

Benefits of early glycaemic control by insulin on sensory neuropathy and cataract in diabetic rats

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While there is an emphasis on the early glycaemic control for its long-term benefits in preventing microvascular complications of diabetes, the biochemical mechanisms responsible for the long-lasting effects are not clearly understood. Therefore the impact of early insulin (EI) versus late insulin (LI) treatment on diabetic sensory neuropathy and cataract in streptozotocin-induced diabetic Wistar male rats were evaluated. EI group received insulin (2.5 IU/animal, once daily) treatment from day 1 to 90 while LI group received insulin from day 60 to 90. Early insulin treatment significantly reduced the biochemical markers like glucose, triglyceride, glycated hemoglobin, thiobarbituric acid reactive substances, advanced glycation end products and ratio of reduced glutathione and oxidized glutathione in diabetic rats. The late insulin treatment failed to resist the biochemical changes in diabetic rats. Diabetic rats developed sensory neuropathy as evidenced by mechanical and thermal hyperalgesia and showed a higher incidence and severity of cataract as revealed by slit lamp examination. Early insulin treatment protected the rats from the development of neuropathy and cataract, but late insulin administration failed to do so. The results demonstrate the benefits of early glycaemic control in preventing neuropathy and cataract development in diabetic rats.

Keywords: Advanced glycation end products, Hyperglycemic memory, Lipid peroxidation, Microvascular complications

Benefits of intensive glycaemic control and the impact of earlier intervention to prevent diabetic complications have been shown¹. The ever increasing armamentarium of antidiabetic medications in the management of hyperglycaemia includes a) insulin secretagogues, b) insulin sensitizers, c) inhibitors of intestinal carbohydrate metabolism and d) agents targeting the entero-insular axis and the incretins². Despite the availability of these clinically effective therapies for diabetes, achieving good glycaemic control in patients with diabetes is not only a tough task but the consequences of poor glycaemic control are reflected in progression of secondary diabetic complications like diabetic neuropathy, retinopathy, nephropathy and cardiovascular problems³.

The diabetes control and complications trial (DCCT) and the results of the epidemiology of

diabetes interventions and complications (EDIC) study convincingly demonstrated that the deleterious effects of hyperglycemia persist for a considerable period, even after glucose levels has been normalized⁴. These studies also emphasize that the early glycaemic control has long-lasting effects in preventing the microvascular complications of diabetes^{4,5}. In the DCCT-EDIC study, the persistent long-lasting effects of insulin therapy on retardation of diabetic complications were found to be originated from reduced advanced glycation end (AGE) products and oxidative stress at the cellular and systemic levels^{5,6}. While animal models of diabetes are ideal to test the long-lasting effects of early glycaemic control, there is lack of such studies in relation to diabetic neuropathy and cataract development. Therefore, in the present study, the effects of early insulin versus late insulin treatment have been investigated in streptozotocin-induced diabetic rats on two specific conditions of sensory neuropathy and cataract development along with markers of AGEs and oxidative stress.

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Materials and Methods

Animals—Male Wistar rats aged 10-12 weeks (body weight, 200-250 g) were obtained from the King Institute (Chennai) and maintained at 22 ± 1 °C under a 12:12 h light-dark cycle. Normal pellet diet (Nutrilab, Bangalore) and water were given *ad libitum*. All experiments were performed in accordance with regulations specified by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) and the project was approved by the Institutional Animal Ethics Committee (IAEC) of the Madras Diabetes Research Foundation, Chennai.

