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Effects of Diltiazem on Electrolytes Homeostasis in Streptozotocin-Induced Diabetic Rats

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Abstract: Calcium concentration plays an important role in the development of diabetic complications in tissues permeable to glucose like liver, blood vessels of retina, kidney and central and peripheral nervous systems. This study was designed to investigate the possible involvement of intracellular electrolytes in antihypertensive effects of diltiazem in diabetes. In diltiazem treated control rats serum, heart, kidney calcium and magnesium were significantly decreased where as RBC, heart potassium and magnesium and Na-K-ATPase activity were significantly increased as compared to control animals. In STZ-induced diabetic rats serum sodium, magnesium, RBC potassium, ATPase activity, heart and kidney potassium were significantly decreased while serum potassium, glucose, RBC and heart sodium were significantly increased. In diltiazem treated diabetic rats serum and heart magnesium and serum glucose were increased where as RBC, heart, kidney sodium, heart, kidney calcium, kidney magnesium and kidney potassium were decreased significantly as compared to control rats. It is assumed that total peripheral resistance, systemic blood pressure and after load is decreased and thus diltiazem is useful in managing angina and hypertension in diabetes by decreasing calcium and sodium in heart and kidney tissues. Diltiazem may be useful in improving the clinical benefits for cardiovascular complications in diabetes.

Key words: Diltiazem, electrolytes homeostasis, diabetic rats, Na-K-ATPase, streptozotocin

INTRODUCTION

It has been shown that increased intracellular calcium concentration plays an important role in the development of long term complications of diabetes in the tissues permeable to glucose such as liver, blood vessels of retina, kidney as well as central and peripheral nervous system (Chan and Junger, 1984). Previous studies showed that intracellular deposition of calcium increases in streptozotocin (STZ)-induced diabetic models (Hightover *et al*., 1980). It has also been reported that intracellular calcium levels of platelets and erythrocytes increased in STZ-induced diabetic rats. In addition to these findings, hyperglycemia inhibits Ca-ATPase and increases intracellular calcium concentration (Dunbar *et al*., 1989). It has been previously hypothesized that glycated proteins which increase in diabetes bind higher amount of calcium (Liang *et al*., 1986). In a previous study three different calcium channel blockers were shown to have positive effects on the general appearance, weight as well as the blood glucose and HbA1c level in STZ-induced diabetic rats. This condition was reported to be due to the calcium channel blockers antioxidant effect and to intracellular calcium homeostasis (Alev *et al*., 1995). It is suggested previously that oxidative stress increased in diabetic kidney and calcium channel blockage can prevent these change (Anjaneyulu and Chopra, 2005). Our previous studies showed the antihypertensive role of intracellular and tissue sodium, potassium, calcium and magnesium in various induced forms of hypertension (Tabassum *et al*., 1996). In order to investigate the possible involvement of intracellular electrolytes in antihypertensive effects of diltiazem in diabetes the present study was designed. Despite the large body of evidences regarding the effects of diltiazem administration on various hormones and other metabolites, very little is known regarding its role in the intracellular and tissue content of sodium, potassium, calcium and magnesium in STZ-induced diabetic rats. In the light of the above findings it is hypothesized that diltiazem's antihypertensive and antioxidant preventive role may be through alteration in the intracellular and tissue electrolytes. The present study describes the role of Na-K-ATPase, serum and intracellular electrolytes in the antihypertensive effects of diltiazem in diabetic rats. The work further elucidates the alterations of sodium, potassium, calcium and magnesium in heart, kidney and liver tissues after diltiazem administration in streptozotocin induced diabetic rats.

MATERIALS AND METHODS

Animals: Male wistar albino rats, weighing 180-250 g body weight were used.

