

Study on the changes of Carbonic Anhydrase activity in insulin resistance and the effect of methylglyoxal

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Abstract

Objectives: To determine the serum levels of methylglyoxal (MG) and carbonic anhydrase (CA) activity in known cases of type-II diabetes mellitus with insulin resistance and to compare them with age-sex matched healthy controls.

Methods: Eighty six participants were enrolled for the present case-controlled study with ages ranging from 25 to 44 years. The analytes measured were fasting blood glucose, serum insulin, methylglyoxal and carbonic anhydrase assay by standard procedures. Further carbonic anhydrase activities were measured in erythrocytes haemolysate and the effect of methylglyoxal on erythrocyte membrane was studied. The effects of methylglyoxal on erythrocyte carbonic anhydrase activity and insulin reactivity were also measured.

Results: The mean serum levels of insulin, fasting blood glucose, carbonic anhydrase activity, methylglyoxal and body mass index (BMI) was significantly higher ($p < 0.001$) in patients compared to the controls. The carbonic anhydrase activity was directly proportional to increasing blood glucose concentration. The haemolysate prepared from the erythrocytes of patients and controls also showed significantly higher carbonic anhydrase activities in patients than the controls. Increased carbonic anhydrase activity in a dose-dependent manner with methylglyoxal was also observed.

Conclusion: Insulin resistance is accompanied by increased activity of carbonic anhydrase which is significantly related to increasing methylglyoxal levels.

Keywords: Essential hypertension, Carbonic anhydrase, Lipid profile (JPMA 62: 417; 2012).

Introduction

Carbonic anhydrase is well-characterised and widely distributed in all living organisms. In mammals, carbonic anhydrases (CAs, EC 4.2.1.1) are found in at least 14 different isoforms localised in cytosol, mitochondria, membrane bound and are also secreted in saliva. CA is a pH-regulating enzyme in most tissues.¹ Changes in CA activity have been associated with altered metabolism, especially in diabetes mellitus. The activities of CA has been widely studied and linked with various diseases where changes in catalytic activities have been demonstrated.²⁻⁵ The catalytic and inhibitory mechanisms of these enzymes are understood in great detail, and this greatly helped to design some potent inhibitors of clinical interest.⁶ The modulations of enzyme kinetics of carbonic anhydrase have been proposed for treatment and prophylaxis of obesity.^{7,8} A study conducted elsewhere suggests the presence of anti-CA antibodies in subjects with metabolic syndrome.⁹ Studies also propose CA activity as a potential candidate for a biomarker of diabetes mellitus for the early detection of insulin resistance because the changes in CA activities were proportional to the severity of diabetes.¹⁰

Since glycation may change the structure and functional properties of CA activities, the patho-physiology of diabetes can be linked to modulation of CA activities in diabetes.¹¹ Studies have been conducted on the role of methylglyoxal in glycation of proteins to yield irreversible advanced glycated end products (AGEs), leading to cross-linking or degradation of proteins causing various chronic complications associated with diabetes.^{12,13}

Studies also report the association of MG levels with oxidative stress, as stress leads to depletion of cellular antioxidants by the overwhelming production of free radicals.¹⁴ Since the glyoxalase enzymatic activities are linked with the antioxidants status, oxidative stress indirectly causes increased MG levels, not merely by increasing its synthesis, but by decreasing the capability of MG degrading enzyme glyoxalase. Various studies have focussed on the toxicity of MG in diabetes, but very few studies have reported its effect on CA activities. Keeping in view all this, we designed the current study to establish the link between changes of CA activities in conditions with insulin resistance and also to observe whether MG could be responsible in those changes. The study tried to determine the serum levels

of MG and CA activity and also the CA activity in the erythrocyte haemolysates in patients having type-II diabetes mellitus with insulin resistance. The results were subsequently compared with age-sex matched healthy controls. Furthermore, in-vitro studies on erythrocytes were conducted to elucidate whether the increased levels of MG had any effect on the CA activity.

Patients and Methods

This case-controlled study was carried out in the Department of Biochemistry, College of Medicine and J. N. M. Hospital, Kalyani, West Bengal University of Health Sciences, India, for a period of one year from November 2009 to October 2010.

