Indian Journal of Experimental Biology
Vol. 49, October 2011, pp. 749-755

Insulin resistance mediated biochemical alterations in eye lens of neonatal streptozotocin-induced diabetic rat

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Received 12 January 2011; revised 29 June 2011

Cataract, the leading cause of blindness worldwide, is associated with many risk factors including diabetes. Impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) states are associated with pre-diabetes and insulin resistance. This condition subsequently leads to the development of type-2 diabetes. Epidemiological studies indicated that not only diabetes but IGT/IFG will also lead to the development of microvascular disorders and cataract. However, there are no studies on the mechanism of insulin resistance induced changes in the eye lens. In the present study, IGT/IFG-induced changes in lens using neonatal-streptozotocin (nSTZ) rat model have been investigated. Though, nSTZ rats showed the signs of IGT and insulin resistance starting from two months old, they did not develop cataract even at the age of 8-months. However, biochemical analysis indicates a three-fold increase in sorbitol levels in nSTZ lens upon prolonged (6-months) IGT and insulin resistance. Also there was an increase in lipid peroxidation and alterations in antioxidant enzymes. Results of this study showed that activation of polyol pathway and increased oxidative stress, commonly associated with long-term complications of diabetes, have been observed in eye lens due to prolonged IGT and insulin resistance which may lead to cataract.

Keywords: Animal models, Cataract, Diabetes, Eye lens, Insulin resistance

Cataract, the opacification of the eye lens, is a major cause of blindness worldwide[1]. Although advances in surgical removal of cataracts have made treatment very effective, no method to halt the formation of a cataract has been shown to be effective. Nevertheless, cataract continues to be a serious public-health problem due to increase in population, and extended life expectancy. Several risk factors have been identified and diabetes mellitus is associated with a 5-fold higher prevalence of cataracts[2,3]. A number of studies have demonstrated the association between diabetes and cataract in different populations. Globally, the number of people with diabetes is projected to rise from 171 million in 2000 to 330 million by the year 2030[4,5]. The long-term complications of diabetes such as blindness due to cataract remain serious problem.

Insulin resistance or pre-diabetes most often precedes the onset of type 2 diabetes (T2D) by many years, is present in a large segment of the general population[6]. Earlier studies indicate that approximately 25% of non-diabetic individuals exhibit insulin resistance within the range of that observed in patients with T2D[7,8]. Deterioration into impaired glucose tolerance (IGT) occur when either insulin resistance increases or the insulin secretary responses decrease, or both. IGT or impaired fasting glucose (IFG) serves as a marker for the state of insulin resistance. In general, prevalence of IGT varies from 4-21%[9-12.] In India, the prevalence of IGT is reported to vary from 9.9% to 16.3%[13,14].

Prolonged exposure to chronic hyperglycemia in diabetes can lead to various complications, both of macro and microvascular nature, like cardiovascular, renal, neurological and ocular[15,16]. Microvascular diseases typically associated with diabetes are also observed in individuals with IGT/IFG[17-19] and at the time of diabetes diagnosis[20,21]. Similarly, association between diabetes and various types of cataracts has been established[2,3]. Studies also suggest that glucose intolerance is common in patients with senile cataract who showed no glycosuria and had a normal fasting blood sugar on routine examination[22,23] and people with IFG have increased risk of cortical cataract[24]. However, there are no studies on the effect of insulin resistance or pre-diabetes on biochemical alterations in the eye lens vis-a-vis development of cataract. Neonatal streptozotocin (nSTZ) induced diabetic rat

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model is very commonly employed for studies on insulin resistance and T2D[25,26]. This animal model is not only useful for screening hypoglycemic or antidiabetic agents[27,28] but also to study the complications like ischemia/cardiomyopathy[29,30]. Therefore, in the present study nSTZ rat model has been used to evaluate biochemical changes in eye lens associated with insulin resistance.

