Introduction

Chronic hyperglycemia is one of the most important initiating factors responsible for the development of long-term diabetic complications. During nonenzymatic glycation a stable ketoamine adduct is formed from glucose and free amino groups of proteins impairing their physiological functions in blood (e.g., fibrinogen, hemoglobin, plasminogen, antithrombin III, apolipoprotein A-I, apolipoprotein B-100, transferrin, erythrocyte spectrin, platelet calmodulin) (5,6,8,13,16,19,21,23,34) and other tissues (cardiac myosin, peripheral nerve myelin, basic fibroblast growth factor, lens crystallins) (9,14,18,35,37).

Moreover, glycation accelerates extensive oxidation reactions appearing in long-lived proteins that lead to tissue damage by cumulative formation of more permanent, irreversible cross-linked condensation products. Accumulation of advanced glycation end products (AGE) showing fluorescence at 440 nm, such as N\(^6\)-(carboxymethyl) lysine (CML), pyrraline, or pentosidine, have been implicated in development of retinopathy, nephropathy, neuropathy, connective tissue pathology, thromboembolic disorders, and atherosclerosis (1,7,15,36,20).

Nonenzymatic glycation and oxidative stress play an important role in the multifactorial development of diabetic and senile cataracts (12,29,30), a major cause of blindness worldwide. The only curative procedure involves replacement of the cataractous lens by a synthetic intraocular implant, as demonstrated in Fig. 1.

Previously published experiments studied cataractogenesis in human or animal lenses collected via intracapsular or extracapsular surgery. The current trend in ophthalmology is to remove cataract using phacoemulsification (ultrasound degradation, emulsification and aspiration of the lens nucleus fragments, cortex aspiration from the eye) and small incision surgery with implantation of the foldable intraocular lenses. The purpose of our study was to determine the amount of glycated proteins and advanced glycation end products (AGE) in cataractous lens homogenates of patients who underwent phacoemulsification, and to define a simple in vitro protein model of glycoxidation. Analysis of 30 cataractous lenses (15 diabetic and 15 non-diabetic) revealed a significant increase in both glycated lens proteins of diabetics compared with the controls (0.15 vs 0.08 nmol/mg protein, P < 0.01) and AGE-linked fluorescence at 440 nm (4.8 vs 2.8 AU/mg protein, P < 0.01). The presence of AGE fluorescence in lenses indicates the role of oxidative stress in cataractogenesis. Fifty-six days incubation of alanine and aspartate aminotransferases, used as model proteins, with 500 mM D-fructose at 25 and 37 °C led to a complete inhibition of ALT and AST activities. The fluorescence of both aminotransferases rose according to the chosen incubation temperature: 37 °C > 25 °C > 4 °C. ALT and AST incubated in a medium containing D-fructose are subject to nonenzymatic glycation followed by a consequent formation of AGE products. Our data: i) support the concept of glycation-glycoxidation pathway appearing in diabetic patients; ii) form a base for determination of the efficiency of various antioxidative compounds in vitro.

Summary: The purpose of our study was to determine the amount of glycated proteins and advanced glycation end products (AGE) in cataractous lens homogenates of patients who underwent phacoemulsification, and to define a simple in vitro protein model of glycoxidation. Analysis of 30 cataractous lenses (15 diabetic and 15 non-diabetic) revealed a significant increase in both glycated lens proteins of diabetics compared with the controls (0.15 vs 0.08 nmol/mg protein, P < 0.01) and AGE-linked fluorescence at 440 nm (4.8 vs 2.8 AU/mg protein, P < 0.01). The presence of AGE fluorescence in lenses indicates the role of oxidative stress in cataractogenesis. Fifty-six days incubation of alanine and aspartate aminotransferases, used as model proteins, with 500 mM D-fructose at 25 and 37 °C led to a complete inhibition of ALT and AST activities. The fluorescence of both aminotransferases rose according to the chosen incubation temperature: 37 °C > 25 °C > 4 °C. ALT and AST incubated in a medium containing D-fructose are subject to nonenzymatic glycation followed by a consequent formation of AGE products. Our data: i) support the concept of glycation-glycoxidation pathway appearing in diabetic patients; ii) form a base for determination of the efficiency of various antioxidative compounds in vitro.

Key words: Glycoxidation; Glycation; Diabetes; Lens; AGE; Fluorescence; Protein; ALT; AST; Catalytic activity
Ophthalmology, University Hospital Hradec Králové with their written informed consent. The study was approved by the Ethical Committee of the Faculty of Medicine in Hradec Králové. The group consisted of 15 diabetics (5 men and 10 women, the median age 74 years, range 64–88) and 15 non-diabetic subjects (7 men and 8 women, the median age 72 years, range 53–89). Serum glucose, serum fructosamine (Hitachi 917, Roche Diagnostics, Mannheim, Germany), and concentrations of glycated hemoglobin (HbA1c) in erythrocytes (Variant II, Bio-Rad, Hercules, USA) were measured in all subjects immediately prior the experiment.