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Induction of diabetes: Rats were housed in individual cages at 20-40°C through out this study period with free access to food and water. Diabetes mellitus was induced by a single subcutaneous injection of STZ in a dose of 60 mg kg^{-1} body weight. STZ was dissolve in citrate buffer at pH 4.5. Control group received only citrate buffer at the same amount. Blood sugar level was monitored after 48 h of injection. The animals whose blood sugar level was higher than 13 mmol at 48 h post injection period were included in the study.

Experimental design: Animals were divided into four experimental groups:

Control, diabetic, drug-treated diabetic and drug treated control animals.

Diltiazem hydrochloride (30 mg kg^{-1} day^{-1}) was initiated 72 h after STZ injection. At the end of four weeks period systolic blood pressure was monitored in terms of mmHg by Harvard indirect rat tail blood pressure system. Rats were placed in restrainer for approximately 10 min before the onset of blood pressure determination. The mean of tree artifact free determination (not differ by 10%) served as an index of SBP.

Sample collection: At the end of the treatment animals were decapitated and blood was sampled from the head wound in the lithium heparin coated tubes. A portion of blood was taken in the separate tube to collect the serum. Heart and kidneys were excised, trimmed of connective tissues, rinse with deionized water to eliminate blood contamination, dried by blotting with filter paper and weighed. The tissues were then kept in freezer until analysis.

Intraerythrocyte sodium and potassium estimation: Serum was separated from the clotted blood by centrifugation, erythrocytes were prepared for the estimation of sodium, potassium as described earlier (Tabassum *et al.*, 1996), simply heparinized blood was centrifuged and plasma was separated. Buffy coat was aspirated and discarded. Erythrocytes were washed three times at room temperature by suspension in the 112 mmol L^{-1} magnesium chloride solution, centrifugation at 450 x g for 5 min and aspiration of the supernatant. Final supernatant was retained for the estimation of sodium and potassium concentration. Neither electrolyte was detectable in the final wash. Washed erythrocytes were then lysed and used for the estimation of intraerythrocyte sodium and potassium.

Erythrocyte membrane preparation: The red cell pack extracted by centrifugation at 4°C were resuspended and diluted in 25 volumes of Tris buffer at pH 7.4. The hemolyzed cells were than centrifuged at 12000 rpm at 4°C and the membrane pellet was suspended in 30 mL of 0.11 mol L^{-1} Tris-HCl buffer. This centrifugation step was repeated three times. The final concentration of the membrane suspension was ~4 mg protein mL^{-1} of Tris buffer. The membrane suspension was stored at -80°C until the assay was performed.

Erythrocyte membrane Na-K-ATPase activity measurement (Raccah *et al.*, 1996): Na-K-ATPase activity was measured in a final volume of 1 mL as follows: membrane (400 μg) was preincubated for 10 min at 37°C in a mixture containing 92 mmol L^{-1} Tris-HCl (pH 7.4), 100 mmol L^{-1} NaCl, 20 mmol L^{-1} KCl, 5 mmol L^{-1} $MgSO_4.H_2O$ and 1 mmol/EDTA. Assays were performed with or without ouabain (1 mmol L^{-1}), a specific inhibitor of Na-K-ATPase. After incubation with 4 mmol L^{-1} ATP at 37°C for 10 min, the reaction was stopped by adding ice cold TCA to a final concentration of 5%. After centrifugation at 4°C, 5500 g for 10 min the amount of inorganic phosphate in the supernatant was determined (Dryer and Tammes, 1957). Na-K-ATPase was calculated as the difference between inorganic phosphate released during 10 min incubation with and without ouabain. Activity was corrected to a nanomolar concentration of inorganic phosphate released mg^{-1} protein h^{-1}. The concentration of protein was determined by Biuret method.

Tissue digestion: Frozen tissues were digested for 3 h at room temperature and then at 70°C for another 3 h in 20 mL deionized water followed by 10 mL of concentrated nitric acid and perchloric acid. The samples were initially heated very gently after foaming subsided the temperature was increased to produce steady boiling. The excess acid was boiled off to near dryness. The digest then cooled to room temperature and analyzed for sodium, potassium, calcium and magnesium content (Kang *et al.*, 1977; Leblondel and Allain, 1988).

