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Lens glutathione, lens protein glycation and electrophoretic patterns of lens proteins in STZ induced diabetic rats

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As diabetes is a very complex disease, with the pathological symptoms varying with age, diabetic type and means of control, it still warrants many *in vivo* and *in vitro* studies. During hyperglycaemia, increases in the sorbitol pathway, nonenzymatic glycosylation of lens proteins and damage to antioxidant systems have been reported to cause opacification of the lens leading to cataract formation. In this study, intracapsular extracts of lenses from STZ induced diabetic female rats were examined. Total protein, glutathione and nonenzymatic glycosylation were determined by the Lowry, Ellman reagent and thiobarbituric acid methods respectively. Laemmli protein electrophoresis was also carried out on the lens homogenates. After a period of as short as 5 weeks, a decrease in lens glutathione, and an increase in nonenzymatic glycosylation of lens proteins were found. The electrophoresis showed an increase in proteins of high molecular weight.

Keywords: lens, diabetes, glutathione, nonenzymatic glycosylation, electrophoresis

Introduction

The lens, which is behind the iris, refracts light entering the eye through the pupil, thus focusing it on the retina. The perfect physiochemical balance of the lens proteins gives it transparency. Any alteration in the optical homogeneity of the lens or decrease in its transparency is known as a cataract [1, 2]. There is an increased incidence of cataract formation in diabetic patients [1, 3]. Since glucose from both the aqueous and vitreous humor diffuses into the lens uncontrolled by the hormone insulin, the lens is one of the body parts most affected in diabetes mellitus. It has been reported that hyperglycaemia promotes the conversion of glucose to sorbitol and that the intracellular accumulation of sorbitol in the lens causes osmotic swelling of the lens fibres and cataract formation [1, 2]. Glutathione is one of the antioxidant factors which protect the lens. A decrease in reduced glutathione during hyperglycaemia causes oxidation of protein sulphydryl groups resulting in alterations in protein linkages, solubility and transparency, and also in free radical induced damage to the lens [1, 4–6].

Nonenzymatic glycosylation of lens proteins increases during hyperglycaemia. This results in protein functional and conformational changes causing opacification of lens proteins [1, 7–10].

This study investigates changes in lens glutathione and protein glycation levels, and in the electrophoretic patterns of lens proteins, in STZ induced acute diabetic rats.

Materials and methods

Fourteen female Wistar Albino rats were rendered diabetic by one intraperitonal injection of 75 mg kg^{-1} STZ (streptozotocin) in a citrate buffer (pH = 4.5). The other eleven rats were used as a nondiabetic control group. All rats were 8 weeks old. Five weeks after STZ injection, samples of cardiac blood were taken from all rats under ether anaesthesia. The rats were then killed by giving them excessive ether and intracapsular extracts were made from their lenses. Blood glucose and glycosylated haemoglobin were determined in the blood samples using a Boehringer Mannheim Reflolux S Glucometer with Haemo-glucotest 20-800R strips and a Sigma Kit (Catalogue No. 440-A) respectively. The left and right

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lenses of each rat were homogenized together in 2 ml of physiological saline and kept in a deep freeze. The total protein, nonenzymatic glycosylation and glutathione levels of the lens homogenates were determined and protein electrophoresis carried out on them.

Lens total protein levels were measured by the method of Lowry [11] and lens protein nonenzymatic glycosylation by the 2-thiobarbituric acid method [12]. The latter involved hydrolysing each 0.5 ml homogenate with 0.5 ml 0.5 M oxalic acid in an autoclave for 1 h at 124 ± 1 °C. To this 0.5 ml 40% trichloracetic acid (w/v) was added, mixed, centrifuged at 1500 × **g** for 10 min, and filtered using filter paper. Its absorbance at 443 nm was recorded. Then, 0.75 ml of supernatant was incubated in 0.25 ml 0.05 M 2-thiobarbutiric acid at 37 °C for 30 min. After standing for 15 min at room temperature, its absorbance was again measured at 443 nm and the differences between the first and second absorbances calculated. The nonenzymatic glycosylation values were expressed as nmol of fructose per mg protein. Commercial fructose (Sigma) was used as a standard.

