The Preventive Effect of L-Lysine on Lysozyme Glycation in Type 2 Diabetes

Hossein Mirmiranpour[1,2] , Shahnaz Khaghani[2], S. Zahra Bathaie[3], Manouchehr Nakhjavani[4], Abbas Kebriaeezadeh[5], Maryam Ebadi[4], Siavash Gerayesh-Nejad[2] , and Mohammad Zangooei[2]

[1] *Department of Biochemistry, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran*

[2] *Department of Clinical Biochemistry, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*

[3] *Department of Clinical Biochemistry, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran*

[4] *Endocrinology and Metabolism Research Center, Vali-Asr Hospital, Tehran University of Medical Sciences, Tehran, Iran*

[5] *Departments of Toxicology/ Pharmacology, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

Received: 25 Jun. 2014; Accepted: 25 Dec. 2014

Abstract- Lysozyme is a bactericidal enzyme whose structure and functions change in diabetes. Chemical chaperones are small molecules including polyamines (e.g. spermine), amino acids (e.g. L-lysine) and polyols (e.g. glycerol). They can improve protein conformation in several stressful conditions such as glycation. In this study, the authors aimed to observe the effect of L-lysine as a chemical chaperone on structure and function of glycated lysozyme. In this study, in vitro and in vivo effects of L-lysine on lysozyme glycation were investigated. Lysozyme was incubated with glucose and/or L-lysine, followed by an investigation of its structure by electrophoresis, fluorescence spectroscopy, and circular dichroism spectroscopy and also assessment of its bactericidal activity against M. lysodeikticus. In the clinical trial, patients with type 2 diabetes mellitus (T2DM) were randomly divided into two groups of 25 (test and control). All patients received metformin and glibenclamide for a three months period. The test group was supplemented with 3 g/day of L-lysine. The quantity and activity of lysozyme and other parameters were then measured. Among the test group, L-lysine was found to reduce the advanced glycation end products (AGEs) in the sera of patients with T2DM and in vitro condition. This chemical chaperone reversed the alteration in lysozyme structure and function due to glycation and resulted in increased lysozyme activity. Structure and function of glycated lysozyme are significantly improved by l-lysine; therefore it can be considered an effective therapeutic supplementation in T2DM, decreasing the risk of infection in these patients.

Acta Med Iran 2016;54(1):24-31.

Keywords: Lysozyme; Chemical chaperone; L-lysine; Type 2 diabetes

Introduction

Protein glycation (non-enzymatic glycosylation) due to hyperglycemic conditions is one of the most important mechanisms modifying proteins, leading to conformational changes and malfunction of the protein (1-3). Various proteins including structural, vascular, cytosolic and nuclear proteins are adversely affected by hyperglycemia associated with type 1 and 2 diabetes mellitus (2-7).

Protein glycation due to binding of its free amino group with the carbonyl group of a reduced sugar yields a Schiff base and Amadori product. Glycated proteins synthesized by this process are transformed into a heterogeneous group of compounds known as advanced glycation end products (AGEs). These products can be identified by various methods, such as fluorescence and cross-linking (8-10). It is well-established that diabetic complications are directly correlated with AGE formation (11). AGE inhibitors and AGE-crosslink breakers protect proteins against the consequences of glycation; therefore can be considered as potential treatment strategies for diabetic complications (3-7,12).

Natural products, defined as curative complements, have been used to prevent protein glycation in patients with diabetes (4,13). Various types of chemicals and natural compounds have been used to prevent this disorder under in vitro and in vivo conditions (3,4,11,14,15). For example, amino acids act as one of the main groups of chemical chaperones. We have previously reported that administration of L-lysine (Lys) to diabetic rats reduces AGE formation, improved lipid

Corresponding Author: Sh. Khaghani
Department of Biochemistry, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 21 88953004, Fax: +98 2164053385, E-mail address: khaghani@tums.ac.ir

profile, induced HSP70 formation and helped preserve the correct folding structure of proteins (4).

