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Effects of Usnic Acid on Hyperglycemia and Renal Function in Streptozotocin-Induced Diabetic Rats

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Abstract

Background: Diabetic hyperglycemia and glomerular hyper filtration play a causative role in the progression of chronic kidney disease. Renal glucose handling via Sodium-Glucose Cotransporter (SGLT)-2 is a targetable approach and SGLT-2 inhibitors have proven therapeutic benefits in diabetic kidney disease. Usnic Acid (UA) is an active constituent of lichen species and symbiotic organism of algae and fungi, which is variously studied in folk medicine. The objective of this study was to demonstrate the beneficial effects of UA on glucose homeostasis and renal function in streptozotocin-induced diabetic Sprague-Dawley rats and to determine whether UA has an effect on regulation of SGLT that may further aid in glucoregulation and renal function.

Methods: Type 1 diabetes was induced in Sprague-Dawley rats with Streptozotocin (STZ, 60mg/kg) by intraperitoneal route on day 0. Diabetic rats were treated with UA (75 mg/kg) from day 15 to 35 via oral gavage. On day 35, urine was collected and Oral Glucose Tolerance Test (OGTT) was performed. After OGTT, blood was collected through cardiac puncture and kidneys were preserved for biochemical analysis. The results are expressed as mean ± standard error of the mean for n=8 rats per study group. The data were subjected to 1-way or 2-way ANOVA with Bonferroni's multiple comparison post hoc test using Graph Pad Prism 5 and were considered significant at $p \leq 0.05$.

Results: Diabetic rats chronically treated with UA had improved hyperphagia, hyperglycemia and glucose intolerance, glomerular hyper filtration, and urinary protein excretion ($p<0.05$). However, UA did not prevent loss of circulating insulin in diabetic rats. UA's blood glucose lowering effect was associated with enhanced diuretic-glucosuric response and decreased protein expression of renal SGLT-1 ($p<0.05$). While protein expression of SGLT-2 was partially increased in the diabetic kidney and it was not decreased by UA. Metabolic corrections with UA treatment occurred in parallel with reductions in uremia and improvement of renal function indices. Additionally, in diabetic rat kidney, UA treatment corrected oxidative changes.

Conclusions: Based on preliminary findings we conclude that chronic treatment of UA may act in an insulin-independent manner in lowering of diabetic hyperglycemia and improvement of renal function.

Keywords: Glucosuria; Hyperglycemia; Renal Function; Sodium-Glucose Cotransporter; Usnic Acid

Introduction

Diabetic hyperglycemia and its renal and cardiovascular complications contribute significantly to mortality and healthcare cost [1-4]. Hyperglycemia has been shown to promote glomerular hyper filtration [5], a phenomenon in diabetes/obesity that serves as an early predictor of chronic kidney diseases [6-8]. The concept of glomerular hyper filtration as a targetable mechanism to control kidney diseases is very old [9]. Nonetheless, hyper filtration is an incompletely and poorly understood process defined as an elevated baseline glomerular filtration rate. It may contribute to uremic toxicity, oxidative stress, inflammation and hypertension that over time results in kidney diseases. Control of hyperglycemia has been shown to significantly lower the incidence of kidney diseases. Therefore, any approach to lower hyperglycemia or hyper filtration will be beneficial.

The importance of renal glucose handling has been documented for many years. Complete reabsorption of filtered glucose by renal proximal tubule cells via luminal SGLT-2 and

SGLT-1 is of greater significance and recently became targetable tool in diabetes. SGLT-2 (low affinity and high capacity, 90-97%) and SGLT-1 (high affinity and low capacity, 3-10%) transporters are located in renal proximal tubule. Active reabsorption of glucose via SGLT isoforms is driven by an electrochemical gradient created by basolateral Na^+-K^+-ATPase where Glucose Transporter (GLUT)-2 facilitates passive transport of glucose back to venous circulation in favor of a glucose gradient. When renal glucose reabsorption is saturated, extra glucose spills in urine, i.e. glucosuria ensues. The intensity of glucosuria depends on glomerular filtration, blood glucose and renal transport capacity.