Lens glutathione levels were determined by using Ellman's reagent [13]. Here a precipitation solution, containing metaphosphoric acid, EDTA and NaCl was added to each 0.2 ml of homogenate. This was mixed with a vortex mixer and centrifuged at 1500 × **g** for 10 min. Then, 0.3 M Na_2HP0_4 was added to an aliquot of the supernatant followed by Ellman's reagent, 0.04% 5,5' dithio-bis 2(nitrobenzoic acid), in 1% sodium citrate. After standing for 5 min at room temperature, the absorbance at 412 nm was recorded. Reduced glutathione (Sigma) was used as a standard. The concentration of lens glutathione was expressed as nmol of glutathione per mg protein.

Electrophoretic examination of lens proteins was carried out by SDS-polyacrylamide gel electrophoresis as described by Laemmli [14]. Schleicher and Schueller Profile System mini electrophorosis was performed and Sigma low molecular weight protein standards (SDS-7, Dalton Mark VII-L) were used. After electrophoresis, scans of Coomassie blue stained protein bands were obtained using a densitometer (Helena Laboratories TCL plus). Peak areas were measured with a planimeter (Placom-Sokkisha, Kp-80N, digital) and the protein percentage calculated in each band.

The results were evaluated using an unpaired *t*-test and regression analysis using the NCSS statistical computer package.

Results

Prior to inducing diabetes the groups were checked for differences in weight and blood glucose but none were found (Table 1). At the end of the 5 weeks the diabetic rats lost weight, the control rats maintained their weight, and in the diabetic group blood glucose and glycosylated haemoglobin were significantly higher than in the control group (Table 1).

Total lens protein was higher in the diabetic group than control (Table 2). Nonenzymatic glycosylation of the lens proteins was also significantly higher in the diabetic group, but the lens glutathione levels were significantly lower than in the controls (Table 2).

Table 1. Mean levels of blood glucose, glycosylated haemoglobin and body weights for control and diabetic groups.

	Control group (n = *11*)		*Diabetic group* (n = *14*)		
	Mean	*SD*	*Mean*	*SD*	p *value*[b]
Blood glucose ($mmol\,L^{-1}$) (Before STZ)	8.85	1.06	8.20	1.01	0.1
Blood glucose ($mmol\,L^{-1}$) (After 5 weeks)	9.27	1.30	24.50	9.99	0.0001
Glycosylated Haemoglobin (%)	4.46	0.97	5.57	0.97	0.009
Weights (g) (Before STZ)	169.6	18.0	171.4	19.4	0.8
Weights (g) (After 5 weeks)	173.2	13.8	151.7	22.7	
p (for weights)[a]	0.6		0.02		

[a] 'before' vs 'after'.
[b] 'control' vs 'diabetic'.
SD, standard deviation.
In Tables 1–4

Table 2. Mean levels of lens total protein, lens glutathione and nonenzymatic glycosylation of lens proteins in control and diabetic groups.

	Control group (n = 11)		*Diabetic group (n = 14)*		
	Mean	*SD*	*Mean*	*SD*	p *value*
Total protein ($mg\,dl^{-1}$)	49.7	5.7	54.4	6.6	0.08
Glutathione (nmol GSH per 1 mg protein)	2.81	0.85	1.56	0.61	0.0001
NEG of lens proteins (nmol fructose per 1 mg protein)	1.25	0.35	3.61	1.88	0.0001

GSH, glutathione; NEG, nonenzymatic glycosylation.

From the SDS polyacrylamide gel electrophoresis of the lens proteins, 19 different protein bands (numbered in order of decreasing molecular weight) were observed, and each band was in the same position for every sample (Figs 1 and 2). The molecular weights of the protein bands were between 14 and 108 kDa (Table 3). The molecular weight of band 1 was estimated as 108 kDa; however, this was only an approximate value extrapolated from the range of protein standards used. In the diabetic group, the intensity of staining of bands 1, 7, 8, 9 and 11 was significantly increased compared with their respective controls. This change was the largest for bands 1, 7 and 8 (108, 42 and 39 kDa). Significant decreases occurred in the staining of bands 3, 16, 18 and 19 in the