Similar to other proteins, studying the etiological role of lysozyme (LZ) in diabetes complications is very important and preventing its glycation would be helpful for patients at risk of increased AGE formation and diabetic complications. LZ is an antibacterial protein with lytic function produced by various cells, including epithelial and blood cells; it is excreted through various fluids such as nasal and salivary secretions, mucus, milk, and serum (16). Due to an abundance of basic amino acids, the isoelectric pH of LZ is approximately 11 and therefore, the protein is classified as a member of the basic protein family (17). LZ has a molecular weight of approximately 14.5 kDa and comprises 129 amino acids (18); its concentration in normal human serum is approximately 4.6 ± 0.8 mg/L (19). The presence of four disulfide bonds in LZ increases its stability and in a limited number of animals, such as steeds, dogs, and birds, it has calcium binding state (18,20). LZ affects the peptidoglycans in the cell wall of gram-positive bacteria and catalyzes its degradation (18,21). LZ is, therefore, considered to be a part of the immune system and is expressed in granulocytes, monocytes, macrophages and bone precursors (20).

It has been previously showed that glycation alters the conformation of LZ (7,22), resulting in marked exposure of its active state to the proteolytic action of trypsin. In addition, the hydrophilic/hydrophobic balance of LZ surface plays an important role in its catalytic activity; glycation affects this balance decreasing the hydrolytic activity of LZ (23). This results in a reduction in the antibacterial function of the protein. Various proteins including structural, vascular, cytosolic, and nuclear proteins are adversely affected by hyperglycemia associated with type 1 and type 2 diabetes (16). These changes are a major factor in the increased prevalence of bacterial infections observed in patients with diabetes (24).

The present study investigated the in vitro effects of Lys on LZ glycation combined by its therapeutic effect on patients with type 2 diabetes (T2DM). This study was a continuation of our previous studies on the effect of chemical chaperones from different families (4,5,12) and natural products (15) on diabetic complications in animal models and the inhibitory effect of these chemical chaperones on the structure and function of proteins (4-7). In addition, this study aimed to confirm the positive results that were observed following Lys administration to diabetic rats (4).

Materials and Methods

Materials

Chicken egg white LZ was purchased from Sigma Chemical Co. (New York, USA), L-lysine monohydrochloride from Sigma Chemical Co. (St. Louis, MO) and Micrococcus lysodeikticus from Sigma Chemical Co. (Koln, Germany). The LZ ELISA Kit was purchased from Immunodiagnostic AG (Bensheim, Germany), the glycated serum protein (fructosamine) enzymatic assay kit from Diazyme Laboratories (Gregg Court Poway, USA) and the glucose oxidase (GOD) assay kit from Teyf Azmoon Pars Co. (Tehran, Iran). All other reagents were obtained from Merck Chemical Co. (Darmstadt, Germany) and were of analytical grade. Sachets containing 1.5 g of Lys and standard additives were purchased from Osvah Pharmaceutical Co. (Tehran, Iran).

Methods

In vitro studies

LZ glycation

Solutions (10 mg/ml) of LZ in phosphate buffer pH 7.4 (0.1 M) were prepared with or without 50 mM of glucose (Glc). In addition, similar solutions were prepared with or without 0.1% w/v of Lys. The final volume of all the samples was 10 ml. Samples were filtered through 0.22 μm Millipore membranes and then incubated in capped vials at 37 °C for up to 4 months. Aliquots (500 μl) were collected weekly from each sample and stored at -80 ºC for analysis by fluorescence spectroscopy, circular dichroism (CD) and polyacrylamide gel electrophoresis (PAGE) (4).

Electrophoresis

The PAGE analyzes were performed according to the protocol for basic proteins (7,25).

Fluorescence spectroscopy

LZ samples (0.5 mg/ml) were prepared and their fluorescence emission was read at 440 nm and with an excitation wavelength of 350 nm (26) using a Shimadzu Spectrofluorometer Model RF-5000 (Japan, Kyoto).

