The ultrastructural alterations in rat corneas with experimentally–induced diabetes mellitus

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ABSTRACT

Objectives: To examine the ultrastructural changes of rat corneas in streptozotocin (STZ) induced diabetes mellitus and the follow-up insulin treatment.

Methods: Sprague-Dawley type rats was used for experimental procedures during the period from January to April 2003 at Baskent University, Ankara, Turkey. Rats were studied in 4 groups; group 1: controls, group 2: sham controls (single dose IV sodium citrate), group 3: STZ-induced diabetes mellitus (single dose 45 mg/kg STZ intravenously), group 4: diabetes mellitus + insulin treatment (8 U/day).

Results: We observed degenerative changes in the epithelial layer, stromal keratocytes and endothelial cells in diabetic group. In contrast, the corneal layers have revealed positive alterations in the insulin-treated group. The statistical analyses showed significant narrowing in the epithelial layer in the diabetic group (p=0.002), whereas thickening was observed in the epithelial basement membrane and Descemet's membrane (p=0.002).

Conclusion: It was determined that diabetes mellitus causes degenerative changes in cornea, which are positively influenced by short-term insulin treatment.

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Diabetes mellitus is a chronic metabolic disease affecting multiple organs in the body.[1] Chemical agents such as streptozotocin (STZ) and alloxan are frequently used to induce experimental diabetes mellitus to study the side effects of this illness on tissues. Streptozotocin causes a reduction in the plasma insulin levels caused by selective necrosis in B cells of pancreas.[2] Therefore, especially STZ–induced diabetic rats make up a proper model for human type I (insulin dependent) diabetes mellitus.[3] Studies based on STZ-induced diabetic rats have described degenerative changes in various organs caused by diabetes.[3,4] Especially, irregular thickening and lamination in basement membranes of renal tubules, glomerules and dermal epithelium,[4] damage in nervous tissue,[5] and structural degenerations in blood vessels[6] have been reported.

Ophthalmologic studies revealed that on diabetic patients, and experimental diabetic models on experimental animals that diabetes causes retinopathy.[7] Although there were various studies on cornea with diabetes mellitus, it is notable that these are generally clinical ones.[8,9]

Specular microscopic studies have reported that hexagonal endothelial cells are reduced in diabetic corneas as compared with the non-diabetic controls.[9,10] The relation of such physiologic alteration of corneal layers in diabetes mellitus with corneal morphology has not been fully clarified yet.[8]

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As a result, various specular microscopic studies of corneal physiology in diabetes focusing solely on the endothelial cells have been found in the literature.[9,11] However, experimental morphologic studies involving all of the corneal layers to explain these changes are insufficient. So, the aim of present study was to evaluate all of the corneal layers ultrastructurally in STZ-induced diabetes in rats, and to investigate the reversibility of these changes with insulin treatment.

Methods. In this study, 24 Sprague-Dawley type rats were used. The experimental protocol was approved by the local Ethical Committee for animal studies and conducted at the Faculty of Medicine, Baskent University in January to April 2003. The rats have been divided in to 4 groups. Group 1: controls (n=6). group 2: sham controls (n=6) (single dose intravenous (I. V.) 0.5 ml sodium citrate, pH 4.5).[12] group 3: STZ-induced diabetes (n=6) (single dose 45 mg/kg I.V. STZ) (Sigma, Deutschland, Germany). Group 4: diabetes (2 mounts) + insulin treatment (3 days) (n=6) (8 U/day NPH, intraperitoneal).

Assessment of blood glucose. In the 3rd day following STZ injection, plasma glucose level of every rat was assessed with glucometer. Plasma glucose levels of the rats in diabetic group were found to be 396-415 mg/dl in average. In the control group, blood sugar values ranged from 95-98 mg/dl in average.

Obtainment of the tissues. Rats were fed by free diet and water for 2 months in all groups and blood glucose levels measured twice in a week by glucometer (Aqucheck, Roche, Germany). Than rats in 1st, 2nd, 3rd groups were sacrificed and their corneas were removed. In the 4th group, before the rats were sacrificed, short term (3 days) 8 U/day I.P. NPH insulin treatment was applied and their corneas were removed.

