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Metabolic Control Improves Baroreflex Sensitivity In Diabetic Rats

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ABSTRACT

Objectives: The present study was undertaken to evaluate the effect of glycemic control on the baroreflex sensitivity, in rats having uncontrolled diabetes.

Methods: Diabetes was induced in healthy male Wistar albino rats by a single intraperitonial injection of Streptozotocin (STZ). Experiments were conducted eight weeks after induction of diabetes. The baroreflex was evaluated by measuring the changes in heart rate (HR) with changes in arterial blood pressure induced by bolus injections of phenylephrine (vasoconstrictor) and sodium nitroprusside (vasodilator).

Results: After 8 weeks of STZ administration the reflex bradycardia and tachycardia response to hypertension and hypotension respectively were impaired in the diabetic group but improved after one month of Insulin therapy. The decreased body weight, heart rate and blood pressure and raised blood sugar in diabetic rats were improved by Insulin therapy. Insulin significantly reduced oxidative stress and inflammatory cytokine levels in diabetic rats.

Conclusions: Results suggest that glycemic control with Insulin improves the altered baroreflex sensitivity in diabetic rats possibly through maintaining endogenous antioxidant enzyme activities and decreasing cytokine levels.

Key-words: Baroreflex sensitivity; Diabetes; Insulin therapy; Oxidative stress; TNF alpha; IL 6

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Introduction

Cardiovascular autonomic neuropathy (CAN) is a serious and common complication of diabetes. It causes damage to the autonomic nerve fibers that innervate the heart and blood vessels, resulting in abnormalities in heart rate control and vascular dynamics[1,2]. The baroreceptor reflex (BR) is one of the body's homeostatic mechanisms to maintain blood pressure. The system relies on specialized neurons (baroreceptors) in the aortic arch and carotid sinuses to monitor changes in blood pressure and relay them to the brainstem. Subsequent changes in blood pressure are mediated by the autonomic nervous system[1]. It provides early detection of autonomic dysfunction in diabetes[3]. The baroreflex control of heart rate has been found to be altered in rats with streptozotocin (STZ)-induced diabetes, in diabetic BB/Wor rats and rabbits[4-7]. It was observed that poor metabolic control is a major determinant of neurological damage and impairment of autonomic control of blood pressure could be reversed when better metabolic control was obtained[8-10]. Although hyperglycemia has been identified as a risk factor for development of diabetic complications, there is no consensus regarding the pathogenic link between hyperglycemia and complications. There are number of hypothesis on the origin of complications most of them are intertwined. Out of these oxidative stress and inflammatory markers are closely related to metabolic disorders and diabetes[3,11-13].

Extensive clinical investigations on cardiac autonomic neuropathy in experimental diabetes have been performed, but scanty information is available on whether treatment with insulin in chronic uncontrolled experimental diabetes is associated with reversal of autonomic nerve abnormalities. In most of the earlier studies Insulin treatment was started early after Streptozotocin (STZ) administration (2-3 days) and its role in preventing autonomic neuropathy was observed[4,14]. Hence the present study was undertaken to evaluate the effect of glycemic control with insulin on the baroreflex sensitivity, in rats having uncontrolled diabetes for 8 weeks and to study the possible association of oxidative stress and inflammatory cytokines with metabolic control and autonomic neuropathy in type I diabetes.

Materials and Methods

Animals

Healthy male Wistar albino rats, weighing between 250-300 g, were obtained from the animal house of University College of Medical Sciences (UCMS), Delhi. They were housed in polyethylene cages and kept in room temperature maintained at 25±2°C with a 12-h light/dark cycle, given food and water *ad libitum.* Experiments were performed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India, after approval by Institutional Animal Ethical Committee, UCMS, Delhi, India.

Chemicals

Streptozotocin (STZ) and urethane were purchased from Sigma Chemical Co., USA. All other chemicals and reagents used in the study were of analytical grade.

