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ORIGINAL ARTICLE

Oxidative Stress Mediated Cytotoxicity of Glycated Albumin: Comparative Analysis of Glycation by Glucose Metabolites

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Abstract The non-enzymatic reaction between reducing sugars and proteins has received increased attention in nutritional and medical research recently. In the current manuscript, effect of glycation in structural changes of human serum albumin (HSA) by the metabolites of glucose such as glyoxal, methylglyoxal and glyceraldehyde was studied using different spectroscopy techniques. Glycation of HSA was monitored by following advanced glycation end-products (AGEs) fluorescence changes, HSA intrinsic fluorescence measurement, extrinsic fluorescence using 8-analino 1-nephthlene sulfonic acid (ANS) dye, and circular dichroism (CD) studies. AGEs were formed within 7 days of incubation with glyoxal, methylglyoxal and glyceraldehyde. However, methylglyoxal induced significant structural changes in HSA compared with glyoxal and glyceraldehydes. Moreover, ANS binding to native and glycated-HSA showed difference in binding pattern of these metabolites to HSA. The CD spectrum revealed changes in the secondary structure of HSA upon glycation when compared to native HSA. Furthermore, the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay established the cytotoxicity of the glycated-HSA towards human liver carcinoma (HepG2) cell lines via the production of reactive oxygen species.

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Introduction

Non-enzymatic glycation is a complex cascade of reactions initiated by condensation of reducing sugars with the free amino groups of proteins to form reversible Schiff's bases, which undergo rearrangement to form relatively stable Amadori products. Amadori products, over a period of time, undergo a series of reactions involving multiple dehydration, fragmentation, and oxidative modification via highly reactive dicarbonyl intermediates to form stable, heterogeneous adducts called advanced glycation end products (AGEs) [1–3].

Although AGEs formation occurs during the normal ageing process, it is accelerated under hyperglycemic conditions. Further, it has been shown that formation of AGEs in vivo contributes to several pathophysiologies associated with ageing and/or diabetes mellitus, for example arthritis, atherosclerosis, chronic renal insufficiency, Alzheimer's disease, nephropathy, neuropathy, and cataracts [4–8]. In addition, many cells have receptors for AGEs (RAGE). Interaction of AGE with RAGE leads to activation of NF-kB, which stimulates generation of the pro-inflammatory and adhesion molecules that underlie the pathology of diabetic vascular complications [9]. Moreover, abnormal modification, including glycation, induces neuronal proteins to misfold and form amyloid fibrils in a stepwise process from prefibrils to fibrils [10].

Tissue and circulating AGE levels are higher in smokers with concurrent increase in inflammatory markers [11]. There is evidence from animal studies that exposure to high levels of exogenous AGEs contributes to renal and vascular complications [12]. AGEs often accumulate intracellularly [13] as a

result of their generation from glucose-derived dicarbonyl precursors [14].

Glycation of proteins interferes with their normal functions by disrupting molecular conformation, altering enzymatic activity, reducing degradation capacity, and interfering with receptor recognition [15]. Glycation-derived free radicals can cause protein fragmentation, and oxidation of nucleic acids and lipids [16]. The amino groups of adenine and guanine bases in DNA are also susceptible to glycation and AGE formation [17].

Glyoxal and methylglyoxal are potent endogenous glycating glucose metabolites. Methylglyoxal is formed spontaneously from triose phosphates in all organisms during anaerobic glycolysis [18] and from other non-enzymatic and enzymatic pathways [19]. Glyoxal is formed by lipid peroxidation and degradation of monosaccharides, saccharide derivatives and glycated proteins [20]. Glyceraldehyde is also involved in the glycation of proteins. It is derived from glyceraldehyde-3-phosphate, an intermediate of glycolysis, through the polyol pathway, or from fructose, during its transformation by fructokinase [21].