Electrolytes estimation: Concentration of sodium and potassium in serum, heart and kidney were analyzed by flame photometer. Serum magnesium was estimated by ion selective electrode using ion meter 3345. Red cell sodium and potassium were estimated by the method of Fortes Meyer and Starkey (1977). Concentration of calcium in serum, heart and kidney tissue was analyzed by Ca ion selective electrode using ion meter 3345.

Statistical analysis: Results are presented as mean±SD. Significance difference from control and test values were evaluated by student's t-test.

RESULTS

Effects of diltiazem on serum electrolytes in control and STZ-induced diabetic rats: A decrease in concentration of serum sodium observed in diltiazem treated and STZ diabetic rats as compared to untreated control animals ($p<0.01$). No significant change was observed in diltiazem treated STZ diabetic rats. Concentration of potassium was increased significantly in diltiazem treated diabetic rats as compared to STZ-induced diabetic controls ($p<0.05$). No significant change in serum calcium was observed in all three groups. Concentration of magnesium was decreases in STZ-induced diabetic group ($p<0.05$) however an increased level was observed in STZ diabetic group receiving diltiazem ($p<0.01$) (Fig. 1).

Effects of diltiazem on glucose, red cell sodium, potassium and Na-K-ATPase activity in control and STZ-induced diabetic rats: Production of diabetes was confirmed by elevated blood glucose in ST-induced diabetic group as compared to control. Diltiazem administration in both normal and diabetic group did not show any significant effect on blood glucose level. STZ-induced diabetes group showed a significant increase in the level of sodium in red blood cell. Diltiazem treatment significantly lowers this elevated level in STZ-induced diabetic group. Red cell potassium level was increased in diltiazem treated group. In STZ-induced diabetic group red cell potassium levels was decreased significantly. Na-K-ATPase activity was increased ($p<0.05$) in diltiazem treated group. In STZ-induced diabetic group a decreased Na-K-ATPase activity was observed ($p<0.05$) but diltiazem treatment did not show any effect (Fig. 2).

Effects of diltiazem treatment on heart electrolyte contents in control and STZ-induced diabetic rats: Diltiazem treatment decreases sodium content in the heart tissue. In STZ-induced diabetic rats an increased sodium content was observed ($p<0.01$). Diltiazem treatment in STZ-induced diabetic group decreased sodium content in the heart tissue. No significant effect of diltiazem or STZ-induced on calcium content of heart tissue was observed. However diltiazem treatment significantly lowered calcium content of heart tissue in STZ-induced diabetic group ($p<0.01$). An increase potassium content of heart tissue was observed in diltiazem treated group as compared to control. In STZ-induced diabetic group potassium content of heart tissue was decreased.