Materials and Methods

Materials—Streptozotocin (STZ), NADPH, NADH, 2-thiobarbituric acid (TBA), 1,1,3, 3-tetraethoxy propane (TEP), DL-glyceraldehyde, lithium sulfate, β-mercaptoethanol, glutathione, glutathione reductase, bovine serum albumin, sorbitol and sorbitol dehydrogenase were purchased from Sigma Chemical Company (St Louis, USA). All other chemicals are of analytical grade and were obtained from local companies.

Neonatal streptozotocin (nSTZ) induced insulin resistance—Two-day old male Wistar-NIN (WNIN) rat pups (16) obtained from the National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India were injected i.p 90 mg/kg body weight with STZ dissolved in 0.1*M* citrate buffer, *p*H 4.5. Control pups (8) received only vehicle. Two out of 16 pups died within 2-3 days of STZ injection. These pups were weaned after 21 days and maintained on stock colony diet in individual cages throughout the experimental period. During this period fasting blood glucose levels were monitored and the experiment was continued for a period of 8 months.

Animal care—Animal care and protocols were in accordance with and approved by the Institutional Animal Ethics Committee (IAEC). Animals were housed in individual cages in a temperature and humidity controlled room with a 12 h L:D cycle. All these animals had free access to water. Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research were followed.

Oral glucose tolerance test—Oral glucose tolerance test (OGTT) was performed at the age of 2 and 4 months on 14 h of fasted rats by administering glucose orally as a bolus, at a dose of 2.5 g/kg body wt. Blood samples were collected from orbital sinus flux at 0, 30, 60, and 120 min for determining plasma glucose and insulin concentrations.

Homeostasis model assessment (HOMA)—Insulin resistance was assessed by homeostasis model assessment (HOMA)-IR as described earlier for rats[31,32] using the following equation: HOMA-IR = [fasting plasma glucose (mg/dl) × fasting plasma insulin (μU/ml)]/2,430. To assess the animals insulin response to a challenge of glucose, the area under the curve (AUC) for insulin and glucose during the OGTT was computed. Rats having OGTT positive were included in this study and maintained up to 8 months to study the impact of IR on eye lens.

Lens examination by slit-lamp biomicroscope—Eyes were examined every week using a slit lamp biomicroscope (Kowa Portable, Japan) on dilated pupils to observe the morphological changes in the lens.

Clinical parameters—Plasma glucose was measured by the glucose oxidase-peroxidase (GOD-POD) method with a commercially available kit (Ozone Biomedicals Pvt. Ltd., New Delhi, India) and plasma insulin by an RIA kit (BRIT-DAE, Mumbai, India).

Lens processing—At the end of 8-months, the animals were euthanized by CO_2 asphyxiation and the lenses were dissected out by the posterior approach and stored at -80°C until further analysis. Lens homogenate (10%) was prepared from 3-5 pooled lenses in a 50 m*M* sodium phosphate buffer, *p*H 7.4. All the biochemical parameters were analyzed in the soluble fraction of the lens homogenate (15,000 × *g* at 4°C) except for lens sorbitol and malondialdehyde (MDA) which were determined in the total homogenate.

Biochemical estimations—Lens MDA was measured as thiobarbituric acid reacting substances (TBARS) as described previously[33,34]. Activity of aldose reductase (AR) and sorbitol levels in the lens was estimated as reported earlier[34]. Specific activity of antioxidant enzymes like, superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glucose-6-phosphate dehydrosenase (G6PD) was assayed as described previously[33,34].

Protein estimation—Protein content of lens tissue (total and soluble) was estimated by the method of Lowry using BSA as the standard.

Fluorescence—Tryptophan fluorescence and advanced glycation end product (AGE) fluorescence was measured in soluble protein (150 μg/ml in 0.05 *M* sodium phosphate buffer, *p*H 7.4). Tryptophan fluorescence spectra were obtained by excitation at 295 nm and emission between 310-400 nm. AGE-

fluorescence spectra were obtained from 400-500 nm with excitation at 370 nm in a Spectrofluorometer (Jasco FP-6500).