Electron microscopic preparation. The sixth rats from each group were sacrificed with a lethal injection of xylazine (50 mg/kg) at the end of the study. After the central parts of corneal tissues, were fixed in a 1/15M phosphate buffered 2% glutaraldehyde solution (Agar Scientific LTD, Cambridge, UK) (pH 7.2). The tissues were then fixed in 1% osmium tetroxide (OsO4) (Agar Scientific LTD, Cambridge, UK) for 1 hour. After the samples turn dark, they were washed in phosphate buffer and dehydrated in a series of alcohol baths. Then they were embedded in a mixture of 10/10/1/0.5 Araldite CY 212 (Agar Scientific LTD, Cambridge, UK), dodecanyl succinic anhydride (Agar Scientific LTD, Cambridge, UK), dibutyl fytalate (Agar Scientific LTD, Cambridge, UK) and benzyl dimethylamine (Agar Scientific LTD, Cambridge, UK). Semi-thin sections stained with toluidine blue were observed by photomicroscope (Nikon, Tokyo, Japan), and thin sections stained uranyl acetate (Agar Scientific LTD, Cambridge, UK) and lead citrate (Fluka Steinheim, Switzerland) and were examined by transmission electron microscopy (EM) (LEO 906E, Oberkochen, Germany).[13]

The criteria for degeneration of cornea were as follows: disruption of junctional complexes and vacuolizations of epithelial and endothelial cell lines, abnormal mitochondria with loss of cristea in this cells, degenerations of epithelial, endothelial cells and keratocyte's organelles, accumulation of electron dense material in intercellular space of epithelial cells, and nuclear degeneration of all types of cells in cornea. Also, the epithelium, basement membrane and Descemet's membrane thickness assessments were performed with the electron microscope and evaluated via statistical analyses.

Statistical analysis. Data were expressed as means ± SD. Differences between groups were considered significant at p=0.002 by Mann-Whitney U test by using Statview computer program as having 4 groups and less than 20 animals in each group.

Results. ***Electron microscopy results.*** In the control group, normal histological structures were present in the corneal multilayered squamous epithelium, epithelial basement membrane, stroma, Descemet's membrane and endothelial cells (data not shown).

In the sham controls, while epithelial layer was observed in normal structures in the cellular junctional complexes and the basement membranes, there were partial vacuolizations in the cells of stratum basale and more prominently in the cells of stratum spinosum. These structures have been considered as changes secondary to the acidic pH of injected sodium citrate. Absence of degeneration in the other organelles and junctional complexes was considered as an indicator of normal function in the epithelial layer. In this group, observations of the stroma revealed longitudinal collagen fibers and keratocytes with normal ultrastructure. The endothelial cell layer was identical to those of the controls (data not shown).

In the 2-months STZ-dependent diabetes group, ultrastructural alterations were observed in all layers of the cornea in every rat. Cellular vacuolization observed in the sham control group was more prominent in the multilayered squamous epithelial layer. Vacuoles occasionally opened into the intercellular space. Crystolysis was observed in the epithelial cell mitochondria. Also, occasional disruption of the junctional complexes was noted in

