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Lens and Skin Protein Glycation, Lipid Peroxidation and Glutathione Levels in Vitamin C Administered Diabetic Rats

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Diabetes mellitus is one of the most common metabolic disorders that causes micro- and macro-vascular complications. The lens and skin, as well as most other organs, suffer as a result of metabolic disturbances caused by diabetes. Early diagnosis of diabetic skin and lens complications can be prevented or treated. Vitamin C ,which is an important antioxidant in human, is capable of scavenging oxygen-derived free radicals. The aim of this study was to investigate the effect of vitamin C on the lens and skin of streptozotocin-induced diabetic rats. Vitamin C, 80 mg/kg/day, i.p., was given to both diabetic and control rats daily, until the end of the experiment. 8 days after the diabetes induction, the total protein, nonenzymatic glycation of proteins (NEG), lipid peroxidation (LPO), and glutathione (GSH) levels in the lens and skin homogenates were determined by the methods of Lowry, thiobarbituric acid, Ledwozwy, and Ellman, respectively. Laemmli SDS polyacrylamide gel electrophoresis was also carried out on the lens or skin homogenates. Uncontrolled induced diabetes caused significant increases in blood glucose ($p<0.01$), NEG of skin and lens proteins, significant increase in skin LPO(respectively, $p<0.01$, $p<0.05$, $p<0.05$) and significant decrease in lens GSH ($p<0.01$). SDS-polyacrylamide gel electrophoresis revealed no significant differences in any protein bands between any of the groups. As vitamin C, in dose we used, did not reduce blood glucose level, it could not reverse the diabetic alterations of the skin and lens parameters in rats.

Key Words: *Diabetes, vitamin C, lens, skin, nonenzymatic glycation, oxidative stress.*

C Vitamini Verilen Diabetik Sıçanların Lens ve Deri Protein Glikasyonu, Lipid Peroksidasyonu ve Glutatyon Düzeyleri

Diabetes mellitus, mikro ve makro vasküler komplikasyonlara neden olan en yaygın metabolik bozukluklardan biridir. Diğer bütün organlar gibi lens ve deri de diyabetin oluşturduğu bu metabolik bozuklukların sonuçlarından etkilenmektedir. Erken teşhis edildiğinde lens ve deri problemlerinin bir kısmı önlenebilir veya tedavi edilebilir. C vitamini oksijenden türeyen serbest radikalleri temizleyen insan vücudu için önemli bir antioksidandır. Bu çalışmada, streptozotosin (STZ) ile diyabet oluşturulan sıçanların lens ve deri parametreleri üzerine C vitamininin etkileri araştırıldı. Diyabet oluşturulduktan sonra C vitamini 80 mg/kg/gün dozunda intraperitonal olarak 8 gün boyunca hergün kontrol ve diyabetik sıçanlara verildi. Lens ve deri homojenatlarında total protein, nonenzimatik glikozilasyon (NEG), lipid peroksidasyon (LPO) ve glutatyon seviyeleri sırasıyla Lowry, tiyobarbitürik asit, Ledwozwy ve Ellman metodları ile tayin edildi. Ayrıca lens ve deri homojenatlerında Laemmli SDS-poliakrilamid jel elektroforezi uygulandı. Kontrolsüz diyabet, kan glukozunda anlamlı artışa neden olurken ($p<0.01$), lens ve deri proteinlerinin NEG'da ve deri LPO'sunda anlamlı artışa (sırasıyla $p< 0.01$, $p<0.05$, $p<0.05$) ve lens GSH'ında anlamlı azalmaya ($p<0.01$) neden oldu. C vitamini verilmesi bu dozda kan şekerinde istatistiki açıdan anlamlı azalmaya neden olmadığından diyabetin lens ve deri parametreleri üzerindeki olumsuz etkilerini geri döndüremedi. SDS-poliakrilamid jel elektroforezi gruplar arasında protein bantları açısından da bir farklılık oluşturmadı.