Human serum albumin (HSA) is the most abundant (40 mg/mL) and quantitatively the most important depot and transport protein in blood plasma. It is also a major antioxidant plays an important role in maintaining osmolarity of plasma and interstitial fluids. In vivo, the proportion of glycated albumin in healthy persons is in the range between 1 and 10 % [21, 22]. However, among individuals with hyperglycemia it can increase two to threefold [23, 24]. For this reason serum albumin has been adopted as a model in many in-vitro studies on glycation [25, 26]. To determine the role of glycation, it was imperative to assess the specific pattern of modifications caused by various glycating agents. In the present study, we attempted to elucidate the specific alterations (Conformational change) in human serum albumin (HSA) caused by glycation with different carbonyl metabolites. Moreover, we also investigate toxicity of glycated-HSA in liver (HepG2) cancer cell lines.

Material and Methods

Glycation of Human Serum Albumin

Human serum albumin (0.5 mg/mL) was incubated in phosphate buffer (50 mM, pH 7.4) with 0.5 mM of glyoxal, methylglyoxal and glyceraldehyde for 7 days at 37 °C. The concentration of protein was estimated using Bradford method. BSA (1 mg/mL) was used as standard.

Tertiary Structure of Native and Glycated HSA: Intrinsic Fluoresence Measurement

Intrinsic fluorescence of native and glycated HSA (2 μM) was monitored by the use of Jasco (FP-750) fluorescence spectrofluorometer. The emission spectrum from 300 to 400 nm was recorded after excitation at 280 nm at 25 °C. Both the excitation and emission slit widths were at 5 nm.

Detection of Advanced Glycation end Products (AGEs)

The AGE-related fluorescence of all incubated samples was obtained on a Jasco (FP-750) fluoresence spectrophotometer by exciting at 320 nm and the emission was recorded between 345 and 600 nm. Excitation and emission slit width was 5 and 10 nm respectively.

Surface Hydrophobicity Analysis: ANS Fluorescence Measurement

A stock solution of 1 mM ANS (Sigma, USA) was prepared in water. Native and glycated HSA (2 μM) and ANS (50 μM) were mixed at room temperature and the fluorescence was subsequently measured by recording the emission spectrum from 400 to 600 nm after excitation at 380 nm.

Circular Dichroism (CD) Analysis of Native and Glycated-HSA

Far-UV CD measurements were performed by the use of a circular dichroism chiroptical spectrometer (Applied Photophysics, Chirascan-Plus, UK). Samples in a 1 mm quartz cuvette were maintained at 25 °C with a circulating water bath. Spectra of native and glycated HSA (0.2 mg/mL) at different times were measured in the range 200–280 nm with a step size of 1.0 nm. Each measurement was repeated thrice and mean values were taken.

Cytotoxicity of Glycated-HSA

Cell Culture HepG2 cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 0.2 % sodium bicarbonate, and antibiotic/antimycotic solution (1 mL/100 mL of medium). The cells were maintained in 5 % CO_2–95 % atmosphere under high humidity at 37 °C.

MTT Assay HepG2 cells were treated with different concentrations of glycated-HSA (1.5–12 μg/mL) for 24 h. The treated cells were studied for cytotoxicity assay by MTT assay. Percent cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described earlier by Siddiqui et al. [27]. Briefly, cells

(1 × 104) were allowed to adhere for 24 h in CO_2 incubator at 37 °C in 96 well culture plates. After 24 h exposure, MTT (5 mg/mL of stock in PBS) was added (10 μl/well in 100 μl of cell suspension) in each well and plates were incubated for 4 h in CO_2 incubator at 37 °C. Then, supernatant was discarded and 200 μl DMSO was added to each well and mixed gently. The developed color was read at 550 nm. Untreated sets were also run under identical conditions and served as control.

Morphological Analysis Morphological changes were observed to determine the alterations induced by glycated-HSA in HepG2 cells. All the cells were exposed to different concentrations (1.5–12 μg/mL) of glycated-HSA for 24 h. The images were taken using an inverted phase contrast microscope at 20× magnification.