Circular dichroism spectroscopy

Far- and near-UV CD were used to analyze changes in secondary and tertiary structures of LZ (0.1 mg/ml), respectively. The analyzes were performed on a JASCO-810 spectropolarimeter (Tokyo, Japan). Data were reported as molar ellipticity, [θ] (deg cm2 dmol−1), based on the average weight of amino acids (112.4). The equation $[\theta]\ \lambda = (\theta \times 112.4)/cl$ shows the molar

ellipticity, where, c is the protein concentration in mg/ml, l is the light path length in cm and θ is the measured ellipticity in degrees at wavelength λ. JASCO J-810 software was used to smooth noise in the spectra. The percentage of secondary structure content of all the samples was calculated using the Protein Secondary Structure Estimation Program (JWSSE-480), J-800 for Windows, using Yong plots as references for α-helixes, β-sheets, β-turns and random coils.

LZ activity assay

Protein functionality was assayed by mixing 0.2 ml of LZ solution (0.1 mg/ml) with 0.98 ml of M. lysodeikticus solution (0.25 mg/ml) in 66 mM of sodium phosphate, pH 6.2, equilibrated at 25 °C. The cuvette was then inverted several times for 15 seconds to mix the samples, and turbidity was measured at 450 nm using a Shimadzu Spectrophotometer Model-3101. The slope of the linear part of the plot was related to the lytic activity of the enzyme, with one unit of activity being equivalent to a decrease in absorbance of 0.0026/min (7,27).

In vivo study

Study design

The in vivo study was performed at the Department of Endocrinology and Metabolism Research Center, Vali-Asr Hospital, Tehran University of Medical Sciences (Tehran, Iran). Administration of Lys to human participants was registered as No. 416.871 by the Ethics Committee of Tehran University of Medical Sciences.

Patient

A total of 50 patients of either sex, aged ≥40 years were enrolled in the *in vivo* study. Inclusion criteria were 1) type 2 diabetes mellitus according to the American Diabetes Association (ADA) criteria (28), 2) glycated haemoglobin (HbA1c) level ≥ 7%, 3) normal serum insulin level (5–19 μU/ml), 4) no history of ketoacidosis, cancer, hyper- or hypothyroidism, diabetic ulcers, chronic renal failure, liver cirrhosis, feverous diseases or corticosteroid use with a serum creatinine level of ≥2 mg/dl and 5) no symptoms of hypoinsulinemia such as ketonuria, weight loss and a serum Glc level of >400 mg/dl. Exclusion criteria included 1) no response to oral agents, 2) insulin therapy and 3) the presence of diabetic ulcers or feverous diseases during treatment. The patients were then randomly divided into 2 groups of 25 each (test and control) according to FBS and clinical symptoms. All patients were prescribed the antidiabetic drugs metformin and glibenclamide for three months, with the test group also orally consuming 3 g/day of Lys. Patients in the test group consumed one sachet containing 1.5 g twice a day.

Sampling

Blood samples were collected from all patients at baseline and after three months, and serum samples were divided into 4 groups (test groups 1 and 2 and control groups 3 and 4 measured at baseline and after 3 months, respectively). Serum LZ levels were assayed by ELISA (sandwich) method (Immundiagnostik AG Kit-Germany) and applying Mindray ELISA reader (MR-96A-Germany) (29) and FBS using the GOD (30) by applying a BT-3000 plus Auto-Analyzer (Biotechnica Co., Italy). Protein activity assay was performed by EnzChek® Lysozyme Assay Kit (E-22013) (Oregon, USA).

Determination of AGEs was performed according to the method described by Kalousova *et al*., Briefly, serum was diluted 1: 50 with PBS, pH 7.4 and maximum fluorescence emission recorded at 440 nm following excitation at 350 nm (26). Fluorescence intensity was expressed as the percent of fluorescent emission (F %) (4). Serum fructosamine levels were measured using a glycated serum protein enzymatic assay kit (Diazyme Laboratories, Gregg Court Poway, USA).

Data were analyzed by ANOVA using SPSS 16.0, with the level of significance set at $P < 0.05$.

Results

In vitro studies

Overall, 16 samples of each type were taken. Figure 1 shows the fluorescence emission of AGE products which was performed at weekly time periods in the four months study. For four sample types; i.e. LZ alone, LZ with Glc, LZ with Lys and finally LZ with both Glc and Lys. The presence of Glc and increased incubation time resulted in enhanced LZ glycation. However, glycophore formation decreased in the presence of Lys up to the end of the four months experiment.