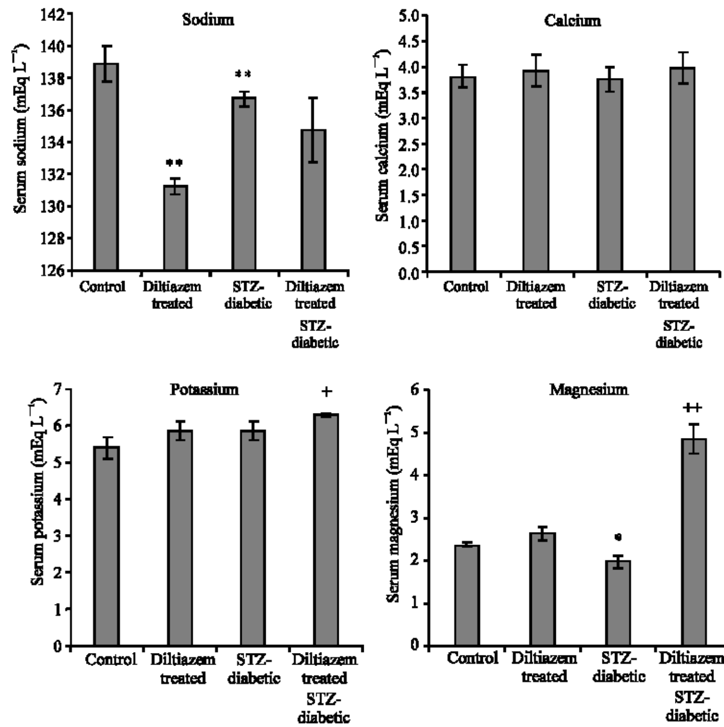

Fig. 1: Effect of diltiazem on serum electrolytes in control and ST-diabetic rats. Values are means±SE (n = 8). Significantly different from control *$p<0.05$ and **$p<0.01$. Significantly different from STZ-diabetic control +$p<0.05$ and ++$p<0.01$

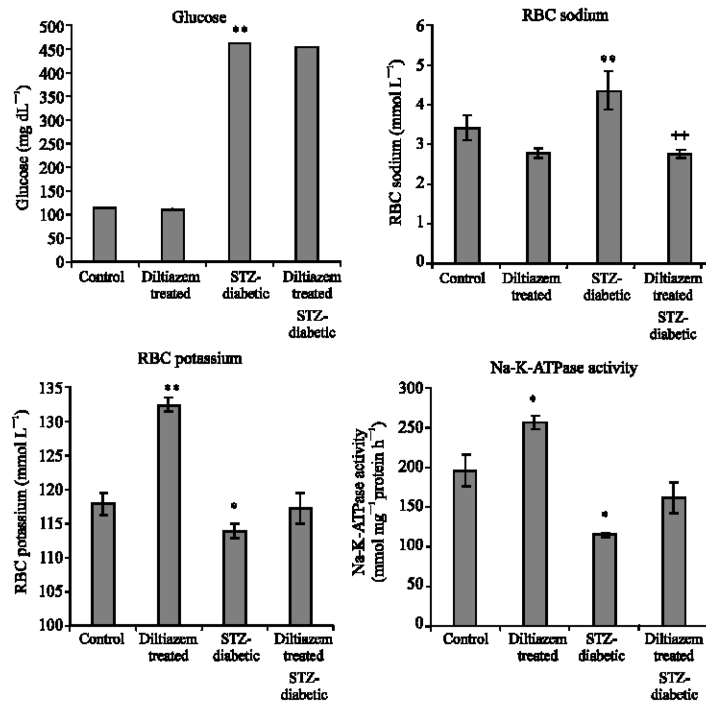

Fig. 2: Effects of diltiazem on glucose, red cell sodium, potassium and Na-K-ATPase activity in control and STZ-diabetic rats. Values are means ± SE (n = 8). Significantly different from control *p<0.05 and **p<0.01. Significantly different from STZ-diabetic control +p<0.05 and ++p<0.01