Lichens are symbiotic organisms formed by fungi and algae. Usnic Acid (UA) is one of the most extensively studied, widely distributed and variously utilized dibenzofuran metabolites of lichens. Multiple evidences have shown that lichen species containing one or both, the (+) and (-) enantiomers, of UA exhibit anti-cancer [10,11] and growth inhibitory actions against several microorganisms [12,13] and plant pathogens [14]. While, Okuyama, et al. [15] showed analgesic and anti-inflammatory properties of lichen components-UA and diffractaic acid in mice, Vijayakumar and colleagues [16] showed acute and chronic anti-inflammatory activity of (+) UA in Wistar rats. Administration of UA also has shown anti-ulcerogenic and anti-oxidative effects in rats [17]. UA containing lichen species have also been used in the treatment of tuberculosis [18] and even in folk medicine [19-21]. These effects have not been attributed to UA, yet. On the other hand, high dose of UA has been reported to cause oxidative hepatic injury [22,23] and uncommon adverse reactions [24-26]. As UA have shown anti-inflammatory, and anti-oxidative properties, we were interested to evaluate its beneficial effects in diabetes. In preliminary results, UA showed inhibition of SGLT in porcine derived proximal tubule cell line LLC-PK_1 (IC_{50} = 29 μM). To allow further therapeutic applications of UA, the present study was focused primarily to demonstrate the beneficial effects of UA on glucose homeostasis and renal function in streptozotocin-induced diabetic Sprague-Dawley rats and to determine whether UA has an effect on regulation of SGLT that may further aid in glucoregulation and renal function.

Material and Methods

Animal Treatment

Male Sprague-Dawley rats were obtained from Taconic Farms, Germantown, NY and were kept in a room maintained at a temperature of 23±1°C and in a 12hr light/dark cycle. All experimental groups were maintained on laboratory rodent diet (LabDiet® 5001, PMI Nutrition International, Brentwood, MO) and drinking water ad libitum. Animals were randomly divided into four groups (n=8 per group): normal rats treated with 10 mM citrate buffer (control, i.p.), pH 4.5; untreated diabetic rats (STZ); diabetic rats treated with UA (STZ+UA; 75 mg/kg/day, oral gavage, days 15-35, once daily). The dose of UA was selected based on earlier reports [15-17,23,27,28]. Experimental type 1 diabetes was induced by a single administration of STZ (60 mg/kg, i.p.) dissolved in citrate buffer (10 mM, pH 4.5) on day 0. STZ solution was freshly prepared each time. After 72 hrs of STZ administration, the non-fasting blood glucose concentration was determined in all animals on day 0,4,7,10,14,21,28 and 35, via the tail pricking method (~1 mm) using a commercially available glucometer (TRUE track, Nipro Diagnostics, Fort Lauderdale, FL) that utilizes the glucose oxidase method. On day 21,28 and 35, blood glucose was monitored at 0,4,8 and 24 hours post UA administration to determine its effectiveness in lowering of blood glucose. Animals having blood glucose over 300 mg/dL were considered diabetic and used for further studies. STZ and UA were purchased from Sigma Aldrich, San Diego, CA. All animal procedures were performed in accordance with guidelines established by the United States Department of Agriculture and were approved by the Institutional Animal Care and Use Committee of St. John's University, NY. Changes in body weight was monitored every week throughout the study. At the end of study (35 days) urine was collected while the animals were kept in metabolic cages to evaluate the effect of UA on excretion of glucose and renal function. On Day 35, Oral Glucose Tolerance Test (OGTT) was performed on all animals to evaluate the effect of UA on glucose intolerance. All overnight fasted animals were received oral challenge with glucose solution (2g/kg). Blood glucose for OGTT was measured via tail pricking method (~1 mm) using a commercially available glucometer before (0 min) and after (15,30,45,60,90,120,240 and 480 min) glucose challenge. After OGTT, the blood sample was collected into EDTA-coated tubes through cardiac puncture under isoflurane anesthesia and animals were euthanized. Blood was processed for plasma preparation (700g,30 min). The kidneys were excised by the freeze clamp technique and stored at -80°C. The kidney homogenate (10%w/v) was prepared in Phosphate Buffered Saline (PBS), pH 7.4, containing protease and phosphatase inhibitors.

