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Correlation of uric acid levels and purine metabolism enzyme activities in plasma and liver tissues of diabetic rats

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The uric acid alteration in human beings causes major health problem due to its pivotal role in the etiology of many systemic diseases. The purine metabolism enzyme activities have a significant role in the process of elevated uric acid in diabetes mellitus. Hence, a study has been undertaken to understand the alteration of these enzyme activities in diabetic condition with an attempt to establish the possible cause for uric acid elevation. Alloxan was administered (150 mg/kg; *i.p.*) to induce diabetes in rats. Thirty days after alloxan induced diabetes, the enzyme activities were assessed in both plasma and liver tissues. The enzyme activities such as 5′-nucleotidase (5′-NT), adenosine deaminase (ADA), xanthine oxidase (XO) in the plasma and liver tissues were assayed by spectrometric technique and uric acid levels were also measured by Caraway procedure. A significant ($P< 0.001$) increase in 5′-NT, ADA and XO enzyme activities in plasma and liver tissue with a concomitant increase ($P< 0.001$) in uric acid levels was observed in diabetic group. The uric acid and the activities of enzymes did not change significantly in control group. From the present study, it can be concluded that an increase in uric acid levels noticed in diabetic condition may be due to increased catabolism of purines as evidenced by increased activities of 5′-NT, ADA and XO enzymes.

Keywords: 5′-Nucleotidase, Adenosine deaminase, Alloxan, Diabetes, Xanthine oxidase

Diabetes mellitus comprises a group of metabolic disorders that are characterized by chronic hyperglycemia associated with disturbances of carbohydrate, fat and protein metabolism due to defects in insulin secretion, decreased insulin sensitivity, or both[1,2]. The incidence of diabetes is gradually increasing mainly due to changes in eating habits and lifestyle which alter the functions of most of the organ systems such as cardiovascular, neuronal, excretory and reproductive systems[3]. Based on 2017 report, 451 million people are associated with diabetes worldwide and 90% of them have type 2 diabetes. It is expected to rise upto 592 million by 2035[1]. Alloxan induced diabetic model is well used to understand the complications as well as to test the therapeutic drugs[4].

The purine bases (both endogenous and exogenous) are constituents of nucleotides, which undergo a continuous process of synthesis, interconversion and breakdown. The catabolic process leads to the free purine bases, adenine and guanine which are enzymatically oxidized to form uric acid (UA) (Fig. 1). The absence, deficiency or exacerbated activities of the enzymes involved result in various inherited metabolic disorders that are characterized by abnormal concentrations of UA, purines and/or other metabolites in cells and body fluids[5].

Serum UA levels are an important risk factor of disease progression for a number of diseases, including those related to lifestyle. The metabolic syndrome and type 2 diabetes mellitus are two specific life style-related diseases, which often lead to common pathological complication[6]. By its antioxidant property serum uric acid exerts neuro-protective effects against Alzheimer's disease caused by oxidative injuries, and lowers the progression risk from impaired cognitive function to dementia[7,8]. Considerable evidence in the recent past has shown elevated UA levels in many diabetic subjects[9]. However, no studies have examined the association between purine metabolism enzymes