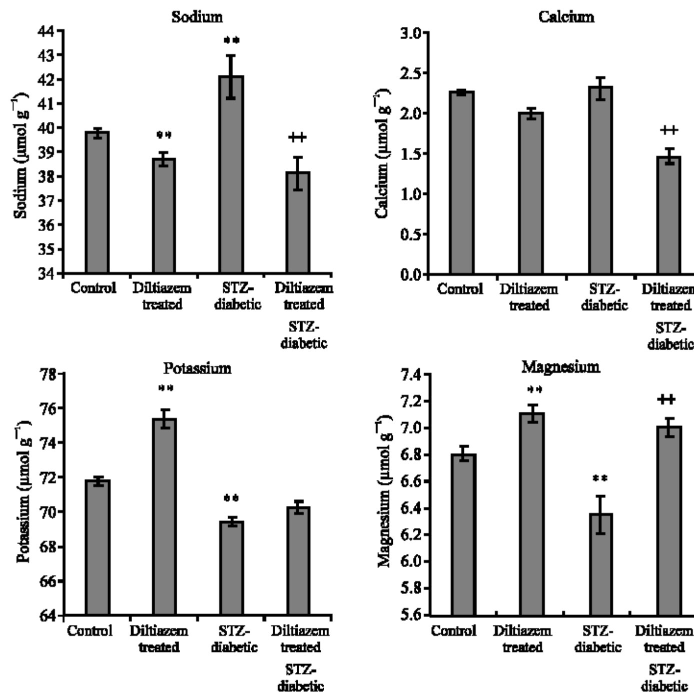

Fig. 3: Effects of diltiazem on heart electrolyte contents in control and STZ-diabetic rats. Values are means±SE (n = 8). Significantly different from control *p<0.05 and **p<0.01. Significantly different from STZ-diabetic control +p<0.05 and ++p<0.01

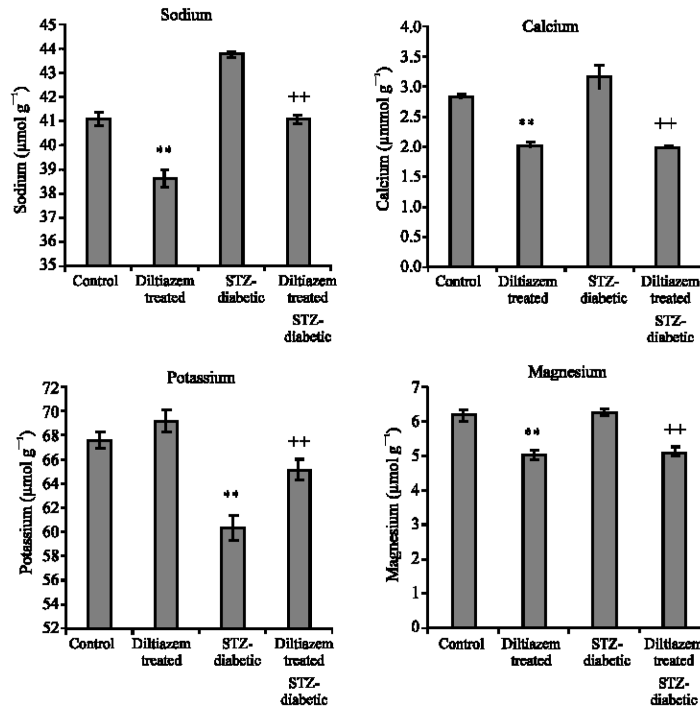

Fig. 4: Effects of diltiazem on kidney electrolyte contents in control and STZ-diabetic rats. Values are means±SE (n = 8). Significantly different from control *p<0.05 and **p<0.01. Significantly different from STZ-diabetic control +p<0.05 and ++p<0.01

Diltiazem treatment increased magnesium content in normal as well STZ-induced diabetic rats (p<0.01). In STZ diabetic group magnesium content in the heart tissue was increased (p<0.01) (Fig. 3).

Effects of diltiazem treatment on kidney electrolyte content in kidney tissue: Diltiazem treatment decreased sodium, calcium and magnesium content in the kidney tissue of both normal and STZ-induced diabetic rats as compared to their respective controls (p<0.01). No significant change in the kidney tissue sodium, calcium and magnesium was observed in STZ-induced diabetic group as compared to control rats. Potassium content of kidney tissue was decreased significantly in STZ-induced diabetic rats. In diltiazem treated STZ-induced diabetic increased potassium content of the kidney was observed (p<0.01) (Fig. 4).

DISCUSSION

Calcium channel blockers improve diabetes-induced cardiomyopathy, hypertension and hyperlipidemia (Saito *et al.*, 1999). This study demonstrates that several alterations in serum and tissue electrolytes occur after diltiazem administration that may have a significant role to improve diabetes induced cardiovascular complications.