Experimental design—Diabetes was induced in rats by ip injection of a freshly prepared solution of streptozotocin (STZ, Sigma-Aldrich, MO, USA) in a dose of 50 mg/kg⁷. Citrate buffer was given to control animals (n=6). Diabetic rats with plasma glucose between 300 and 500 mg/dL, 3 days after STZ injection, were selected and randomized into following three different groups of 6 each: diabetic rats without intervention, diabetic rats under early insulin (EI) treatment and diabetic rats under late insulin (LI) treatment. 'One shot' Insultard (Novonordisk, Bangalore) which is a long-acting insulin was used. EI group was given insulin 2.5 IU/day/animal from day 1 to 90 while LI group received the same dose of insulin only between days 60 to 90. The insulin injection was standardized at a dose of 2.5 IU/day that resulted in a significant reduction of nonfasting hyperglycemia (from 338 ± 34 to 175 ± 19 mg/dL) without hypoglycemia in the STZ-diabetic animals. Considering the long-acting nature of the insulin given, the status of good glycemic control was checked 8 h after the insulin administration for every fortnight throughout the study i.e., 6 instances in early insulin (EI) group and 2 instances in late insulin (LI) group. Sham injection with normal saline was administered to control rats and untreated diabetic rats. To prevent weight loss and death due to keto acidosis, 0.2 IU insulin/animal was given weekly thrice to diabetic and LI groups. At the end of the study, rats were sacrificed by CO₂ asphyxiation and body fluids/tissues stored immediately at -80 °C.

Biochemical estimations—Animals fasted for 12 h from each group were bled by retro orbital plexus puncture and approximately 0.4 mL of blood was collected in 10% EDTA tubes. Plasma was separated upon centrifugation at 3000 rpm for 15 min and stored

at -80 °C until the assays performed. Biochemical analyses were carried out on a Cobas Integra 400 plus auto analyzer (Roche Diagnostics Mannheim, Germany). Fasting plasma glucose by glucose oxidase-peroxidase (GOD-POD) method and triglycerides by glycerol phosphate oxidase-peroxidase-amidopyrine (GPO-PAP) method were estimated. Glycated hemoglobin (HbA1c) was estimated from whole blood by high-pressure liquid chromatography using the Variant machine (Bio-Rad, Hercules, CA, USA).

Estimation of advanced glycation index—Serum was diluted 50 times in phosphate-buffered saline and filtered (0.22 µm, Millex-GV; Millipore, Bedford, MA, USA). Hemolytic sera were excluded from advanced glycation end product (AGE) measurements, and the intrinsic AGE-specific fluorescence was monitored spectrofluorimetrically (Fluoromax-3, Jobin Yvon Horiba, NJ, USA) by exciting the samples at 370 nm and collecting the emission readouts at 440 nm. Fluorescence estimations at these wavelengths represent measurement of Maillard-specific AGE peptides in circulation⁸⁻¹¹. The concentrations of the AGE products were directly proportional to the fluorescence intensity, and the increase in fluorescence intensity at each addition of serum sample was curve fitted to a linear regression line¹². The slope of the regression line was termed advanced glycation index (AGI) and expressed in arbitrary units.

Lipid peroxidation—Plasma levels of malondialdehyde (MDA), a marker of lipid peroxidation, was measured by thiobarbituric acid reactive substances (TBARS) using a fluorescence methodology¹³. Briefly, 0.1 mL of plasma was mixed with 10 % sodium dodecyl sulfate (SDS), 20 % acetic acid and 0.53 % thiobarbituric acid (TBA) and boiled at 100 °C for 1 h. To this, butanol:pyridine (49:1) was added, mixed and centrifuged at 3000 rpm for 10 min. The organic solvent was removed and the sample was read at excitation 535 nm and emission 552 nm. Absolute MDA levels were calculated by regression parameters using different concentrations of the standard, 1,1',3,3'- tetramethoxypropane.

Measurement of GSH and GSSG—Oxidized (GSSG) and reduced glutathione (GSH) were estimated from whole blood according to the method of Sampath Kumar *et al*¹⁴ with necessary modifications. Blood (0.2 mL) was lysed with

0.1 M EDTA and precipitated with metaphosphoric acid. The filtrate was used to measure GSH and GSSG. For the extract used for GSSG, N-ethylmaleimide (NEM) was used to prevent reduction of GSSG to GSH. Derivatization of the sample with orthothalaldehyde (OPA) was carried out in phosphate EDTA buffer for GSH and 0.1 N NaOH for GSSG and GSH/GSSG levels were estimated using fluorimeter at an excitation of 350 nm and an emission of 420 nm and the reduction of GSH/GSSG ratio was reported as a reliable marker of oxidative stress.

