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SHORT COMMUNICATION

Beneficial role of amino acids in mitigating cytoskeletal actin glycation and improving F-actin content: *In vitro*

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Aims: **The actin filaments present in circulating leukocytes facilitate their passage through microvenules and capillaries by helping in their deformability. Decreased deformability of granulocytes is now known to cause occlusion of the retinal microcapillaries leading to hypoxia and the subsequent development of diabetic retinopathy. Structural and functional loss of proteins, due to non-enzymatic glycation and glycoxidation, has been reported to cause diabetic pathogenesis. As amino acids have been earlier reported to have antidiabetic properties, the present study involves the investigation of the susceptibility of the cytoskeletal actin to glycation and its mitigation by free amino acids. This study also involves quantifying F-actin in cultured mononuclear cells obtained from diabetic and normal healthy volunteers and on the effect of glucose and free amino acids on F-actin content.** ***Methods:*** **Commercial non-muscle actin and actin immuno-precipitated from granulocytes obtained from (a) normal healthy human volunteers and (b) patients with type 2 diabetes mellitus were subjected to glycation studies using [U] ^{14}C glucose. The effect of free amino acids, as antiglycating agents, was determined using various concentrations of lysine, arginine, alanine, aspartic acid and glutamic acid. F-actin content in cultured mononuclear cells was estimated by flow cytometry using fluorescein isothiocynate (FITC)-Phalloidin.** ***Results:*** **Commercial actin at physiological conditions of pH and temperature was found to undergo non-enzymatic glycation. The extent of *in vitro* glycation was significantly low ($P < 0.001$) in actin isolated from patients with type2 diabetes when compared to the non-diabetic group, suggesting an increased *in vitro* structural modification of actin in patients with diabetes. All the free amino acids tested were found to have varying degrees of antiglycating effect. The F-actin content in the intact mononuclear cells obtained from diabetic patients was found to be low when compared with normal healthy volunteers ($P < 0.001$). Similarly the F-actin content was significantly low when the normal mononuclear cells were incubated with glucose. This effect was reversed upon the addition of free amino acids to the incubation mixture.** ***Conclusions:*** **Free amino acids can play a positive role in improving leukocyte deformability by mitigating cytoskeletal actin glycation and improving F-actin content.**

Keywords: glycation, antiglycation, cytoskeletal actin, free amino acids and diabetes mellitus

Introduction

Diabetes mellitus (DM) represents a major medical problem affecting millions of people all over the world. One of the most devastating microvascular complications of diabetes is diabetic retinopathy (DR) [1]. Despite a significant improvement over the past two decades in our understanding and treatment of ocular complications of DM, many aspects about DR need to be elucidated, because the management and treatment of DR still remains one of the most formidable challenges before the medical profession. It is now evident that structural and functional loss of proteins due to non-enzymatic glycation/glycoxidation is the common event in the multi cascade process leading to DR [2]. Accumulation of advanced glycation end products (AGEs) has been associated with the pathogenesis of micro- and macrovascular complications of DM [3]. Intervention of glycation may prove to be beneficial to patients suffering from DM [3].

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Decreased granulocyte deformability has been implicated in the pathogenesis of DR [4]. Granulocytes contain abundant actin filaments, which aid in cell-cell interaction, focal adhesion, cell motility, cell shape regulation and inter cellular signaling [5]. In addition to this, glycation of axonal cytoskeletal actin has been attributed to the pathogenesis of diabetic neuropathy [6]. Moreover, the content of F-actin in diabetes has been reported to be decreased [7]. We hypothesize that non-enzymatic glycation may impair the structural and functional integrity of cytoskeletal actin, which may be responsible for the decreased deformability of granulocytes. The present study has been undertaken to find out the degree of susceptibility of cytoskeletal actin to non-enzymatic glycation, and the effect of free amino acids on this glycation. We used amino acids to inhibit non-enzymatic glycation, as free amino acids are known to mitigate the glycation of lens protein, delay cataractogenesis and bring down blood sugar in diabetic rats [8,9]. In addition to this, our findings include the levels of F-actin content in isolated mononuclear cells obtained from diabetic and non diabetic individuals, and the effect of free amino acids on the level of F-actin content in cultured cells from these subjects.

