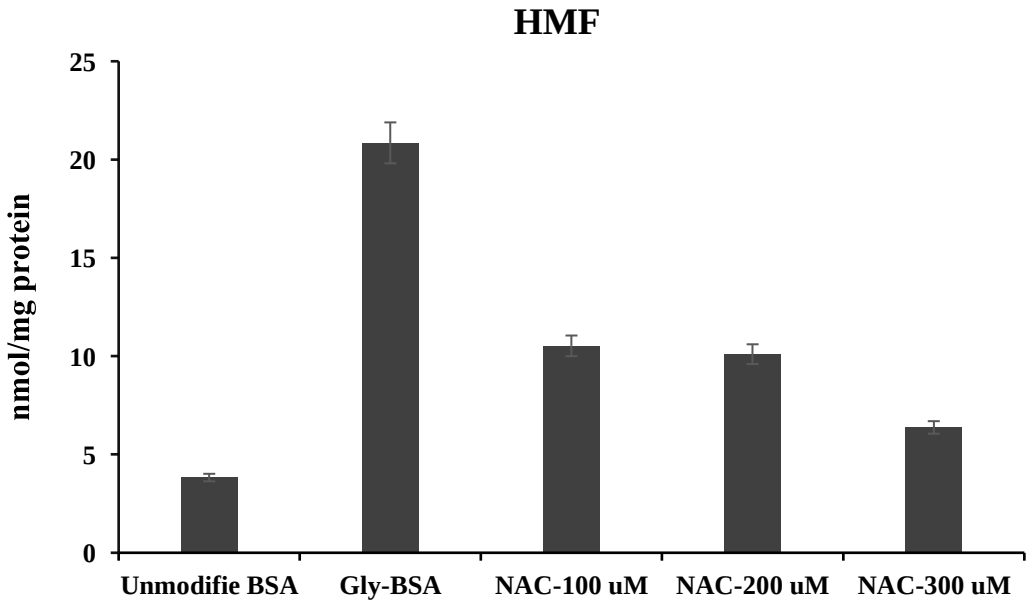

Figure 16. HMF content of unmodified BSA, Gly-BSA and NAC treated GlyBSA. The data represented are the mean ± SD of three independent experiments.

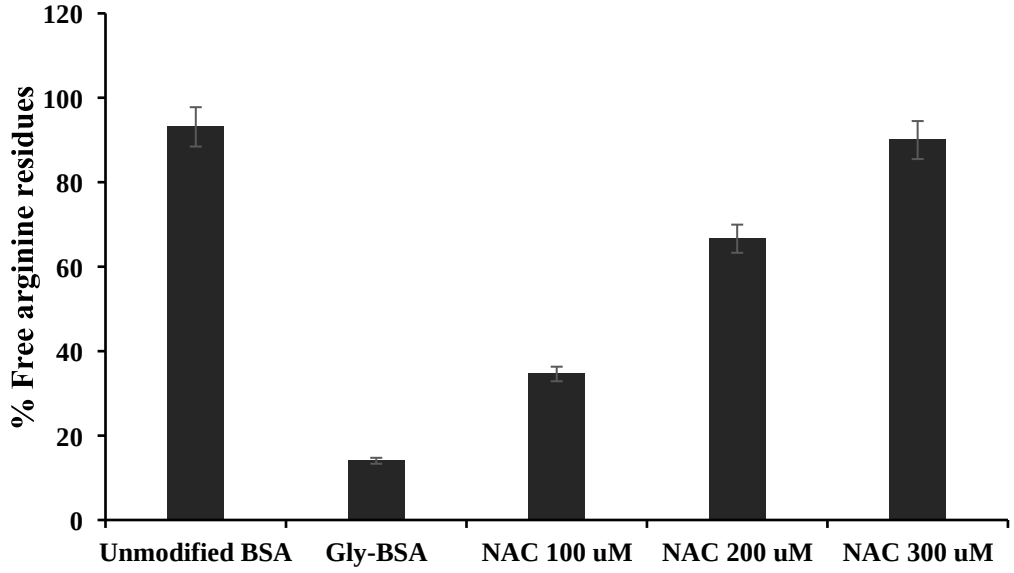

Figure 17. Free arginine residues in unmodified BSA, Gly-BSA and NAC treated Gly-BSA. The data represented are the mean ± SD of three independent experiments.