Reactive Oxygen Species (ROS) Generation ROS generation was assessed using 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma–Aldrich, USA) dye as a fluorescence agent following the protocol [28]. After the exposure of

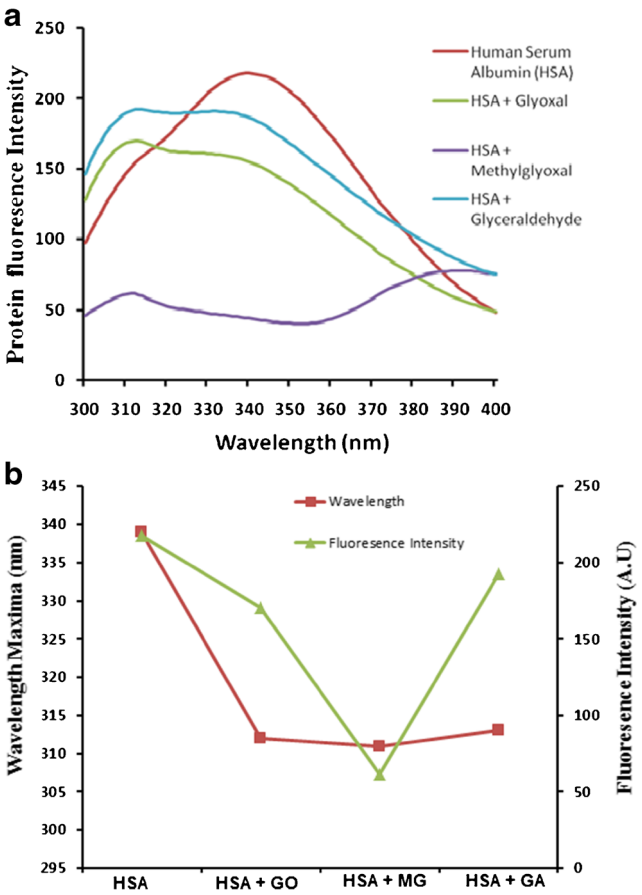

Fig. 1 Intrinsic Fluorescence measurement of native and Glycated-HSA. **a** The emission spectrum from 300 to 400 nm was recorded after excitation at 280 nm at 25 °C. Both the excitation and emission slit widths were at 5 nm. 2 μM of Native and glycated-HSA was used for fluorescence measurement. **b** Change in wavelength maxima was plotted against glycating agent