Materials and methods

Subjects

Blood samples from 11(F = 4, M = 7) individuals with type-2 diabetes mellitus and 11(F = 5,M = 6) non-diabetic individuals were used for the study. The clinical and demographic data of patients with Type 2 diabetes mellitus i.e age, duration of diabetes and plasma postprandial blood sugar were 56 ± 3.2 (yrs), 12 ± 5 (yrs) and 15.5 ± 4.3 (mM) respectively. For non-diabetic healthy volunteers it was 52 ± 4.8 (yrs) and 5 ± 1.6 (mM). All the values mentioned above are mean $\pm$ SD. The volunteers chosen were non-obese, non-alcoholic, non-smoking and free from any systemic disease. Consent was obtained from all the patients and volunteers for the use of their blood in the isolation of actin in our experiments. Approval was obtained from the Research Board and Advisory Committee of the institution for this project. All procedures were carried out in terms of the tenets of the Helsinki declaration.

All the chemicals used in this study were from Sigma chemical company, USA, unless otherwise specified.

Isolation of granulocytes

Peripheral blood samples were collected from the anticubital vein, using 5-ml syringe containing 100 units of heparin. The blood was diluted with an equal volume of PBS pH 7.4. The diluted blood was overlaid over an equal volume of percoll density gradient (Amersham Pharmacia, USA) and the granulocytes were isolated as per the manufacturer's protocol. The presence of granulocytes was confirmed by performing Leishman stain on a cytospin smear [10]. The yield of the cells was quantified using Neubauer counting chamber.

Immunoprecipitation of actin

Granulocytes (2×10^5cells) were suspended in 1.0 ml of radio immuno precipitation assay buffer (RIPA) containing 1% nonidet-p40, 0.1% sodium dodecyl sulphate, 0.5% sodium deoxy cholate, 30 µl/ml aprotinin, and 10 µl/ml phenyl methyl sulphonyl fluoride. The cells were incubated for 30 min in ice cold condition, then lysed by repeated aspiration and dispersion by passing through a 21-gauge needle, then incubated again in ice cold condition for 30 min and centrifuged at 10,000 rpm for 15 min at 4°C.

One ml of total cell lysate (with known protein concentration) was incubated with 0.2 µg of polyclonal goat actin antibody (Santa Cruz Biotechnology Inc., USA) for an hour at 4°C. This was followed by addition of 20 µl of protein A/G agarose conjugate (Santa Cruz Biotechnology Inc., USA) and continued incubation for 18 hrs at 4°C in rocker platform. The immunoprecipitate thus obtained was washed thrice in RIPA buffer, by centrifuging each time at 3,000 rpm 3–5 min at 4°C. Finally the actin molecule was retrieved from the immunoprecipitate by the method described earlier [12]. At every step during isolation of actin, protein estimation was done by Lowry method [11].

The protein profile of the cell lysate was analysed by silver stain after performing12% SDS PAGE [13,14]. The purity of actin in the immunoprecipitate was assessed by performing Western blot.

Glycation studies on actin

Glycation studies on actin were carried out as described earlier [8]. Briefly 20 μg of actin (either commercially available non-muscle actin or immunoprecipitated actin from granulocytes) was incubated with either 5 or 10 mM glucose, along with 0.2 μci of $U^{14}C$ glucose in phosphate buffer pH 7.4, and 0.01% sodium azide for 72 hours at 37°C. The total volume of the incubation mixture was 1.5 ml. After the incubation period, the contents were dialyzed against milli Q water using a cellulose membrane to remove the free glucose. The process was continued until the solution outside the membrane gave nil disintegration per minute (DPM) of radioactivity. The contents were then transferred to scintillation vials along with 4 ml of scintillation cocktail, and the amount of $U^{14}C$ glucose incorporated in the protein was measured by a Beckman liquid scintillation system (LS 6500). The extent of glycation was quantified and expressed in terms of mg glucose incorporated mg^{-1} actin, and a percentage of glucose incorporated.

Anti- glycation studies were carried out as described above, except that the incubation mixture contained 10 mM glucose along with either 2,5,10 mM solutions of arginine, aspartic acid, glutamic acid, and alanine or mixture of all these free amino acids. Quantification of glycation was done as described above.