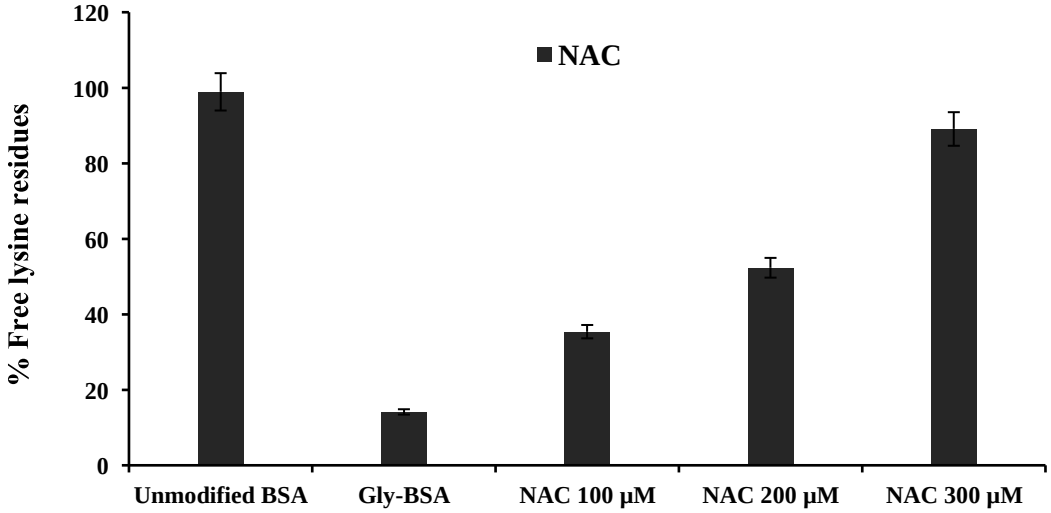

Figure 18: Free lysine residues in unmodified BSA, Gly-BSA and NAC treated Gly-BSA. The data represented are the mean ± SD of three independent experiments.