Heat-induced aggregation of crystallins—Total lens soluble protein (1.0 mg/ml) in 50 m*M* phosphate buffer, *p*H 7.2 was heated at 65°C using a circulating water bath[35]. Light scattering due to aggregation was measured at 360 nm in a UV/Vis Spectrophotometer (Perkin Elmer *Lambda 35).*

Statistical analysis—One-way ANOVA was used for testing statistical significance between groups of data, and individual pair difference was tested by means of Duncan's multiple-range test. Heterogeneity of variance was tested by the nonparametric Mann-Whitney test. $P< 0.05$ was considered significant.

Results

Food intake and body weights—There was no difference in food intake and body weights of rats between the two groups throughout the experimental period (data not shown).

Glucose—Fasting glucose levels of control and nSTZ group animals are presented in Table 1. Although, the nSTZ rats showed mild hyperglycemia as evidenced by a marginal increase in blood glucose at the age of 4 months compared to control group, the glucose levels were gradually reduced and were comparable between control and nSTZ rats at the end of 8-months of age (Table 1).

Insulin resistance—Degree of insulin resistance was calculated in both control and nSTZ group at the age of 2 and 4 months by HOMA-IR. During OGTT, glucose and insulin response indicated that there was increased impaired glucose intolerance in nSTZ group compared to control animals at both 2 (data not shown) and 4 months (Fig. 1). Interestingly, the AUC for glucose, AUC for insulin, and HOMA-IR index were significantly ($P<0.01$) higher in nSTZ rats compared to control rats (Table 2) indicating that the nSTZ group animals have developed insulin resistance.

Slit-lamp observation—Lenses were examined for opacity from the age of 2 months till the end of the experiment. During this period no morphological changes (lens opacification) were observed in the lenses of both the groups.

Lens weight and protein content—There was no significant difference in lens weight and protein (total and soluble) content between the groups. However, there was a decrease in the percentage of soluble protein in the nSTZ group (Table 3).

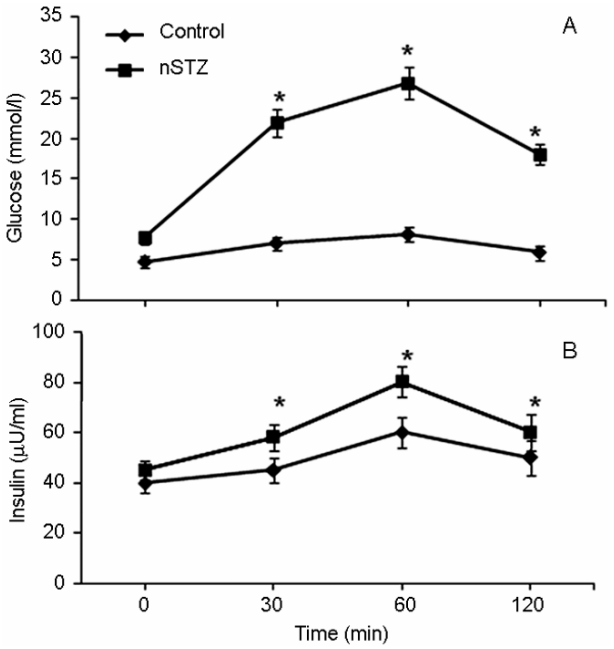

Fig.1—Glucose (A) and Insulin (B) response during OGTT at the age of 4 months. [Values are mean ± SD of 6-14 animals/ group; $^{*}P<0.01$ as compared to control group]

Table 2—Area under curve for glucose, insulin during OGTT and HOMA-IR
[Values are mean ± SD of 6-14 animals/group]

Parameter	Control	nSTZ
AUC/h Glucose	11.04±3.06	33.43±5.55*
AUC/h Insulin	46.95±10.02	69.75±7.43*
HOMA-IR	1.42±0.23	2.22±0.34*