Figure 2 demonstrates the molar ellipticities of the four types of samples; i.e., LZ alone or incubated with Glc, Lys or both, respectively. Molar ellipticities of glycated LZ in the far- and near-UV regions changed according to those of native protein. However, after the addition of Lys these alterations decreased to almost normal values. Table 1 shows the percentage of secondary structures of LZ under these conditions.

The PAGE pattern of LZ under the above

conditions is shown in Figure 3. Since LZ is a cationic protein, its electrophoretic mobility was from the anode towards the cathode. The movement of the glycated protein in lanes 3 and 7 (duplicate sample) was lower than others. As observed in lanes 4 and 8, the addition of Lys to the medium partially compensated for the effect of Glc.

Figure 4 shows the activity of LZ under the four mentioned conditions. Activity decreased in the presence of 50 mM of Glc, whereas it increased and neared to normal following the addition of Lys to the solution.

In vivo study

This study investigated the effect of Lys supplementation in conventional treatment in patients with type 2 diabetic. Table 2 shows FBS, LZ level and activity and AGE and fructosamine levels in the test groups (Groups 1 and 2) and control groups (Groups 3 and 4) at baseline and after three months of treatment, respectively. Significant changes (P<0.05) were observed in all these parameters in the group treated with Lys for three months.

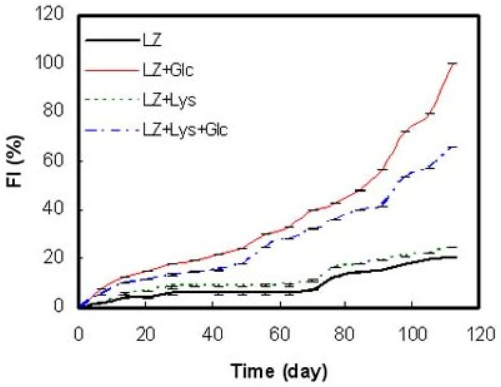

Figure 1. Percentage of fluorescence intensity (FI %) of glycophores due to incubation of LZ with Glc in the presence or absence of Lys
The excitation and emission wavelengths were 350 nm and 440 nm, respectively. Symbols are described in the Figure

Table 1. Percentage of secondary structures of LZ after four months incubation with or without Glc and in the presence or absence of Lys

Protein (Mean ± S.D.)	α-Helix %	β-Sheet %	β-turn %	Random Coil %
LZ	27.20 ± 0.07	13.30 ± 0/14	24.40 ± 0.14	35.10 ± 0.14
LZ + Glc	17.10 ± 0.07	19.80 ± 0.07	22.20 ± 0.07	40.90 ± 0.00
LZ + Lys	26.60 ± 0.07	15.80 ± 0.07	24.90 ± 0.07	32.70 ± 0.07
LZ + Glc + Lys	24.60 ± 0.14	16.10 ± 0.07	25.20 ± 0.07	34.10 ± 0.14

Table 2. FBS, LZ concentration and activity, the percentage of fluorescence intensity (FI %) of AGEs and concentration of fructosamine in patients with type 2 diabetes. Groups 1 and 2 represent the test groups, and groups 3 and 4 are the control groups, before and after treatment, respectively

Parameter (Mean ± S.D.)	Groups			
	1	2	3	4
FBS (mg/dl)	184.32 ± 38.92	138.48 ± 30.91*	187.96 ± 41.90	171.24 ± 39.25
LZ (ng/ml)	856.00 ± 47.95	913.20 ± 73.18*	861.60 ± 45.15	864.40 ± 64.87
LZ activity (%)	98.54 ± 0.79	99.23 ± 0.77*	98.54 ± 0.35	98.35 ± 1.14
AGEs (FI %)	69.35 ± 4.51	63.97 ± 8.43 *	70.77 ± 5.11	69.20± 9.84
Fructosamine (µmol/L)	408.24 ± 67.91	317.16 ± 100.14*	409.08 ± 57.56	395.08 ± 87.31

* Versus the same measure in group 1 (P < 0.05).