Anahtar Kelimeler: *Diyabet, C vitamini, lens, deri, nonenzimatik glikasyon, oksidatif stres.*

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Introduction

Oxidative stress is involved in the pathophysiology of diabetes mellitus. In diabetes, increased nonenzymatic glycosylation (NEG) of proteins and lipid peroxidation (LPO) and decreased glutathione (GSH) may reduce the resistance of skin to harmful substances (1-4) and cause opacification of the lens and cataract formation (5-9).

Diabetes can affect every part of the body, including the skin. As many as 33 percent of people with diabetes will have a skin disorder caused or affected by diabetes at some time in their lives. In fact, such problems are sometimes the first sign that a person has diabetes.

Studies revealed that the retina has high amount of polyunsaturated fatty acids and the highest glucose oxidation which can lead to increased oxidative stress. Impaired activities of antioxidant defence enzymes such as superoxide dismutase (SOD) appear to be one of the possible sources of oxidative stress in diabetes (10).

Vitamin C is an important antioxidant in human, capable of scavenging oxygen-derived free radicals. It is structurally similar to glucose and can replace it in many chemical reactions, and thus is effective in prevention of non-enzymatic glycosylation of proteins (11). Several studies showed decreased basal vitamin C level in diabetic patients and also it is suggested that oxidative stress is increased in diabetes. Vitamin C is necessary for the formation of collagen, a type of protein that's needed to make skin, cartilage, tendons, and blood vessels. An inadequate intake of vitamin C can result in rough, dry, and scaly skin, not to mention bleeding gums, dry hair, and nosebleeds (11, 12). Researchers found that using only one therapy alone did not produce the positive results, but together the results were very clear. Vitamin C and insulin used in combination stopped the damage to blood vessels in type 1 diabetes patients with poor blood sugar control (13).

The aim of the study, therefore, was to investigate the effect of vitamin C on lenses and skin in terms of NEG, LPO and GSH in STZ-induced diabetic rats.

Materials and Methods

A total of 40 male Wistar albino rats, 200-250 g, were randomly divided into 4 groups, two diabetic and two control. They were kept at a constant temperature (22 ± 1°C) with 12 h light and dark cycles. The diabetic groups were rendered diabetic at day 0 by intraperitoneal injection of STZ (65 mg/kg, citrate buffer pH 4.5) (14). Two days later than the STZ injection blood glucose levels were determined. The rats with blood glucose below 150 mg/dL were discarded from the experiment. After diabet induction one of the control and one of the diabetic groups were given 80 mg/kg vitamin C (Roche, Turkey) by the intraperitoneal injection daily until the end of the experiment at day 8. Saline solution was given to the rest of control and diabetic groups intraperitoneally. On day 8, cardiac blood samples were taken from all rats under ether anaesthesia. All experiments were carried out in accordance with the guidelines of the Animal Care and Use Committee of Istanbul University, The Institute of Experimental Medicine (DETAE).

The rats were then killed by administering excess ether and their lenses extracted intracapsularily. The right and left lenses were both homogenized together with physiological saline. Skin samples were taken from the back of each rat after removing local fur. After the epidermis was removed, the skin samples were homogenized in physiological saline. Blood glucose was measured by the RANDOX glucose kit (RANDOX, GL3981, United Kingdom).

Protein assay

In alkali medium, proteins are reacted with cupper ions than reduced by pholine reactive (phosphomobydic-phosphotungstic acid). The absorbance of the blue colored product at 500 nm was evaluated Bovine serum albumin was used as a standard. Total protein level was expressed as % mg (15).

