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Inhibitory Action of Vit C and Mannitol on Induced Cytotoxic Effect of Glycated Protein-metal Ion on Rat Hepatocyte

[1]Mohammad Taghi Goodarzi, [2] Mohammad Reza Safari and [3]Fatemeh Zal
[1]Department of Biochemistry, Medical School,
Hamadan University of Medical Sciences Hamadan Iran
[2]Department of Laboratory Science, Para-Medical School,
Hamadan University of Medical Sciences Hamadan Iran
[3]Department of Biochemistry, Medical School,
Shiraz University of Medical Sciences Shiraz Iran

Abstract: In this study we aimed to examine the inhibitory activity of Vit C and mannitol on induced cytotoxic effect of "glycated protien-metal ion" system. Albumin was glycated by treatment with glucose for 2, 4 and 6 week incubation. After measurement of glycation, glycated albumin was added to rat-hepatocye suspension in presence and absence of either manntiol and Vit C. Produced MDA was measured in these systems as an indicator of oxidative stress and the results were analysed using descriptive statistics and Wilcocson matched pairs test. The obtained data showed that presence of Vit C in cell suspension leads to reduction of MDA ($p = 0.000$). Similar effect was observed for mannitol. Both substances showed stronger activity at 1.0 micromolare concentration. We concluded that using antioxidant such as Vit C and mannitol it is possible to diminish reactive oxygen species production *in vitro*. To extrapolate this effect in diabetic patients *in vivo* experiments are needed.

Key words: Mannitol, ascorbic acid, glycation, rat hepatocytes

INTRODUCTION

Chronic hyperglyceamia is a major initiator of diabetic microvascular complications (e.g., retinopathy, neuropathy and nephropathy). Glucose processing uses a variety of divers' metabolic pathways; hence chronic hyperglycemia can induce multiple cellular changes leading to complications (Sheetz and King, 2003). Free radicals are formed disproportionately in diabetes by glucose oxidation, nonenzymatic glycation of proteins the subsequent oxidative degradation of glycated proteins. Abnormally high level of free radicals and the simultaneous decline of antioxidant defense mechanism can lead to damage of cellular organelles and enzymes, increased lipid peroxidation and development of insulin resistance (Wendt *et al*., 2003). Advanced Glycation End products (AGE), formed via the Millard reaction alter the structure and function of molecules and increase oxidative stress in biological systems (Valassor and Palace, 2003). Yim *et al*. (2001) reported that glycated proteins accumulated *in vivo* provide stable active sites for catalyzing the formation of free radicals. There is evidence showing that nonoxidative (glycation) and oxidative (glycoxidation) reactions lead to conversion of Low Density Lipoprotein (LDL), to a form that is recognized by the scavenger receptors of macrophages (Knott *et al*., 2003). This results in the accumulation of cholesterol and cholesteryl esters within macrophages and the formation of foam cells, a hallmark of atherosclerosis. Sajithlal *et al*. (1998) reported that presence of metal ion can accelerate the development of Millard reaction on collagen, and also metal catalyzed oxidation reaction play a major role in the cross-linking of collagen by glucose. In diabetes an increase in lenticular glucose induces glycation with the release of copper ion from copper containing enzymes, thus increasing the concentration of lenticular copper ion (Lin, 1997). As a result, superoxide scavengering activity is reduced and peroxide lipid concentration is increased.

AGE toxicity may be adverted by promising dietary and pharmacological strategies. Prevention of excessive glycoxidation could be a goal of recommendations designed to control the tissular alterations occurring in aging (Meli *et al*., 2003). Vitamins antioxidants have been

Corresponding Author: Dr. Goodarzi MT, Department of Biochemistry, Medical School,
Hamadan University of Medical Sciences, Hamadan, Iran
Tel : +98 811-8276293-4 Fax: +98 811- 8276299

Table 1: Effect of Vit C on ROS formation (produced MDA) induced by glycated-Fe+++ system

Glycation* µmol HMF mg^{-1} protein	Vit C (µmol L^{-1}) 0	0.1	1	10
5.02	32.92±1.94	28.3±2.10	25.74±1.76	24.36±1.80
31.3	52.98±2.96	40.23±2.36	35.65±2.54	27.35±2.38
53.2	53.95±2.38	42.51±2.36	28.68±2.12	24.77±2.31
123.2	85.89±3.68	76.35±3.31	59.52±2.78	46.56±3.13

* In Table 1-4 albumin was glycated by treatment with 100 mM glucose for 2, 4 and 6 weeks. Figures are X±SD of produced MDA (umol mg^{-1} protein) in absence and presence of three different concentration of Vit C