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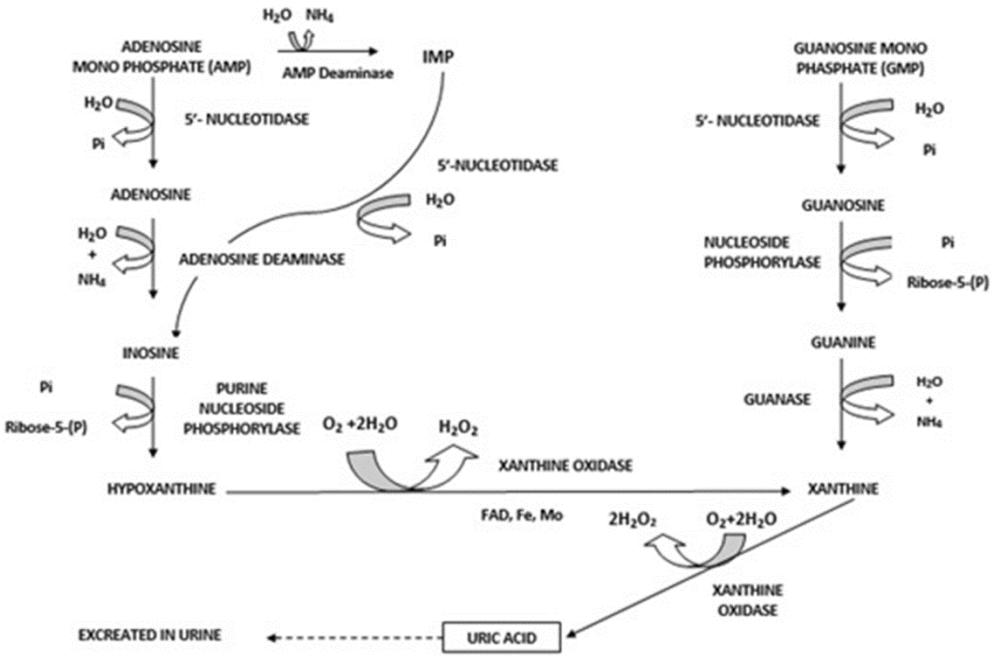

Fig. 1 — Purine nucleotide catabolic pathway

(5′-NT, ADA and XO) activities and the risk of developing hyperuricemia in diabetes.

Enzyme 5′-nucleotidase (NT) catalyzes the conversion of nucleoside monophosphates or deoxynucleoside monophosphates into nucleosides and deoxynucleosides by releasing more inorganic phosphates *via* hydrolysis of a phosphodiester bond. This enzyme, together with nucleotide kinase, regulates the pool of the nucleotides in cells[10]. Adenosine deaminase (ADA) is an important cytosolic enzyme involved in the metabolism of purine nucleosides; it catalyzes the irreversible hydrolytic deamination of both adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine, respectively[11]. Xanthine oxidase (XO) is an iron-sulfur molybdenum-containing cytosolic enzyme that plays a role in nucleotide metabolism. It is involved in the sequential oxidation of hypoxanthine to xanthine, and xanthine to uric acid and hydrogen peroxide in the purine catabolic pathway. XO is housekeeping and the rate-limiting enzyme in purine catabolism[12] (Fig. 1).

Some reports indicate that uric acid elevation is related to diabetic complications, whereas a few claimed that uric acid elevation is also seen in prediabetic condition[13]. The reason for this elevation of uric acid and a possible role of insulin in this regard are obscure and the data is inconclusive and controversial. Hence, this study explored the link between the alteration in 5′-NT, ADA and XO enzyme activities and uric acid levels in plasma and liver tissues of diabetic rats.

Materials and Methods

Chemicals

Alloxan monohydrate was procured from Sigma Aldrich chemicals (St. Louis, U.S.A). Adenosine deaminase and xanthine oxidase were purchased from Thermo Fisher (USA).Other chemicals and solvents were of Analytical Reagent (AR) grade, purchased from Randox Laboratories (UK).

Animals

Randomly selected healthy male Albino Wistar rats weighing 150-180 g were housed in polycarbonate cages in normal day-night cycle and temperature maintained at 22±2°C. The rats were fed with commercial rat feed (Amruth Rat Feed, supplied by Pranav Agro Industries, Pune, India) and had free access to water *ad libitum*. The animal experiments were conducted as per CPCSEA guidelines and this work was approved by Institutional Animal Ethics Committee (IAEC) of Basaveshwara Medical College and Hospital, Chitradurga (BMCH/IAEC/05 Biochem/2015, dated 04.06.2015).

Experimental design

For the experimental work, the animals were divided into 2 groups consisting of 6 animals in each group.

The rats which received normal saline (1 mL/kg b.w) orally were served as normal control; For another group of rats, diabetes was induced by using freshly prepared solution of Alloxan monohydrate dissolved in normal saline to 18 h fasted rats as a single dose (150 mg/kg; intraperitonally-*i.p.*). Animals were given 5-10% glucose to drink after alloxan injection to prevent hypoglycaemia. Hyperglycemia was confirmed by confirming elevated glucose levels in blood determined at 48 h after injection and the animals tested for glucose positive for 3 consecutive days. Rats with blood-glucose levels above 250 mg/dL were considered diabetic and selected for the study[14].