Induction of Diabetes with STZ

Diabetes was induced by a single intraperitonial injection of STZ (65mg/kg, SIGMA) dissolved in citrate buffer[15]. This dose induces type I diabetes mellitus in animals. The control animals were injected with equal volume of vehicle. After 7 days of STZ administration, blood was collected and serum samples were analyzed for blood glucose with Accu-chek glucometer. Animals showing blood glucose higher than 250

mg/dl were considered as diabetic rats and were used for the study. The animals were weighed and blood sugar estimated weekly.

Grouping of animals

Animals were randomly divided into 3 groups each having 7 rats.

Group 1: Normal control rats injected with vehicle (citrate buffer) and fed normal pellet diet (control group),

Group 2: Rats were injected STZ intraperitoneally and kept for 8 weeks (diabetic group),

Group 3: STZ-diabetic rats treated with NPH Insulin 6-8 IU/day subcutaneously for 30 days after 8 weeks of STZ administration (Therapeutic Insulin).

Insulin dose

Initial doses of insulin were started with 2 IU and increased by 1IU on alternate day till glycemic control was achieved. Depending on the extent of glycemic control dose was adjusted for each rat which ranged from 6-8 IU per day.

Hemodynamic analysis

Arterial blood pressure and heart rate

Rat was anesthetized with urethane dissolved in distilled water and injected intraperitoneally (i.p.) at a dose of 1gm/kg body weight. Disappearance of pedal reflexes indicated adequate anesthesia. Rat was placed on a small table and secured by tying the limbs. A middle incision was given in the neck region; retracting the pretracheal muscle exposing trachea and a transverse incision was given in between two rings. A cannula was introduced into the opening to allow free breathing without obstruction. Body temperature of the rat was maintained at 37-38^{o}C. Femoral artery of one side was exposed and a polyethylene catheter filled with heparin solution (500 IU/ml, v/v) was inserted in the artery through a small incision for recording arterial blood pressure (ABP). The catheter was attached to 23-gauge needle connected via three-way stopcock to a pressure transducer (Statham-P23D). Femoral vein of the other limb was cannulated for injecting drugs.

Prior to recording Arterial blood pressure (ABP), catheter was flushed with heparinized saline solution (500 IU/ml, v/v) to prevent formation of any blood clot, which might interfere with normal recording of ABP. The pressure recording system was calibrated with the help of a mercury manometer before each experiment. Arterial blood pressure was measured after 20 min of stabilization period. Systolic, diastolic, mean arterial pressures and heart rate were displayed and recorded on Power Lab data-acquisition system (4SP, AD Instruments, Australia) with a computerized analysis programme (Lab Chart 7, AD Instruments, Australia).

Measurement of baroreflex sensitivity (BRS)

Baroreflex sensitivity was assessed by administering increasing doses of vasoconstrictor phenylephrine (20µg/ml/kg) and vasodilator sodium nitroprusside (20µg/ml/kg) as bolus injections through a venous catheter. An appropriate interval (15 minutes) between doses was allowed for ABP and heart rate (HR) to return to basal levels. The resultant changes in HR at corresponding rise or fall in systolic blood pressure were measured at different time intervals. The relationship between increase in systolic blood pressure evoked by phenylephrine and associated bradycardia or decrease in systolic blood pressure evoked by sodium nitroprusside and associated tachycardia was assessed by regression analysis for individual animals. The regression coefficient (slope of regression line), expressed as beats per minute

per millimeter of mercury (beats/min/mm Hg), was taken as an index of baroreflex sensitivity measurement.

Biochemical Estimations:

After completion of haemodynamic studies, blood sample was collected. Serum was subsequently harvested by centrifuging and stored at -20°C for biochemical assays. Lipid peroxidation marker malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), Catalase and inflammatory marker tumour necrosis factor-alpha (TNF-α) and IL6 were measured in serum using appropriate kits.