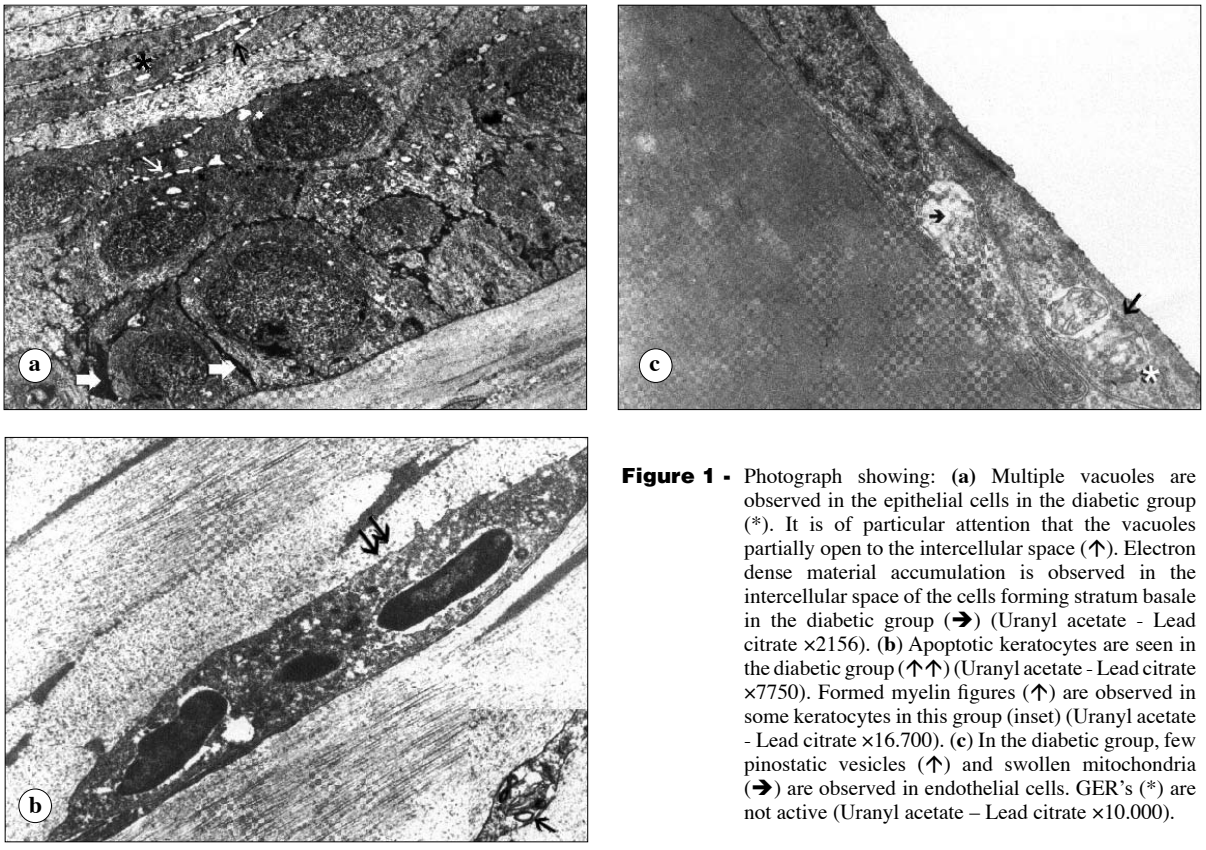

Figure 1 - Photograph showing: **(a)** Multiple vacuoles are observed in the epithelial cells in the diabetic group (*). It is of particular attention that the vacuoles partially open to the intercellular space (↑). Electron dense material accumulation is observed in the intercellular space of the cells forming stratum basale in the diabetic group (➔) (Uranyl acetate - Lead citrate ×2156). (**b**) Apoptotic keratocytes are seen in the diabetic group (↑↑) (Uranyl acetate - Lead citrate ×7750). Formed myelin figures (↑) are observed in some keratocytes in this group (inset) (Uranyl acetate - Lead citrate ×16.700). (**c**) In the diabetic group, few pinostatic vesicles (↑) and swollen mitochondria (➔) are observed in endothelial cells. GER's (*) are not active (Uranyl acetate – Lead citrate ×10.000).