Nonenzymatic glycation assay

Protein glycation was assed by the 2-thiobarbituric acid method. The latter involved hydrolysing each 0,5 ml homogenate with 0.5 ml of 0.5 M oxalic acid in an autoclave for 1 h at 124±1 ^{o}C. To this, 0,5 ml 40% trichloracetic acid (w/v) was added, mixed, centrifuged at 1500x g for 10 min, and filtered using fitler paper. Absorbance at 443 nm was recorded. Then 0.75 ml of supernatant was incubated in 0.25 ml of 0.05 M 2-thiobarbituric acid at 37 ^{o}C for 30 min. After standing for 15 min at room temperature, absorbance was again measured at 443 nm and the differences between the first and second absorbances were calculated. The protein glycation values were expressed as nmol of fructose per mg protein. Commercial fructose (Sigma) was used as a standard (16).

Glutathione assay

The GSH levels were determined according to Beutler's method using Ellman's reagent. The procedure is based on the reduction of Ellman's reagent by SH groups to form 5,5′-dithiobis (2-nitrobenzoic acid) with an intense yellow color, measured spectrophotometrically at 412 nm using a Shimadzu spectrophotometer. Results were expressed as µmol GSH/g tissue (17).

Lipidperoxidation assay

Tissue samples were homogenized with ice-cold saline solution (0.9 %) for the determination of malondialdehyde (MDA) and glutathione levels. LPO levels were estimated by Ledwozyw's method (18). In brief, the adducts formed following boiled tissue sample with thiobarbutiric acid is extracted with n-butanol. The difference in optical density at 532 nm is measured in terms of the tisuue malondialdehyde (MDA) content, also of TBARS, which is undertaken as an index of lipid peroxidation. Results were expressed as µmol MDA g protein^{-1}.

SDS-Polyacrlyamide gel electrophoresis

Protein electrophoresis was carried out as described by Laemmli (19). Schleicher and Schueller Profile System mini-electrophoresis was performed with Sigma low molecular and high molecular weight protein standards (SDS-7,Dalton Mark VII-L and Hemocyanin crosslinked standart, Sigma) After electrophoresis, scans of Coomassie blue stained protein bands were obtained using a densitometer (Helena Laboratories TCL plus). Peak areas were measured with a plannimeter (Placom-Sokkisha, Kp-90N, digital) and the protein percentage calculated in each band.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of $P < 0.05$ were regarded as significant.

Results

The mean levels of the blood glucose and body weight for the six groups are shown in Table 1. Prior to inducing diabetes (day 0), the groups were checked for the differences in weight and blood glucose, but none were found. As expected, after 8 d of diabetes, the blood glucose levels of all the diabetic groups were significantly higher than those at day 0. Vitamin C administration did not decrease blood glucose level in diabetic group (Table 1).

Macroscopic evaluation revealed yellow porosities on skins and thickening of the fur on all rats in both diabetic groups. Figure 1 shows the differences between groups for skin NEG and LPO at the end of the experiment. NEG and LPO was significantly higher than than controls in the diabetic group. Vitamin C administration did not significantly change the NEG skin proteins and LPO levels in any group.

Figure 2 shows the differences between groups for lens NEG and GSH at the end of the experiment. Although macroscopic evaluation revealed no opafication of rat lenses in any groups NEG was significantly higher than controls in both diabetic groups (p<0,001). GSH was significantly lower than control in the diabetic group (p<0.001). Vitamin C administration did not significantly change the NEG of lens proteins and GSH levels in any group.

Table 1. Mean levels of body weights and blood glucose.

	Control (1) (n=10)		Control+Vit C (2) (n=9)		Diabetic (3) (n=10)		Diabetic+Vit C (4) (n=11)		p ANOVA
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Body weights (g)	243,90±36,18		243,33±38,31		202,80±36,70 [a]		194,72±36,23 [a]		0,0086
Blood glucose (mg/dL)	149,70±19,38		124,77±28,71		254,10±115,23 [a]		247,18±101,83 [a]		0,0011

Values are given as mean ± SD [a] : p<0.01 significantly different from control and control+vitamin C groups