Paw pressure-withdrawal test—The Randall and Selitto test was performed as described by Berti-Mattera *et al*¹⁵. The nociceptive flexion reflex was evaluated in all animals using an analgesy meter LE7306 (PanLab, Spain). Increasing pressures (at a linear rate of 10 g/s with the cut-off of 200 g to avoid tissue injury) was applied to the center of the hind paw. When the animal displayed pain by withdrawal of the paw, the applied paw pressure was registered by an analgesia meter and expressed in mass units (g). Two readings per animal were taken at 5 min intervals and subsequently averaged; the mean±SE of the individual responses was considered the threshold for each group.

Tail flick test—Pain threshold was measured using a standard hot-water tail-flick assay¹⁶. The dependent variable was the latency (in seconds) for the rat to flick its tail from the hot-water bath. The water was maintained at 55 °C in a constant-temperature water bath and was monitored using an electronic thermometer. The distal third of the rat's tail was immersed in the water bath and the time required for the rat to remove its tail was measured by use of a stopwatch. The tail-flick latency score was calculated as the mean of the last two of three trials, separated by 30 sec intervals. Each trial was terminated at 30 sec if no withdrawal response occurred. Water at 55 °C did not induce tissue damage to the tail. The experimenter conducting the tail-flick assay was blind to the experimental treatments of the rats.

Slit lamp examination and cataract classification—Eyes were examined at the end of the study (90 days) using a slit lamp biomicroscope (Kowa Portable, Japan) with dilated pupils. Eye pupils were dilated with atropine (1 % atropine sulfate). Initiation and progression of lenticular opacity was graded into four stages as documented previously¹⁷. Briefly, stage 0-clear lenses and no vacuoles present; stage

1-vacuoles cover approximately one-half of the surface of the anterior pole forming a sub capsular cataract; stage 2-some vacuoles have disappeared and the cortex exhibits a hazy opacity; stage 3-a hazy cortex remains and dense nuclear opacity is present; and stage 4-a mature cataract as visualized as a dense opacity in both cortex and nucleus. One rat each from the EI and LI group was not used for cataract score due to slight bleeding in the eyes. Therefore, cataract assessment made in EI and LI group was with an 'n' size of 5 each.

Collection of lens and biochemical assays—The animals were sacrificed by CO₂ asphyxiation and the lenses were dissected by the posterior approach and stored at -80 °C until further analysis. A 10 % lens homogenate was prepared from two-three pooled lenses in a 50 mM phosphate buffer (pH 7.4). The activities of aldose reductase (AR)¹⁸ and glutathione peroxidase (GPx)¹⁹ were assayed. Protein carbonyls content were measured in pooled total soluble protein (TSP) as per Uchida *et al*²⁰.

Statistical analysis—Experimental values are expressed as mean±SD or mean±SE. Comparison between groups were performed using Student's t-test and one way ANOVA followed by Dunnett's multiple comparison test with $P<0.05$ considered as a significant. All analysis was done using GraphPad Prism and Windows based SPSS statistical package (Version 10.0, Chicago, IL).

Results

Effect of insulin on metabolic parameters—Diabetic rats had elevated levels of plasma glucose ($P<0.001$), triglycerides ($P<0.05$) and higher HbA1c ($P<0.05$) values compared to control animals (Table 1). Fasting plasma glucose ($P<0.05$) and HbA1c ($P<0.05$) levels were significantly decreased in early insulin (EI) treated rats compared to untreated diabetic rats. In EI group, triglyceride levels showed a decreasing trend but without statistical significance. However all the metabolic markers remained higher in late insulin (LI) group. Compared to control rats, body weight was decreased in STZ-induced diabetic rats. However, insulin treated groups showed increased body weight. Compared to control rats, fasting plasma glucose values were seen elevated (albeit statistically non-significant) in EI treated diabetic rats (Table 1) on day 90. During the similar period, LI treated diabetic rats showed elevated plasma glucose levels ($P<0.001$) compared to control