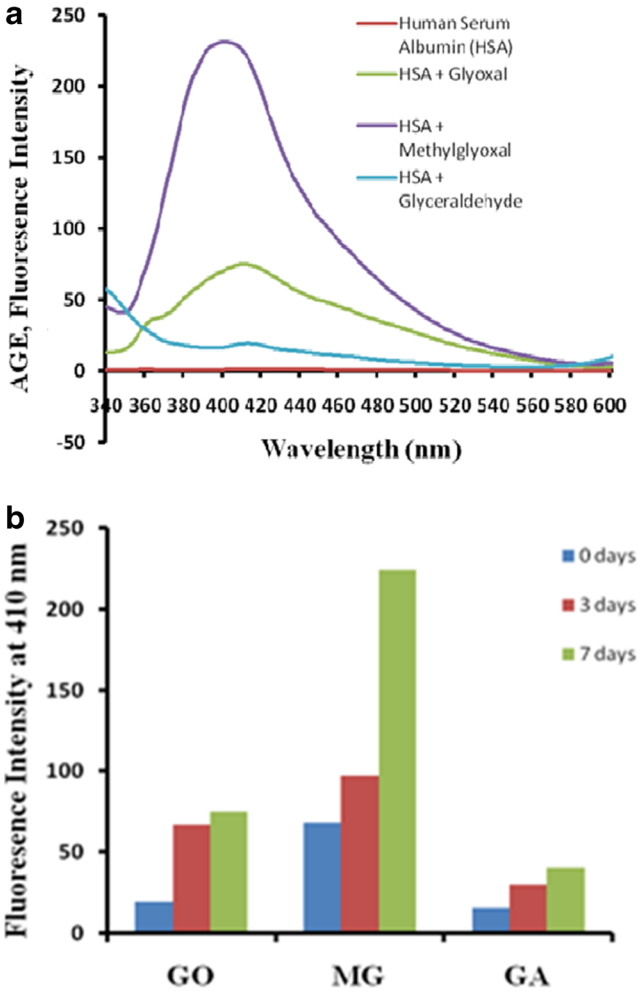

Fig. 2 Detection of advanced glycation end product (AGE). **a** Fluoresence was measured by exciting at 320 nm and the emission was recorded between 345 and 600 nm. Excitation and emission slit width was 5 and 10 nm respectively. **b** Time dependent AGEs formation

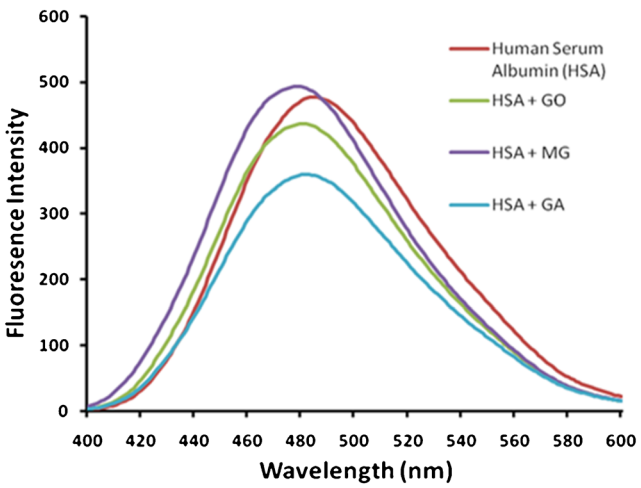

Fig. 3 ANS (8-anilino 1-napthalene sulphonic acid) fluorescence measurement. ANS, fluorescence was measured by recording the emission spectrum from 400 to 600 nm after excitation at 380 nm

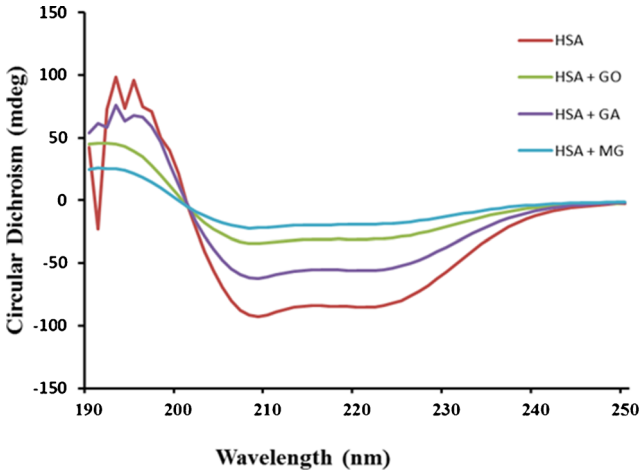

Fig. 4 Circular Dichroism (CD) measurement of native and Glycated-HSA. Spectra of native and glycated HSA (0.2 mg/mL) after 7 days incubation were measured in the range of 200–280 nm with a step size of 1.0 nm

glycated-HSA for 24 h, cells were washed with PBS and were incubated for 60 min in DCFH-DA (20 μM) containing incomplete culture medium in dark at 37 °C. Then, the cells were analyzed for intracellular fluorescence using fluorescence microscope.

Results and Discussion

Glycation of proteins by glucose metabolites such as glyoxal, methylglyoxal and glyceraldehyde have been reported to play a critical role in ageing [29], diabetes [16, 17], renal failure [18, 19], Alzheimer's disease [10], tumourigenesis and multidrug resistance in cancer chemotherapy [30].