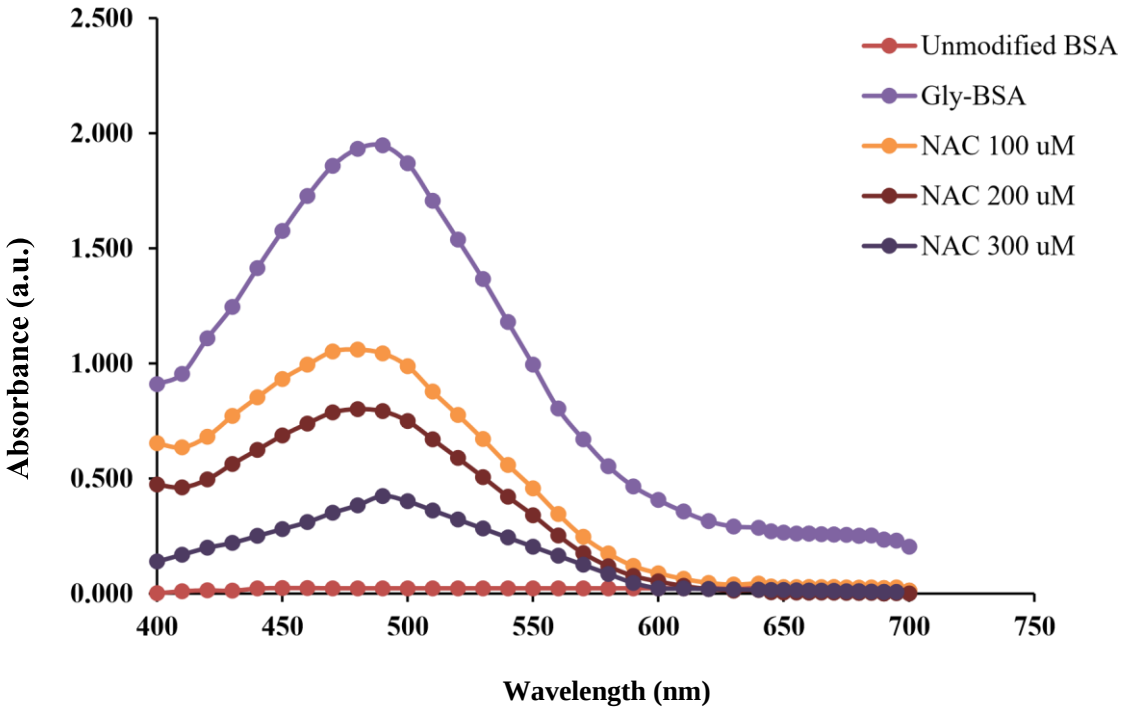

Figure 19. Congo Red binding in unmodified BSA, Gly-BSA and NAC treated Gly-BSA. Absorbance was recorded in the range of 400–700 nm in Eppendorf UVVis spectroscopy.

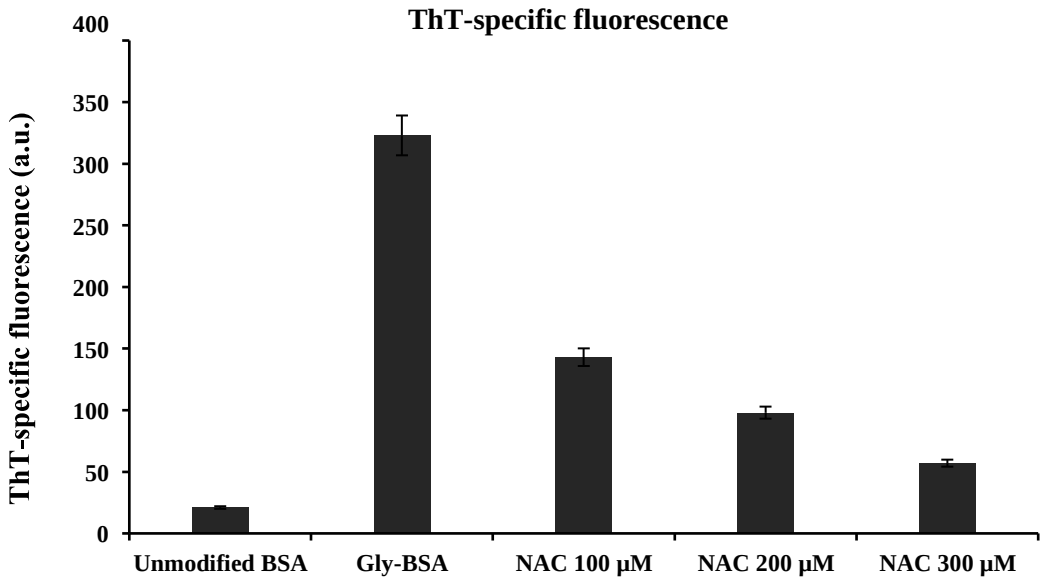

Figure 20: Thioflavin T-specific fluorescence in NAC treated Gly-BSA samples). The data represented are the mean ± SD of three independent experiments.