Eighty-six participants (46 males; 40 females) were enrolled for the study with ages ranging from 25 to 44 years. Forty-six insulin-resistant diabetic patients (Type II, NIDDM; 26 men and 20 women, 29-44 years of age) and 40 healthy controls (22 men and 18 women, 25-39 years of age) were recruited for the study. The patients were selected randomly from among those referred from the OPD for investigation. The sample size (n) was determined using the calculation $n = 4 \times (SD)^2/L^2$ (Mahajan, BK fifth edition)¹⁵ where allowable error (L) was taken as 1 and SD (of CA activity) taken as 3. The study was pre-approved by the Ethical Committee of the Institution Review Board. Informed consent from both patients and controls was obtained before their recruitment for the study. The inclusion criteria entailed patients with fasting blood glucose levels >126 mg/dl and BMI ≥ 25 kg/m². Patients with hypertension, dyslipidaemia, current smokers and those on insulin therapy were excluded. Also, patients on lipid-lowering drugs and antioxidant vitamin supplements were also excluded.

Twelve-hour fasting blood samples were collected from healthy volunteers and patients with insulin resistance. The patients selected for the study were registered in the Out Patients Department (OPD) of the College of Medicine & JNM Hospital, Kalyani. Ten ml of blood samples were collected from the participants, of which 5ml blood was collected in a sterile test tube, allowed to clot and then carefully centrifuged at 3000 rpm for 10 minutes. Clear serum was collected and kept in -4°C until tests were performed. Serum samples obtained were used for analysis of biochemical parameters. For in vitro study in RBC model, packed cells were prepared from the remaining 5 ml of blood collected with heparin. The blood samples were centrifuged at 3000 rpm for 10 minutes and the plasma and buffy coat were removed. After the packed red cells were washed with NaCl (0.9%), the erythrocytes were haemolysed with chilled distilled water. The ghost and intact cells were removed by centrifugation at 4°C , 20,000 rpm for 30 minutes. Fasting blood glucose (FBG) was assayed with the glucose oxidase

method. The insulin levels were determined from the patient's serum using ELISA method of assay. Methylglyoxals were estimated according to the modified form of Racker.¹⁶ Twenty-five μl of serum samples were added to 350 μl of DNPH [0.1% DNPH in 2N HCl]. Then to each tube 2.125 ml of distilled water was added. Then it was incubated for 15 minutes at 37°C . After the incubation, 1.5 ml 10% NaOH was added and absorbance was read at 576 nm using Microlab 300 semi-auto analyser. For CA, the assay system consisted of 100 μl of sample (serum) containing 1.4 mL of 0.05 M Tris-SO₄ buffer (pH 7.4) and 1.5 mL of 3mM p-nitrophenyl acetate. The change in absorbance at 348 nm was measured over a period of 3 minutes, before and after adding the sample. One unit of enzyme activity was expressed as 1 μmol of released p-nitrophenol per minute at room temperature.

Further, the erythrocytes were washed with normal saline, and haemolysate was prepared from the erythrocytes with distilled water and the CA activity was determined in the haemolysate from the patients and the healthy controls.

Further in vitro investigations were undertaken to demonstrate the effect of MG on insulin reactivity, on erythrocytes membrane integrity and erythrocyte CA activity:

Two experiments were done to study the effect of methylglyoxal RBC membrane. Two ml of packed cells were prepared from the heparinised blood of a normal volunteer. The packed erythrocytes were washed three times with normal saline. In the first experiment the packed RBCs were diluted (1:10) with the normal saline. One ml each of diluted packed cells was taken in six test tubes. Serial dilutions of MG were added to all the test tubes except for the first test tube where only normal saline was added. These were incubated at room temperature for one hour. Lactate dehydrogenase (LDH) levels of each of the samples were measured to assess the amount of haemolysis as a result of erythrocyte membrane damage using standardised reagent kits (E-Merck).

Evidence of RBC membrane damage was further confirmed by making hanging drop preparations from each of the test tubes and observing them under a microscope.

For in vitro effect of MG on erythrocyte CA activity, each of the above test tubes was centrifuged and the CA activity measured in the partially haemolysed contents as per the assay method already described.

For in vitro assessment of erythrocyte CA activity, the pH of the haemolysate was brought to 8.5 with solid Tris and thereafter assayed as described above. Acetazolamide inhibitor was simultaneously used in a parallel set of test tube in the dose of 10 microgram/ml to confirm the observation on the CA activity.

Methylglyoxal was procured from Sigma Chemicals, USA. Dinitrophenyl Hydrazine was from Merck chemicals

and the rest of the chemicals used were of analytical grade and purchased locally.

The data from the patients and the controls were compared by using the Student's t-test. The values were expressed as mean \pm standard deviation (SD). Microsoft Excel for Windows 2003 was used for statistical analysis. P-values <0.05 were considered as indicating statistical significance.