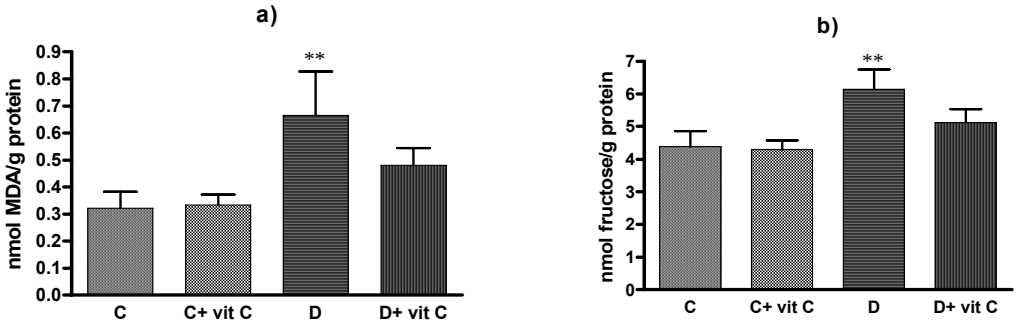

Figure 1. a) Lipid peroxidation (LPO) b) Nonenzymatic glycation (NEG) levels in the skin tissues of saline or Vitamin C treated control and diabetic groups. ** p<0.05 significantly different from C groups. (C: Control group, C+Vit C: Vitamin C treated control group, D:Diabetic group, D+Vit C : Vitamin C treated diabetic group)

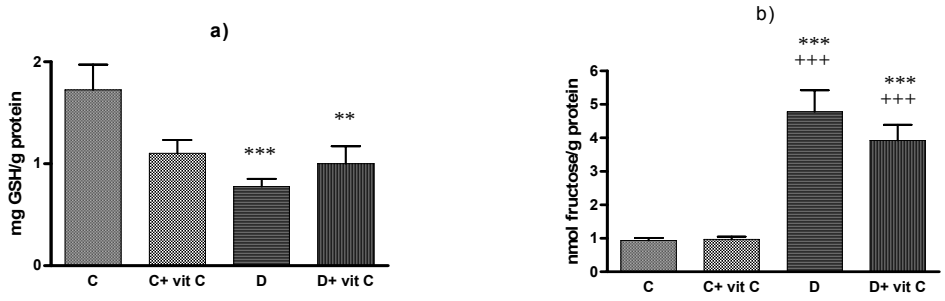

Figure 2. a) Glutathione (GSH) b) Nonenzymatic glycation (NEG) levels in the lens tissues of saline or Vitamin C treated control and diabetic groups. *** p<0.001 significantly different from C group, ** p<0.05 significantly different from C group, +++ p<0,001 significantly different from C+VitC group. C: Control group, C+Vit C: Vitamin C treated control group, D:Diabetic group, D+Vit C : Vitamin C treated diabetic group

Total protein levels of skin and lens samples were not found to differ significantly between all groups (data not shown). The protein bands obtained by Laemmli SDS-PAGE were in the same position for every sample and found at the same molecular weights (Data not shown).

Discussion

Diabetes mellitus (DM) is one of the most common metabolic disorders that causes micro and macrovascular complications (11). Oxidative stress, the situation in which an imbalance between the levels of reactive oxygen species and antioxidants exists, can lead to disturbed glucose metabolism and hyperglycemia. Oxidative stress is consistently observed in patients with diabetes and causes many complications in all body parts including skin and lens (20) .

Vitamin C has antioxidant properties that may be protective against diabetes. Vitamin C is present at high concentrations in the lens, cornea, retinal pigment epithelium of humans, monkeys and many other animals. It readily scavenges reactive oxygen and nitrogen species, such as superoxide and hydroperoxyl radicals, aqueous peroxyl radicals, singlet oxygen, ozone, peroxy nitrite, nitrogen dioxide, nitroxide radicals, and hypochlorous acid (20, 21).

In our experimental diabetes model, we observed an increase in blood glucose and a decrease in body weights. We also found an increase in NEG of lens and skin proteins and skin LPO and a decrease in lens GSH in diabetic rats. These findings are consistent with the other studies (22, 23).