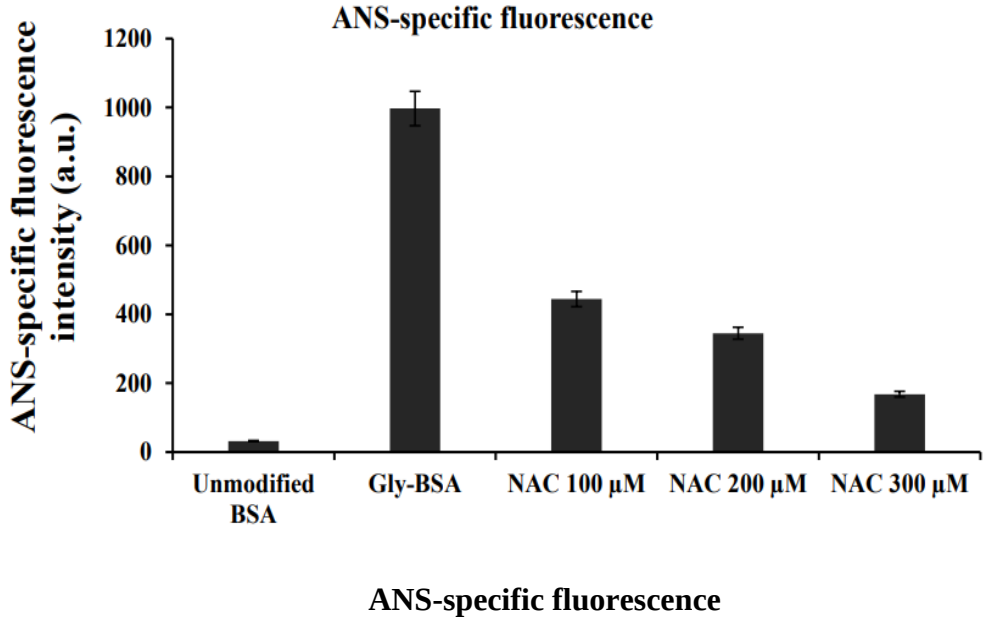

Figure 21: ANS-specific fluorescence in NAC treated Gly-BSA. The data represented are the mean ± SD of three independent experiments

Discussion

Non-enzymatic glycation of macromolecules, especially proteins leading to their oxidation is increased in patients with DM due to hyperglycemia and plays an important role in diabetic complications. Protein glycation mostly occurs in intra-chain lysine residues resulting in the formation of early-stage Amadori products which are finally converted to AGEs. A growing body of data demonstrates that AGEs are ultimately involved in the pathophysiology of ASCVD by stimulating inflammation, contributing to atheroma formation and modulating vascular stiffness (Prasad et al. 2012). There is considerable interest in antiglycation agents because of their therapeutic potential in reducing the morbidity and mortality associated with diabetes and associated diseases. Different strategies have been proposed to reverse the effects of AGEs, focusing on inhibition of AGE formation, removal of AGEs or interference with cellular effects of AGEs (Elosta et al., 2012).

This is the first study to report the inhibitory effect of NAC on D-ribose mediated BSA glycation. The structural changes induced in BSA were studied by UV Spectra (Ansari et al., 2009). Our results from UV-VIS spectroscopic analysis demonstrated that glycation with D-ribose resulted in increased hyperchromicity of BSA on the 19th day which was due to the perturbations in protein structures upon glycation. This enhanced hyperchromicity was reduced after incubation of Gly-BSA with NAC at varying concentrations. Moreover, being a potent natural antioxidant NAC also showed a marked regression in the hyperchromicity by 93.43% and 89.29% in case of Gly-BSA, when compared to respective untreated Gly-BSA. These ameliorative effects might have accompanied by the interference of aforesaid compound with the initial attachment of D-ribose to the amino groups of proteins, therefore, blocking the formation of Gly-BSA. In the present study, the inhibitory effect of NAC on glycation-induced structural alteration was estimated on the 9th day because, after 9 days of the incubation, the level of early glycation end products (EGEs) became negligible as these EGEs are known to transform into AGEs, an indication of complete glycation.

Our findings advocate the beneficial effects of NAC in combating AGEs induced diabetic secondary complications.

AGEs are a heterogeneous group of high molecular weight aggregates and have specific characteristic fluorescence spectra. Thus, we used auto-fluorescence to detect the formation of AGEs in all the samples. Our results demonstrated that the glycation of BSA resulted in the substantially enhanced level of fluorescent AGEs as depicted by increased intrinsic FI by 99.29% which are well in agreement with previous studies documenting the enhanced level of fluorescent AGEs in macromolecules after Maillard reaction (Nabi et al., 2020). Development of fluorescent AGEs is attributed to the formation of heterogeneous cyclic structures during the process of glycation which makes adducts fluoresce. Our data provide the evidence that protein-AGEs could interfere with normal physiological functions and might contribute to the initiation of diabetes-induced atherosclerosis and other secondary cardiovascular and diabetic problems including diabetic nephropathy (Nabi et al., 2019). The reported role of AGEs in vascular complications of diabetes and atherosclerotic cardiovascular disease (ASCVD) also prompted the development of novel pharmacological AGEs inhibitors. In this order, we found that the level of fluorescent AGEs in Gly-BSA were markedly decreased when treated with different concentration of test inhibitor (NAC), which was possibly resulted due to the potent antioxidant and/or free radical (ROS) scavenging activity of compound leading to lesser hyperchromicity, fluorescent AGEs and structural perturbations in Gly-BSA, hence, enhanced protein stability. In addition, NAC also inhibits either the formation of heterogeneous cyclic structures capable of fluoresces or eliminates the highly reactive open chain form of D-ribose. On the basis of above findings, we found that our inhibitor, NAC showed significant reduction in hyperchromicity and fluorescent AGEs in Gly-BSA.