Effects of diltiazem on serum sodium and potassium levels in control and STZ-induced diabetic rats: Present study shows that serum sodium levels were significantly (p<0.01) decrease in STZ-induced diabetic rats, whereas, serum potassium levels were increase non-significantly, (Fig. 1) as compared to control animals.

It has been previously reported that there is an inverse relationship exist between sodium and potassium levels. This disorder may be based on the movement of electrolytes between intra and extra cellular spaces dependent on impaired insulin action as well as hyperosmolality (Orskov *et al.*, 1994). These results are supported by another study (Khadouri *et al.*, 1994) which concluded that serum sodium decreased significantly while serum potassium increased in diabetic patients results in slight but significant reduction in nerve conduction velocity.

In STZ-induced diabetic rats hyperglycemia is characterized by decreased in serum insulin associated with increase in serum concentration of mineralocorticoids and glucocorticoids, a decrease in serum concentration of thyroid hormones an increase in serum potassium and a decrease in total body K^+ (Fein *et al.*, 1980; Kindermann *et al.*, 1986). Thus it can be suggested that plasma glucose decreases the insulin activity which in turn increases the serum potassium levels in STZ-induced

diabetic rats. The other possible mechanism is that in STZ-induced diabetic rats angiotensin II and plasma aldosterone concentration were lowered (Levy *et al*., 1994).

Effect of diltiazem on serum calcium levels in control and STZ-induced diabetic rats: In the present study, the serum concentration of calcium decreased in STZ-diabetic rats whereas an increased calcium level was observed in diltiazem-treated rats (Fig. 1).

Previous studies proposed that abnormal intracellular calcium metabolism causes insulin resistance and impair insulin secretion resulting in hypertension and obesity and these abnormalities can be observed in red blood cells and platelets of diabetic patients (Mazzanti *et al*., 1989). It has been reported that erythrocytes from diabetic patients have a higher intracellular Na^+ concentration (Hallam and Rink, 1985). Calcium was inversely related to Na-K-ATPase activity so that the decrease of the latter might raise calcium through increased intracellular sodium (Ward *et al*., 2001) producing activation of Na-Ca exchange.

In diabetic rats the decrease in calcium concentration may be due to elevated GFR with raised urinary output and reduced calcium reabsorption (Denis and John, 1981). It is previously reported that the renal tubular reabsorption of glucose is essential to augment calcium and magnesium excretion (Budriesi *et al*., 2007). Since an enhanced urinary output of Ca and Mg is a characteristic of early human and experimental diabetes.

Previously it has been reported that calcium levels were significantly increase in hypertension (Sunaga and Ogihara, 1990). The observed decreased calcium level in STZ-induced diabetic rats may have a role in the reduction of blood pressure after diltiazem treatment.

Effects of diltiazem on serum magnesium levels in control and STZ-induced diabetic rats: Our present results shows that there is a significant ($p<0.01$) decrease in the serum magnesium concentration in diabetic rats as compared with control whereas, significantly ($p<0.01$) increased magnesium was observed in diltiazem treated STZ-induced diabetic rats (Fig. 1).

Magnesium depletion is a common feature of diabetes mellitus, apparently related to glycemic control (Resnick *et al*., 1993). The Mg deficiency that has been demonstrated in insulin-resistant states such as hypertension and diabetes may contribute to suppressed glucose metabolism and action (Paolisso and Barbagallo, 1997; Sales and Pedrosa, 2006) results in inhibition of both basal and insulin stimulated glucose use.

The increase in serum magnesium in STZ-induced diabetic rats after diltiazem treatment may exert a beneficial effect. Magnesium ion also acts as a cofactor for Na-K-ATPase. Magnesium also appears to be a special kind of calcium antagonist in vascular smooth muscle. At vascular membrane it can lower peripheral and cerebral vascular resistance and lower arterial pressure (Kaymaz *et al*., 1995). Thus the observed increase in magnesium level may have a role in lowering of blood pressure in STZ-induced diabetic rats.