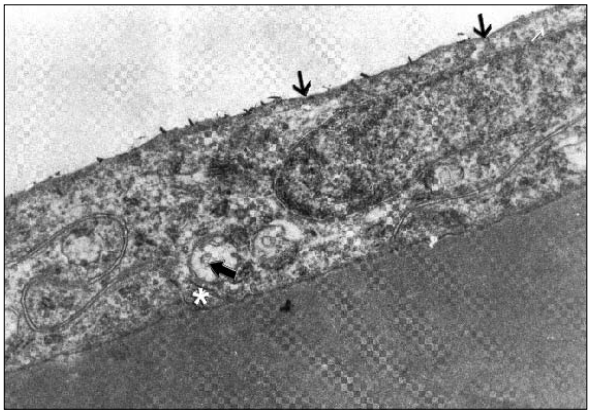

Figure 2 - Endothelial cells in the insulin-treated group are seen in nearly normal structure with mitochondria (➔), active GER (*). The pinostatic vesicles are increased in number (↑) as compared with the diabetic group (Uranyl acetate – Lead citrate ×10.000).

this group. Disruption of the junctional complexes was found to be more frequent among the cells of stratum granulosum layer. Also, electron dense material accumulation was observed in the intercellular space of the stratum basale cells (**Figure 1a**). Examination of the stroma in the diabetic group revealed normal longitudinal collagen fiber structure. Though there were normal keratocytes in the stroma, degenerative cells were also detected. These cells displayed chromatin condensation and fragmentation in their nuclei (**Figure 1b**). In the keratocytes, vacuolization, and particularly in high magnifications, myelin figures formed in the peripheral region of cytoplasm were noted (**Figure 1b inset**). In the diabetic group, the Descemet's membrane was normal in structure whereas ultrastructural alterations were observed in the endothelial cells. There were less pinostatic vesicles in the cell cytoplasms as compared with those of the controls, and some mitochondria were found to be swollen (**Figure 1c**).

In the group with 3-days insulin treatment following the 2-months diabetes period, a more regular ultrastructure was observed in all corneal

layers as compared with the diabetic group without insulin treatment. In the multilayered squamous epithelium, vacuolization in the epithelial cell cytoplasms was seen to be less than that in the diabetic group. However, the structure was not yet identical to that seen in the controls. While the junctional complexes were partially normal in structure, regional disruption was observed. The electron dense material accumulation in the intercellular space was reduced (data not shown).

In this group, stromal collagen fibers have been observed in normal structure. Though this region of the keratocytes was normal in appearance, large vacuoles have been noted in some keratocytes, particularly at the peripheral regions of the cytoplasms (data not shown) The organelle compositions and ultrastructure of the endothelial layer in this group were similar to those of the controls (**Figure 2**).

Statistical results. In this study, thickness was measured with electron microscopy in the corneal epithelium, basement membrane, and the Descemet's membrane, and evaluated statistically.

It has been found that when the epithelial thickness was measured at the level of electron microscopy, there was no statistical difference between the thickness values in control and sham control groups (p=0.937) (**Table 1**). In the diabetic group, epithelial thickness was statistically found to be reduced as compared with both control and sham control groups (p=0.002) (**Table 2**). On the other hand, insulin treatment had a positive influence on the epithelial thickness, and statistically the values were closer to those of the controls and sham controls (p=0.002) (**Table 4**). When the insulin-treatment group was compared with the controls, the reduction in epithelial thickness was found to be less than that in the diabetic group (p=0.015) (**Table 3**).

The epithelial basement membrane thickness values were similar in control and sham control groups (p=0.818) (**Table 1**). In the diabetic group, epithelial basement membrane thickness was observed to be increased as compared with the controls and sham controls (p=0.002) (**Table 2**). However, the epithelial basement membrane thickness in the insulin-treatment group was found to be reduced as compared with that of the diabetic group (p=0.002) (**Table 3**), and was closer to, those of both the controls and sham controls (p=0.009) (**Table 4**).