Statistical analysis

Analysis was done on SPSS 20.0 statistical package. The data are presented as mean ± SEM. The three groups were compared by one way ANOVA with Tukey test at 5% level of significance. Pearson correlation was used to determine the relationship of blood glucose with baroreflex sensitivity, heart rate and BP parameters. P value of less than 0.05 was considered to be significant.

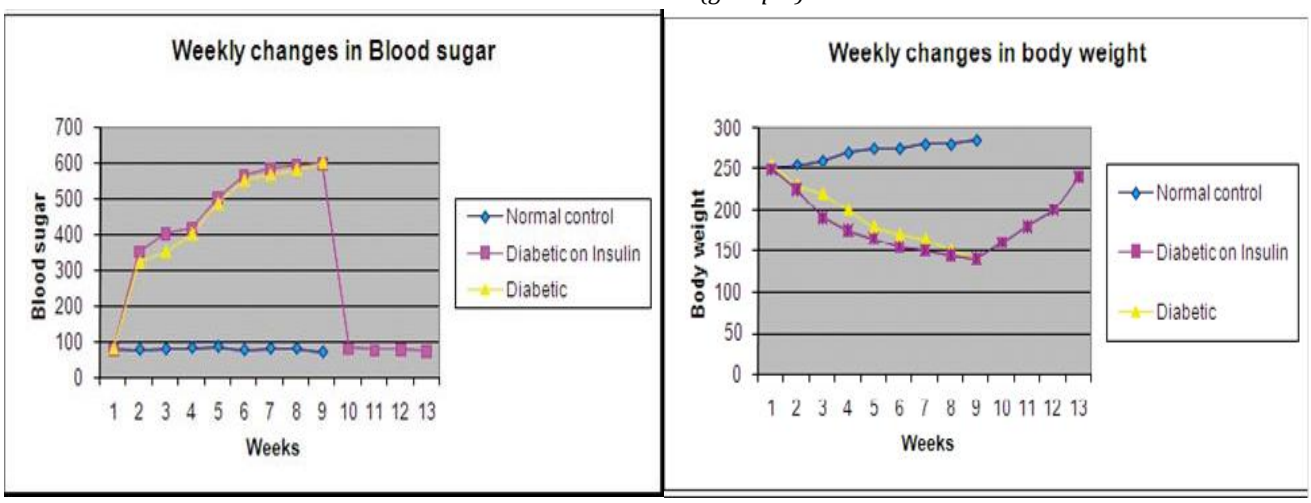
Results:

All the rats that received STZ became diabetic. The mortality rate was 20% in diabetic rats. These rats died within one week of STZ administration.

Body Weight and Blood sugar

Body weights were similar in all the three groups at baseline. The body weights of 8 week diabetic rats (185.71± 8.75gms) were significantly lower than non diabetic controls (267.14± 8.65gms). The rats of group 3 started regaining weight after instituting insulin therapy and reached their baseline levels within one month (252.14± 4.61gms). The blood glucose levels measured after STZ injection (group 2 & 3) were higher (550.57± 68.08 mg%) than after citrate buffer injection (85.14±9.07mg%) at all times till 8 weeks. There after it decreased in group 3 after starting insulin therapy (76.43± 7.25mg %). The weekly changes in body weight and blood glucose are shown in Figure 1.

Figure 1: *Weekly changes in body weight (gms) and blood sugar (mg%) in control (group 1), diabetic (group 2) and diabetic on insulin treatment (group 3).*



Arterial pressure and Heart rate

Diabetic rats had significantly lower systolic, diastolic, mean arterial pressure and heart rate as compared to normal controls. The systolic, diastolic, mean arterial pressure and heart rate increased significantly

after insulin therapy and was similar to its values in normal controls (Table 1). There was a significant negative correlation of SBP, DBP, MBP and heart rate with blood glucose (P<0.05).