Biochemical Analysis

Plasma insulin content was measured by solid-phase, two-side, direct sandwich ELISA (Calbiotech Inc., Spring Valley, CA). The urinary glucose excretion was measured using colorimetric assay kit (Procedure No. 510, Sigma Chemical Co., St. Louis, MO) representing a minor modification of the method [29]. Renal expression of sodium-glucose cotransporter (SGLT)-1 and SGLT-2 was quantified via direct sandwich ELISA (Mybiosource Inc., San Diego, CA). Urea Nitrogen (UN) and Creatinine (Cr) in plasma and urine were measured using colorimetric kit (Stanbio Laboratory, Boerne, TX) to estimate indices of renal function such as Glomerular Filtration Rate (eGFR), fractional excretion of

urea nitrogen (FE_{UN} (%) = 100X (urine urea/plasma urea)/ (urine creatinine/plasma creatinine)), blood urea nitrogen to creatinine ratio (BUN-to-Cr) and urine-to-plasma creatinine ratio (UP_{cr}). GFR was estimated as described earlier by Pestel [30]. Plasma and kidney homogenate samples were deproteinized using phosphoric acid and used for measurement of reduced (GSH) and oxidized glutathione (GSSG). The concentrations of GSH and GSSG in kidney and plasma was measured by a fluorometric method that uses o-phthalaldehyde as a fluorescent reagent [31]. Reduced and oxidized glutathione values were reported in micromole/mg tissue. Activities of catalase (CAT), Glutathione Peroxidase (GPx) and Super Oxide Dismutase (SOD) were determined by the methods of Aebi [32], Günzler and Flohé [33] and Misra [34], respectively, and reported as U/mg protein/min.

Statistical Analysis

The experimental results are expressed as mean ± S.E.M. (standard error of the mean) for n=8 per group. The data were subjected to 1-way or 2-way ANOVA with Bonferroni's multiple comparison post hoc test using Graph Pad Prism 5 and were considered significant at $p \leq 0.05$.

Results

Effect of UA on Body Weight, Food Intake, Plasma Insulin and Blood Glucose

Control rats showed normal body growth (mean: 290±5 g at day 0 to 368±11 g at day 35), while body weight of untreated or UA-treated diabetic rats remained statistically unchanged during study (Figure 1A). Similarly, type 1 diabetic rats showed significant hyperphagic response (STZ: 31.4±2.2-34.2±1.8 vs. control: 25.3±1.8-24.4±2.1 g/rat/day, days 4-35), which was reduced by UA (24.8±1.6-27.7±1.9 g/rat/day; days 4-35) (Figure 1B). Diabetic rats also showed typical STZ-induced hypoinsulinemia as compared to control rats (STZ: 7.5±0.1 vs. control: 15.4±3.4 mU/L). Treatment with UA did not prevent loss of insulin (Figure 1C). In diabetic rats, STZ-induced hypoinsulinemia was associated with persistent hyperglycemia throughout study period (STZ: 360±16-536±18 vs. control: 91±2- 121±6 mg/dL) (Figure 1D). On day 21, i.e. after one week of treatment, UA was able to control rise in blood glucose significantly (301±11, 302±10 and 392±50 mg/dL at 4, 8 and 24 hour) which remained similar on day 28 (316±16, 333±13 and 372±37 mg/dL at 4, 8 and 24 hour) and day 35 (300±15, 326±8 and 369±16 mg/dL at 4, 8 and 24 hour).

