Transmission electron microscopy and autofluorescence findings in the cornea of diabetic rats treated with aminoguanidine

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ABSTRACT • RÉSUMÉ

Background: The accumulation of advanced glycation end products (AGEs) has been implicated in the pathogenesis of diabetic keratopathy. The present study was aimed to understand if aminoguanidine (AG), an AGE inhibitor, was protective against the development of corneal complications in a diabetic rat model.

Methods: Wistar rats were divided into three experimental groups: control, diabetic, and AG-treated diabetic. Diabetes was induced in rats via a single intraperitoneal injection (60 mg/kg) of streptozocin (STZ) and AG was administered in drinking water at a dose of 1 g/L. All animals were sacrificed at the end of 10 weeks and corneas from diabetic and nondiabetic rats were analyzed via transmission electron microscopy (TEM). Corneal autofluorescence measurements were also performed in all experimental groups.

Results: Electron microscopic evaluation revealed that aminoguanidine treatment in diabetic rats prevented the formation of intracellular spaces between neighbouring cells in the superficial corneal epithelium. Hyperglycemia-induced degeneration of intracellular organelles and formation of cytoplasmic vacuoles in the corneal stroma was also prevented with the treatment of AG. Corneal autofluorescence detected in the diabetic group (5.98 ± 2.17 Fi/mg protein) was found to be significantly greater than the control (3.92 ± 0.56 Fi/mg protein) and the AG-treated diabetic group (4.18 ± 0.59 Fi/mg protein) (p < 0.05).

Interpretation: The presented data provide evidence that AG is preventive against corneal alterations in experimental diabetes.

Contexte : L’accumulation de produits terminaux de glycation (PTG) a été impliquée dans la pathogénèse de la kératopathie diabétique. La présente étude avait pour objet de comprendre si l’aminoguanidine (AG), inhibiteur de PTG, protégeait du développement de complications cornéennes dans un modèle expérimental aux rats.

Méthodes : Des rats Wistar ont été répartis en trois groupes expérimentaux : témoin, diabétique et diabétique traité à l’AG. Le diabète a été induit chez les rats avec une seule injection intrapéritonéale (60 mg/kg) de streptozocine (STZ). L’AG était administrée dans l’eau à boire à une dose de 1 g/L. Tous les animaux furent...
Corneal complications are often associated with diabetes mellitus and can be vision-threatening. Corneal abnormalities that occur in patients with diabetes mellitus (DM) are identified as diabetic keratopathy. Histological findings have demonstrated subepithelial deposits, thickening of the subepithelial basement membrane, and altered morphological appearance in the corneal epithelium and endothelium of patients with DM. Although pathogenic mechanisms underlying these corneal abnormalities are uncertain, the accumulation of advanced glycation end products (AGEs) has been implicated in the pathogenesis of diabetic keratopathy.

In hyperglycemia, reducing sugars such as glucose, react nonenzymatically with amino groups in proteins, forming Schiff bases and Amadori products to produce AGEs. This process, also known as the Maillard reaction, was described in the early 1900s when it was noticed that amino acids heated in the presence of reducing sugars developed a characteristic yellow-brown color. The accumulation of reactive dicarbonyl lipids and nucleic acids precursors as intermediate products was also observed during the Maillard reaction.

Nonenzymatic glycation of proteins is an important phenomenon in the development of diabetic complications like macro- and microangiopathy. The formation of AGEs leads to protein cross-linking and causes the destruction of cellular structures. Indeed, AGE inhibitors, such as pyridoxamine (PM) and aminoguanidine (AG), have been reported to retard the development of diabetic complications in animal models by preventing the formation of AGEs in various proteins including collagen.

The present study was aimed at understanding the effect of AG in the formation of corneal disorders observed in diabetic keratopathy. Thus, transmission electron microscopy and autofluorescence measurements were carried out in the cornea of diabetic rat models that were treated with AG.