**Lens Analysis**

Cataractous lenses were extracted with the PHACO-Emulsifier Aspirator (Alcon Laboratories, Fort Worth, USA) and saline lens homogenates were stored at −20 °C until use. Thawed homogenates were disintegrated using a Tissue Tearor 985 (Biospec, London, UK). Ammonium sulphate (14 g) was added to 20 ml of the homogenate to precipitate proteins for 20 h at 30 °C. Centrifugation at 10,000 g for 25 min followed. The supernatant was removed and the dried sediment was reconstituted in 1.5 ml of 10 % trichloracetic acid and hydrolysed for 20 h at 5 °C. After centrifugation (the same conditions as above), the protein pellet was received. The protein was weighed out into twist-top vials. The amount of early glycation products was determined by the 2-thiobarbituric acid (TBA) method according to the protocol of Blakytny et al. (3). The absorbance was recorded at 443 nm. Glycation was determined from a standard graph obtained using D-fructose (Sigma, St. Louis, USA) standards. The values were expressed as nmol of fructose per mg protein.

The second part of the lens protein pellet was used for determination of AGE-linked fluorescence. The lens protein was digested with 20 µl of proteinase K (Roche) at a concentration of 20 mg/ml in 1 ml of 0.1 M phosphate buffer, pH 7.4 at 70 °C for 90 min. After 10 min centrifugation at 10,000 g the supernatant was used for fluorescence scanning in the Perkin-Elmer LS 50B spectrofluorometer. The excitation/emission wavelengths were set at 370 and 440 nm, respectively. Excitation and emission slits were 10 nm. The instrument was zeroed with 0.1 M phosphate buffer containing 20 µl of proteinase K only. The fluorescence was expressed in arbitrary optical units (AU) per milligram protein.

**In vitro experiment**

Purified alanine aminotransferase (ALT, EC 2.6.1.2) and aspartate aminotransferase (AST, EC 2.6.1.1) (Sigma) were incubated in the presence of 50 mM or 500 mM D-fructose for 56 days. D-fructose was chosen because of its higher glycation activity in comparison with D-glucose (31). Incubation temperatures were 4, 25 or 37 °C. After incubation, changes in catalytic activities of both enzymes were measured using the Hitachi 917 analyzer. AGE-linked fluorescence was performed as above. The appropriate enzyme in 0.1 M phosphate buffer (pH 7.4) incubated for 56 days as above was used as a control sample.

**Statistical analysis**

Because the data were not normally distributed, all measured variables in diabetic and non-diabetic subjects were compared using the Wilcoxon rank sum test. Results in tables are expressed as the medians and ranges. Differences are considered significant at the 5 % level.

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**Fig. 1:** Cataractous eyes before and after phacoemulsification. Slitlamp photo 1A shows the left eye of a woman, 73 years, suffering from mature cataract. Photo 1B demonstrates the clinical status two months after cataract surgery with the foldable intraocular lens.
Results

The laboratory data describing glycemic control parameters of diabetic and non-diabetic subjects, both suffering from the cataractous lens, are summarized in Table 1. As expected, the diabetic group has statistically higher levels of serum glucose, serum fructosamine, and HbA1c in erythrocytes than non-diabetics (P < 0.01). Analysis of lenses revealed a significant increase in both glycated lens proteins of diabetics compared with the controls (0.15 vs 0.08 nmol/mg protein, P < 0.01) and AGE-linked fluorescence at 440 nm (4.8 vs 2.8 AU/mg protein, P < 0.01). There were no associations between the AGE fluorescence and the concentration of serum glucose (r=0.23, NS), fructosamine (r=0.25, NS), HbA1c (r=0.15, NS), or lens glycation (r=-0.42, NS). Also, we did not find any relationship between the level of lens glycation and serum glucose (r=0.26, NS), fructosamine (r=0.29, NS), or HbA1c (r=-0.17, NS). Fig. 2 presents typical emission spectra of AGE products formed in the cataractous lenses.

Table 2 shows residual aminotransferase activities and AGE fluorescence in the samples after 56 days of incubation in vitro. Residual catalytic activities are related to the initial activities measured on day 0.