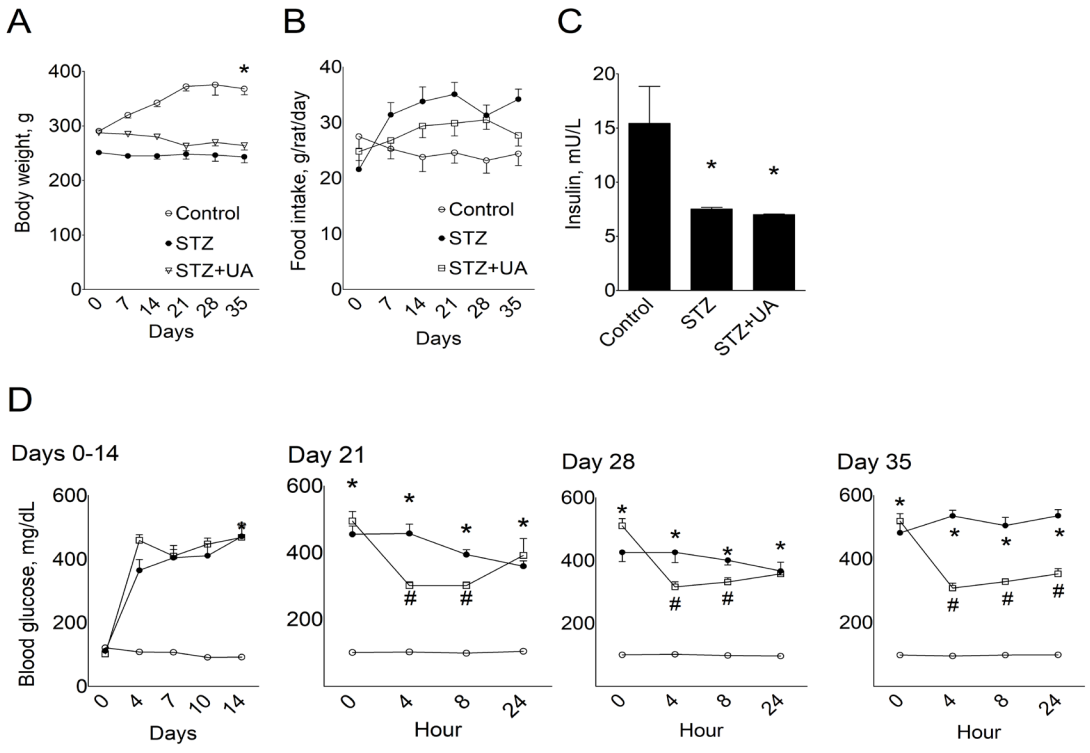

Figure 1: Effect on **(A)** body weight, **(B)** food consumption, **(C)** plasma insulin and **(D)** blood glucose before (days 0-14) and after (day 21, 28 and 35) treatment with Usnic Acid (UA) in Streptozotocin (STZ)-induced diabetic rats. Results are expressed as mean ± S.E.M. for n=8 per group. The results were subjected to 1-way (C) or 2-way (A, B, D) ANOVA with Bonferroni's multiple comparison post hoc test using Graph Pad Prism 5 and were considered significant at $p \leq 0.05$; *vs. control, #vs. STZ.

Effect of UA on Oral Glucose Tolerance Test

Diabetic rats showed significant glucose intolerance, i.e. higher blood glucose (554±15-442±18 mg/dL) persisted longer for 480 minute (Figure 2). Whereas normal control rats showed only modest increases in blood glucose (90±3 -174±13 mg/dL) which was normalized completely at 90 minute. Glucose intolerance in diabetic rats was significantly r-educed with UA treatment (325±23-472±24 mg/dL).

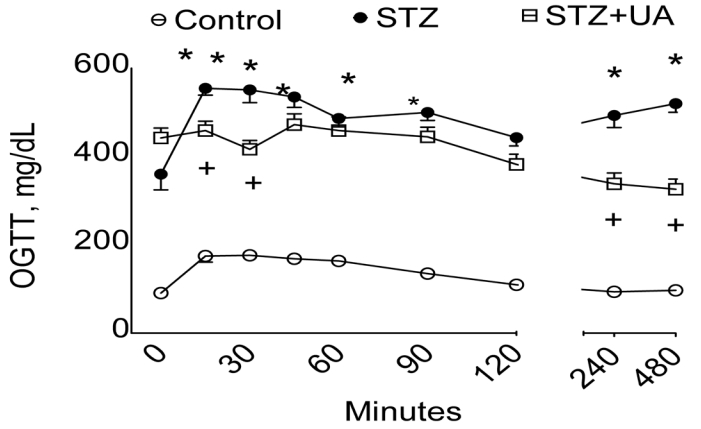

Figure 2: Effect of usnic acid on Oral Glucose Tolerance Test (OGTT) in Streptozotocin (STZ)-induced diabetic rats. On Day 35, overnight fasted animals were administered with UA or vehicle control 15 min before oral challenge with glucose solution (2 g/kg). Glucose levels were determined at 0,15,30,45,60,90,120,240 and 480 minute after oral glucose challenge. Results are expressed as mean ± S.E.M. for n=8 per group. The results were subjected to 2-way ANOVA with Bonferroni's multiple comparison post hoc test using Graph Pad Prism 5 and were considered significant at $p \leq 0.05$; *vs. control, +vs. STZ.