**Methods**

**Animals**

All animal procedures were reviewed and approved by the animal ethics committee at Akdeniz University Medical School. Twenty-four male Wistar rats weighing 220 to 330 g, aged 10 weeks, were used in the study. Standard rat chow and water were given ad libitum, and animals were housed in groups of 4 to 5 rats at standard conditions (24 ± 2°C and 50 ± 5% relative humidity) with 12 h light–dark cycles. Animals were randomly divided into three groups of eight rats. Group 1 served as age-matched controls; group 2 received a single intraperitoneal (IP) injection (60 mg/kg) of streptozocin (STZ) (Sigma Chemical Co., St. Louis, Mo.) dissolved in 0.1 mol/L citrate buffer (pH 4.5) with 12 h light–dark cycles. Animals were randomly divided into three groups of eight rats. Group 1 served as age-matched controls; group 2 received a single intraperitoneal (IP) injection (60 mg/kg) of streptozocin (STZ) (Sigma Chemical Co., St. Louis, Mo.) dissolved in 0.1 mol/L citrate buffer (pH 4.5). To prevent hypoglycemia, 2 mL of 5% glucose was administered orally and drinking water was supplemented with 10% sucrose for the first 24 h after STZ injection. Group 3 rats received IP injection of STZ as described above and were subsequently given aminoguanidine (AG) hydrochloride (Sigma Chemical Co.) in drinking water at a dose of 1 g/L, as described previously.

Blood glucose levels were determined with a blood glucose meter (Hypoguard Supreme, Minneapolis, Minn.) 3 days after IP injection of STZ and rats were...
considered diabetic if blood glucose levels were greater than 250 mg/dL.\textsuperscript{12}

**Transmission electron microscopy**

All animals were sacrificed at the end of 10 weeks and corneas from diabetic and nondiabetic rats were prefixed by immersion in 2.2\% glutaraldehyde in 0.1 mol/L (pH 7.4) Sorensen's phosphate buffer for 4 h and postfixed in 1\% osmium tetroxide in 0.1 mol/L (pH 7.2) sodium phosphate buffer. The samples were dehydrated in graded ethyl alcohol series and embedded in Araldite CY212. Ultrathin sections were contrasted with uranyl acetate and lead citrate for examination by transmission electron microscope (LEO 906E, Oberkochen, Germany).

**Corneal autofluorescence measurements**

Corneas obtained from all experimental groups were homogenized in phosphate-buffered saline (PBS) and autofluorescence was determined at 340 nm excitation and 460 nm emission wavelength using a Shimadzu RF 5000 spectrofluorometer (Kyoto, Japan)\textsuperscript{13} and reported as fluorescence intensity per milligram of protein (Fi/mg protein). Protein concentrations in all samples were measured spectrophotometrically by a Lowry assay\textsuperscript{14} with bovine serum albumin as a standard.

**Statistical analysis**

The mean corneal autofluorescence was compared among the experimental groups by Mann–Whitney $U$ tests via the SPSS statistical program. The $p$ value of 0.05 was set for statistical significance.

**RESULTS**

**Plasma glucose**

Aminoguanidine treatment had no effect upon plasma glucose concentrations. Final plasma glucose levels measured (mean $\pm$ SD) in control, diabetic, and diabetic + aminoguanidine-treated rats were 127.4 $\pm$ 15.8, 542 $\pm$ 73.1 and 574 $\pm$ 68 mg/dL, respectively.

**Transmission electron microscopy findings**

Fig. 1 shows transmission electron micrographs of cornea from the control group. Starting out from the superficial squamous cell layer, corneal epithelium is tightly packed with no intercellular spaces between

![Fig. 1—Transmission electron micrographs of cornea from control rats. Top: Superficial cells of the corneal epithelium. Asterisks indicate cell nuclei. Middle: Epithelial basal cells (EB) of the cornea. Arrowheads show the basement membrane and S indicates stroma. Bottom: Electron micrograph of the corneal stroma.](image)
neighbouring cells. Numerous hemidesmosomes are present in the subepithelial basement membrane. Regularly spaced, compact collagen fibres can be seen enfolding stromal keratocytes.

Fig. 2 shows transmission electron micrographs of cornea from diabetic rats. Intracellular spaces are present between neighbouring cells in the superficial corneal epithelium, while deformation is observed in lower layers of epithelial cells. Stromal keratocytes display degeneration of intracellular organelles and cytoplasmic vacuoles of various sizes.Disconnected collagen fibres have resulted in structural disintegration in the corneal stroma.

Fig. 3 shows transmission electron micrographs of cornea from diabetic rats treated with AG. Like in the control group, no intercellular gaps are present between neighbouring cells in the superficial corneal epithelium. The structural unity of the stratified epithelium is unaltered and stromal keratocytes display an active cytoplasm.
Corneal autofluorescence

Corneal autofluorescence obtained from the three experimental groups are shown in Fig. 4. Autofluorescence detected in the diabetic group (5.98 ± 2.17 Fi/mg protein) was found to be significantly greater than that in the control (3.92 ± 0.56 Fi/mg protein) (p = 0.04) and AG-treated diabetic group (4.18 ± 0.59 Fi/mg protein) (p = 0.04). No significant difference was found between the control and AG-treated diabetic group.