***Table 1**: Blood pressure parameters and heart rate in control (group 1), diabetic (group 2) and diabetic on insulin treatment (group 3):*

	Group 1 (normal controls) n=7	*Group 2 (Diabetic controls) n=7*	*Group 3 (Diabetic on Insulin treatment) n=7*	*P value*
SBP mmHg	*119.00±1.40*	*105.86±3.14**	*116.86±2.30*	*0.002*
DBP mmHg	*80.29±1.76*	*67.57±2.62**	*77.14±1.94*	*0.002*
PP mmHg	*39.43±0.84*	*38.29±1.70*	*39.71±1.65*	*0.649*
MBP mmHg	*93.43±1.64*	*80.29±2.77**	*90.43±1.87*	*0.001*
HR beats/min	*345.43±12.57*	*292±35.22**	*335±6.95*	*0.009*

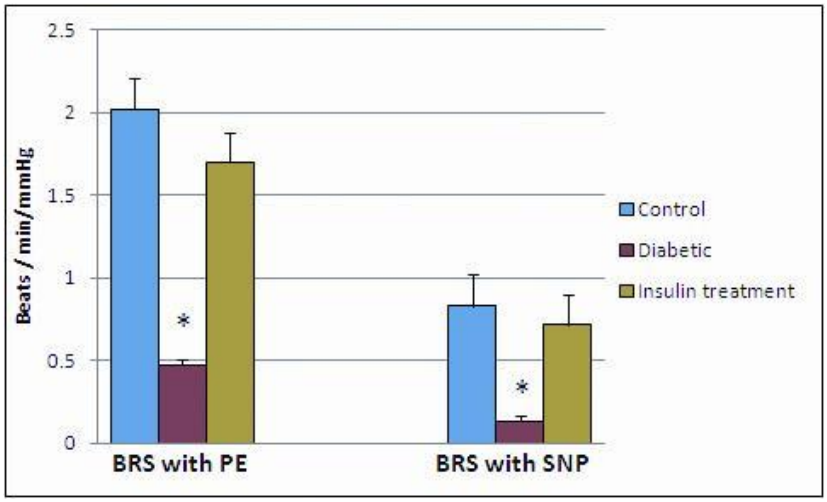
Data are mean ± SEM

**Significance of group 2 with group 1 &3 (*P<0.05)*

Baroreflex sensitivity

Baroreflex sensitivity was measured as ratio of bradycardia response to rise in arterial pressure by phenylephrine and ratio of tachycardia response to fall in arterial pressure by sodium nitroprusside. The BRS was markedly reduced in diabetic rats as compared to normal rats of the control group, as indicated by a significant (P< 0.05) fall in both reflex bradycardia(0.47±0.05 as compared to 2.02±0.22 in controls, P 0.001, Figure 2) and tachycardia (0.13±0.03 as compared to 0.83±0.19 in controls, P 0.006, Figure 2) response elicited by phenylephrine and sodium nitroprusside, respectively. Both the aspects of BRS were significantly improved after one month of Insulin therapy (Figure 2).

***Figure 2:** Baroreflex sensitivity in control (group 1), diabetic (group 2) and diabetic on insulin treatment (group 3). All values are expressed as mean ± SEM (n=7). *P<0.05 v/s group 1 and 3.*



Relationship between baroreflex sensitivity and blood glucose

A negative correlation was observed between blood glucose and baroreflex mediated bradycardia due to Phenylephrine (P <0.05) and tachycardia due to sodium nitroprusside (P< 0.05).

Biochemical estimations:

A significant rise in the level of MDA (P 0.001), and decline in GSH (P 0.004), SOD (P 0.001) and Catalase (P 0.006) was observed in the serum of STZ induced diabetic rats compared to controls. Administration of Insulin brought back all these levels to near normal status (Figure 3). Similarly inflammatory markers

TNF-α (P 0.008) and IL6 (P 0.000) were observed to be significantly increased in diabetic rats and reverted back to normal after insulin therapy (Figure 4).