Effect of UA on Diuresis and Indices of Renal Function

In comparison to normal control rats, diabetic rats as expected showed significant diuresis (STZ: 68±5 vs. control: 15±1 mL/day) which was further increased by UA (81±4 mL/day) (Figure 3A). Correspondingly, diabetic rats also showed significant urinary protein excretion (STZ: 3.7±0.4 vs. control: 1.0±0.1 mg/h) which was reduced with UA treatment (2.8±0.3 mg/h) (Figure 3B). Untreated diabetic rats exhibited glomerular hyper filtration as determined by eGFR (STZ: 59.3±6.2 vs. control: 9.7±0.4 µL/min) which was reduced significantly with UA (41.8±2.8 µL/min) (Figure 3C). In the same way, index of renal excretory and reabsorptive capacity FE_{UN} (STZ: 240±37 vs. control: 105±6 %) (Figure 3D) and an index of acute kidney injury BUN-to-Cr ratio (STZ: 95±10 vs. control: 66±4) (Figure 3E) were markedly increased in diabetic rats which were normalized with UA treatment (FE_{UN}: 85±6; BUN-to-Cr: 61±6). However, UP_{cr}, an index of distal tubular water handling remained statistically unchanged in study groups (Figure 3F).

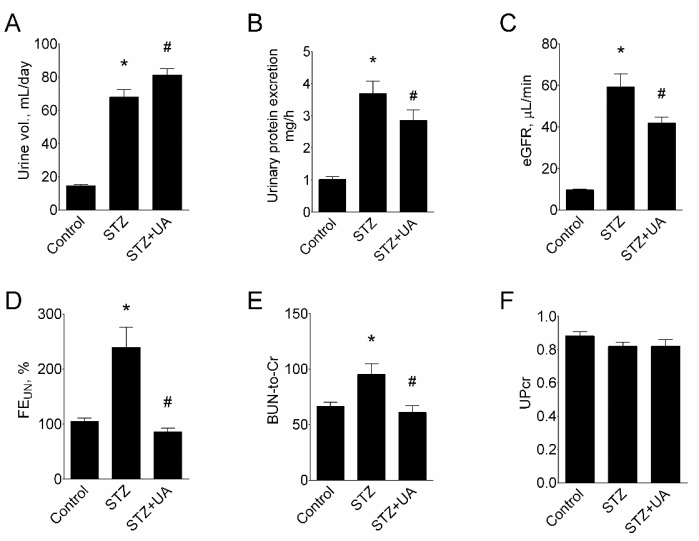

Figure 3: Effects of chronic treatment of usnic acid on **(A)** urine volume, **(B)** urinary protein excretion, **(C)** estimated glomerular filtration rate (eGFR), **(D)** fractional excretion of urea nitrogen (FEUN, %), **(E)** Blood Urea Nitrogen-to-Creatinine ratio (BUN-to-Cr) and **(F)** Urine-to-Plasma Creatinine ratio (UP_{cr}) in Streptozotocin (STZ)-induced diabetic rats. Results are expressed as mean ± S.E.M. for n=8 per group. The results were subjected to 1-way ANOVA with Bonferroni's multiple comparison post hoc test using Graph Pad Prism 5 and were considered significant at $p \leq 0.05$; *vs. control, #vs. STZ.

Effect of UA on Glucosuria and Renal Expression of SGLT-1 and SGLT-2

As expected, normal control rats showed negligible glucosuria, i.e. urinary excretion of glucose (1.9±0.2 mg/h) which was found significantly elevated in diabetic rats (20.3±2.5 mg/h) (Figure 4A). UA treatment increased glucosuric response further (32.0±0.8 mg/h) in diabetic rats. However, renal protein expression of SGLT-2, a predominant isoform responsible for glucosuric response remained statistically unchanged in study animals (Figure 4B). On contrary, protein expression of SGLT-1 was found increased in diabetes (STZ: 3.49±0.35 vs. control: 2.85±0.26 ng/mg protein) which was reduced with UA treatment (2.48±0.20 ng/mg protein) (Figure 4C).