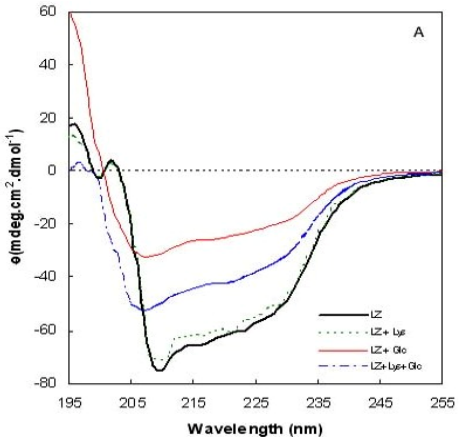

Figure 2. The CD spectra of LZ under different conditions, as explained in the text. (A) far UV region and (B) near UV region. Symbols are described in the Figure.

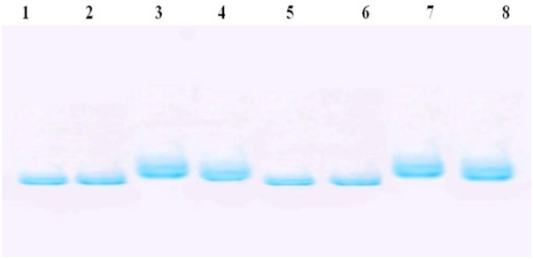

Figure 3. Electrophoretic mobility of LZ under different conditions, as explained in the text. Lanes 1 & 5, LZ ; lanes 2 & 6, LZ + Lys ; lanes 3 & 7, LZ + Glc ; lanes 4 & 8, LZ + Glc + Lys (as duplicate samples).

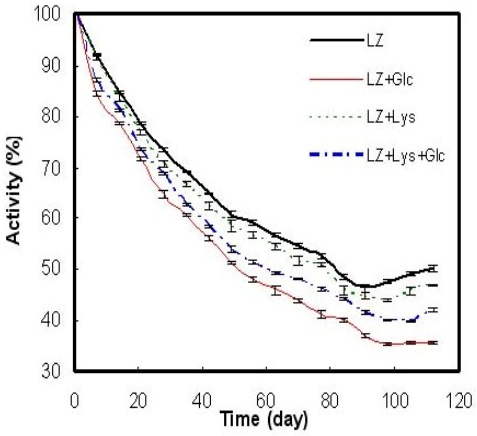

Figure 4. Percentage lytic activity of LZ under different conditions, as explained in the text. Symbols are described in the Figure.

Discussion

Hyperglycemia is an important marker for the development of complications of diabetes mellitus. Protein glycation due to increased Glc concentrations leads to the formation of AGEs, which in turn, increases the risk and rate of development of diabetic complications.

The pathogenicity of AGEs, especially in connection with diabetes and age-related diseases has been extensively studied, and the severity of complications has been shown to be directly related to AGEs levels. Physical and structural properties of tissue components are directly altered by their reaction with AGE precursors and associated production of oxidative free radicals. Decreasing the level of these compounds by inhibiting their formation, increasing degradation or enhancing renal excretion delays their progression

toward glycation (31,32). Therefore, reduction in serum levels of AGEs and consequently protein glycation in patients with diabetes is of great value. Fluorometry is the best method for determining AGE products (8-10). Our fluorescence spectroscopy studies showed LZ glycation in the presence of high Glc concentration resulted in the formation of AGE glycophores both in vivo (Table 2) and in vitro (Figure 1). Similar findings have been reported in diabetic rats and with both bovine and human serum albumin (4) and also in the study of Mirmiranpour et al (33). Plots in (Figure 1) indicate the increase in fluorescence emission due to AGE formation after three months incubation with LZ and Glc. In the current study, Glycophore (AGE) formation was potentially inhibited by Lys, whereas LZ alone failed to show the same pattern. A similar effect has been observed with glycerol (7).

LZ consists of 4 alpha helices, 8 beta pleated sheets, 6 coiled domains, and 2 major turns (34). As mentioned above, the conformation of LZ is modified by glycation. In this study, it was observed that LZ secondary structure alters due to glycation (Figure 2A and Table 1); additionally, CD spectrum of the tertiary structure of the glycated protein altered by incubation with Glc (Figure 2B). Interestingly, all these adverse affects caused by glycation diminished once Lys was added, with both the far- and near-UV CD spectra of glycated protein in the presence of this chemical chaperone being close to that of the native protein. The authors recently reported similar results for albumin, fibrinogen, and LZ, indicating that Lys inhibited albumin and fibrinogen glycation and that glycerol-preserved the structure of LZ from harmful alterations (4,7).