rats. However as mentioned in the methods section, the status of glycemic control was checked 8 h after the insulin administration for every fortnight throughout the study i.e., 6 instances in early insulin group and 2 instances in late insulin group. During this time, the mean reduction of glucose was similar in early insulin (170 ± 13 mg/dL) and late insulin groups (161 ± 5 mg/dL) clearly emphasizing the fact that good glycemic control was achieved in both the groups during the periods of insulin administration, with the only difference of 2 months of persistent hyperglycemia in the late-insulin treated group.

Effect of insulin on biochemical markers—Table 2 presents levels of biochemical markers in the study groups. The mean AGI values in serum from diabetic animals were significantly ($P < 0.05$) higher as compared to control animals. The EI group showed significantly ($P < 0.05$) lower AGI values compared to diabetes animals without any treatment. Despite the treatment, late insulin group showed highly elevated AGE levels. Compared to control animals, the levels of TBARS were significantly ($P < 0.05$) higher in diabetic animals. While the plasma levels of TBARS were significantly lowered in EI group, these lipid peroxidation levels remain higher in LI group. While the ratio of GSH/GSSG significantly ($P < 0.05$) decreased in STZ-diabetic animals, early insulin, but not late insulin treatment, normalized this antioxidant status.

Assessment of hyperalgesia—On day 0, the paw-withdraw threshold for all the groups were similar (160 to 165 g). Diabetic rats developed mechanical

hyperalgesia as evidenced by an approximately 50 % decrease in the paw withdrawal threshold to mechanical stimuli (Fig. 1A). While in EI group the paw threshold values were similar to control animals (164 ± 4.2 , and 172 ± 10 g, respectively), paw threshold

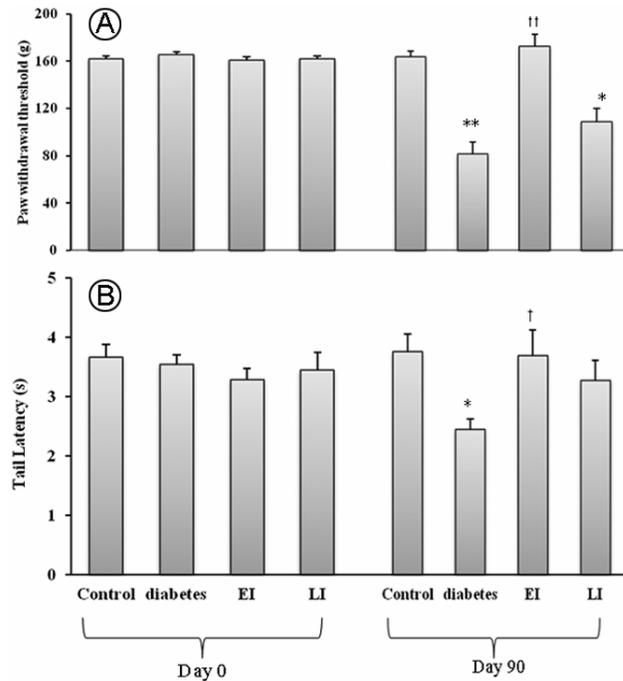


Fig. 1—Cumulative data (mean+SE) of paw-withdrawal threshold measured by Randall-Sellitto test (A) and tail flick latencies in response to thermal noxious stimuli (B) in the study groups. *P* values: * <0.05 compared to control rats, ** <0.001 compared to control rats, † <0.05 compared to diabetes rats without treatment, †† <0.001 compared to diabetes rats without treatment.