Effects of diltiazem on glucose, red cell sodium, potassium and Na-K-ATPase activity in control and STZ-induced diabetic rats: Results showed that serum glucose levels were slightly decreased in STZ-induced treated diabetic rats (Fig. 2).

In diabetic animals, the blood sugar levels were significantly higher at the end of the experiment than the initial levels. However, such an increment of blood glucose was not observed in animals treated with calcium channel blockers. In other words, the increase of glycemia in the diltiazem treated group was not of the same magnitude as the STZ-induced diabetic group (Chellingsworth *et al*., 1989; Grafter and Baxter, 1989).

Diltiazem in STZ-induced diabetic rats increased Na-K-ATPase activity, serum Mg and decreased sodium and increased K in red blood cells (Fig. 1, 2).

It has been shown that cellular magnesium deficiency can alter the activity of the membrane bound Na-K-ATPase, which is involved in maintenance of gradients of Na and K and in glucose transport (Suzuki *et al*., 2006). These findings are supported by the previous studies that Mg deficiency causes alteration in the activity of Na-K-ATPase, which results in K efflux and Na influx into the cells and this decrease the intracellular K content in diabetes.

After the administration of diltiazem in STZ-diabetic rats it is possible that the observed diltiazem induced increase in serum magnesium may increase Na-K-ATPase activity leading to increased intracellular potassium and decreased sodium. It may be possible that the inhibition of calcium influx with diltiazem may alter the intracellular ratio of sodium to calcium, which in turn may activate the Na-K-ATPase as a compensatory mechanism. It may be possible that changes in the membrane conformation caused by diltiazem binding could directly stimulate the ouabain-sensitive pump activity.

Effect of diltiazem in control and STZ-induced diabetic rats on heart, kidney and liver electrolyte content: In the present study, it was observed that Na and Ca content

of heart and kidney were decreased in both normal and STZ-induced diabetic rats after diltiazem treatment as shown in (Fig. 3, 4). In kidney tissues an increase in K and decrease in Mg content was observed (Fig. 4).

The widely accepted mechanism of these actions as an inhibition of transmembrane Ca influx in both cardiac and vascular smooth muscles leading to reduction in the force of cardiac contraction and peripheral vascular resistance (Frey and Fleckenstein, 1985). The entry of extracellular calcium ions is more important in initiating the contraction of myocardial cells, while the release of calcium ions from intracellular storage sites also participates in vascular smooth muscle cells (Chellingsworth *et al.*, 1989). On the other hand, there is some evidence demonstrating that using calcium channel blockers (Balasubramaniam *et al.*, 2004) might reduce intracellular Ca levels. Recently, it is proposed that it is similar to verapamil in that it inhibits the influx of extracellular calcium across both the myocardial and vascular smooth muscle cell membrane. In coronary and peripheral arterial smooth muscle and the heart, inhibition of calcium entry blunts the ability of calcium to serve as an intracellular messenger. Thus, diltiazem a smooth muscle dilator and have a negative ionotropic effect on the working myocardial cells of the atria and ventricles (Kinoshita *et al.*, 1979).

The tissue Na may be related to renin-angiotensin system which diltiazem reported to antagonize, which in turn decrease the calcium influx through Na-Ca exchange mechanism and caused a decreased intracellular calcium content and peripheral vascular resistance (Buhler *et al.*, 1985). Recently, it is proposed that diltiazem inhibit smooth muscle cell proliferation in addition to their effects on vascular tone.

CONCLUSION

In conclusion, we assume that total peripheral resistance, systemic blood pressure and after load are decreased and thus diltiazem is useful in managing angina and hypertension in diabetic patients by decreasing calcium and sodium in heart and kidney tissues. The alterations in the electrolytes particularly decreased red cell sodium, increased potassium, increased serum magnesium and increased membrane Na-K-ATPase activity in STZ-induced diabetic rats treated with diltiazem shows that diltazem may be used as a useful treatment for improving the clinical benefits of therapy for cardiovascular complications in diabetic patients.

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