Intrinsic fluorescence analysis showed quenching/decrease in fluorescence intenisty of Glycated-HSA compared to native HSA as indicated in Fig. 1(a). Glyceraldehyde and Glyoxal induced marginal changes in HSA while methylglyoxal displayed maximum decrease in the intrinsic fluorescence intensity. This can be attributed to conformational changes, which may expose tryptophan residues to extrinsic quenchers. Glycation induced conformational change has been earlier reported in various proteins including HSA, α-Synuclein, ovalbumin, lysozyme and hemoglobin [31, 32].

Changes in the emission wavelength maxima were also observed in HSA during glycation. A blue shift was observed in HSA treated with glyoxal, methylglyoxal and glyceraldehyde Fig. 1(b). However, maximum decrease in fluorescence intensity and a blue shift of 30 nm was found in methylglyoxal treated-HSA when compared to HSA incubated with Glyoxal and Glyceraldehyde. Blue shift in Glycated-HSA suggested that human serum albumin may get converted into more compact and transition aggregate-like structures. Glycation induced protein aggregation has been reported previously in insulin [33], hemoglobin [31] and other proteins [10].

Advanced glycation end-products (AGEs) formation was detected using fluorescence spectroscopy techniques [10]. AGEs give emission spectra between 400 and 410 nm after excitation at 320 nm. Our results (Fig. 2a and b) illustrated formation of AGEs in the presence of glyoxal, methylglyoxal and glyceraldehyde as evident from the increase in fluorescence around 400 nm. Increase was more prominent in HSA treated with methylglyoxal. Higher levels of AGEs were formed in the presence of methylglyoxal, suggesting dicarbonyl are more efficient glycating agent in comparision to glyceraldehyde.

1-anilinonaphthalene-8-sulfonate (ANS) is an extrinsic dye to measure protein variation in hydrophobicity. ANS binds to

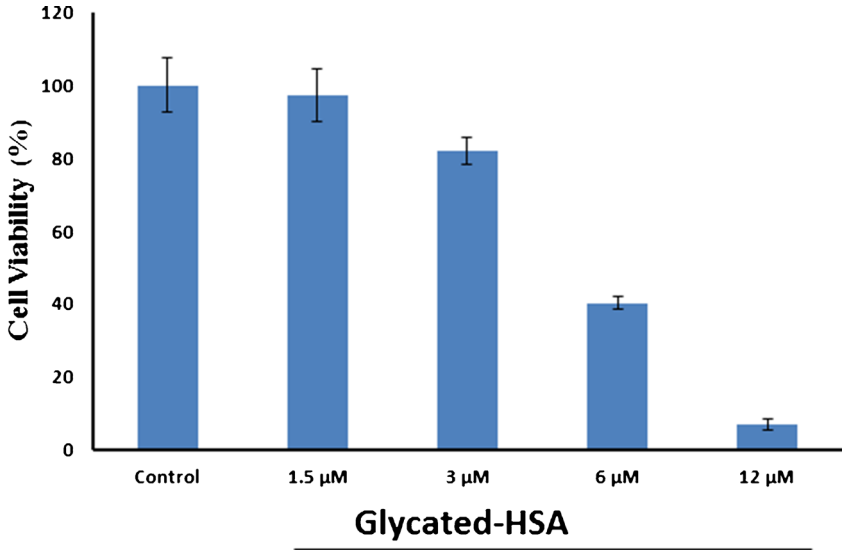

Fig. 5 Cytotoxicity of glycated-HSA: MTT reduction assay. Percent viability of HepG2 was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