Table 1—Effect of early and late insulin treatment on metabolic parameters in rats
[Values are mean \pm SD from 6 rats in each group]

Parameters	Control	Diabetic	Diabetic rats treated with early insulin	Diabetic rats treated with late insulin
Fasting plasma glucose (mg/dL)	95 \pm 4.4	372 \pm 48**	200 \pm 48 [†]	296 \pm 32**
Plasma triglycerides (mg/dL)	100 \pm 4	184 \pm 9*	159 \pm 21	257 \pm 29*
HbA1c (%)	2.8 \pm 0.2	4.3 \pm 0.71*	2.5 \pm 0.3 [†]	4.8 \pm 0.43*
Body weight (g)	254 \pm 10	178 \pm 15*	271 \pm 12 [†]	226 \pm 17

P values: * <0.05 compared to control; ** <0.001 , compared to control rats; † <0.05 compared to diabetic rats without treatment

Table 2—Effect of early and late insulin treatment on biochemical and stress parameters in rats
[Values are mean \pm SE from 6 rats in each group]

Parameters	Control	Diabetic	Diabetic rats treated with early insulin	Diabetic rats treated with late insulin
Advanced glycation index	13 \pm 0.3	27 \pm 2.3*	17 \pm 0.6 [†]	37 \pm 6 [†]
TBARS (nM/mL)	3.6 \pm 0.3	8.7 \pm 1.4**	4.9 \pm 0.9* [†]	8.5 \pm 0.7*
GSH/GSSG ratio	2.8 \pm 0.1	1.9 \pm 0.2**	2.5 \pm 0.1 [†]	2.2 \pm 0.2*

P values: * <0.05 compared to control; ** <0.001 , compared to control rats; † <0.05 compared to diabetic rats without treatment

levels of diabetes rats (82 ± 7 g) and LI group (109 ± 11 g) were significantly lower. The nociceptive threshold in tail immersion test was carried out on day 0 and at the end of the treatment (day 90). On day 0, all four groups showed almost same tail latency times. On day 90, diabetic animals showed significant ($P < 0.05$) decrease in tail latency time compared to control animals (Fig. 1B). EI treatment normalized the tail latency time almost equal to that seen in control animals. However, LI group showed higher tail latency.

Cataract assessment—Control rats showed no (0 %) cataract while all the diabetic rats showed cataract in stage 3 (Fig. 2). In EI group, cataract was not seen in 40 % of the lenses while in the rest only

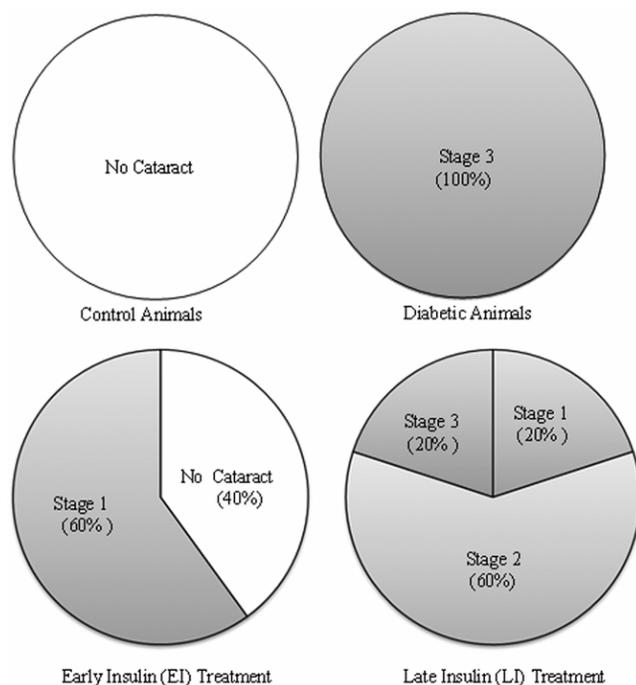


Fig. 2—Incident (%) of cataract with severity of various stages of cataract (Stage 1 to 3) in the study groups.

stage 1 cataract was seen. Among the LI treated rats, 20 % of the lenses had stage 1; 60 % stage 2; and 20 % stage 3 cataract. Compared to control animals, the STZ-induced diabetic rats showed a marked progression of opacity in lenses as evidenced by the increased average cataract score (> 2.5) (Table 3). Cataract score was considerably lower in EI rats. However in LI treated rats cataract scores were much higher.