The Descemet's membrane thickness measurements with electron microscopy revealed no statistically significant difference between the controls and sham controls (p=0.015) (**Table 1**). Descemet's membrane thickness in the diabetic group was statistically different as compared with the controls (p=0.002) (**Table 2**). In the insulin-treated group, the Descemet's membrane thickness was found to be

Table 1 - The statistical data of epithelium, basement membrane and Descemet's membrane thickness values in control and sham control groups

Structures	Group 1		Group 2		Z	*P*
	Mean	SD	Mean	SD		
Epithelium	42.74	0.49	42.94	0.583	-0.160	0.937
Basement membrane thickness	0.090	0.0068	0.089	0.0072	-0.320	0.818
Descemet's membrane	4.41	0.15	4.20	0.0717	-2.402	0.015

Table 2 - The statistical data of epithelium, basement membrane and Descemet's membrane thickness values in control and diabetic groups

Structures	Group 1		Group 3		Z	*P*
	Mean	SD	Mean	SD		
Epithelium	42.74	0.49	31.36	0.97	-2.882	0.002
Basement membrane thickness	0.090	0.0068	0.17	0.008	-2.882	0.002
Descemet's membrane	4.41	0.15	5.30	0.18	-2.882	0.002

Table 3 - The statistical data of epithelium, basement membrane and Descemet's membrane thickness values in control and insulin-treatment groups

Structures	Group 1		Group 4		Z	*P*
	Mean	SD	Mean	SD		
Epithelium	42.74	0.49	41.754	0.424	-2.402	0.15
Basement membrane thickness	0.090	0.0068	0.101	0.0043	-2.562	0.009
Descemet's membrane	4.41	0.15	4.249	0.063	-1.601	0.132

Table 4 - The statistical data of epithelium, basement membrane and Descemet's membrane thickness values in diabetic and insulin-treatment groups

Structures	Group 3		Group 4		Z	*P*
	Mean	SD	Mean	SD		
Epithelium	31.36	0.97	41.754	0.424	-2.882	0.002
Basement membrane thickness	0.17	0.008	0.101	0.0043	-2.882	0.002
Descemet's membrane	5.30	0.18	4.249	0.063	-2.882	0.002

reduced as compared to the diabetic cornea (p=0.002) (**Table 3**) whereas closer to that of the controls and not statistically different (p=0.132) (**Table 4**).

Discussion. Diabetes mellitus is a syndrome characterized with irregularity of blood glucose levels, causing diffuse structural and functional damage in human and animal tissues.[4,8] Studies on diabetic subjects and experimental diabetes models showed that diabetes affects the structure of eyes.[14,15] Among the complications of diabetes, the most commonly recognized one is diabetic retinopathy, but also corneal erosions, keratitis and various epithelial defects were reported.[16,17]

Friend et al[18] studied the epithelial layer of cornea with electron microscopy in STZ-induced diabetic rats and reported increased intracytoplasmic tonofilament and glycogen content in the epithelial cells. Researchers stressed that long-term diabetes cause degeneration in the basal cell layer, and basement membrane display thickening and folding. Jiang et al[19] in their studies on STZ-induced diabetic rabbit corneas, reported changes in accordance to Friend's et al[18] findings in corneal epithelium secondary to diabetes. They also pointed out increased mitochondrion and glycogen content in the epithelial cells and thickening of basement membranes. Yamamoto et al[20] reported that in experimental diabetic rats, the corneal epithelium was thinner at the end of the 1st month of diabetes, and cellular proliferation was reduced, as compared with the controls. In this study, when the corneal epithelial layer in rats with STZ-induced diabetes for 2 months examined, which the epithelial layer thickness was found to be significantly thinner than that in the controls, supported the observations of Yamamato et al[20] after the insulin treatment following the 2-months diabetic period, epithelial thickness was similar to that of the controls. These observations revealed that the reduction of thickness in the epithelial cell layer reported by Yamamato et al[20] at the end of the first month is also present in the second month. Therefore, it was concluded that diabetes chronically reduced epithelial cell proliferation. Also, in this study various degenerative changes were observed in the epithelial cells, such as mitochondrial crystolysis, prominent vacuolization in the cytoplasm, and detachments of the attachment complexes. Such alterations were seen to be highly common in the basal cell layer supporting the reports of Friend et al.[18] However, the increment of mitochondrion content reported by Jiang et al[19] in the epithelial cells was not observed in our study, but we found crystolysis in the mitochondria. Further, electron dense material accumulation was noted in the intercellular zone, and there was more prominent in the basal cell layer. Which, accumulation together with the degenerative changes in the cells, suggested a disruption of the cellular osmotic balance secondary to diabetes. It was also noted that all alterations observed in diabetes were reverted to normal with insulin treatment.