Determination of F-actin content

All experiments were performed on mononuclear cells isolated from healthy and diabetic subjects. The peripheral blood mononuclear cells were isolated by ficoll hypaque density gradient centrifugation technique [15]. Cell density was adjusted to 2×10^5/ml using RPMI 1640 medium supplemented with antibiotics and 5%FCS. They were incubated with or without 10 mM glucose along with 10 mM mixture of amino acids in sterile eppendrof tubes at 37°C under 5% CO_2 environment for 24 hrs. The F-actin content was estimated by flow cytometry using fluorescein isothiocynate (FITC)-phalloidin [16]. The cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and labelled with FITC-phalloidin for 30 min at room temperature. The samples were then immediately analysed using FACS (Becton & Dickinson). Fluorescence emission for FITC was detected by selectively collecting at 480nm to 515nm on 10,000 labeled cells gated to include mononuclear cells to exclude lymphocytes, other non-monocytic cells. The data were collected using logarithmic amplification of 10,000 cells excluding cell debris by a combination of a forward and side scatters. The total number of cells fluorescing positively/10,000 cells was expressed as percentage F-actin content. The data were analysed using Cell Quest software (Becton & Dickinson & Co.).

Statistical analysis

Students *t* test was used to assess the statistical significance of the data obtained. P value < 0.05 was considered significant.

Results

Protein content in cell lysates of control and diabetic group were 3.2 ± 0.3 mg/ml and 2.8 ± 0.2 mg/ml respectively. Protein content in the immunoprecipitates of control and diabetic group were 0.22 ± 0.02 mg/mg protein and 0.17 ± 0.01 mg/mg protein respectively. Final yields of actin retrieved from immunoprecipitate were 0.138 ± 0.02 mg actin/mg protein and 0.104 ± 0.01 mg actin/mg protein in control and diabetic groups. These results indicate a significant difference in the synthesis and yield of actin between the two groups studied.

In vitro glycation of actin

The results are given in Table 1. The amount of glucose incorporated in actin was maximum in standard actin, followed by actin isolated from healthy volunteers and least in diabetic patients.

Effect of free amino acids on glycation of actin

The results are summarized and presented in Table 2. The results established significantly lower levels of glycation in the presence of the free amino acids used in the study. This effect was found to be dose dependent on various concentrations of amino acids tested. Though all amino acids used in this study were found to have varying degrees of antiglycating effect, alanine was found to be most effective, followed by the mixture of free amino acids. Arginine was found to have the least effect.

F-actin content

The F-actin content as a total percentage of mononuclear cells fluorescing, is given in the form of a histogram (Figure 1). The F-actin content in diabetic mononuclear cells was found to be $40.23 \pm 13.64\%$, while in normal cells obtained from healthy volunteers it was $87 \pm 7.5\%$. There was a significant decrease in the content of F-actin ($33.7 \pm 13.7\%$) in the normal cells when they were cultured in the presence of 10 mM of glucose. Further the F-actin content was improved to $83 \pm 8.5\%$ when they were cultured in the presence of 10 mM glucose and 10 mM of mixture of free amino acids.

Discussion

In this study, we have demonstrated that free amino acids decrease the *in vitro* glycation of cytoskeletal actin. They not

Table 1. Extent of *in vitro* incorporation of glucose in standard actin and actin isolated from granulocytes of individuals with/without diabetes.

	5 mM glucose		*10 mM glucose*	
Actin source	*mg of glucose incorporated/ mg of actin*	*% of glucose incorporated*	*mg of glucose incorporated/ mg of actin*	*% of glucose incorporated*
Sigma standard	4.2 ± 0.01	5.67	11.2 ± 0.7	7.5
Actin isolated from patients with diabetes n = 11	0.78 ± 0.01	1.04	2.4 ± 0.2	1.6
Actin isolated from non diabetic individuals n = 11	3.3 ± 0.02	4.4	9.7 ± 0.4	6.4

Values are Mean ± SD. Values when compared between standard and diabetes were found to be statistically significant ($P < 0.001$). Similar results were obtained between diabetes and control ($P < 0.001$).

Table 2. The effect of 5mM free amino acids on *in vitro* glycation of standard actin, actin isolated from granulocytes of patients with diabetes and normal healthy subjects.

Effect of 5 mM amino acid with 10 mM glucose	*Standard actin incorporation of glucose*		*Actin isolated from diabetic group incorporation of glucose*		*Actin isolated from controls incorporation of glucose*	
	mg Glucose mg protein	*% of glucose*	*mg Glucose mg protein*	*% of glycation*	*mg Glucose mg protein*	*% of glycation*
10 mM glucose alone	11.2 ± 0.7	7.5	2.4 ± 0.2	1.6	9.7 ± 0.4	6.5
Lysine	0.8 ± 0.2	0.55	1.0 ± 0.1	0.7	1.7 ± 0.6	1.1
Arginine	1.0 ± 0.2	0.7	1.3 ± 0.1	0.9	2.5 ± 0.4	1.7
Aspartic acid	0.5 ± 0.1	0.33	0.8 ± 0.2	0.53	1.9 ± 0.3	1.3
Glutamic acid	0.9 ± 0.1	0.6	0.9 ± 0.3	0.63	0.9 ± 0.2	0.6
Alanine	0.5 ± 0.1	0.32	0.6 ± 0.1	0.43	0.7 ± 0.2	0.46
Mixture of all the above amino acids	0.8 ± 0.3	0.5	0.7 ± 0.1	0.44	0.6 ± 0.1	0.4