Lens enzyme activities and protein carbonyl content—Lens aldose reductase activity, glutathione peroxidase (GPX) activity and protein carbonyl content are presented in Table 3. Compared to control animals, STZ-diabetic rats exhibited higher aldose reductase activity. Both the early and late insulin treatment reduced the lens aldose reductase activity. The activity of GPX, an endogenous antioxidant, was significantly reduced in lenses of diabetic rats. While EI treatment restored the GPX activity near to normal values, LI treatment did not improve the GPX status. The level of lens protein carbonyls is an important marker for the lens oxidative stress. Compared to control rats, diabetic rats exhibited increased protein carbonyls in their lenses. While EI treatment decreased the lens protein carbonyl levels near to the normal values, LI treatment did not offer any significant protection against protein carbonyl accumulation in the lenses.

Discussion

The findings of the present study demonstrate that the STZ-treated diabetic rats without insulin treatment developed sensory neuropathy (thermal hyperalgesia and mechanical hyperalgesia) and cataract within 3 months; these diabetic rats showed systemic elevation of lipid peroxidation/advanced glycation and reduced glutathione at the systemic level and increased protein carbonyls, aldose reductase activity and decreased

Table 3—Cataract score and biochemical alterations in the lens from the study group
[Values are mean \pm SE from 6 rats in each group]

Parameters	Control	Diabetic	Diabetic rats treated with early insulin	Diabetic rats treated with late insulin
Cataract score	0	$2.7 \pm 0.12^{**}$	$0.5 \pm 0.22^{\dagger}$	$1.8 \pm 0.25^{**\dagger}$
Aldose reductase activity (AR)	39 ± 1.4	$50 \pm 3.8^*$	45 ± 4.4	$35 \pm 0.9^{\dagger}$
Glutathione peroxidase activity (GPx)	27 ± 1.2	$19 \pm 2.5^*$	$24 \pm 1.0^{\dagger}$	19 ± 4.7
Protein carbonyl content (%) (pooled samples of each group)	100	131	100	111

P values: * < 0.05 compared to control; ** < 0.001 , compared to control rats; $^{\dagger} < 0.05$ compared to diabetic rats without treatment
AR and GPX activity expressed as μmol of NADPH oxidized/h/100 mg of protein

glutathione peroxidase activity in rat lenses. Early insulin treatment lowered the biochemical parameters towards normal and resisted the development of sensory neuropathy and cataract. However, these metabolic changes were not seen in late-insulin treated diabetic rats.

Neuropathic pain and abnormal sensory perceptions occur in a significant proportion of patients with diabetes²¹. However the mechanisms underlying these disorders remain unclear. Assessment of behavioural responses to external stimuli in diabetic rodents provides valuable information regarding the mechanisms of abnormal sensation and pain associated with diabetes²². Diabetic rats displayed altered thermal and mechanical analgesia. They also exhibited systemic level elevation of TBARS (lipid peroxidation), advanced glycation end products and decreased glutathione. While diabetic rats demonstrated reduced latencies of both tail-flick and paw-withdrawal thresholds, early insulin treatment (but not late insulin) normalized these sensory responses and biochemical aberrations. Thermal and mechanical hyperalgesia in diabetic rats due to biochemical aberrations and their correction by antioxidants and other therapeutic measures have been reported²³⁻²⁸. The DCCT-EDIC study also demonstrated the benefits of intensive insulin treatment on reductions in the incidence of diabetic neuropathy which persisted for 13-14 years even after DCCT closeout period²⁹.

Cataractogenesis is one of the earliest secondary complications of diabetes mellitus particularly in rodents. Since extracellular glucose diffuses into the lens uncontrolled by the hormone insulin, the lens is one of the most affected body parts in diabetes mellitus. Cataract formation in STZ-diabetic rats is a common complication of poorly controlled diabetes³⁰. Clark *et al.*³¹ have demonstrated degeneration swelling and disruption of lens fibers, which are typical cataract-like changes in the lens of STZ-diabetic rats. The proteins of the lens have a long life and there is virtually no protein turnover, which provides great opportunities for post-translational modification to occur. Multiple mechanisms have been implicated in the development of cataract in diabetes^{32,33}.