Results

The mean serum levels of insulin, fasting blood glucose, CA activity, MG and BMI was significantly higher ($p < 0.001$) in patients compared to the controls (Table). When analysed for relationship of CA activity with glucose concentration, it was found to be directly proportional to increasing blood glucose concentration (Figure-1). The haemolysate prepared from the erythrocytes of patients and controls also showed significantly higher ($p < 0.01$, two tailed, unpaired student's t test) CA activities (Unit/mgHb) in patients (12.4 ± 2.3) than controls (22 ± 2.8). A similar set of experiments done simultaneously with the same haemolysate samples after incubation with acetazolamide showed comparatively reduced CA activity in patients (2.9 ± 1.2) and controls (5.3 ± 1.6) (Figure-2).

When further in-vitro studies were conducted to

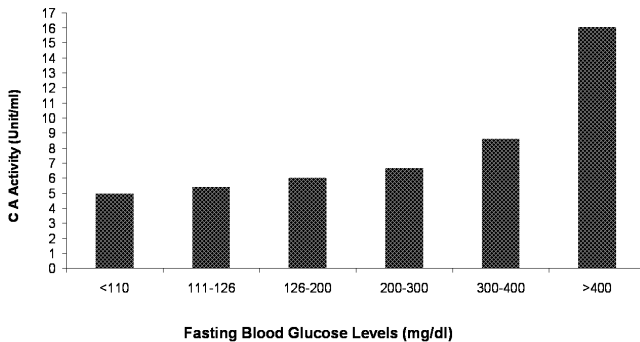


Figure-1: Serum Carbonic anhydrase activity in relation to different fasting blood glucose levels.

Table-1: Clinical and Laboratory parameters of Study Subjects.

Parameter	Healthy Controls (n=40)	Patients (n=46)	p Value
Age (yrs)	41.5 \pm 1.5	42.2 \pm 1.8	NS
Sex (M/F)	26/20	22/18	NS
Fasting Blood Glucose (mg/dl)	74.0 \pm 12.0	275.4 \pm 10.8	<0.001
BMI (kg/m ²)	21.67 \pm 2.87	25.87 \pm 3.98	<0.001
Insulin (Units/L)	5.3 \pm 1.1	23 \pm 0.5	<0.001
Carbonic anhydrase (Units/ml)	5.8 \pm 0.6	8.1 \pm 1.3	<0.001
Methylglyoxal (μ mol/L)	51.9 \pm 4.5	89.64 \pm 6.8	<0.001

FBG indicate Fasting Blood Glucose, AC indicate Carbonic Anhydrase Activity, MG indicate Methyl Glyoxal; NS indicates no significance; P 0.05 considered significant by unpaired, two tailed, student's t test.

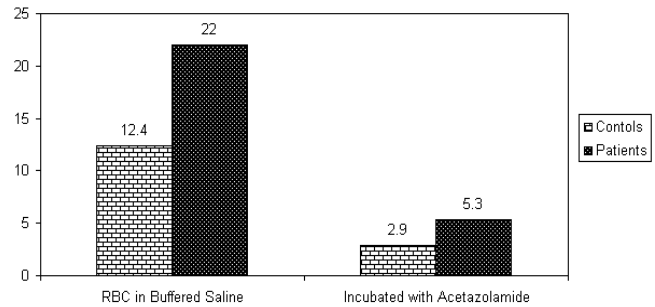


Figure-2: Carbonic anhydrase in hemolysate (Unit/mg Hb) prepared from patients and control RBC's.

Carbonic Anhydrase Activity in Haemolysates (U/mg Hb)

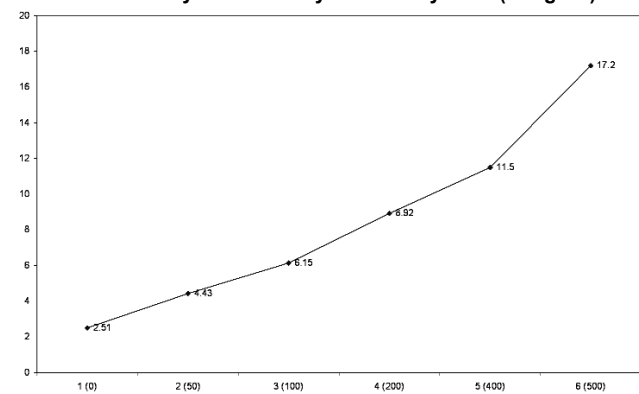


Figure-3: Carbonic anhydrase activity in haemolysate (U/mgHb) prepared from RBC's incubated with different concentrations of methylglyoxal.