Previous studies have also shown glycation reduces the number of α-helixes and increases the β-sheet content of proteins (4,5,7,35). Similar changes were observed in this study (Table 1).

The overall negative charge density of protein increases with protein glycation, as Glc covers the positive charges of Lys residues. The electrophoretic mobility of glycated proteins towards the anode would, therefore, be greater than native proteins. It is important to note that LZ is a basic protein, with the direction of electrophoretic mobility being reversed in comparison with ordinary PAGE. Lanes 3 and 7 in (Figure 3) show increased mobility of LZ towards the anode due to glycation after 4 months incubation with Glc. The presence of Lys in the incubation mixture had no effect on the mobility of protein alone (Lanes 2 and 6), but restored the mobility of protein plus Glc to approximately normal values (Lanes 4 and 8). These findings are very similar to those we obtained previously by adding Lys to medium containing albumin or fibrinogen in the presence of Glc (4). The authors observed that the activity of LZ also becomes less with glycation (Figure 4). Similar observations were reported by in vitro investigations performed by Saito *et al*., and Bathaie *et al*., (7,22). Binazzi *et al*., also showed a reduction in LZ function in the skin of patients with clinical diabetes, with this change being suggested as a factor in the cutaneous infections that develop in these patients (36). A reduction in LZ activity due to glycation has also been reported by other researchers (37,38). As mentioned above, the pathologic process that leads to a failure to kill gram-positive bacteria and increased susceptibility to bacterial infection in diabetic patients is essentially started by functional alterations to LZ caused by glycation (18,20,21,36). An increase in LZ secretion caused by administration of glibenclamide, a sulfonylurea drug, has been reported previously by several authors (39). In contrast, a study on the effects of metformin, a biguanidine drug, showed a decrease in the activity of LZ in serum (40).

As shown in (Table 2), both the quantity and activity of LZ in diabetic patients treated with Lys in combination with ordinary treatment was significantly greater than the control group receiving ordinary treatment only. Attention to the inseparable association between secretion and function of LZ (41), illustrates their coordinated increasing changes in recent protein due to Lys effect (Table 2). However the human LZ (in vivo study) consists of noticeable different amino acids in comparison with enzyme belonging to hen-egg-white (in vitro study), but both of two present studies indicate the similarity between the findings related to alterations of LZ activity obtained from in vivo and in vitro analysis (Table 2 and Figure 4).

The beneficial effects of Lys have been shown in animal models of diabetes (4). The usefulness of Lys therapy in human infectious diseases induced by herpes simplex virus (42) has also been reported. This study showed that Lys was non-toxic and compatible with the entire human metabolic system. The findings summarized in Table 2 of beneficial changes in FBS, AGEs and fructosamine provide convincing evidence on the usefulness of Lys supplementation as a new adjunct therapy for diabetes. These data are supported by in vitro data and confirm the involvement of glycated proteins in the pathogenesis of disease and also the usefulness of glycation inhibitors.

As the limitation of this study, the authors could point to lack of providing the control group in the

clinical study with placebo therapy; therefore more extensive double-blinded studies in a larger patient population are recommended to better justify present results.

In summary, Lys, acting as a chemical chaperone, inhibits LZ glycation, thereby conserves its folding structure and function. Supplementation with Lys may, therefore, attenuate some of the pathological changes associated with diabetes.

Acknowledgment

The authors are very thankful to the Vice-Chancellor of Tehran University of Medical Sciences, Research Council of Tarbiat Modares University and Osvah Pharmaceutical Co for supporting this project. Additionally, we would like to express our gratitude for Dr. Alireza Esteghamati's invaluable revision of the manuscript.