Figure 3: *Oxidative stress parameters (Catalase, MDA, GSH and SOD) in the control (group 1), diabetic (group 2) and diabetic on insulin treatment (group 3). All values are expressed as mean ± SEM (n=7). *P<0.05 v/s group 1and 3.*

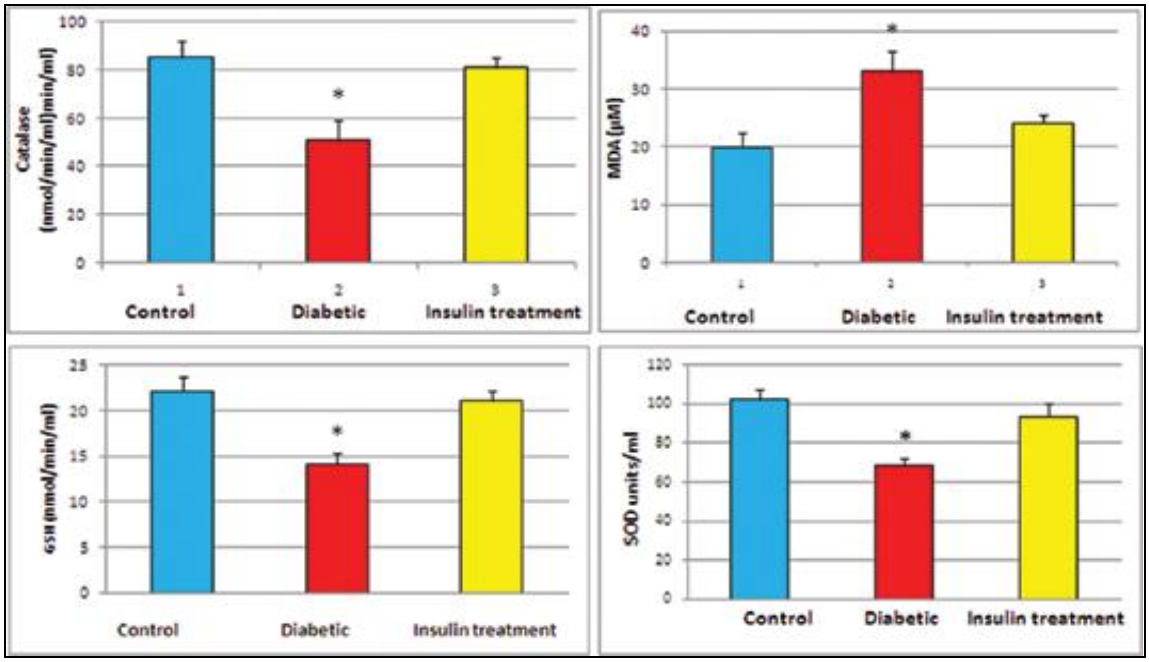
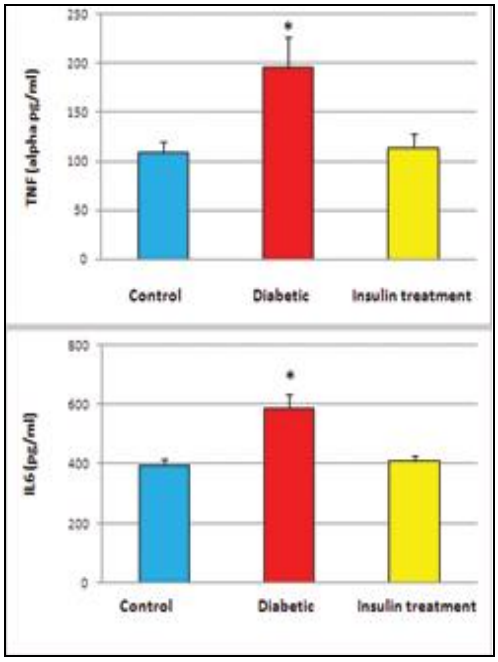


Figure 4: *Inflammatory markers (TNF alpha and interleukin 6) in the control (group 1), diabetic (group 2) and diabetic on insulin treatment (group 3). All values are expressed as mean ± SEM (n=7). *P<0.05 v/s group 1and 3.*



Discussion

In the present study there was a progressive fall in body weight and rise of blood sugar levels after STZ administration. We observed that 8 week diabetic rats had significantly reduced systolic, diastolic, mean arterial pressure, heart rate and baroreflex along with increase in oxidative stress and pro inflammatory cytokines as compared to controls. These parameters returned to normal levels after insulin therapy emphasizing the role of glycemic control in uncontrolled diabetes.