Note: n = 5 in each group. $P < 0.001$ between 10 mM glucose and various amino acid treatment to the actin obtained from a commercial source, normal healthy or diabetic patients. Experiments when repeated with 10 mM glucose, with 2 or 10 mM amino acids revealed similar results. Values are mean ± SD.

only decreased the glycation of actin, but also improved the F-actin content in the mononuclear cells (Figure 1). The damage done by glucose to actin was found to be inhibited by free amino acids.

The low yields of protein in cell lysate from the diabetic group in our study could be attributed to the fact that in diabetes the synthesis of cellular proteins is impaired [17]. The decreased yield of immunoprecipitate and actin molecules retrieved from immunoprecipitate in diabetic patients, could be due to *in vivo* glycation and other related structural alterations such as post glycation modifications, glycoxidation etc., [18]. In the in vitro experiments using radioactive glucose, for the same concentrations of actin isolated from samples belonging to both the groups, incorporation of glucose into actin obtained from normal controls was four-fold higher (Table 1) when compared to actin isolated from diabetic subjects. Significantly low *in vitro* incorporation of glucose in the diabetic group could be due to increased *in vivo* alterations taking place in the hyperglycemic environment. The above results could be attributed to: glycation in hyperglycemic condition of one or more of the 20 free amino groups/ molecule of actin (19 free ε amino groups of lysine residues and one free N terminal amino group) [19]. Besides, post glycation changes in the form of intramolecular rearrangements, hemiacetal formation with more glucose molecules, condensation reactions between aldehyde group of glucose and free hydroxyl groups of serine and threonine residues of actin, hydrogen bonding, etc., which could have also caused severe structural impairment in diabetic patients.

The efficacy of aminoguanidine (an antiglycating agent) and its role in mitigating the complications of diabetes mellitus has been a subject under immense investigation for the past decade [20]. However, it is important to stress that, while aminoguanidine inhibits the glycation at a later stage in the multi-step glycation pathway and prevents protein–protein cross-linking, free amino acids inhibit the first step in the pathway of glycation cascade, by competitive inhibition [reviewed in reference 21]. A recent clinical trail undertaken by us with the supplementation of 1 g of free amino acid per day to type 2-DM patients did not reveal any side effect or abnormal renal or hepatic functions. In addition, their postprandial blood sugar was found to be significantly decreased when compared to placebo group. It is reported that supplementation of 40 g of amino acid to elderly individuals, improved their nitrogen balance and tissue protein synthesis with no adverse side effect [22]. These findings substantiate our use of free amino acid concentrations of 2, 5 and 10 mM, in our *in vitro* studies, which cannot be deemed high or unphysiological. It is also pertinent to note that we have used only physiological concentrations of glucose in our studies. pH and temperature were also maintained at 7.4 and 37°C respectively.

F-actin is the functional form of actin. Adequate energy is necessary to assemble G-actin (the monomer unit) into F-actin. For want of energy in the form of ATP, the assembly of G form to F form may be impaired in diabetic cells [23]. In our present study we have found that F-actin content was significantly low in patients with diabetes when compared to non diabetic subjects. This was improved by addition of free amino acids. Therefore it is concluded that non-enzymatic glycation of actin at least in part, may be responsible for the decreased deformability of leukocytes. Unlike erythrocytes, leukocytes, owing to their size, need more deformability to pass through microcapillaries particularly in the retina. Therefore this might result in capillary occlusion in retina and subsequent development of DR.