Table 2: Effect of Vit C on ROS formation (produced MDA) induced by glycated-Cu++ system

Glycation µmol HMF mg^{-1} protein	Vit C (µmol L^{-1}) 0	0.1	1	10
5.02	37.6±1.84	31.54±2.65	29.54±2.64	24.13±2.71
31.3	58.9±2.28	48.58±3.28	42.58±3.91	36.58±3.12
53.2	57.9±1.89	48.35±2.36	36.53±2.94	35.24±2.88
123.2	88.9±2.83	76.35±4.45	63.57±3.77	57.85±3.56

shown o be effective therapy in experimental models in reducing free radical species and inhibiting the oxidative stress in diabetes subjects. This study was aimed to test that whether Vitamin C and mannitol can reduce formation of Reactive Oxygen Species (ROS) in presence of glycated protein-metal ions.

MATERIALS AND METHODS

Preparation of glycated albumin: The glycated albumin was prepared according to the method of Monnier *et al.* (1990) with minor modification. Briefly aliquots of bovine serum albumin (0.10 gm L^{-1}) in 0.3 M phosphate buffer (pH = 7.4) containing 0.04% sodium azide in dialysis bag was incubated with 100 mM D-glucose at 37°C for either 2, 4 or 6 weeks. Controls were treated under the same condition without glucose. On days 10, 20 and 30 from the beginning of incubation, microbiological testing of the samples was carried out to confirm the absence of microbiological contamination. To avoid the interference by glucose the samples were dialyzed overnight against 0.01 M Phosphate Buffer Saline PBS (pH = 7.4). The samples were subsequently applied to an endotoxin-absorption column (Pyrosep, Daicel Chemical Japan). The column was equilibrated with 0.01 M PBS and the samples in 5 mg mL^{-1} concentration were applied to the column. The fraction was monitored at 280 nm in a spectrophotometer.

Measurement of the level of glycation: Glycation of albumin was measured using Thiobarbitoric Acid (TBA) colorimetric reaction (Furth, 1988). The colorimetric method with TBA is based on the hydrolysis of the glycated proteins using oxalic acid at 100°C yielding 5-hydroxymethyl furfural (5-HMF) which react with TBA. The absorbance was measured at 443 nm. 5-HMF was used as standard and glycation of albumin was calculated and expressed as µmol HMF per mg protein.

Preparation of rat hepatocytes suspension: Wistar rats weighing 250-300 g were sacrificed and their liver were removed, minced and homogenized at 4°C in 3 mL^{-1}g liver in 20 mM Tris buffer (pH = 7.4) containing 0.15 mM NaCl, 1 mM $CaCl_2$ and 1 mM PMSF. The samples were centrifuged at 800 g for 10 min and the supernatant was removed and filtered through Miracloth. The resulting filtrate was quickly frozen in liquid nitrogen and stored at -20°C for performing the other experiments.

Treatment of rat hepatocyte suspension with glycated albumin - transition metal ion: Glycated and non-glycated albumin were treated with rat hepatocye suspension in presence of different concentration (0, 20 and 40 µmol L^{-1}) of either $FeCl_3$ or $CuCl_2$. This cocktail was incubated at 37°C for 30 min and then the samples were centrifuged at 4000 g for 20 min and supernatants were isolated.

Measurement of cytotoxic effects of glycated albumin-transition metal-ion on rat hepatocytes in presence of vitamin C and mannitols: The cytotoxic effect of glycated albumin-metal ion was estimated by measuring produced Malondialdehyde (MDA) using thiobarbitoric acid colorimetric reaction (Gutteride and Quinlan, 1998). The above prepared cocktail (0.1 mL) was mixed with 1 mL of 0.67% TBA and 0.5 mL of trichloroacetic acid and incubated at 100°C for 20 min. After cooling, the reaction mixture was centrifuged at 4000 rpm for 5 min and the absorbance of supernatant was read at 532 nm. This experiment was repeated in presence of 1, 0.1 and 10 µM either mannitol or Vit C. The concentration of produced ROS was calculated and expressed as nmol of MDA per

Table 3: Effect of mannitol on ROS formation (produced MDA) induced by glycated-Fe+++ system

Glycation μmol HMF mg^{-1} protein	Mannitol (μmol L^{-1})			
	0	0.1	1	10
5.02	32.92±1.94	33.3±1.96	27.8± 1.6	25.6±1.30
31.3	52.9±2.69	44.56±1.96	30.30±1.62	28.3± 2.34
53.2	53.9±2.38	43.16±1.96	28.3±1.92	26.25±2.21
123.2	85.9±3.68	70.33±3.52	61.35±2.65	59.25±3.21