Experimental diabetes induced by STZ has been used by several investigators to study disorders of autonomic control of cardiovascular system. Rats treated with STZ display many features seen in human subjects with uncontrolled diabetes, including hyperglycemia, hypoinsulinemia, increased urinary glucose levels and consequently polyurea as well as weight loss. The high levels of blood glucose confirmed the efficacy of STZ in producing experimental chronic (8 weeks) diabetes in rats. STZ enters the B cell *via* a glucose transporter (GLUT2) and causes alkylation of DNA. DNA damage induces activation of poly ADP-ribosylation, depletion of cellular NAD+ and ATP and formation of superoxide radicals. Furthermore, STZ liberates toxic amounts of nitric oxide that inhibits aconitase activity and participates in DNA damage. As a result of the STZ action, B cells undergo destruction by necrosis[15]. In the present study there was progressive fall in body weight and rise of blood sugar levels after STZ administration. The body weight was regained after Insulin therapy. It is known that diabetes is associated with caloric deficiency which results in marked reduction in body weight[7,8,14,16].

There are conflicting reports of blood pressure in experimental diabetes. Hypotension[7], hypertension[17]and normotension[4] have been reported. Differences in rat strain, duration of diabetes, anesthesia or techniques for measuring blood pressure could explain the conflicting data[18]. We observed that 8 week diabetic rats had significantly lower systolic, diastolic and mean arterial pressure as compared to controls. These values returned to control levels after insulin therapy. This is in agreement with earlier studies where consistent hypotension is reported with direct measurement of BP in animal models independent of duration of diabetes[8,14]. The reduction in arterial pressure may be attributed to decreased cardiac output in diabetic rats due to hypovolemia caused by hyperglycemic osmotic diuresis. In most of the earlier studies Insulin treatment was started within 2-3 days of STZ administration[4,14]. While in the present study we allowed diabetes to progress for 8 weeks and then studied the reversal with Insulin treatment.

The present study confirms the previous findings of reduced heart rate in diabetes. Various mechanisms have been reported for the reduction in heart rate. Change in the sinoatrial node activity[16], autonomic dysfunction indicated by increase in vagal tone or decline in sympathetic tone[19], metabolic injury to pacemaker cells[6] have all been reported. Chang and Lund (1986) also observed that insulin treatment restores normal cardiovascular and baroreflex function, at least when given during the early stages of diabetes. These data suggest that these early phenomena constitute a stage of neurological dysfunction distinct from “true” neuropathy[20].

The impaired bradycardiac and tachycardiac response to arterial pressure changes in diabetic rats observed in the present study are in contrast with others who reported variable[4] or no change[5,6, 21] in one or both the components of autonomic nervous system. The controversial data regarding changesin baroreflex sensitivity after diabetes may be attributed todifferences in experimental and analytical approaches. The timeafter diabetes induction[4,14] and the methodological analysis ofbaroreceptor

sensitivity[11] as well as the animal model used[22]can change the interpretation of the data. More over the weight loss observed in our diabetic rats may have partially contributed to impaired baroreflex since it is known that weight loss affects autonomic functions in humans[22].

There is a body of evidence that insulin modifies the diabetic state, changing the course of different complications of experimental diabetes[21,23]. In a morphological study of the autonomic nervous system, Schmidt et al [24] showed amelioration of neuroaxonal dystrophy by early treatment of diabetic rats with insulin, by pancreatic islet transplant or by administration of the aldose reductase inhibitor sorbinyl. However, when these treatments were instituted later, that is after neuropathy was established, inhibition of progression was attained, but the parameters studied did not normalize[25].