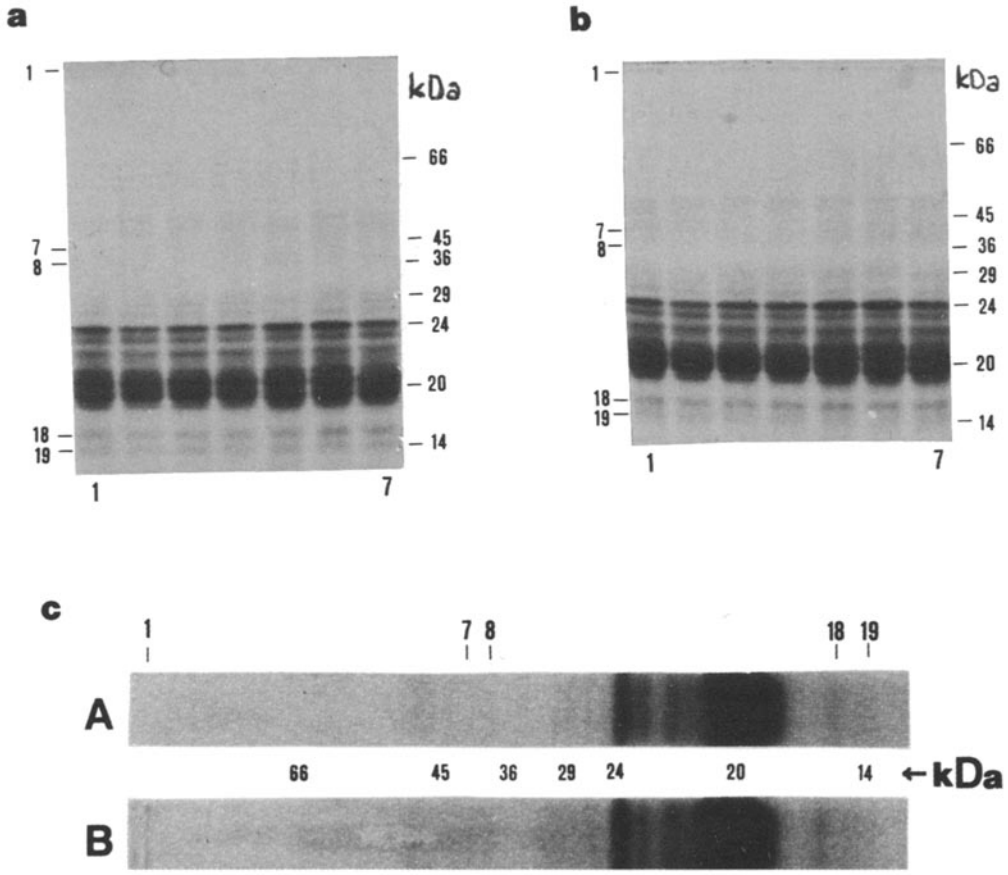

Figure 1. Typical SDS-polyacrylamide gel electrophoretic patterns of lens proteins: (a) control group; (b) diabetic group; (c) enlargement of typical tracks; A, control and B, diabetic.

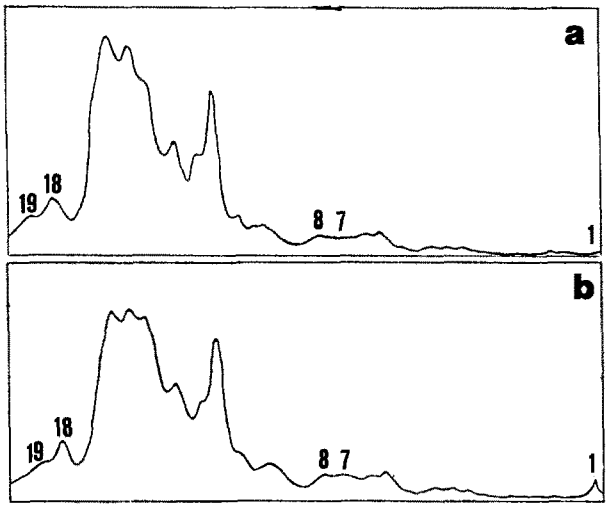

Figure 2. Typical SDS-PAGE densitograms of lens proteins: (a) control; (b) diabetic.

diabetic group, with the decreases in bands 18 and 19 being more marked (Table 4). No significant correlations were found between any of the other parameters.

Discussion

The lens is one of the areas most affected during hyperglycaemia in diabetes. The prevalence of cataract formation and blindness in diabetic patients is higher than normal. Since the factors leading to cataract formation are multiple, studies have generally focused on the changes and their prevention in lens metabolism, during cataractogenesis.