*$P<0.01$ as compared to control group

Table 1—Fasting blood glucose levels (mg/dL) during the experiment
[Values are mean ± SD of 6-14 animals/group]

	Duration (months)				
Groups	I	II	IV	VI	VIII
Control	97.59±8.65	82.17±7.35	86.67±11.68	66.5±3.54	74.1±1.14
nSTZ	107±40.72	92±7.06	120±23.48#	106±12.5#	91±13.17

#$P<0.05$ as compared to control group

Polyol pathway—During diabetes, activation of a polyol pathway has been linked to several pathological changes in insulin-insensitive tissues such as blood vessels, peripheral nerves, renal medulla and ocular lens. Aldose reductase (AR), a key enzyme of polyol pathway, converts excess glucose to sorbitol which is associated with many secondary complications including cataract and retinopathy. In the present study, activity of AR was not significantly different in the lens of nSTZ rats compared to control rats (Fig. 2A). However, a significant increase in sorbitol formation was observed in nSTZ rat lens (Fig. 2B) which indicates insulin resistance/IGT state leads to accumulation of intracellular sorbitol.

Oxidative stress and antioxidant enzyme status—MDA, an end product of lipid peroxidation, is an index of oxidative stress in many diseased/stress conditions. There was a marginal increase in MDA in nSTZ group lens compared to control group indicating increased oxidative stress in the lens of pre-diabetic rats. Similarly, activity of SOD, an antioxidant enzyme which is essential for the free radical scavenging is significantly increased and GST activity was decreased in the lens of nSTZ rats compared to control rats. This further substantiated the oxidative stress due to insulin resistance. However, there was no change in the specific activity of GPx and G-6-PD in both the groups (Table 4).

Tryptophan and advanced glycation end product (AGE) fluorescence—Tryptophan fluorescence spectra (Fig. 3A) showed a marginal decrease in nSTZ compared to control indicating alterations in protein microenvironment and possibly protein oxidation due to insulin resistance. There was a marginal increase in AGE fluorescence (Fig. 3B) in nSTZ group compared to control indicating glycation of lens proteins due to impaired glucose metabolism for a long period.

Heat-induced aggregation—The implicit assumption is that the turbidity of proteins which develop in the test tube due to a variety of treatments such as oxidation, UV irradiation, elevated temperatures and glycation in a few minutes to few

Table 3—Lens weight and protein content
[Values are mean ± SD of 6-14 animals/group]

Parameter	Control lens	nSTZ lens
Lens weight (mg)	50.6±5.7	51.6±1.3
Total protein (mg/g lens)	531±7.0	560±24
Soluble protein (mg/g lens)	357±14	352±15
% Soluble protein	67.32	62.78