Glycation is not only a major cause of AGE-mediated protein modification, but it also induces oxidation-dependent tissue damage, leading to the development of complications of diseases including diabetes. Protein-bound carbonyls represent a marker of global protein oxidation as it plays a key role in AGE formation and together with glycation generate protein-bound carbonyl groups, which have been detected in

human tissues (Nabi et al., 2018; 2020). ROS generation during glycation and glycoxidation is able to oxidize side chains of amino acid residues in protein to form carbonyl derivatives, which are well reckoned to mediate free radicalinduced damage to various biological macromolecules (Alvi et al., 2021). In this context, we also evaluated the CC and HMF content of BSA samples for the confirmation of early glycation end products (EGEs) generation, as these are the most commonly used biomarker of protein oxidation and EGEs/AGEs formation.

In the present study, the level of HMF and CC have been estimated on 9th day because it has been previously noted that the level of ketoamine and HMF content are at the maximum on day 9th and tend to decrease after 9th day in case of 80mM D-ribose Gly-BSA as both the HMF and CC are considered to be the part of the Amadori products (EGEs) and transform into AGEs with the course of time. CC for Gly-BSA, determined with dinitrophenylhydrazine (DNPH) method, was found to be the highest on day 9th which was inhibited in samples treated with NAC expressing their *in-vitro* anti-glycation potential. The decreased level of CC by NAC possibly resulted due to the carbonyl scavenging activity as well as the blocked formation of Amadori products during the process of glycation (Nabi et al., 2018).

HMF that might have formed in the early glycation of BSA was also determined as TBA reactive substance (TBARS) after hydrolysis. The HMF content in Gly-BSA was reported to be maximum at 9th day, when compared to HMF level of native BSA. We reported a marked amelioration of aforesaid changes after treatment with glycation inhibitor, NAC that might be resulted due to the free radical scavenging properties of this compound (Ahmad et al., 2019). Our finding clearly indicated that NAC may play an important role in the decreased pathogenesis of AGE-induced cardiovascular and micro/macro complications of diabetes via decreasing the ability of Gly-protein to complex with arterial wall proteoglycans, hence, reducing subsequent atherosclerotic events.

Further, the potent sites in proteins that are responsible for the glycation process are arginine and lysine residues (Nabi et al., 2020; Alvi et al., 2021) and most

strikingly BSA consists of a total of 59 lysine and 23 arginine residues, making it more prone to glycation (Nabi et al., 2020; Alvi et al., 2021). Recent in-vitro and invivo studies revealed that protein glycation is responsible for the structural alteration of proteins (Nabi et al., 2020; Alvi et al., 2021). In the same context, we also observed that the percent free arginine and lysine content was very smaller in GlyBSA, when compared to unmodified BSA, suggesting that most of the arginine and lysine resideus (83.71% and 83.78%, respectively) in Gly-BSA reacted and masked by D-Ribose molecules as a result of glycation. Further, we reported that treatment with NAC at different concentrations significantly increased the percent free arginine and lysine content in D-Ribose-exposed BSA, when compared to untreated GlyBSA. Such beneficial effects of NAC suggest that it might have combined with the residues of BSA and thereby reducing the extent of glycation and subsequent ROS generation and preventing the amino-acid residues from getting oxidized to form dicarbonyls (Nabi et al., 2020; Alvi et al., 2021).