Glutathione is one of the antioxidant factors which have been investigated. Glutathione decrease can be prevented in the presence of aldose reductase inhibitors [15]. Although it has been reported that osmotic stress is not itself responsible for cataract formation, glutathione can prevent or diminish the severity of sugar cataractogenesis [16]. It has been suggested that the accumulation of sorbitol may be a basic mechanism underlying diabetic complications leading to cataract formation [17]. Oxidative risk factors such as reduced and oxidized glutathione, malondialdehyde, vitamin A, and glucose-6-phosphate dehydrogenase have been studied and found to play a possible role in the pathogenesis of cataracts in diabetics [18]. No significant difference has been found in lens glutathione levels between senile cataract patients, with or without diabetes [19]. However, the anticancer agent, adriamycin, given to rats on a riboflavin free diet, has been found to accelerate glutathione decrease [20].

In the present study, the only antioxidant factor studied was glutathione. Lens glutathione was found to decrease by 50% in the diabetic rats in 5 weeks. This is consistent with some of the above mentioned studies.

Table 3. Molecular weights (MW, kDa) of protein bands obtained by Laemmli SDS-polyacrylamide gel electrophoresis.

Band No.	*MW*	*Band No.*	*MW*	*Band No.*	*MW*	*Band No.*	*MW*
1	108[a]	6	47	11	27	16	20
2	66	7	42	12	25	17	18
3	62	8	39	13	24	18	15
4	59	9	31	14	22	19	14
5	49	10	29	15	21		

[a]Obtained by extrapolation.

Table 4. Comparison of diabetic and control group protein bands (%) which differed significantly.

	Control group (n = *11*)		*Diabetic group* (n =*14*)		
Band No.	*Mean*	*SD*	*Mean*	*SD*	p *value*
1	0.09	0.31	0.72	0.28	0.0001
3	0.55	0.19	0.36	0.22	0.03
7	0.10	0.01	1.3	0.56	0.0001
8	0.11	0.01	1.3	0.61	0.0001
9	1.3	0.4	1.9	0.66	0.02
11	2.2	0.46	2.6	0.46	0.02
16	18.5	3.6	15.6	2.42	0.025
18	6.7	1.1	5.6	0.42	0.006
19	5.7	1.6	3.6	0.54	0.0001

Also investigated in this study was the nonenzymatic glycosylation of lens proteins, which is considered to be one of the factors leading to cataract formation in diabetics [21] and the aged. Lens protein glycosylation occurs on exposure to high glucose levels [1]. *In vitro* studies have reported that the number of disulfide bonds increased in lens crystallins when they were incubated with glucose, and also after long incubation in glucose, nonenzymatic browning was seen similar to that observed in the aged lenses [1, 22]. Glycosylation has been reported as being time and concentration dependent, and hyperglycaemia-accelerated nonenzymatic-glycosylation of lens proteins from diabetic senile cataractous patients was higher than in non-diabetic senile cataractous patients [22]. An investigation of the relationship between senile cataracts and glucose tolerance showed glucose intolerance to be associated with senile cataract formation, suggesting a possible independent role of nonenzymatic glycosylation in cataract pathogenesis [23]. Many studies have reported nonenzymatic glycosylation as playing a role in the conformational change of lens proteins [1, 22, 24, 25]. Nonenzymatic glycosylation of lens proteins by glucosamine has also been studied, in which glucosamine was found to bind to B crystallins [26]. The findings of Patrick *et al*. [27] are in conflict with other studies, in that they found that nonenzymatic glycosylation of proteins did not increase with age. This suggests that nonenzymatic glycosylation is not an age-dependent chemical modification of long-lived proteins but that it reaches a plateau with the passage of time [27].

In this study, the TBA method was used to measure nonenzymatic glycosylation in lens homogenates. We found a three-fold increase in nonenzymatic glycosylation levels of diabetic rat lenses. These results are consistent with the above studies.

In cataract pathogenesis, lens proteins are modified by glycosylation, ageing, heat, pH changes, radiation, free radicals and several chemical substances [1–2]. Modified proteins lose their biological activity, no longer perform their metabolic role and cause inflammation, necrosis or autoimmunity [28].

In the present study, 19 different protein bands were detected using Laemmli SDS-polyacrylamid gel electrophoresis. The significant increase in levels of the higher molecular weight protein bands and the significant decrease in the levels of the lower molecular weight protein bands may be attributed to advanced glycation end product formation [29]. Again these results are consistent with other studies.

In summary, uncontrolled induced diabetes after periods of as short as 5 weeks was found to affect lenses causing a decrease in lens glutathione and an increase in nonenzymatic glycosylation of lens proteins.

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