CA Activity in Haemolysate (Unit/mg Hb)

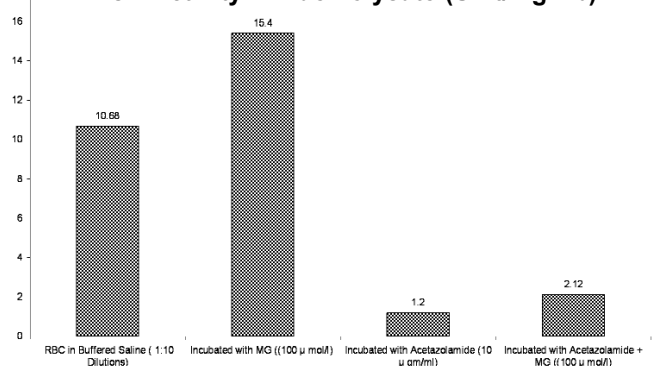


Figure-4: Carbonic anhydrase activity in haemolysate (U/mgHb) prepared from RBC's incubated with Normal saline, methylglyoxal and acetazolamide.

observe the role of MG on CA activity, we observed increased CA activity in a dose-dependent manner with methylglyoxal (Figure-3).

When haemolysate was prepared after RBC's incubated with methylglyoxal, the CA activity (Unit/mgHb) was found to increase (15.4 ± 1.8) significantly ($p < 0.05$,

unpaired t test) than the activity of the enzyme (10.68 ± 1.4), while the same RBCs incubated in the buffered saline and the esterase activity was purely due to the CA enzyme per se was proved by incubation with acetazolamide, which decreased the esterase activity (1.2 ± 0.45). Further, the activity of esterase was slightly increased (2.12 ± 0.72) when incubated with acetazolamide and methylglyoxal (Figure-4).

Discussion

The study observed significantly higher ($p < 0.0001$) levels of blood glucose and insulin levels in patients compared to the healthy controls which was similar to the observations of an earlier study conducted which had observed higher insulin and blood glucose levels in American Blacks.¹⁷

Our study showed that insulin resistant patients had higher BMI (overweight) which is supported by another study which had also observed similar findings and concluded that though obesity was an important risk factor for ischaemic heart disease, variations in BMI alone poorly reflected the risk of IHD associated with features of insulin resistance syndrome.¹⁸

Elevated methylglyoxal levels have been reported in insulin-resistance syndrome in previous studies. Our study also observed serum MG levels in insulin resistant patients to be significantly higher compared to the controls. Berlanga et al, 2005, earlier reported the toxicity of methylglyoxal in insulin resistance. Their study demonstrated that the toxicity of MG on rats by prolonged exposure resulted in impairment of wound healing and diabetes-like vascular damage. Their study also demonstrated the usefulness of MG in arresting cellular growth, but it also increased serum creatinine levels, induced hypercholesterolaemia (all $p < 0.05$) and impaired vasodilation ($p < 0.01$) compared with controls.^{1,19} This oxoaldehyde compound is formed significantly in diabetes, causes rapid glycation of proteins and thus deforms the functional properties of proteins.²⁰

The consistently elevated blood glucose flows to sorbitol pathway causes increased utilisation of Nicotinamide Adenine Dinucleotide Phosphate-Oxidase (NADPH), which subsequently increases trios-phosphate concentration and thus generates excessive amounts of MG.²¹ Our study demonstrated the lysis of erythrocytes in insulin-resistant patients by in vitro experiment with increasing concentrations of MG, supported by the increasing concentration of lactate dehydrogenase release from lysed erythrocytes.

Research based on MG toxicity on insulin signaling pathway has earlier demonstrated that MG impairs signal transduction independently by the formation of intracellular reactive oxygen species.²² Another study²³ has linked the role

of MG to different insulin resistance states, including diabetes and hypertension. That study also investigated the effects of MG on insulin signaling transduction. The experimental study involved Sprague-Dawley (SD) rats who were fed with fructose for nine weeks resulting in an insulin-resistant state, accompanied with increased triglyceride and insulin levels, high blood pressure and decreased insulin-stimulated glucose uptake by adipose tissue. Their study reported a close correlation between insulin resistance with elevated MG levels in serum and adipose tissue. It concluded that the increase in endogenous MG impairs insulin-signaling pathway and decreases insulin-stimulated glucose uptake in adipose tissue, which may contribute to the development of insulin resistance.²³