After a stipulated period of 30 days, the animals were anesthetized using isoflurane, and blood was collected in plain and heparinized tubes by retro-orbital puncture. The rats were weighed and sacrificed by injecting with sodium pentobarbitone immediately after sacrifice for assays. Blood plasma was separated by centrifugation (1700 × g, 15 min, 8°C) and immediately stored at −80°C. Liver tissue was aseptically removed and washed with saline and tissue homogenate was prepared using phosphate-buffered saline (PBS), pH 7.4 and maintained at −4°C until further assay. The plasma and liver tissues were then employed for the estimation of UA and for the assay of 5′-NT, ADA and XO activities.

Estimation of uric acid

Uric acid was estimated according to the method of Caraway[15]. To 5.4 mL of diluted tungstic acid, 0.6 mL of plasma sample was added, mixed well and centrifuged. To the test tubes 3.0 mL of supernatant, 0.6 mL of sodium carbonate and 0.6 mL of phosphotungstic acid reagent were added, mixed and placed in a 25°C water bath for 10 min. The blue colour developed was read at 660 nm. The uric acid levels were expressed as mg/dL. The liver tissue (100 mg) was homogenized in 100 mM Tri-HCl buffer (pH 7.4), with ten times the volume of tissue using a tissue homogenizer. A part of the homogenate was used to measure protein concentration and uric acid was analysed in cytosolic fraction (20 µg of protein) by phosphotungstic acid method and expressed as µg/mg protein.

Estimation of xanthine oxidase

The activity of XO was assayed by the method of Hashimoto *et al*.[16]. The liver tissue was homogenized in 9 volumes of ice-cold 0.25M sucrose. The sonicated homogenate was centrifuged at 4°C (10000 g for 20 min), and the supernatant was dialyzed against 100 volumes of the same solution at 4°C for 24 h, and assessed for protein content by Bradford method. 3.0 mL of incubation mixture included 150 µM phosphate buffer, 0.2 µM xanthine, 0.3 µM potassium oxonate and 10 µg of protein. The mixtures were incubated at 30°C for 30 min, added with 0.1 mL of 100% of TCA (trichloro acetic acid), mixed well, then centrifuged at 10000 × g for 15 min and supernatant was analysed for xanthine oxidase activity. The test samples and standards were read against the blank at 292 nm. One unit (U) of this enzyme is defined as the amount of enzyme forming 1 µM uric acid/min/mg protein. The xanthine oxidase activity in plasma was assayed with an assay kit (Biolab kits) and expressed as mU/mL.

Assay of adenosine deaminase

The activity of ADA enzyme was measured using adenosine substrate based on the colorimetric method described by Giusti and Galanti[17]. The absorbance of blue-colored complex formed at the end of reaction was measured using semi automated chemistry analyzer at 620 nm.

Assay of 5′-nucleotidase

The enzymatic assay was carried out in a reaction medium containing 10 mM $MgCl_2$, 100 mM NaCl, 5 mM KCl, 6 mM glucose and 50 mM Tris–HCl buffer, pH 7.4, at a final volume of 200 µL as described by Lunkes[18]. Twenty microliters of the enzyme preparation (8-10 µg of protein) was added to the reaction mixture and the pre-incubation proceeded for 10 min at 37°C. The reaction was initiated by the addition of AMP (Adenosine monophosphate) at a final concentration of 2.0 mM, and the time of incubation was 60 min. The reaction was stopped by the addition of 200 µL 10% TCA (final concentration 5%). The results are expressed as nm Pi released/ min/mg protein.

Statistical analysis

Data obtained from the control and experimental rats (n=6/group) are expressed as Mean ± SEM. For statistical analysis, data were subjected to Student's *t*-test. The Dunnett post hoc analysis was performed to compare between the groups. A level of $P < 0.05$ was taken as significant. The statistical analysis was done using the SPSS statistical package (version 22.0).

Results

Experimental data demonstrating the ADA activity in the plasma and liver of diabetic subjects are shown in (Fig. 2). These findings indicate that plasma and liver ADA activity in the diabetic group was significantly higher ($P < 0.001$) in comparison with control group.