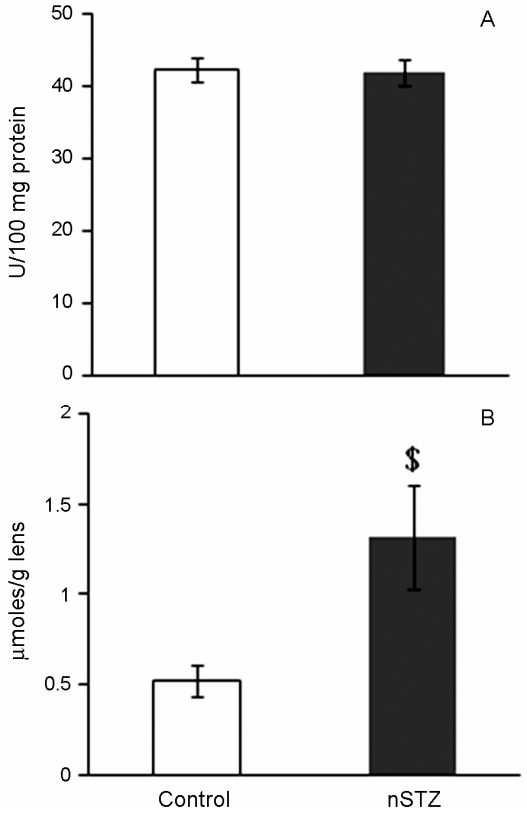

Fig. 2—Aldose reductase activity (A) and sorbitol levels (B) in the lens. AR activity was expressed as µmoles NADPH oxidized/h/100 mg protein and sorbitol was expressed as µmoles/g lens. [Values are mean ± SD of 6-14 animals/group, $^{\$}P<0.001$ as compared to control group]

Table 4—Oxidative stress and activities of antioxidant enzyme in the lens
[Values are mean ± SD of 6-14 animals/ group]

Parameter	Control lens	nSTZ lens
MDA (nmol/g lens)	10.65±0.27	12.24±0.83
SOD (units/100mg protein)	33.60±2.32	44.37±4.81*
GPx (µmoles of NADPH oxidized/h/100 mg protein)	19.56±1.61	20.16±2.11
GST (µmoles of CDNB-GSH formed /h/100 mg protein)	30.70±2.135	27.43±2.38
G6PD (µmoles of NADPH oxidized/h/100 mg protein)	11.61±0.64	12.52±0.97

*$P<0.01$ as compared to control group

hours, represents an accelerated version of the situation which might arise in the lenses over greater periods of time due to the same conditions. Therefore, susceptibility of total soluble proteins (TSP) consisting of major lens crystallins to heat-induced aggregation was studied by light scattering methods. The extent of aggregation between the groups was compared by using same amount of protein from both the groups. Lens TSP from nSTZ group showed significantly higher aggregation at 65°C compared to control group (Fig. 4) indicating insulin resistance exposed lens proteins are more susceptible to various insults.

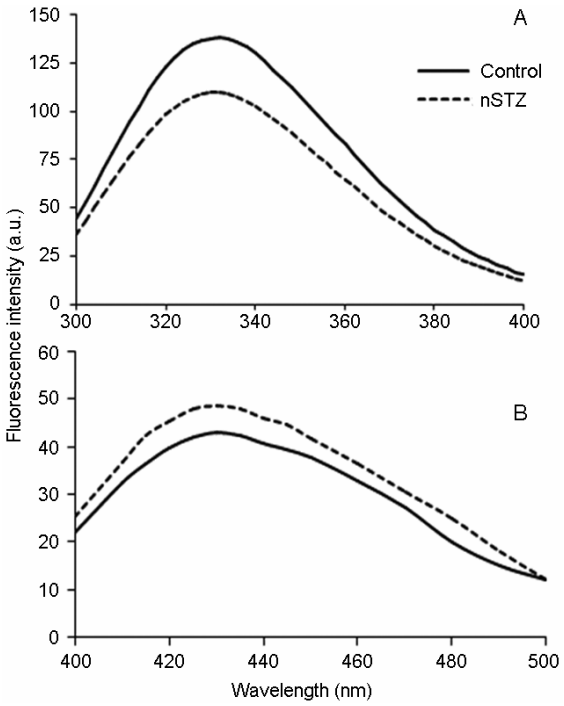

Fig. 3—Tryptophan (A) and AGE (B) fluorescence of lens total soluble proteins. [Values are mean ± SD of 6-14 animals/group]

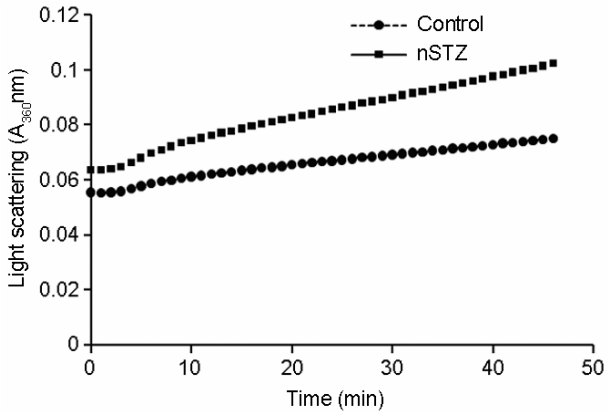

Fig. 4—Heat induced aggregation of lens total soluble proteins. [Values are mean ± SD of 6-14 animals/group]