Lopes et al has stated that the role of free radicals in the pathogenesis of diabetic retinopathy and the potential therapeutic effects of vitamin C in the treatment of diabetic eye disease remain open to debate. They also have revealed that cataracts associated with diabetes are caused by the degeneration of proteins in the lenses of the eyes et al.(24). Vitamin C is also required for collagen synthesis (25) and the addition of vitamin C increases collagen production in human skin fibroblasts (26). At the same time it may reduce production of elastin by an unknown mechanism (27).

Several studies showed decreased basal vitamin C level in diabetic patients and also it is suggested that oxidative stress is increased in diabetes. (28-31). Therefore, we evaluated the effects of 80 mg/kg/day vitamin C on blood glucose, skin and lens NEG, lens GSH and skin LPO. Administration of vitamin C, in a dose used, did not ameliorate any of tested skin and lens parameters.

As vitamin C, in dose we used, did not reduce blood glucose level, it could not reverse the diabetic alterations of the skin and lens parameters in rats. Further investigations are required for testing the effects of different doses of vitamin C on skin and lens in experimental diabetes. Although the primary focus of this study was the effect of vitamin C, further studies will be needed to clarify which vitamin has the most deleterious effect on glycoxidation both on skin and lens.

References

1. Sibbald GR, Landolt SJ. Skin and diabetes. Endocrinol Metab Clin North Am 1996; 25: 463-471.
2. Tentolouris N. Overview of diabetes. In: Katsilambros N, *et al*. (Editors). Diabetes in Clinical Practice. Editors:, John Wiley &Sons, Ltd England, 2006: 1-23.
3. King GL, Branskota NK. Mechanism of diabetic microvascular complications. In: CR Kahn and GC Weir (Editors) Joslin's diabetes mellitus. Lea & Febiger, A Waverly Company, Philadelphia, Baltimore. 1994: 631-647.
4. Makrilakis K. Skin disorders in diabetes. In: Diabetes in Clinical Practice. Katsilambros N, *et al,* (Editors) John Wiley &Sons, Ltd England, 2006: 243-251.
5. Diakoumopoulou E. Diabetic retinopathy. In: Diabetes in Clinical Practice. Katsilambros N, *et al*. (Editors). John Wiley &Sons, Ltd England, 2006: 159-173.
6. Aiello LM, Cavallerano JD. Ocular complications of diabetes mellitus. In: C.R. Kahn and G.C. Weir (Editors). Joslin's diabetes mellitus., Lea & Febiger, A Waverly company, Philadelphia, Baltimore. 1994: 771-793
7. Yarat A, Yanardağ R, Tunali T,*et al.* Effects of Glibornuride versus metformin on eye lenses and skin in experimental diabetes. Arzneim.-Forsch/ Drug Res 2006; 56(7):541-546.
8. Yarat A, Tunali T, Yanardag R, *et al.* The effect of Glurenorm (gliquidone) on lenses and skin in experimental diabetes. Free Rad Biol Med 2001; 31: 1038-1042.
9. Balasubramanyam M, Rema M, Premanand C. Biochemical and molecular mechanisms of diabetic retinopathy. Curr Sci 2002; 83: 1506-1514.
10. Srivatsan R, Das S, Gadde R, *et al.* Antioxidants and Lipid Peroxidation Status in Diabetic Patients with and without Complications. Arch Iranian Med 2009; 12: 121–127.
11. Ardekani MA, Ardekani AS . Effect of vitamin C on blood glucose, serum lipids & serum insulin in type 2 diabetes patients. Indian J Med Res 2007; 126: 471-474.
12. Rasik AM, Shukla A. Antioxidant status in delayed healing type of wounds. Int J Exp Pathol 2000; 81: 257-263.
13. Ceriello A, Esposito K, Ihnat M, Thorpe J, Giugliano D. Long-term glycemic control influences the long-lasting effect of hyperglycemia on endothelial function in type 1 diabetes J Clin Endocrinol Metab 2009; 94: 2751-276.
14. Jound A, Lambert AE, Stauffacher W, Renold AE, Diabetogenic action of streptozotocin Relationship of dose to metabolic response. J Clin Invest 1969; 48: 2129-2139.