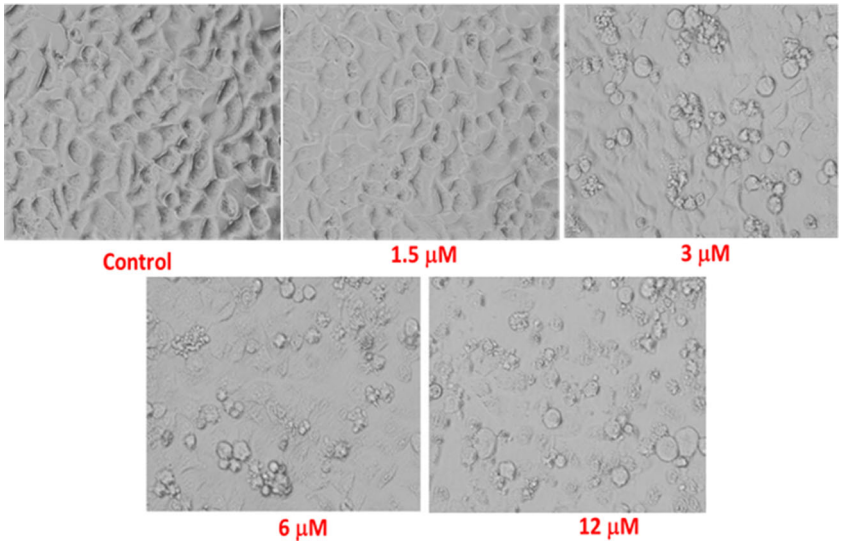

Fig. 6 Morphological analysis of HepG2 cell lines. Alterations induced by glycated-HSA in HepG2 cells. The images were taken using an inverted phase contrast microscope at 20× magnification

hydrophobic domains in protein structure to measure the extent of hydrophobicity in protein. Protein bound ANS (Fig. 3) fluorescence intensity of Glycated-HSA in the presence of glyoxal and methylglyoxal was similar compared to native HSA, suggesting similarity in the mode of binding towards hydrophobic amino acids. However, ANS fluorescence intensity of glyceraldehyde modified-HSA decreased, indicating a conformational changes in HSA from glyceraldehyde interaction resulting in concealing the hydrophobic domains. Hydrophobic domains are perturbed in glycated-HSA, suggesting that the hydrophobic domains might be self-associated to form aggregate-like structure.

Far-UV CD spectrum of HSA is characterized by the presence of two strong negative bands at 208 and 222 nm, suggesting α-helical structure in HSA, which changes significantly after glycation (Fig. 4). Secondary structure of proteins diminished in glycated protein [31, 32].

The cytotoxicity of the glycated-HSA formed under methylglyoxal treatment was examined by adding aliquots of the glycated product, in the range of 1.5–12 μg/mL to HepG2 cell culture media. Cytotoxicity of the glycated HSA was evaluated by MTT reduction inhibition assay. Reduction in cell viability was measured in the presence of glycated-HSA (Fig. 5). 6–12 μg/mL of glycated-HSA showed maximum reduction in cell viability.

The morphology of the untreated and treated HepG2 cells was examined by inverted phase contrast microscope to provide physical evidence and ascertain the toxic effect of glycated product. The cultured cells showed changed morphological patterns upon the treatment with glycated -HSA when compared to the control (Fig. 6).

To examine if the glycated-HSA led biological toxicity was mediated by free radicals, reactive oxygen species (ROS) was measured. There was a high rise in green fluorescence (Fig. 7) indicating huge ROS production in culture cells treated with glycated-HSA compared to control cells. These results suggested that the toxicity of glycated product is largely mediated by the production of ROS. The cause of cytotoxicity might be interaction of protein aggregate with cell membranes of the cultured cells, although some other mechanism(s) for the same cannot be ruled out. The similar trend of cytotoxicity of glycated protein was earlier observed in neurotypic (SH-SY5Y) cell line [34].