A negative correlation between blood glucose and baroreflex sensitivity make us suggest a definitive role of metabolic decompensation in the genesis of these autonomic changes in agreement with earlier studies[9,17]. Several mechanisms have been considered to be involved in the pathogenesis of diabetic neuropathy in the STZ-diabetic rat, but hyperglycemia is always implicated. It has been reported that structural changes occurring in peripheral nerves resemble those of human diabetic neuropathy and are preceded by hyperglycemia- induced biochemical abnormalities[26,27]. Our results are supported by Burger et al[28] who reported the reversibility of early cardiac autonomic neuropathy assessed by power spectral analysis of HR variability by glycemic control in type 1 diabetic patients, and comparable results were also published by Muhr-Becker et al[29] in a study evaluating cardiac sympathetic dysinnervation scintigraphically in a similar group of patients.

Persistent hyperglycemia has been reported to cause increased production of free radicals through glucose auto oxidation and non enzymatic glycation. High levels of free radicals and simultaneous decline of antioxidant defense mechanisms has been suggested to play key role in the pathogenesis of various complications in diabetes[11,12].MDA serves as a reliable marker for the assessment of free radical induced damage to tissues. In the present study a significant rise in the level of MDA was observed in the serum of STZ induced diabetic rats which were significantly reduced after Insulin treatment. Decreased activity of antioxidant system that is GSH, SOD and Catalase was observed in the serum of STZ induced diabetic rats in agreement with previous studies[30,31].The altered balance of the antioxidant enzymes caused by decrease in Catalase, SOD and GSH activities may be responsible for the inadequacy of the antioxidant defenses in combating free radical mediated damage. These enzymes have been suggested as playing an important role in maintaining physiological levels of oxygen and hydrogen peroxide by hastening the dismutation of oxygen radicals and eliminating organic peroxides and hydroperoxides generated by STZ administration[15]. These findings indicated that the improvement of BRS with Insulin can be attributed to its antioxidant activity at least in part.

Pro inflammatory cytokines are capable of modulating cardiovascular functions by various mechanisms[13]. They are secreted in response to stress even in the absence of systemic immune activation. In the present study TNF-α and IL6 were observed to be significantly increased in diabetic rats but reverted back to normal after insulin therapy suggesting anti-inflammatory activity of insulin. This is in agreement with earlier studies[13, 32]. Both these cytokines are known to have cardiodepressive property[13]. This may be one of the reasons of hypotension and bradycardia observed in our diabetic rats.
We agree with earlier reports that autonomic changes may be correlated with the metabolic control or diabetes *per se* and are not related to the direct action of STZ[14]. Results obtained by Schmidt et al[24], who

studied rats treated with STZ receiving insulin, and by Chang and Lund[4], who studied rats which received STZ and did not develop diabetes, strongly suggest that the lesions demonstrated by these investigators in STZ-induced diabetic animals were produced by diabetes and were not the result of a direct toxic effect of the diabetogenic agent.

Several studies demonstrated that insulin can prevent, or even reverse, the derangements caused by chronic diabetes[34-36]. The mechanism responsible for this protective effect is still unknown, because diabetes is a long-standing metabolic disorder with several outcomes. It has been demonstrated in normal cardiac myocytes that insulin speeds the glucose transport into the cell. However, it has been demonstrated also that insulin promotes a positive inotropic effect independent of glucose uptake[34]. In the present study since Insulin is regulating oxidative stress and levels of inflammatory markers, it can be said that Insulin is improving cardiac autonomic functions by above mechanisms in addition to controlling hyperglycaemia.

Conclusion

Metabolic control with Insulin enhances baroreflex sensitivity in diabetic rats possibly through maintaining endogenous antioxidant enzyme activities and decreasing cytokine levels.

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