![Emission spectra of AGE products in diabetic (solid line) and non-diabetic (dashed line) cataractous lenses after phacoemulsification and protein extraction. Both the curves show the emission maximum at 440 nm.](image-url)

Tab. 1: Biochemical variables in subjects underwent phacoemulsification of cataractous lenses. The values are expressed as the medians and ranges.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fructose (mmol/l)</th>
<th>Incubation temperature (°C)</th>
<th>Residual catalytic activity (%)</th>
<th>AGE products (AU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>AST</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>4</td>
<td>59</td>
<td>7.5</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>4</td>
<td>30</td>
<td>114.3</td>
</tr>
<tr>
<td>3</td>
<td>500</td>
<td>4</td>
<td>25</td>
<td>120</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>25</td>
<td>55</td>
<td>159.0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>37</td>
<td>98</td>
<td>8.4</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>37</td>
<td>0</td>
<td>65.4</td>
</tr>
<tr>
<td>7</td>
<td>500</td>
<td>37</td>
<td>0</td>
<td>280.5</td>
</tr>
<tr>
<td>8</td>
<td>500</td>
<td>4</td>
<td>108</td>
<td>85.8</td>
</tr>
<tr>
<td>9</td>
<td>500</td>
<td>25</td>
<td>0</td>
<td>280.5</td>
</tr>
</tbody>
</table>

* P < 0.01; Physiological ranges: glucose 3.6–6.2 mmol/l (Hitachi 917, Roche); fructosamine 205–285 umol/l (Hitachi 917, Roche); HbA1c 4.5–6.2 % (Variant II, Bio-Rad, Hercules, USA)

Tab. 2: Experimental values determined in the aminotransferase samples after 56 days of incubation in vitro. Residual catalytic activities are related to the initial activities measured on day 0.

![Emission spectra of AGE products in diabetic (solid line) and non-diabetic (dashed line) cataractous lenses after phacoemulsification and protein extraction. Both the curves show the emission maximum at 440 nm.](image-url)
Discussion

Lens opacification is a complex phenomenon. The mechanisms underlying the apparent increased oxidative stress in diabetes are not entirely clear. Besides glycoxidation, other processes have been postulated to explain the role of diabetes in accelerating of cataract formation (polyol pathway, altered cell and glutathione redox status and ascorbic acid metabolism, perturbations in nitric oxide and prostaglandin metabolism, and lipid peroxidation) (4,17).

AGE products accumulate in the lens over many years. In our study, the level of lens fluorescence in diabetics exposed to higher concentrations of glucose was almost two-fold compared with the non-diabetic subjects (P < 0.01). This fact indicates the role of oxidative stress in cataractogenesis. On the other hand, we did not find any significant associations between AGE-linked fluorescence or the amount of glycated proteins, and other examined biochemical parameters. We explain it by good diabetic control of the patients before the operation (see Table 1). Three of them were on insulin treatment, five were treated with oral agents, and seven were on diet.

As an in vitro model of glycoxidation we have chosen aminotransferases whose inhibition of catalytic activity reflects the degree of glycation (2,11). Our results demonstrate that both ALT and AST incubated in a medium containing D-fructose, one of the most potent in vitro glycation agents, are subject to nonenzymatic glycation followed by a consequent formation of AGE products. AGE-linked fluorescence at 440 nm increased according to the used fructose concentration in the sample and to the incubation temperature. 500 mM D-fructose induced a hundredfold elevation of fluorescence in relation to the sugar-free samples at 37 °C. Our data support the concept of glycation-glycoxidation pathway appearing in diabetics.

Since our measurement of AGE products in the samples was impeded by the lack of specificity, we were not able to establish how the process of D-fructose autooxidation contributes to the AGE fluorescence during the incubation. Using more advanced analytical techniques, such as enzyme-linked immunosorbent assay (10), radiotracer measurements (24), HPLC (22), or mass spectrometry (25,32) should solve that question in the future.

A lot of substances preventing AGE formation in the body has been found and examined (aminoguanidine, ascorbic acid, tocopherol, lipoic acid, salicylic acid, indomethacin, carvedilol, captopril, superoxide dismutase mimetics, poly(ADP-ribose) polymerase inhibitors, or tenilsetam) (26–28,33). The fundamental application of our in vitro model should be to determine the efficiency of various compounds to reduce oxidation changes in proteins. We believe that in vitro models of browning reactions could be a hopeful strategy how to simply characterize new antioxidative drugs.

Acknowledgement

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References


Fig. 3: Synchronous fluorescence spectra of ALT after 56-day incubation at the presence of 50 mM or 500 mM D-fructose at 4 °C (3A) or 37 °C (3B). Excitation wavelength: 370 nm; emission wavelength: 440 nm. The intensity of fluorescence depends on the chosen incubation temperature.


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