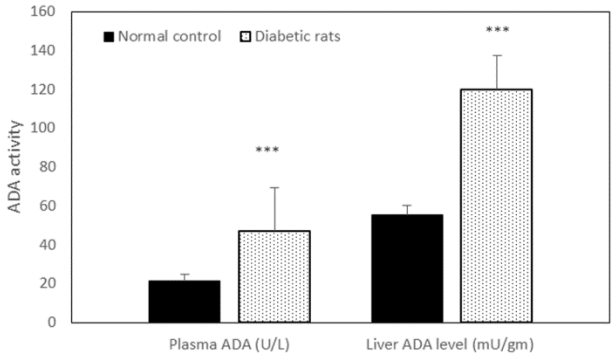

Fig. 2 — This graph depicts the plasma and liver ADA activities in experimental rats. Results are expressed as Mean ± SEM (n=6); *** P< 0.001 significantly different as compared to normal control group

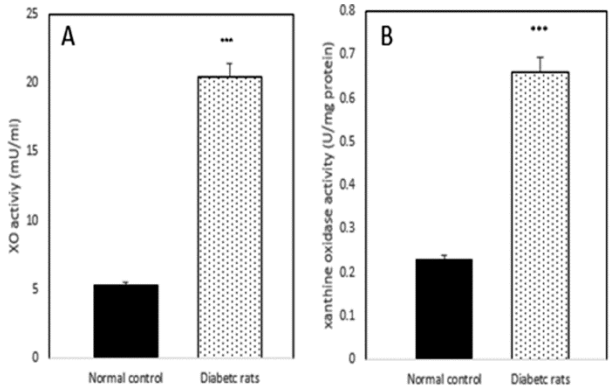

Fig. 3 — Depicts the xanthine oxidase activity in (A) liver tissue and; (B) plasma of normal control and diabetic rats. Results were expressed as Mean ± SEM (n=6) animals. *** P< 0.001 statistically significant as compared with normal control rats

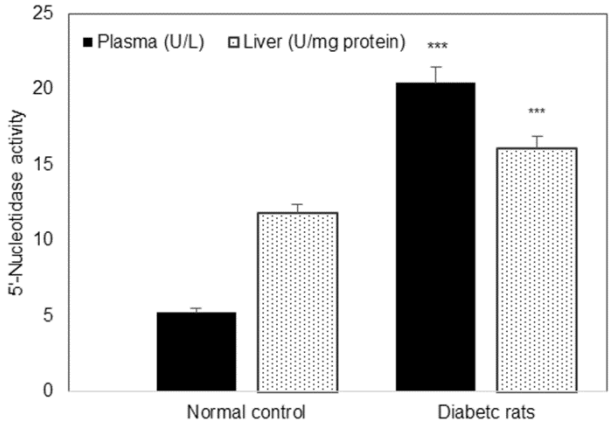

Fig. 4 — This graph depicts the 5′-nucleotidase activities in liver tissue and plasma of normal control and diabetic rats. Results are expressed as Mean ± SD (n=6); *** P< 0.001 statistically significant as compared to normal control group

We observed that there was significant (P< 0.001) increase in xanthine oxidase and 5′-nucleotidase activities in liver and plasma of diabetic rats when compared to the control group (Figs. 3A & B and 4).

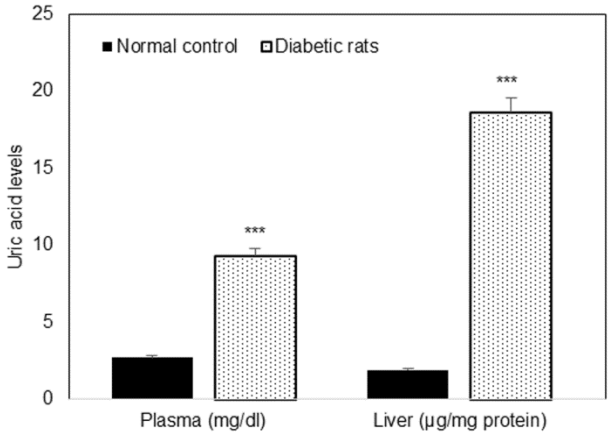

Fig. 5 — This graph depicts the plasma and liver uric acid levels in normal control and diabetic rats. Results are expressed as Mean ± SD (n=6); ***P< 0.001 as compared to normal control group

The results of the uric acid level in liver and plasma are shown in (Fig. 5). Diabetic rats showed a significant (P< 0.001) increase in uric acid levels than normal control group. The uric acid elevation is very well correlated with hyperglycemic condition.