INTERPRETATION

In the present study we have evaluated the role of AG in the development of corneal disorders observed in diabetes. Aminoguanidine treatment in diabetic rats prevented the formation of intracellular spaces between neighbouring cells in the superficial corneal epithelium. Hyperglycemia-induced degeneration of intracellular organelles and formation of cytoplasmic vacuoles in the corneal stroma was also prevented with the treatment of AG (Fig. 3).
Aminoguanidine is a therapeutic agent used for the prevention of AGE formation. It has two reaction centers; the hydrazine group (–NHNH₂) and the guanidino group (–C(=NH)NH₂). These two groups react with dicarbonyl groups and thus function as scavengers of glycating agents.¹⁵ In the absence of AG, the reactive dicarbonyl groups would otherwise form AGEs. The observed results in this study indicate that glycation and accumulation of AGE can be linked to the development of diabetic keratopathy.

Apart from being a scavenger of dicarbonyl compounds, AG is also a potent inhibitor of inducible nitric oxide synthase (NOS) and a weak inhibitor of neuronal and endothelial NOS.¹⁶ The average peak concentration of AG in the plasma of diabetic rats given 1 g/L, as in the present study, is reported to be 47 µmol/L.¹⁷ Tissue concentration of AG at the reported dose of 1 g/L administration is 40–60 µmol/L in the retina and 200 µmol/L in the lens.¹⁸ At these concentrations, AG not only prevents tissue glycation but also inhibits iNOS. Knowing that the presence of iNOS has been documented in corneal diseases,¹⁹ it can be suggested that AG prevents diabetic disease progression in the cornea also via iNOS inhibition.

Corneal autofluorescence, determined at 340 nm excitation and 460 nm emission wavelengths, reflects fluorescence characteristics of AGEs.¹³ In diabetic humans, corneal autofluorescence has been measured in vivo by means of optical methods and has been found to be higher in diabetics compared with controls.²⁰⁻²³ The reported increase of corneal autofluorescence detected in the diabetic group (Fig. 4) is in accordance with previous findings and supports the accumulation of AGEs in the cornea of hyperglycemic rats. No significant difference was found in corneal autofluorescence between the control and AG-treated diabetic group. This result is in agreement with electron microscopy findings and confirms the decrease of AGEs via AG treatment.

Aminoguanidine treatment in experimental diabetes has been reported to be protective against diabetic complications like nephropathy, neuropathy, retinopathy, and cataract. In fact, AG was shown to inhibit the development of albuminuria and mesangial expansion in STZ-induced diabetic rats.²⁴,²⁵ Similarly, AG prevented decreased nerve blood flow and improved nerve conduction velocity in STZ diabetic rats.²⁶ Likewise, a 5-year study of diabetic dogs has shown that AG treatment prevented the development of retinopathy.²⁷ A pyridoxal–aminoguanidine adduct has also been shown to delay the time of cataract development in STZ diabetic rats and decrease the opacification of lenses in culture medium containing high glucose levels.²⁸ Data reported herein now show that AG therapy is also protective against diabetic keratopathy.

Two clinical trials, ACTION I and ACTION II, were carried out to determine if AG ameliorates diabetic nephropathy.²⁹,³⁰ Both studies were randomized, double-blinded, and placebo-controlled. ACTION I was performed in 690 patients with type 1 diabetes and ACTION II enrolled 599 patients with type 2 diabetes mellitus. In ACTION I and II, 33% of patients were randomized to placebo and 67% were randomized to either a low or high dose of AG (50–300 or 100–600 mg/d). The primary endpoint in both studies was the time to doubling of serum creatinine; the secondary end points included evaluations of proteinuria, kidney function, and retinopathy. ACTION 1 did not demonstrate a statistically significant beneficial effect of AG on the progression of overt nephropathy. However, it provided the first clinical proof of the concept that inhibiting advanced glycation end product formation can result in a clinically important attenuation of the complications of type 1 diabetes.²⁹ The External Safety Monitoring Committee recommended the early termination of the ACTION II trial owing to safety concerns and apparent lack of efficacy.¹⁸

In summary, this study provides the first evidence that AG is preventive against corneal complications in experimental diabetes. The use of AG as a pharma-
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A logical agent to prevent AGE accumulation in diabetic rats also provides support for the importance of glycation and dicarbonyl formation in the pathogenesis of diabetic keratopathy.

REFERENCES