References

1. Tan KC, Chow WS, Ai VH, et al. Advanced glycation end products and endothelial dysfunction in type 2 diabetes. Diabetes Care 2002;25(6):1055-9.
2. Sensi M, Bruno MR, Valente L, et al. Retinol-binding protein: a short half life determinant of protein non-enzymatic glycation in diabetes. Diabetes Res 1990;13(4):195-8.
3. Rahbar S, Yerneni KK, Scott S, et al. Novel inhibitors of advanced glycation end product (part I). Mol Cell Biol Res Commun 2000;3(6):360-6.
4. Jafarnejad A, Bathaie SZ, Nakhjavani M, et al. The improvement effect of L-Lys as a chemical chaperone on STZ-induced diabetic rats, protein structure and function. Diabetes Metab Res Rev 2008;24(1):64-73.
5. Jafarnejad A, Bathaie SZ, Nakhjavani M, et al. Investigation of the mechanisms involved in the high-dose and long-term acetylsalicylic acid therapy of type I diabetic rats. J Pharmacol Exp Ther 2008;324(2):850-7.
6. Rahmanpour R, Bathaie SZ. Histone H1 structural changes and its interaction with DNA in the presence of high glucose concentration In vivo and In vitro. J Biomol Struct Dyn 2011;28(4):575-86.
7. Bathaie SZ, Nobakht BB, Mirmiranpour H, et al. Effect of chemical chaperones on glucose-induced lysozyme modifications. Protein J 2011;30(7):480-9.
8. Peppa M, Uribarri J, Vlassara H. Glucose, advanced glycation end products, and diabetes complications. Clin Diabetes 2003;21(4):186-7.
9. Lapolla A, Traldi P, Fedele D. Importance of measuring products of non-enzymatic glycation of proteins. Clin Biochem 2005;38(2):103-15.
10. Yanagisawa K, Makitam Z, Shiroshita K, et al. Specific fluorescence assay for advanced glycatio n end products in blood and urine of diabetic patients. Metabolism 1998;47(11):1348-53.
11. Agardh CD, Stenram U, Torffvit O, et al. Effects of inhibition of glycation and oxidative stress on the development of diabetic nephropathy in rats. J Diabetes Complicat 2002;16(6):395-400.
12. Jafarnejad A, Bathaie SZ, Nakhjavani M, et al. Effect of spermine on lipid profile and HDL functionality in the streptozotocin-induced diabetic rat model. Life Sci 2008;82(5-6):301-7.
13. Gugliucci A, Menini T. The polyamines spermine and spermidine protect proteins from structural and functional damage by AGE precursors: a new role for old molecules? Life Sci 2003;72(23):2603-16.
14. Perlmutter DH. Chemical chaperones: a pharmacological strategy for disorders of protein folding and trafficking. Pediatr Res 2002;52(6):832-6.
15. Shirali S, Bathaie SZ, Nakhjavani N. Effects of saffron aqueous extract on some biochemical factors of serum in streptozotocin-induced diabetic rats. Iran J Med Aromatic Plants 2012;28(2):293-308.
16. Yeboah FK, Alli I, Yaylayan VA, et al. Effect of limited solid-state glycation on the conformation of lysozyme by ESI-MSMS peptide mapping and molecular modeling. Bioconjug Chem 2004;15(1):27-34.
17. Masuda T, Ide N, Kitabatake N. Effects of chemical modification of lysine residues on the sweetness of lysozyme. Chem Senses 2005;30(3):253-64.
18. Ibrahim HR, Aoki T, Pillegrini A. Strategies for new antimicrobial proteins and peptides: Lysozyme and aprotinin as model molecules. Curr Pharm Des 2002;8(9):671-93.
19. Peeters TL, Depraetere YR, Vantrappen GR. Radioimmunoassay for urinary lysozyme in human serum from leukemic patients. Clin Chem 1978;24(12):2155-7.
20. Frare E, Polverino De Laureto P, Zurdo J, et al. A highly amyloidogenic region of hen lysozyme. J Mol Biol 2004;340(5):1153-65.
21. Powroznik B, Gharbi M, Dandrifosse G, et al. Enhancement of lysozyme stability and activity by polyamines. Biochime 2004;86(9-10):651-6.
22. Saito A, Sako Y, Usui M, et al. Functional properties of glycosylated lysozyme secreted in Pichia pastoris. Biosci Biotechnol Biochem 2003;67(11):2334-43.
23. Longo MA, Combes D. A novel chemoenzymatic glycosylation strategy: application to lysozyme modification. FEBS Lett 1995;37(1-2):63-6.