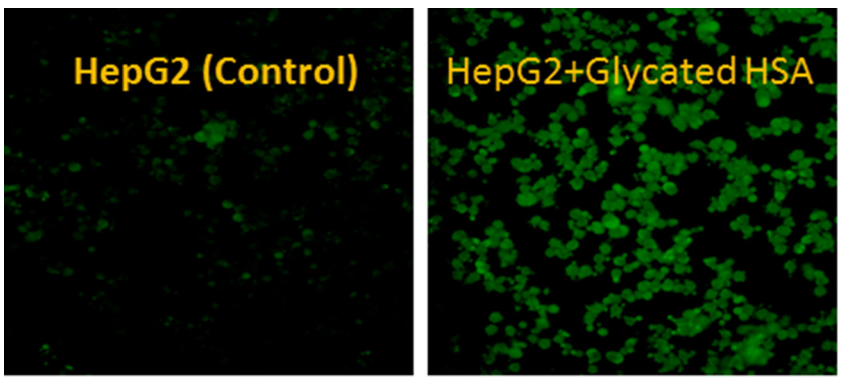

Fig. 7 Reactive Oxygen measurement in HepG2 cell lines. ROS generation was assessed using 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) dye as a fluorescence agent

Conclusion

Glycation induced various conformational changes in human serum albumin. HSA is rapidly glycated in the presence of glucose metabolites generating aggregate like structure which caused cell oxidative stress and resulted in high cytotoxicty towards HepG2 cell lines. Future studies on specific protein domains and mode of interactions would be helpful in the development of anti-glycating agents.