15. Lowry OH, Rosebrough WI, Farr AL Randal RJ, Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.

16. Parker KM, England JD, Casto JD, Hessel R, Goldstein PE. Improved colorimetric assay for glycosylated hemoglobin. Clin Chem 1981; 27: 669-672.

17. Beutler E. Glutathione in red blood cell metabolism: a manual of biochemical methods. In: (2nd ed.), Grune and Stratton, New York 1975: 112-114.

18. Ledwozwy A, Michalak J, Stepien A, Kadziolka A, The relationship plasma triglycerides, cholesterol, total lipids, and lipid peroxidation products during human atherosclerosis. Clin Chim Acta 1986; 155: 275-284.

19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriofage T4. Nature 1970; 227: 680-685.

20. Harding AH, Wareham NJ, Bingham SA,*et al*. Plasma Vitamin C Level, Fruit and Vegetable Consumption, and the Risk of New-Onset Type 2 Diabetes Mellitus. Arch Intern Med 2008; 168: 1493-1499.

21. Carr A C, Frei B. Toward a new recommended dietary allowance for vitamin C based on antioxidant and health effects in humans. Am J Clin Nutr 1999; 6:1086-107.

22. Özçelik F, Yarat, A, Yanardağ R, *et al.* Limited effects of parsley (petroselinum crispum) on protein glycation and glutathione in lenses of streptozotocin induced diabetic rats. Pharm Biol 2001; 39: 230-233.

23. Tunalı T, Yarat A, Yanardağ, R, *et al.* The effect of chard (Beta vulgaris L.var cicla) on the skin of STZ induced diabetic rats. Pharmazie 1998; 53: 638-640.

24. Lopes JCC, Atallah ÁN, Valente O, Fernandes Moça Trevisani V. Vitamin C and superoxide dismutase (SOD) for diabetic retinopathy. Cochrane Database of Systematic Reviews, Issue 1. Art. No.: CD006695. DOI: 10.1002/14651858, 2008.

25. Kivirikko KI, Myllyla R. Post-translational processing of procollagens. Ann NY Acad Sci 1985; 460: 187-201.

26. Geesin JC, Darr D, Kaufman R, *et al.* Ascorbic acid specifically increases type I and type III procollagen messenger RNA levels in human skin fibroblast. J Invest Dermatol 1988; 90: 420-424.

27. Davidson JM, LuValle PA, Zoia O. Ascorbate differentially regulates elastin and collagen biosynthesis in vascular smooth muscle cells and skin fibroblasts by pretranslational mechanisms. J Biol Chem 1997; 272: 345-352.

28. Chen MS, Hutchinson ML, Pecoraro RE, *et al.* Hyperglycemic-induced intracellular depletion of ascorbic acid content in adults with insulin-dependent diabetes mellitus consuming adequate dietary vitamin C. Metabolism 1991; 40: 146-149.

29. Dyer RG, Stewart MW, Metcheson J, *et al*. Ketocholesterol, a specific indicator of lipoprotein oxidation and malondialdehyde in non-insulin dependent diabetes and peripheral vascular diasease. Clin Chim Acta 1997; 260: 1-13.

30. Evans M, Anderson RA, Smith JC, *et al*. Effects of insulin lispro and chronic vitamin C therapy on postprandial lipaemia, oxidative stress and endothelial function in patients with type 2 diabetes mellitus. Eur J Clin Invest 2003; 33: 231-238.

31. Tousoulis D, Antoniades C, Tountas C, *et al*. Vitamin C affects thrombosis/fibrinolysis system and reactive hyperemia patients with type 2 diabetes and coronary artery diseases. Diabetes Care 2003; 26: 2749-2753.