Finally, aggregation specific dye Congo red was put into use to further validate the assumption and detect the formation of aggregates for 80mM sugar incubated BSA samples. It is clear from the results that the formation of amyloid-like aggregates of BSA is exclusively based on the type, concentration and time of incubation with a sugar, hinting at the risk posed by diabetic patients towards the formation of protein aggregates in their bodies. Our results demonstrated the enhanced Congo red-specific absorbance in Gly-BSA due to the formation for high molecular weight protein aggregates. However, treatment with NAC dosedependently reduced the formation of high molecular weight aggregates as evident by the reduced Congo red-specific absorbance in NAC treated samples. These results are consistent with the previous reports showing the antiglycation effects of other natural compounds like iridin (Nabi et al., 2020) and glycyrrhizic acid (Alvi et al., 2021).

Further, the amyloid fibrils can also be readily detected using ThT and ABS dyes, small molecules that give strong fluorescence upon binding to amyloids/fibrils (Nabi et al., 2020; Alvi et al., 2021). Our results from ThT and ANS-specific

fluorescence assays also showed that Gly-BSA possess more binding with ANS and ThT dye than unmofied BSA, suggesting the formation of amyloids and fibrillar aggregates upon D-Ribose-induced glycation. Our findings are well justified by previously published reports advocating the use of ThT dye for the monitoring of protein aggregation (Nabi et al., 2020; Alvi et al., 2021). However, treatment with NAC resulted in a marked decline in both ThT and ANS-specific fluorescence. These findings suggest that NAC has the potential to prevent the AGEs-induced protein damage and aggregation under hyperglycemia possibly through interaction with glycation prone sites of the protein.

CONCLUSION

The present study gives an insight into the *in-vitro* anti-glycation effects of NAC against D-Ribose-induced BSA glycation. Our results exhibited that NAC, being a strong antioxidant, has potent protective effects against D-ribose-induced *in-vitro* BSA glycation and which was achieved at multiple stages during the glycation process leading to the formation of AGEs. One of the mechanisms attributed to the anti-glycation potential of NAC was the hampering of free radical generation upon protein glycation and subsequent inhibition of protein modification. Based on our various *in-vitro* biophysical and biochemical assays, we concluded that NAC was capable to effectively prevent the process of glycation via reducing hyperchromicity, formation of EGPs and fluorescent AGEs, carbonyl content (CC), hydroxymethyl furfural (HMF) content, increasing the free lysine and free arginine content, reduced binding of congo red (CR), and reduced thioflavin T (ThT) and 8-aninilo- 1napthalene sulphonate (ANS)-specific fuorescence in Gly-BSA. Based on our initial findings, we concluded that NAC possesses the significant anti-glycation potential and might be established as a remarkable anti-AGEs therapeutic agent. Further *invivo* and clinical studies are still warranted to uncover the therapeutic effects of NAC against age-related as well as metabolic diseases.

REFERENCES

Ahmed N., Advanced glycation endproducts—role in pathology of diabetic complications, Diabetes Res. Clin. Pract. 67 (1) (2005) 3–21.

Ahmed N., O.K. Argirov, H.S. Minhas, C.A. Cordeiro, P.J. Thornalley, Biochem. J. 364 (2002) 1–14.

Alvi S.S., I.A. Ansari, M.K. Ahmad, J. Iqbal, M.S. Khan, Lycopene amends LPS induced oxidative stress and hypertriglyceridemia via modulating PCSK-9 expression and Apo-CIII mediated lipoprotein lipase activity, Biomed. Pharmacother. 96 (2017) 1082–1093,

Baynes J. W., "Chemical modification of proteins by lipids in diabetes," Clinical Chemistry and Laboratory Medicine, vol. 41, no. 9, pp. 1159–1165, 2003.

Biemel K.M., D.A. Friedl, M.O. Lederer, Identification and quantification of major Maillard cross-links in human serum albumin and lens protein, J. Biol. Chem. 277 (2002) 24907–24915.

Bierhaus A., et al. "Understanding RAGE, the receptor for advanced glycation end products". Journal of Molecular Medicine (Berl) 83.11 (2005): 876-886.

Busch, M.; Franke, S.; Rüster, C.; Wolf, G. Advanced Glycation End-Products and the Kidney. Eur. J. Clin. Investig. 2010, 40, 742–755.

Byun K., Y. Yoo, M. Son, J. Lee, G.B. Jeong, Y.M. Park, G.H. Salekdeh, B. Lee, Advanced glycation end-products produced systemically and by macrophages: a common contributor to inflammation and degenerative diseases, Pharmacol. Ther. 177 (2017) 44–55.