Discussion

Although, vascular and other complications including cataract have been reported under pre-diabetic conditions, biochemical and molecular basis for complications is not completely understood. Further, development of lens opacity or lens abnormalities under experimental conditions has not been reported. nSTZ-induced diabetes is a well-established model to study insulin resistance and T2D. Therefore, this model has been used to understand the lens abnormalities as well as lens opacity if insulin resistance is maintained for a longer period.

As expected, nSTZ rats developed insulin resistance after 2 months and continued for rest of the experimental period. Though, some studies reported that rats develop hyperglycemia at 2-4 months after injection of STZ to 2-day old pups[36-38], in this study no overt hyperglycemia was observed in WNIN strain rats even at 8 months which is in agreement with other studies[39-41]. No lens opacity was observed in WNIN rats which are maintained on insulin resistance for 8 months. These results indicate that the duration and degree of insulin resistance may not be sufficient to cause lens opacification. However, various biochemical alterations that are commonly associated with cataract development and progression were investigated, such as oxidative stress/antioxidant system, polyol pathway, protein oxidation and glycation.

In general there is a decrease of protein (both soluble and insoluble) in various types of cataractous lens due to the leakage of these proteins into aqueous humor[33,34,42]. Although, no changes either in lens weight or protein content (total protein) were observed, there was decline of soluble protein in nSTZ group indicating initiation of protein insolubilization. Activation of polyol pathway, non-enzymatic glycation and oxidative stress are the major pathways implicated in various long term complications of diabetes. Previously, osmotic[43,45,47] and oxidative stress[44-47] was reported in the diabetic precataractous lenses but not in pre-diabetic lenses. Increased osmotic stress in these conditions could be mainly due to accumulation of excess sugar alcohol through activation of polyol pathway. In the present study there was an accumulation of excessive sorbitol in the lens of nSTZ rats indicating osmotic stress due to activation of sorbitol pathway. However, 3 fold increase of sorbitol may not be sufficient to induce lens opacification but this could trigger the development of cataract. These results are similar to

some of the observations made in diabetes-induced pre-cataractous lenses[45-47].

Oxidative stress due to activation of polyol pathway and non-enzymatic glycation was known to contribute to the development of many age related complications including diabetic cataract. Activation of polyol pathway in the diabetic cataract lenses was mainly attributed to hyperglycemia in diabetic conditions. However, in the present study, accumulation of intracellular sorbitol in pre-diabetic lens indicated that activation of polyol pathway is initiated even in pre-diabetic conditions. Several epidemiological surveys have reported a correlation between oxidative stress and insulin resistance[48-50]. In the present study, there was an increased oxidative stress in insulin resistance lens as indicated by enhanced lipid peroxidation levels along with alterations in activities of some antioxidant enzymes. Similar results were observed in precataractous lens of high fructose fed[51] or STZ-treated rats[45-47].

In conclusion, insulin resistance or pre-diabetic condition activates some of the key pathways like polyol pathway and oxidative stress in the lens which are commonly associated with long-term complications of diabetes. Therefore, nSTZ rat model may help to study insulin resistance induced changes in lens biochemistry to understand the mechanism of lens opacity associated with insulin resistance.

Acknowledgement

Part of this work is supported by the Department of Science and Technology (DST) and Indian Council of Medical Research (ICMR), Govt. of India. Authors thank Dr. N. Balakrishna (Statistics Division, National Institute of Nutrition) for his help in statistical analysis.

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