In tissues such as the lens, nerve, retina and kidney, in which secondary complications of diabetes occur, increased aldose reductase activity has been reported with the development of diabetes³⁴. In the present

study, STZ-diabetic rats exhibited higher aldose reductase activity and this was normalized both by the early and late insulin treatments. In the early insulin group, the normalization of aldose reductase activity very well correlated to the improved biochemical aberrations and prevention of cataract. However, despite the normalization of aldose reductase activity, the late-insulin group showed several biochemical aberrations along with cataract most likely due to the uncontrolled hyperglycemia-mediated polyol pathway for a period of two months—which is a clear reflection of metabolic memory. Alternatively, failure of cataract protection in late insulin treatment group might be originated from augmented oxidative and carbonyl stress independent of aldose reductase activity. In support of this, Randazzo *et al.*³⁵ have recently reported that orally active multifunctional antioxidants delayed cataract formation in STZ-treated rats despite the sorbitol accumulation. The accumulation of carbonyl groups in proteins is generally attributed to oxidative damage and is thought to contribute to general protein dysfunction³⁶. The biochemical analyses of eye lens proteins showed significant elevation of carbonyl groups in diabetic animals in comparison to healthy controls (Table 3). The accumulation of protein carbonyls was reduced by early insulin treatment. These results point to the pro-oxidant role of hyperglycemia and are in agreement with findings of other studies^{37,38}. Human studies and *in vitro* and *in vivo* animal experiments strongly suggest that there is an association between increased oxidative stress and the development of cataract³⁹⁻⁴³. The increased activity of aldose reductase under hyperglycemia leads to the depletion of cellular NADPH, which compromises antioxidative defenses, because NADPH is an essential cofactor for reduction of oxidized glutathione (GSSG) by glutathione reductase⁴⁵. In addition, Nishikawa *et al.*⁴⁴ have reported a potential relationship between polyol pathway-induced redox changes and the accumulation of AGE products underlying diabetic complications. In this context, it is suggested that the development of sensory neuropathy, cataract and the increased lipid peroxidation, AGEs and altered glutathione turnover seen in late insulin treated animals is a reflection of 'hyperglycemic memory'. Future work should focus on epigenetic mechanisms because these metabolic memory prototypical changes appear to be regulated through DNA methylation and histone modifications⁴⁶.

The higher HbA1c in late-insulin group in the present study represents a cumulative response of 2 months of hyperglycemia plus one-month of good glycemic control by insulin. It has also been shown that it takes a few weeks for the insulin to reduce the fasting glucose levels⁴⁷. In the present study, long-acting insulin, Insultard was used to make sure that relatively good glycemic control was achieved in both the EI and LI groups during the periods of insulin administration all through the study, with the only difference of 2 months of persistent hyperglycemia in the late-insulin treated group. Therefore, the sensory neuropathy, cataract and biochemical aberrations seen in late insulin treated rats represent a clear reflection of harmful cellular 'metabolic memory'. Bixler *et al.*⁴⁸ have studied the retinal transcriptome in STZ-diabetic animals after 3 months in absence or presence of insulin treatment (insulin treatment after 45 days of diabetes induction). Similar to the present study, it has been found that genes related to inflammatory processes, microvascular integrity, and neuronal functions were not normalized by the late insulin treatment. Bixler *et al.*⁴⁸ also concluded that gene expression changes not rescued or prevented by insulin treatment may be critical to the pathogenesis of diabetic retinopathy and are prototypical of metabolic memory. Future studies should be performed with longer-duration to investigate and delineate the 'legacy' and 'weaning' effects of metabolic memory.

To conclude, early insulin treatment in the present study prevented sensory neuropathy and cataract development in STZ-induced diabetic rats. Late insulin treatment failed to reverse these changes.

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Conflict of interest

There was no conflict of interest.

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