Changes in activities of CA have been associated with altered metabolism, especially in diabetes mellitus. A study which was conducted to investigate the possible role of CA in erythrocytes from normotensive and essential hypertensive subjects, reported CA activities to be statistically significant between normotensive and essential hypertensive. The CA activities were much lower in patients than the normotensive controls.²⁴

The CA activity as the total esterase activity in serum, observed in our study with insulin-resistant patients, was higher and increased with corresponding increase in blood glucose levels. This is in line with an earlier study which reported similar lines of observation.²⁵ Our findings also showed that the CA activity in the erythrocyte haemolysate was significantly higher in the patients with insulin resistance compared to the controls.

Our in vitro study showed that the haemolysate from control erythrocytes incubated with MG had increased CA activity in dose-dependant manner. In another set of experiment with specific CA inhibitor acetazolamide, the above findings were further confirmed. Thus MG mediated increase of CA activity is associated with the insulin resistance state. This alteration of the CA activity could be involved in changes of intra cellular micro pH level and consequently may alter the activity of any of the several enzymes involved in the insulin signaling pathway. Further extensive studies, however, are required on this aspect.

Our in vitro study also illustrated the dose-dependant decrease of insulin reactivity after incubation with MG. An earlier study demonstrated the formation of methylglyoxal-insulin adducts at an arginine residues of insulin molecule, when MG was incubated with insulin. A significant decrease of glucose uptake induced by MG-insulin adducts was also observed in skeletal muscle cells. MG alone had no effect on glucose uptake or the transcriptional expression of insulin receptor. Unlike native insulin, MG-insulin adducts did not inhibit insulin release from pancreatic β -cells. The

degradation of MG-insulin through liver cells was also decreased. Hence, MG modifies insulin by attaching to internal arginine residue in β -chain of insulin. The formation of this MG-insulin adduct decreases insulin-mediated glucose uptake, impairs autocrine control of insulin secretion, and decreases insulin clearance. These structural and functional abnormalities of insulin molecule may contribute to the pathogenesis of insulin resistance.¹² A study involving type-I diabetes mellitus patients looked for the toxic effects of reactive aldehydes MG and glyoxal in young, complication-free type-I patients by assessing activity of the ubiquitous membrane enzyme, Na⁺/K⁺ ATPase. Erythrocyte membrane Na⁺/K⁺ ATPase activity was elevated in diabetes mellitus patients compared to the controls which suffices the effect of methylglyoxal in erythrocyte membrane enzymes.²⁶

However, with the information based on the research on CA activity in insulin-resistant patients and with the observation from our study it is suggested that MG is a toxic compound whose synthesis corresponds with higher blood sugar levels, especially in diabetic with insulin resistance. The increased amount of MG involves glycation of proteins, which changes the structural and functional properties as evidenced by lysis of erythrocytes membrane, insulin reactivity and elevated CA activity in our study.

Conclusion

Insulin resistance is accompanied by increased activity of carbonic anhydrase which is significantly related to increasing methylglyoxal levels. Therefore, apart for normal routine blood glucose determination, it is suggested to measure serum methylglyoxal and carbonic anhydrase activity as well in patients with insulin resistance. Further study on CA activities and the effect of MG in these patients may help to find out new therapeutic targets which can modulate resistance to insulin.