Clinical studies presented that diabetic subjects were prone to development of edema in the corneal stroma following invasive operations such as vitrectomy.[8,17,21]

In our study, examination of the corneal stroma revealed partial apoptotic changes in the keratocytes while collagen fibres were intact in structure. This lack of disturbance in the collagen structure in the presence of apoptotic changes in keratocytes was attributed to the continuity of fiber production by keratocytes lacking apoptosis. Various researchers reported that diabetes particularly caused thickening in the basement membranes. The studies revealed thickening and lamination of basement membranes particularly in dermal blood vessels, renal glomerules and tubules.[4,22] Friend et al[18] reported that the basement membrane of corneal epithelium was thickened with diabetes, and displays partial folding. Our study also revealed statistically significant thickening of epithelial basement membrane in diabetes. In the insulin-treated group, the thickness of basement membrane was found to be near to normal.

Descemet's membrane is the basement membrane of the corneal endothelial cells, and there are very few studies on this structure. Rehany et al[4] in a study published in 2000, reported that in rats with diabetes induced by 65 mg/kg STZ, the collagen fibers of the Descemet's membrane were pleomorphic at the end of a 2-months diabetic period, and this pleomorphism increases with longer periods.[4]

In contrast with the report of Rehany et al[4] after close examination of the Descemet's membranes in rats with diabetes induced by 45 mg/kg STZ at the end of the 2-month period, we found that there was no structural alteration in the collagen fiber. However, statistical evaluation of these electron microscopic studies revealed thickening in the Descemet's membrane of the diabetic group, as compared with the controls and sham controls. Lack of irregularity in the collagen fiber structure has been attributed to the lower dosage of STZ used than that in the literature, therefore it has been suggested that structural alterations can occur in a period longer than that reported in the literature. Insulin treatment for 3-days following 2-months diabetes period resulted in statistically significant structural improvement. The Descemet's membrane thickness was close to normal. This observation revealed that insulin treatment in

early phase of diabetes could revert the morphology of Descemet's membrane to normal. This reversion in the Descemet's membrane suggested that similar events could take place in the other basement membranes.

Diabetes in chronic state brings along microvascular or macrovascular complications.[8] The corneal endothelial cells share a common origin with the endothelial cells of blood vessels, and various changes are expected in diabetes. Corneal endothelial cells do not have mitotic activity.[23] In the literature, various studies generally evaluated the endothelial cells in diabetes using specular microscopy.[23,24] Using specular microscopy, De la Messeliere and Renard[23] studied the corneal endothelial cells in 101 diabetic subjects in 1987, and reported that diabetes causes damage in corneal endothelium but no cell destruction occurred. In a similar research performed by Schultz et al[9] corneal endothelial cells of subjects with both type I and type II diabetes were examined with specular microscope. The researchers reported findings paralleling to those of Messeliere, and concluded that cell loss was higher in type I diabetes than that in type II diabetes.

In this study, mitochondrial swelling and reduction in pinostatic vesicles were noted in the endothelial cells of diabetic rats. In literature, it was reported that diabetic subjects were more prone to developing edema in the stroma following invasive intraocular operations. In our study, it has been suggested that the reduction in pinostatic vesicles can be related with the stromal edema. Insulin treatment has been shown revising the endothelial cell morphology to normal.

In conclusion, as administration of insulin prevented corneal endothelial cells and stromal keratocytes degeneration, and inhibited the generation of collagen irregularity, reduced the corneal epithelial thickness of both basement and Descemet's membranes, insulin appears to play a cytoprotective role in the cornea insulted by diabetes mellitus. It seems likely that insulin with its efficiency as a protector is a potential therapeutic agent in diabetic corneal injury.

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