Cai W, Uribarri J, Zhu I, Chen X, Swamy S, et al. (2014) Oral glycotoxins are a modifiable cause of dementia and metabolic syndrome in mice and humans. Proc Natl Acad Sci USA 111: 4940-4945.

Cordain L., S. B. Eaton, A. Sebastian et al., “Origins and evolution of the western diet: health implications for the 21st century,” The American Journal of Clinical Nutrition, vol. 81, no. 2, pp. 341–354, 2005.

Deemter M. van, T. L. Ponsioen, R. A. Bank et al., “Pentosidine accumulates in the aging vitreous body: a gender effect,” Experimental Eye Research, vol. 88, no. 6, pp. 1043–1050, 2009.

Dludla P.V., S.E. Mazibuko-Mbeje, T.M. Nyambuya, V. Mxinwa, L. Tiano, F. Marcheggiani, et al., The beneficial effects of N-acetyl cysteine (NAC) against obesity associated complications: a systematic review of pre-clinical studies, Pharmacol. Res. 146 (2019) 104332.

Egaña-Gorroño L., R. López-Díez, G. Yepuri, L.S. Ramirez, S. Reverdatto, P.F. Gugger, A. Shekhtman, R. Ramasamy, A.M. Schmidt, Receptor for advanced glycation end products (RAGE) and mechanisms and therapeutic opportunities in 49 diabetes and cardiovascular disease: insights from human subjects and animal models, Front. Cardiovasc. Med. 7 (2020) 37

Gebhardt C., J. N´emeth, P. Angel, and J. Hess, “S100A8 and S100A9 in inflammation and cancer,” Biochemical Pharmacology, vol. 72, no. 11, pp. 1622– 1631, 2006.

Gill V., V. Kumar, K. Singh, A. Kumar, J.J. Kim, Advanced glycation end products (AGEs) may be a striking link between modern diet and health, Biomolecules 9 (12) (2019) 888.

Grzegorczyk-Karolak I., et al. “Inhibition of Advanced Glycation End-Product Formation and Antioxidant Activity by Extracts and Polyphenols from Scutellaria alpina L. and S. altissima L”. Molecules 21.6 (2016.).

Ilieva EV, Naudi A, Kichev A, Ferrer I, Pamplona R, et al. (2010) Depletion of oxidative and endoplasmic reticulum stress regulators in Pick disease. Free radic Biol Med 48: 1302-10.

Jakuš V., M. Sapák, J. Kostolanská, Circulating TGF-β1, glycation, and oxidation in children with diabetes mellitus type 1, Exp. Diabetes Res. 2012 (2012) 510902, ,

Maillard L-C. Action of amino acids on sugars. Formation of melanoidins in a methodical way. CR Hebd Acad Sci 1912; 154: 66–8.

Nabi R., S.S. Alvi, M. Saeed, S. Ahmad, M.S. Khan, Glycation and HMG-CoA reductase inhibitors: implication in diabetes and associated complications, Curr. Diabetes Rev. 15 (2019) 213–223.

Nabi, R., Alvi, S. S., Saeed, M., Ahmad, S., & Khan, M. S. (2019). Glycation and HMG-CoA reductase inhibitors: implication in diabetes and associated complications. Current diabetes reviews, 15(3), 213-223.

Nabi, R., Alvi, S. S., Saeed, M., Ahmad, S., & Khan, M. S. (2019). Glycation and HMG-CoA reductase inhibitors: implication in diabetes and associated complications. Current diabetes reviews, 15(3), 213-223.

Nabi, R., Alvi, S. S., Shah, M. S., Ahmad, S., Faisal, M., Alatar, A. A., & Khan, M. S. (2020). A biochemical & biophysical study on in-vitro anti-glycating potential of iridin against D-Ribose modified BSA. Archives of Biochemistry and Biophysics, 686, 108373.https://doi.org/10.1016/j.abb.2020.108373.

Rabbani N, Thornalley PJ (2015). Dicarbonyl stress in cell and tissue dysfunction contributing to ageing and disease. Biochem Biophys Res Commun, 458:221-25.