In conclusion, we have demonstrated that cytoskeletal actin is prone to non-enzymatic glycation and free amino acids mitigate this glycation. In addition to this effect, free amino

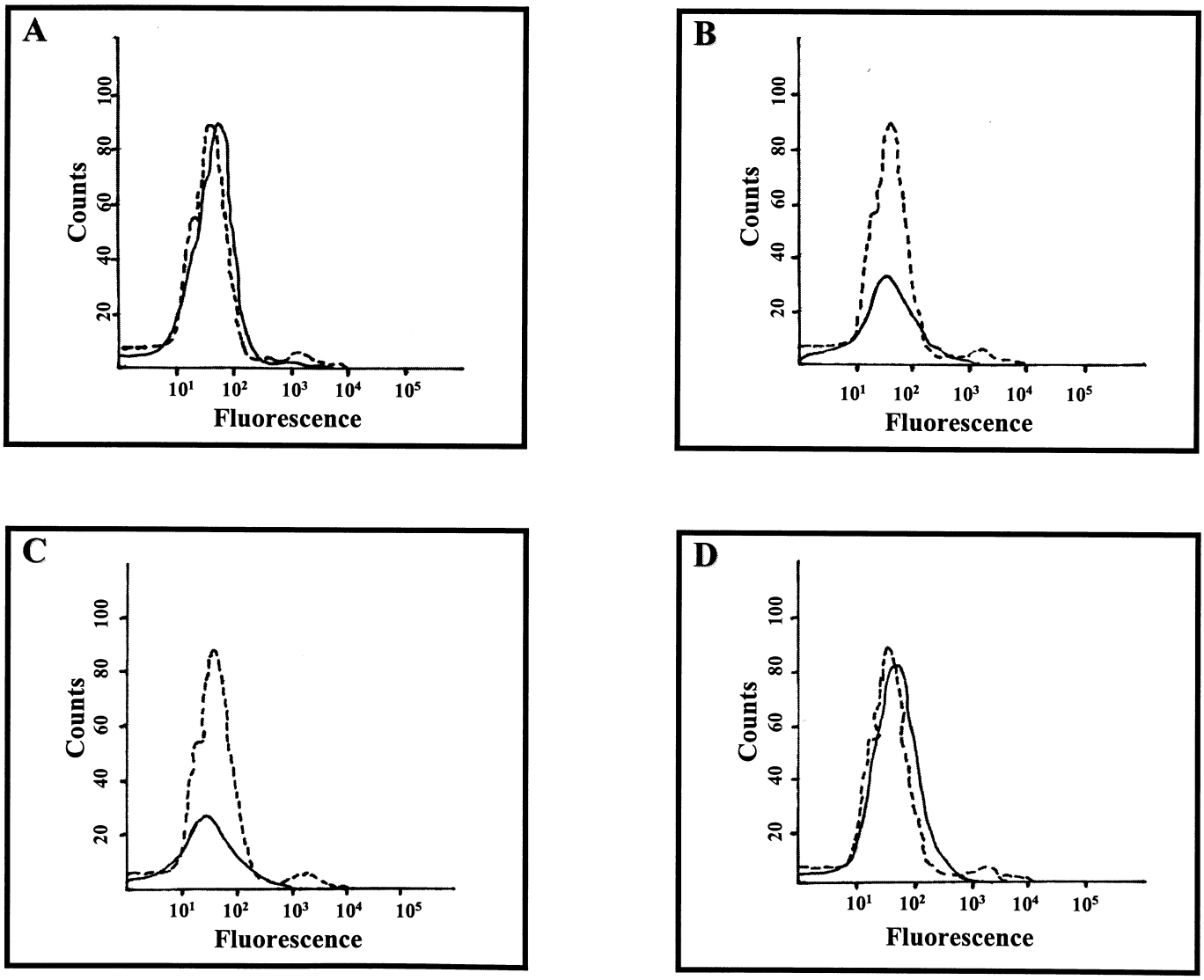

Figure 1. F-actin content in mononuclear cells isolated from peripheral blood of normal (n = 5) and diabetic patients ($n = 5$) and the effect of *in vitro* treatment of glucose and free amino acids on the same. The dotted lines represent the normal base line photo multiplier (PMT) setting, while the bold line represents the actual F-actin content. **A:** the F-actin content in normal healthy volunteers was 87.5 ± 7.5%. **B:** F-actin content in diabetic patients: 40.23 ± 13.64%. **C:** F-actin content in mononuclear cells obtained form normal healthy volunteers treated with 10 mM glucose: 33.7 ± 13.67%, **D:** The F-actin content: 83 ± 8.5% in mononuclear obtained from normal healthy volunteers treated with 10 mM glucose and 10 mM mixture of free amino acids. Values are expressed as mean ± SD obtained from 3 individual experiments.

acid lysine improves the F-actin content in leukocytes. This study has also provided us the impetus for further investigation on glycation of actin, and the molecular mechanisms of free amino acids in mitigating the glycation of cytosketelal actin in retinal pigment epithelial cells, pericytes and endothelial cells, since these are directly involved in the pathogenesis of DR.