Table 4: Effect of mannitol on ROS formation (produced MDA) induced by glycated-Cu++ system

Glycation μmol HMF mg^{-1} protein	Mannitol (μmol L^{-1})			
	0	0.1	1	10
5.02	37.60±1.84	36.3±2.44	36.21±2.58	35.16±2.45
31.3	58.91±2.28	48.16±2.85	35.9± 3.01	34.68±2.81
53.2	57.95±2.62	45.16±2.96	38.94±2.92	36.85±2.55
123.2	88.94±2.83	69.85±3.96	60.33±3.65	58.35±2.98

mg protein using a freshly diluted solution of 1,1,3,3-tetraethoxypropane for preparing standard curve.

Statistical analysis: The obtained data were analyzed using descriptive statistics and Wilcocson matched pairs test. The $p<0.05$ were considered significant.

RESULTS

As Table 1 shows ROS production in presence of Vit C was lower compared to control (absence of Vit C) ($p = 0.0000$). Vit C in higher concentration (10 μmol L^{-1}) showed higher effect. Also in higher degree of glycation, produced ROS was higher and Vit C showed stronger effect.

Similar effect was observed for Vit C in presence of coppric ion (Table 2). Treatment of the hepatocyte suspension containing glycated albumin-copper ion with vit C leaded to reduction of ROS production compared with those of untreated with Vit C ($p = 0.001$).

Mannitol reduced the ROS production in glycated-ferric ion system ($p = 0.000$). Higher effect was observed in higher concentration of mannitol (1.0 μmol L^{-1}) (Table 3). However 10 μmol L^{-1} of mannitol shows higher activity, the difference was not statistically significant.

As Table 4 shows, presence of cupric ion increased the ROS production by glycated protein and adding mannitol reduced the production of ROS ($p = 0.001$). Also there was difference between the effects of 0.1 and 1.0 μM mannitol ($p = 0.01$). In 10 μM, it showed higher effect but the difference was not significant.

DISCUSSION

This is well known that hyperglyceamia is considered a key causal factor in the development of diabetic vascular complications and can mediate its adverse effects through multiple pathways. Advanced Glycation End Products (AGEs) are a heterogenous group of compounds that have been implicated in diabetes related complications (Stitt, 2001). However at present it is not known if they are the cause or the consequence of the complications observed. Nevertheless the routs of treatment of advanced glycated end product accumulation have been the interest of many investigators during recent years. Hyperglycaemia-induced oxidative stress has been shown to result in decreased nerve condition velocity and decreased endoneural blood flow both precursors for neuropathy (Sigh *et al*., 2001; Dickenson, 2002). The agents that limit AGE formation, increase the catabolism of these species, or antagonize their binding to RAGE may provide new targets for vascular protection (Wauteier and Schmidt, 2004). The antioxidant agents also have been used to reduce the produced oxidative stress in diabetes. In this study we showed that presence of two common antioxidant i.e., Vit C and manitol can lead to the reduction in induced oxidative stress on rat hepatocytes. We reported previously that presence of metal ion promotes oxidative stress in glycated protein (Goodarzi *et al*., 2005). In this study we showed that treatment of this system with vit C and mannitol can reduce the oxidative damage. We studied produced MDA in an *in vitro* model i.e., rat hepatocytes exposed with transition metal ion-glycated albumin. The most frequently used method to assess lipid peroxidation is measurement of thiobarbituric acid reactive substance, since MDA and other aldehydes react with TBA, MDA is a characteristic of oxidative stress. Cheng *et al.* (2005) showed that high glucose concentration heightened the oxidative suseptiblity of LDL and α-tocopherol enrichment reduced from undergoing architectural modification.

There is a need to continue to explore the relationship between free radicals, diabetes and its

complications and to elucidate the mechanisms by which increased oxidative stress accelerates the development of diabetic complications, in an effort to expand treatment option (Cheng *et al*., 2005). Vit C is the most potent intra- and extra-cellular antioxidant. It scavengers superoxide, hydroxyl and peroxyl radicals, react with hypochlorite and singlet oxygen (Therond *et al*., 2000) . Vit C is a powerful water-soluble antioxidant and at physiological concentrations, probably dose not produce reactive intermediates. It protects low density lipoproteins from oxidation, reduces harmful oxidants in the stomach and promotes iron absorption. Its antioxidant role *in vivo* is, however, unclear (Padayatty and levine, 2001; Akhilender, 2003). Also pharmacological properties of mannitol have been known for many years (Kim *et al*., 2002). Some of these properties are due to the antioxidant effect of mannitol. Furthermore, it was used as a reagent for long storage of red blood cells (Hees and Greenwatt, 2002). In this study we indicated that inhibitory action of mannitol and Vit C leads to diminish the ROS production on treated hepatocyte with metal-ion glycated protein. According to these data mannitol can be suggested as an additive in diet, however scientists still believe that aetiological and prevention studies with dietary antioxidant from foods are needed.