Rabbani, G., Ahmad, E., Zaidi, N. and Khan, R.H. pH-dependent conformational transitions in conalbumin (ovotransferrin), a metalloproteinase from hen egg white. Cell Biochem. Biophys. 61 (2011) 551–560.

Rahbar S, Natarajan R, Yerneni K, et al. (2000). Evidence that pioglitazone, metformin and pentoxifylline are inhibitors of glycation. Clin Chim Acta 301:65–77.

Ramasamy R., S.F. Yan, A.M. Schmidt, RAGE: therapeutic target and biomarker of the inflammatory response-the evidence mounts, J. Leukoc. Biol. 86 (2009).

Salahuddin P, Rabbani G, Khan RH (2014). The role of advanced glycation end products in various types of neurodegenerative disease: a therapeutic approach. Cell Mol Biol Lett, 19:407-37.

Schalkwijk C.G., T. Miyata, Early- and advanced non-enzymatic glycation in diabetic vascular complications: the search for therapeutics, Amino Acids 42 (4) (2012) 1193–1204.

Sergi D., L.M. Williams, Potential relationship between dietary long-chain saturated fatty acids and hypothalamic dysfunction in obesity, Nutr. Rev. 78 (2020) 261– 277, p. e1900934-e1900934.

Simionescu M., Endothelial cell—A key player in all stages of atherosclerosis, in: M. Simionescu, A. Sima, D. Popov (Eds.), Cellular dysfunction in atherosclerosis and diabetes, Romanian Academy Publishing House, 2005, pp. 73–96.

Stitt AW, Curtis Tm (2011) Diabetes - related adduct formation and retinopathy. J Occul Biol Dis Infor 4: 10-18.

Suliman M. E., O. Heimbürger, P. Bárány et al., “Plasma pentosidine is associated with inflammation and malnutrition in end-stage renal disease patients starting on dialysis therapy,” Journal of the American Society of Nephrology, vol. 14, no. 6, pp. 1614–1622, 2003.

Tan D, Wang Y, Lo CY, Ho CT. Methylglyoxal: its presence and potential scavengers. Asia Pac J Clin Nutr 2008;17(Suppl 1):261e4

Tan, A.L.; Forbes, J.M.; Cooper, M.E. AGE, RAGE, and ROS in Diabetic Nephropathy. Semin. Nephrol. 2007, 27, 130–143.

Thornalley P.J., Glycation in diabetic neuropathy: characteristics, consequences, causes and therapeutic options, Int. Rev. Neurobiol. 50 (2002) 37–57.

Tian J., A. M. Avalos, S.-Y. Mao et al., “Toll-like receptor 9- dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE,” Nature Immunology, vol. 8, no. 5, pp. 487–496, 2007.

Vistoli G., D. De Maddis, A. Cipak, N. Zarkovic, M. Carini, and G. Aldini, “Advanced glycoxidation and lipoxidation end products (AGEs and ALEs): an overview of their

mechanisms of formation," Free Radical Research, vol. 47, Supplement 1, pp. 3–27, 2013.

Vlassara H., G.E. Striker, AGE restriction in diabetes mellitus: a paradigm shift, Nat. Rev. Endocrinol. 7 (9) (2011) 526–539.

Wang X., J. Liu, Y. Yang, X. Zhang, An update on the potential role of advanced glycation end products in glycolipid metabolism, Life Sci. 245 (2020), 117344.

Yamagishi, S.; Matsui, T. Advanced Glycation End Products, Oxidative Stress and Diabetic Nephropathy. Oxid. Med. Cell. Longev. 2010, 3, 101–108.

Yamamoto Y., et al. "Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice". Journal of Clinical Investigation 108.2 (2001): 261-268.

Yoshinaga E, Kawada A, Ono K, Fujimoto E, Wachi H, Harumiya S, et al (2012). N(ε)-(carboxymethyl)lysine modification of elastin alters its biological properties: implications for the accumulation of abnormal elastic fibers in actinic elastosis. J Invest Dermatol, 132:315-23.

Zhang Q, Ames JM, Smith RD, Baynes JW, Metz TO. A perspective on the Maillard reaction and the analysis of protein glycation by mass spectrometry: probing the pathogenesis of chronic disease. J Proteome Res 2009; 8: 754–69.