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References

1 Aiello LP, Gardner TW, King GL, Blankenship G, Cavallerano JD, Ferris FL, Klein R, Diabetic retinopathy, *Diabetes Care* **21**, 143–156 (1998).

2 Baynes JW, Thrope SR, Role of oxidative stress in diabetic complications: a new perspective on an old paradigm, *Diabetes* **48**, 1–9 (1999).

3 Vlassara H, Recent progress in advanced glycation end products and diabetic complications, *Diabetes* **46**, S19–25 (1997).
4 Schroder S, Palinski W, Schonbein GW, Activated monocytes and granulocytes, capillary non-perfusion and neovascularization in diabetic retinopathy, *Am J Pathol* **139**, 81–100 (1991).
5 Ests JE, Higgins PJ, Actin: biophysics, biochemistry and cell biology, *Adv Exp Med Biol* **358**, 175–192 (1992).
6 Mc Leun G, The role of axonal cytoskeleton in diabetic neuropathy, *Neurochemical Research* **22**, 951–9569 (1997).
7 Tater D, Tepaut B, Bercoi JB, Youino P, Polymorphonuclear cell derangement in type I diabetes, *Diabetes Horm Metab Res* **19**, 642–647 (1987).
8 Ramakrishnan S, Sulochana KN, Decrease in glycation of lens proteins by lysine and glucose by scavenging of glucose and possible mitigation of cataract, *Exp Eye Res* **57**, 623–628 (1993).
9 Sulochana KN, Punitham R, Ramakrishnan S, Beneficial effect of lysine and amino acids on cataractogenesis in experimental diabetes through possible antiglycation of lens proteins, *Exp Eye Res* **67**, 597–601 (1998).
10 Buin BJ, Basic hematological techniques. In *Practical Hematology*, edited by Daice JV and Lewis SM, 8th edition, (Churchill Livingston, Hong Kong, 1998), pp. 59–60.
11 Lowry OH, Roseberg NJ, Farr AL, Randell RJ, Protein measurement with Folin phenol reagent, *J Biol Chem* 193, 265–275 (1951).
12 Saxena AK, Saxena P, Wu X, Obernovich M, Weiss MF, Monnier V, Protein aging by carboxymethylation of lysine generates sites for divalent metal and redox active copper binding: relevance to diseases of glycoxidation stress, *Biochem Biophy Res Comm* **260**, 332–338 (1999).
13 Laemmli U, Cleavage of structural proteins during assembly of the head of bacteriophage T4, *Nature* **227**, 680–685 (1970).
14 Morisey JH, Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity, *Anal Biochem* **117**, 307–310 (1981).
15 English D, Andersen BR, Single step separation of red blood cells, granulocytes and mononuclear leukocytes on discontinuous density gradients of ficoll hypaque, *J Immunological Methods* **5**, 249–252 (1974).
16 Rao MK, Currie MS, Padmanabhan J, Cohen H, Age related alterations in actin cytoskeleton and receptor expression in leukocyte, *J Gerentology* **47**, B37–44 (1992).
17 Gougeon R, Pencharaz PB, Marliss EB, Effect of NIDDM on the kinetics of whole body protein metabolism, *Diabetes* **43**, 318–238 (1994).
18 Shin DB, Hayre F, Kato H, Polymerization of proteins caused by the reaction with sugars, *Agric Biol Chem* **57**, 1451–1458 (1988).
19 Sherterline D, Clayton J, Sparrow JC, In *Actin Structure, Protein Profile: Actin*, 4th edition, (Oxford Press, USA, 1998), pp. 24–95.
20 Nilson BO, Biological effect of aminoguanidine: an update, *Inflammation Research* 48, 509–515 (1999).
21 Sulochana KN, Ramakrishnan S, Rajesh M, Coral K, Badrinath SS, Diabetic retinopathy: molecular mechanisms, present regime of treatment and future perspectives, *Current Science* **80**, 133–142 (2001).
22 Volpi E, Mittendorfer B, Wolf SE, Wolfe RR, Oral amino acids stimulate muscle protein anabolism in the elderly despite higher first-pass splanchic extraction, *Am J Physiol* **277**, E513–E520 (1999).
23 Allison JB, Bruhing SP, Crass MF, Shipp JC, Reduced high-energy phosphates in rat heart: effect of alloxan diabetes, *Am J Physiol* **230**, 1744–1750 (1976).

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