REFERENCES

Akhilender, N. K., 2003. Vitamin C in human health and disease is still a mystery? An overview. Nutr. J., 2: 7-17.

Cheng, C.J., R.H. Hsieh, H.F. Wang, M.Y. Chin and S.Y. Huang, 2005. Effects of glucose and α-tocopherol on low-density lipoprotein oxidation and glycation. Ann. N.Y. Acad. Sci., 1042: 294-302.

Dickinson, P.J., A.L. Carrington, G.S. Frost and A.J. Boulton, 2002 Neurovascular disease, antioxidants and glycation in diabetes. Diabetes Metab. Res. Rev., 18: 260-272.

Furth, A.J., 1988. Methods for assaying nonenzymatic glycosylation. Anal. Biochem., 175: 347-360

Gutteride, J.U.C. and G.J. Quinlan, 1998. MDA formation from lipid peroxidation in the TBA test: The role of lipid radicals, iron salts and metal chelator. J. Biochem., 5: 293-299.

Goodarzi, M.T., M.R. Safari and F. Zal, 2005. Cytotoxic effect of "glycated albumin- transition metal ion" on rat hepatocytes. Iranian Biomed. J. (submitted).

Hess, J.R. and T.G. Greenwalt, 2002. Storage of red blood cells: New approaches. Transfus. Med. Rev., 16: 283-295.

Kim ,H., J.Y. Seo and K.H. Kim, 2002. Effect of mannitol on Helicobacter pylori-induced cyclooxygenase-2 expression in gastric epithelial AGS cells. Pharmacology, 66: 182-189.

Knott, H.M., B.E. Brown, M.J. Davies, R.T. Dean, 2003. Glycation and glycoxidation of low-density lipoproteins by glucose and low-molecular mass aldehydes. Formation of modified and oxidized particles. Eur. J. Biochem., 270: 3572-3582.

Lin, J., 1997. Pathophysiology of cataracts: copper ion and peroxidation in diabetics. Jpn. J. Ophtalmol., 41: 130-137.

Monnier, V.M., 1990. Non-enzymatic glycosylation: The Maillard reaction and the ageing process. J. Gerontol., 45: B105-111.

Meli, M., J. Frey and C. Perier 2003. Native protein glycoxidation and aging. J. Nutr. Health Aging, 7: 263-266.

Padayatty, S.J. and M. Levine, 2001. New insights into the physiology and pharmacology of Vitamin C. CMAJ., 164: 353-355.

Sajithlal, G.B., P. Chithra and G. Chandrakasan, 1998. The role of metal-catalyzed oxidation in the formation of advanced glycation end products: An *in vitro* study on collagen. Free Radic. Biol. Med., 28: 265-269.

Sigh, R., A. Barden, T. Mort and L. Bellin, 2001. Advanced glycation end-products: A review. Diabetologica, 44: 129-146.

Stitt, AW., 2001. Advanced glycation: An important pathological event in diabetic and related ocular disease. Br. J. Ophtalmol., 85: 746-753.

Sheetz, M.J. and G.I. King, 2003. Molecular understanding of hyperglycemia`s adverse effects for diabetic complications. JAMA., 289: 1779-1780.

Therond, P., D. Bonefont-Rousselot, Davit-Spraul, M. Conti and A. Legrand 2000. Biomarkers of oxidative stress: An analytical approach. Curr. Opin. Clin. Nutr. Metab. Care, 3: 373-384.

Valassar, H. and M.R. Palace, 2003. Glycoxidation: The menace of diabetes and aging. Mt. Sinai. J. Med., 70: 232-241.

Wendt, T., N. Tanji, J. Gou and B.I. Hudson, 2003. Glucose, Glycation and RAGE: Implication for amplification of cellular dysfunction in diabetic nephropathy. J. Am. Soc. Nephrol., 14: 1383-1395.

Wauteier J.L. and A.M. Schmidt, 2004. Protein glycation: A firm link to endothelial cell dysfunction. Circ. Res., 95: 233-238.

Yim, M., B. Yim, H.S. C. Lee, S.O. Kang and P.B. Chock, 2001. Protein glycation: creation of catalytic sites for free radical generation. Ann. N.Y. Acad. Sci., 928: 48-53.