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References

1. Sing R, Barden A, Mori T (2001) Advanced glycation end products: a review. Diabetologia 44:129–146
2. Baynes JW, Watkins NG, Fisher CI (1989) The amadori product on protein: structure and reactions. Prog Clin Biol Res 304:43–67
3. Monnier VM (1989) Structure elucidation of a senescence cross-link from human extracellular matrix: implication of pentoses in the aging process. J Biol Chem 264:21597–21602
4. Vlassara H (1996) Advanced glycation end products and atherosclerosis. Ann Med 28:419–426
5. Lyons TJ, Silvestri G, Dunn JA (1991) Role of glycation in modification of lens crystallins in diabetic and nondiabetic senile cataracts. Diabetes 40:1010–1015
6. Brownlee M (1995) Advanced protein glycosylation in diabetes and aging. Annu Rev Med 46:223–234
7. Shuvaev VV, Laffont I, Serot JM (2001) Increased protein glycation in cerebrospinal fluid of Alzheimer's disease. Neurobiol Aging 22: 397–402
8. Kumar MS, Reddy PY, Kumar PA (2004) Effect of dicarbonyl induced browning on α-crystallin chaperone-like activity: physiological significance and caveats of in vitro aggregation assays. Biochem J 379:273–282
9. Mrudula T, Suryanarayana P, Srinivas PNBS (2007) Effect of curcumin on VEGF expression in diabetic retina. Biochem Biophys Res Commun 361:528–532
10. Khan MS, Dwivedi S, Priyadarshini M, et al (2013) Ribosylation of bovine serum albumin induces ROS accumulation and cell death in cancer line (MCF-7). Eur Biophys J 42:811–818
11. Nicholl ID, Stitt AW, Moore JE, Ritchie AJ, Archer DB, Bucala R (1998) Increased levels of advanced glycation endproducts in the lenses and blood vessels of cigarette smokers. Mol Med 4:594–601
12. Zheng F, He C, Cai W, Hattori M, Steffes M, Vlassara H (2002) Prevention of diabetic nephropathy in mice by a diet low in glycoxidation products. Diabetes Metab Res Rev 18:224–237
13. Giardino I, Edelstein D, Brownlee M (1994) Nonenzymatic glycosylation in vitro and in bovine endothelial cells alters basic fibroblast growth factor activity. A Model for Intracellular Glycosylation in Diabetes. J Clin Investig 94:110–117
14. Brownlee M (2001) Biochemistry and molecular cell biology of diabetic complications. Nature 414:813–820
15. Hsieh CL, Yang MH, Chyau CC, Chiu CH, Wang HE, Lin YC, Chiu WT, Peng RY (2007) Kinetic analysis on the sensitivity of glucose- or glyoxal-induced LDL glycation to the inhibitory effect of psidium guajava extract in a physiomimic system. Bio Systems 88:92–100
16. Baynes JW (1991) Role of oxidative stress in development of complications in diabetes. Diabetes 40:405–412
17. Baynes JW (2002) The maillard hypothesis on aging: time to focus on DNA. Ann N Y Acad Sci 959:360–367
18. Phillips SA, Thornalley PJ (1993) The formation of methylglyoxal from triose phosphates. Investigation Using a Specific Assay for Methylglyoxal. Eur J Biochem 212:101–105
19. Thornalley PJ (1993) The glyoxalase system in health and disease. Mol Aspects Med. 14:287–371
20. Loidl-Stahlhofen A, Spiteller G (1994) α-hydroxyaldehydes, products of lipid peroxidation. Biochim Biophys Acta 1211:156–160
21. Shaklai N, Garlick RL, Bunn HF (1984) Nonenzymatic glycosylation of human serum albumin alters its conformation and function. J Biol Chem 259:3812–3817
22. Peters TJ (1996) All about albumin—biochemistry, genetics, and medical applications. Academic Press, San Diego
23. Woodside JV, Yarnell JW, McMaster D, et al (1998) Effect of B-group vitamins and antioxidant vitamins on hyperhomocysteinemia: a double-blind, randomized, factorial-design, controlled trial. Am J Clin Nutr 67:858–866
24. Bourdon E, Loreau N, Blache D (1999) Glucose and free radicals impair the antioxidant properties of serum albumin. FASEB J 13: 233–244
25. Syrovy I (1994) Glycation of albumin: reaction with glucose, fructose, galactose, ribose or glyceraldehyde measured using four methods. J Biochem Biophys Methods 28:115–121
26. Culbertson SM, Vassilenko EI, Morrison LD, Ingold KU (2003) Paradoxical impact of antioxidants on post-amadori glycoxidation: counterintuitive increase in the yields of pentosidine and nepsilon-carboxymethyllysine using a novel multifunctional pyridoxamine derivative. J Biol Chem 278:38384–38394
27. Siddiqui MA, Ahmad J, Farshori NN, Saquib Q, Jahan S, Kashyap MP, Ahamed M, Musarrat J, Al-Khedhairy A (2013) Rotenone-induced oxidative stress and apoptosis in human liver HepG2 cells. Mol Cell Biochem 384:59–69
28. Siddiqui MA, Singh G, Kashyap MP, Khanna VK, Yadav S, Chandra D, Pant AB (2008) Influence of cytotoxic doses of 4-hydroxynonenal on selected neurotransmitter receptors in PC-12 cells. Toxicol in Vitro 22:1681–1688
29. Turk Z, Ljubic S, Turk N, Benko B (2001) Detection of autoantibodies against advanced glycation endproducts and AGE-immune complexes in serum of patients with diabetes mellitus. Clin Chim Acta 303:105–115
30. Van-Heijst JW, Niessen HW, Hoekman K, Schalkwijk CG (2005) Advanced glycation end products in human cancer tissues: detection of N-epsilon-(carboxymethyl) lysine and argpyrimidine. Ann N Y Acad Sci 1043:725–733
31. Iram A, Alam T, Khan JM, Khan TA, Khan RH, Naeem A (2013) Molten globule of hemoglobin proceeds into aggregates and advanced glycated end products. PLoS ONE 8(8):e72075
32. Fazili NA, Bhat WF, Naeem A (2014) Induction of amyloidogenicity in wild type HEWL by a dialdehyde: analysis involving multi dimensional approach. Int J Biol Macromol 64: 36–44
33. Oliveira MALM, Lages A, Gomes RA, et al (2011) Insulin glycation by methylglyoxal results in native-like aggregation and inhibition of fibril formation. BMC Biochem 12:41
34. Yan W, Lan C, Ji C, Lin G, Rong Qiao H (2009) Rapid glycation with D-ribose induces globular amyloid-like aggregations of BSA with high cytotoxicity to SH-SY5Y cells. BMC Cell Biol 10:10