References

- Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nat Rev Drug Discov* 2008; 7: 168-81.
- Berlanga J, Cibrian D, Guillen I, Feeyee F, Lopez-Saura P, Merino N, et al. Methylglyoxal administration induces diabetes-like microvascular changes and perturbs the healing process of cutaneous wounds. *Clin Sci* 2005; 109: 83-95.
- Puhlmann U, Schäfer D, Ziemann C. Update on COX-2 inhibitor patents with a focus on optimised formulation and therapeutic scope of drug combinations making use of COX-2 inhibitors. *Expert Opinion on Therapeutic Patents* 2006; 16: 403-30.
- Bonapace G, Iuliano F, Molica S, Peta A, Strisciuglio P. Cytosolic carbonic anhydrase activity in chronic myeloid disorders with different clinical phenotype. *Biochim Biophys Acta* 2004; 1689: 179-81.
- Parui R, Gambhir KK, Mehrotra PP. Changes in carbonic anhydrase may be the initial step of altered metabolism in hypertension. *Biochem Int* 1991; 23: 779-89.
- Gitto R, Agnello S, Ferro S, Vullo D, Supuran CT, Chimirri A. Identification of Potent and Selective Human Carbonic Anhydrase VII (hCAVII) Inhibitors. *Chem Med Chem* 2010; 5: 823-6.
- De Simone G, Supuran CT. Antiobesity carbonic anhydrase inhibitors. *Curr Top Med Chem* 2007; 7: 879-84.
- Supuran CT, Di Fiore A, De Simone G. Carbonic anhydrase inhibitors as emerging drugs for the treatment of obesity. *Expert Opin Emerg Drugs* 2008; 13: 383-92.
- Alvera A, Mente?ea A, Ereml C, De?era O, Koçakb M, Kehaa EE. Serum carbonic anhydrase autoantibodies in metabolic syndrome. *Diabetes and Metabolic Syndrome: Clinical Research and Reviews* 2009; 3: 211-3.
- Gambhir K K, Ornasir J, Headings V, Bonar A. Decreased total carbonic anhydrase esterase activity and decreased levels of carbonic anhydrase I isozyme in erythrocytes of type II diabetic patients. *J Biochem Genetics* 2007; 45: 431-9.
- Malheiro AJ, Gambhir KK, Bland GN, Abrams CK, Headings V, Abdul-Rahman M. Erythrocyte Total Carbonic Anhydrase Esterase Activity in African American Obese Children: Reduction Starts at a Young Age. *Biochem Genetics* 2009; 47: 322-8.
- Jia X, Olson DJH, Ross ARS, Wu L. Structural and functional changes in human insulin induced by methylglyoxal. *The FASEB J* 2006; 20: 1555-7.
- Derham BK, Harding JJ. Effects of modifications of α -crystallin on its chaperone and other properties. *Biochem J* 2002; 364: 711-7.
- Beisswenger PJ, Drummond KS, Nelson RG, Howell SK, Szweggold BS, Mauer M. Susceptibility to diabetic nephropathy is related to dicarbonyl and oxidative stress. *Diabetes* 2005; 54: 3274-81.
- Mahajan BK. *Methods in Biostatistics For Medical Students and Research Workers*. 6th ed. New Delhi: Jaypee Brothers; 1997: pp 92.
- Racker E. In Colowick SP and Kaplan NO (Editors). *Methods in Enzymology*. Vol. ZZZ. New York: Academic Press 1963; pp 283.
- Mack R, Skumick B, Sterling-Jean Y, Pedra-Nobre M, Bigg D. Fasting Insulin Levels as a Measure of Insulin Resistance in American Blacks. *J Applied Res* 2004; 4: 90-4.
- St-Pierre AC, Cantin B, Mauriège P, Bergeron J, Dagenais GR, Després JP, et al. Insulin resistance syndrome, body mass index and the risk of ischemic heart disease. *CMAJ* 2005; 172: 1301-5.
- Sheader EA, Benson RSP, Best L. Cytotoxic action of methylglyoxal on insulin-secreting cells. *Biochem Pharmacol* 2001; 61: 1381-6.
- Thornalley PJ. Methylglyoxal, glyoxalases and the development of diabetic complications. *Amino acids* 1994; 6: 15-23.
- Barski OA, Tipparaju SM, Bhatnagar A. The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. *Drug Metab Rev* 2008; 40: 553-624.
- Riboulet-Chavey A, Pierron A, Durand I, Murdaca J, Giudicelli J, Van Obberghen E. Methylglyoxal impairs the insulin signaling pathways independently of the formation of intracellular reactive oxygen species. *Diabetes* 2006; 55: 1289-99.
- Jial X, Wu L. Accumulation of endogenous methylglyoxal impaired insulin signaling in adipose tissue of fructose-fed rats. *Molecular and Cellular Biochemistry* 2007; 306: 133-9.
- Parui R, Gambhir KK, Mehrotra PP. Changes in carbonic anhydrase may be the initial step of altered metabolism in hypertension. *Biochem Int* 1991; 23: 779-89.
- Özdemir H, Kufrevioglu OI, Cetinkay R. Effects of Glycation on Erythrocyte Carbonic Anhydrase-I and II in Patients With Diabetes Mellitus. *Turk J Med Sci* 2000; 30: 135-41.
- Han Y, Randell E, Vasdev S, Gill V, Gadag V, Newhook LA, et al. Plasma methylglyoxal and glyoxal are elevated and related to early membrane alteration in young, complication-free patients with Type 1 diabetes. *J Molecular and Cellular Biochemistry* 2007; 305: 123-31.