Discussion

Uric acid (UA) is a purine derivative formed by the breakdown of purines and by direct synthesis from 5-phosphoribosyl pyrophosphate and glutamine. Elevated serum levels of UA have been shown to play an important role in many disease states including gout and articular degenerative disorders as well as vascular inflammation and atherosclerosis[19]. In Alzheimer's disease serum UA level has been significantly reduced, which support oxidative injuries could play an important role in the pathogenesis of AD[20]. Raised levels of uric acid in serum has been proposed to induce insulin-resistant state in the body at various conditions[21].

Uric acid formation and excretion are driven by several enzymatic pathways which occur *via* different genetically-defined isoforms, and are highly regulated by pathophysiological determinants including metabolic products and free radical species. Xanthine oxidase reductase represents the most relevant pathway involved in UA overproduction and offers significant perspectives for a better pharmacological approach for treating hyperuricemia-related vascular and non-vascular disorders in diabetes mellitus condition[22]. In this study, we investigated the connection between elevated UA levels in liver and plasma and the alterations in nucleoside metabolic enzymes.

ADA, 5′NT and XO are enzymes of purine nucleoside metabolism that play an important role in

the regulation of adenosine levels[23]. Adenosine deaminase is one of the key enzymes of purine nucleoside metabolism, participating in the conversion of adenosine to inosine and 2′-deoxyadenosine. These enzymes are able to regulate the extracellular concentrations of adenine nucleotides and nucleosides and play an important role in the maintenance of normal hemostasis and thrombogenesis[24]. Corroborating with the current results, a significant elevation of ADA activity in the tissues of diabetic rats induced with streptozotocin was noted by many researchers. Previous studies have proved that the insulin is involved in the regulation of ADA activity in diabetes[25]. However, it is not clear whether changes in ADA activity are the cause or result of altered insulin resistance. Increased ADA enzyme activity may reflect the decrease in adenosine circulation, leading to the declining of sensitivity by insulin and consequently the increase of glucose[26].

The enzyme ecto-5′-Nucleotidase is located in the tissues promoting the hydrolysis of AMP to its nucleoside, adenosine, in the extracellular medium[27]. Many studies reported that 5′NT expression was 60% higher in diabetes mellitus than the control, which is probably related with the thromboregulation process[28]. Significantly elevated 5′-Nucleotidase activity was also reported in the hypertensive (60%) and type 2 diabetic/hypertensive (53%) groups ($P< 0.01$) and compared with control and type 2 diabetic group[18]. This evidence supports the findings of present research study.

Experimental and clinical studies have proven that hyperuricemia is not only associated with gout but also diabetes mellitus. Uric acid is the reduced form of xanthine oxidoreductase (XOR) (known as xanthine oxidase (XO), and xanthine dehydrogenase (XDH), respectively) from hypoxanthine and xanthine which is generated *via* the purine degradation pathway[29]. Most of the xanthine oxidase enzyme is derived from the liver throughout the body in humans, during liver inflammation; XDH is released into the circulation and is subsequently converted into xanthine oxidase (XO) by proteases in the peripheral vasculature. Importantly, XO locally produces a considerable amount of reactive oxygen species (ROS), such as H_2O_2 and O_2[30]. In this context, we analysed the higher levels of uric acid with higher activity of XO in plasma and also in liver tissues, thereby uncovering hidden risks for the altered insulin action in type 2 diabetes mellitus.

Conclusion

In the present study, the elevation of adenosine deaminase, xanthine oxidase and 5′-nucleotidase activity was significantly correlated with insulin secretion and liver dysfunction. The changes in these enzyme activities were associated with high levels of uric acid in liver tissue and plasma of diabetic rats. An extended prospective study is required to establish the purine catabolism alterations and correlation with diabetes mellitus.

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