24. Li YM. Glycation ligand binding motif in lactoferrin. Implications in diabetic infection. Adv Exp Med Biol 1998;443:57-63.
25. Reisfeld R, Lewis U, Williams D. Disk electrophoresis of basic proteins and peptides on polyacrylamide gel. Nature 1962;195(4838):281-3.
26. Kalousova M, Skrha J, Zima T. Advanced glycation endproducts and advanced oxidation protein products in patients with diabetes mellitus. Physiol Res 2002;51(6):597-604.
27. Goldberg M, Rudolph R, Jaenicke R. A kinetic study of the competition between renaturation and aggregation during the refolding of denatured-reduced egg white lysozyme. Biochemistry 1991;30(11):2790-7.
28. American Diabetes Association. Screening for diabetes (position statement). Diabetes Care 2001;24(Suppl 1):S21-4.
29. Spinozzi F, Velardi A, Rambotti P, et al. Circulating immune complexes and serum lysozyme levels in untreated Hodgkin's disease. Their relationship to immune function. J Clin Lab Immunol 1983;12(2):87-92.
30. Vittal BG, Praveen G, Deepak P. A study of body mass index in healthy individuals and its relationship with fasting blood sugar. J Clin Diag Res 2010;4(6):3421-4.
31. Zheng F, Cai W, Mitsuhashi T. Lysozyme enhances renal excretion of advanced glycation end products in vivo and suppresses adverse AGE-mediated cellular effects in vitro: a potential AGE sequestration therapy for diabetic nephropathy. Mol Med 2001;7(3):737-47.
32. Monnier VM. Intervention against the Maillard reaction in vivo. Arch Biochem Biophys 2003;419(1):1-15.
33. Mirmiranpour H, Bathaie SZ, Khaghani S. Investigation of the mechanism(s) involved in decreasing increased fibrinogen 2 activity in hyperglycemic conditions using L-lysine supplementation. Thrombosis Res 2012;130(3):e13-9.
34. Masuda T, Ide N, Kitabatake N. Structure–sweetness relationship in egg white lysozyme: role of lysine and arginine residues on the elicitation of lysozyme sweetness. Chem Senses 2005;30(8):667-81.
35. GhoshMoulick R, Bhattacharya J. Compensatory secondary structure alterations in protein glycation. Biochim Biophys Acta (BBA) 2007;1774(2):233-42.
36. Binazzi M, Boncio L, Marconi P, et al. Serum and skin lysozyme activity in non-diabetic and diabetic subjects. Arch Dermatol Res 1978;262(3):239-45.
37. Zemliakova ZM, Kravets EB. Activity of nonspecific immunological factors and serum immunoglobulin levels in children with diabetes mellitas. Probl Endokrinol (Mosk) 1981;27(6):6-9.
38. Lechowski R, Lenarcik M, Degòrski A, et al. Serum lysozyme activity and Nitroblue tetrazolium reduction test in dogs with diabetes mellitus. Zentralbl Veterinarmed A 1991;38(7):530-3.
39. Joo NS, Lee DJ, Winges KM, et al. Regulation of antiprotease and antimicrobial protein secretion by airway submucosal gland serous cells. J Biol Chem 2004;279(37):38854-60.
40. Naglaa ZHE, Hesham AM, Hosny AF, et al. Impact of metformin on immunity and male fertility in rabbits with alloxan- induced diabetes. J Am Sci 2010;6(11):417-26.
41. Pang G, Clancy R, Cong M, et al. Influenza virus inhibits lysozyme secretion by sputum neutrophils in subjects with chronic bronchial sepsis. Am J Respir Crit Care Med 2000;161(3 Pt 1):718-22.
42. Griffith RS, Walsh DE, Myrmel KH, et al. Success of L-lysine therapy in frequently recurrent herpes simplex infection. Treatment and prophylaxis. Dermatologica 1987;175(4):183-90.