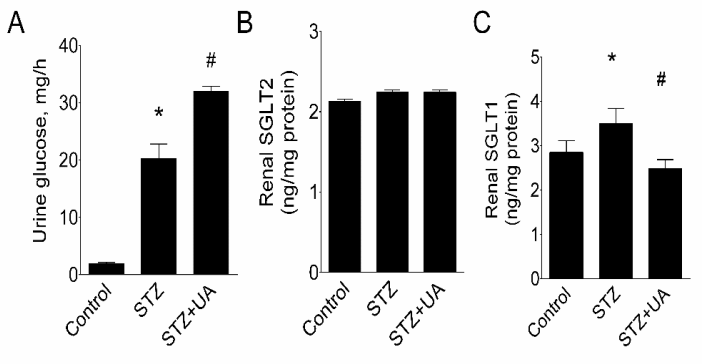

Figure 4: Effects of chronic treatment of usnic acid on **(A)** urinary excretion of glucose and renal expression of **(B)** SGLT-2 and **(C)** SGLT-1 protein in Streptozotocin (STZ)-induced diabetic rats. Results are expressed as mean± S.E.M. for n=8 per group. The results were subjected to 1-way ANOVA with Bonferroni's multiple comparison post hoc test using Graph Pad Prism 5 and were considered significant at $p \leq 0.05$; *vs. control, #vs. STZ.

Effect of UA on Indices of Oxidative Stress in Kidney

In general, oxidative stress in untreated diabetic rat kidney was found modestly affected in comparison to normal control rat kidney. In diabetic kidney, activity of SOD was found increased (STZ: 6.25±0.89 vs. control: 5.13±0.47 U/mg protein/min) which was reduced with UA treatment (4.65±1.23 U/mg protein/min) (Figure 5A). But activity of CAT was found decreased in diabetic kidney (STZ: 0.36±0.08 vs. control: 0.53±0.03 U/mg protein/min) and UA treatment prevented decreases in CAT activity (0.53±0.14 U/mg protein/min) (Figure 5B). Activity of GPx was found increased in diabetic kidney (STZ: 11.3±1.7 vs. control: 7.4±0.4 U/mg protein/min) which was normalized with UA treatment (6.6±1.1 U/mg protein/min) (Figure 5C). Of physiological merit, the GSH: GSSG ratio in diabetic kidney was decreased as compared to control (STZ: 0.29±0.02 vs. control: 0.33±0.03) which was again prevented with UA (0.37±0.04) (Figure 5D).

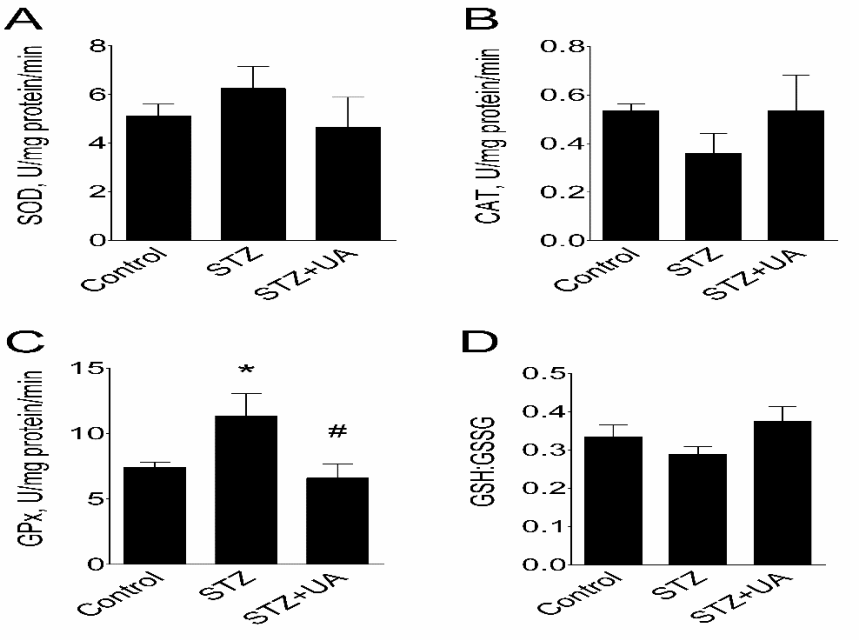

Figure 5: Effects of chronic treatment of usnic acid on activities of **(A)** Superoxide Dismutase (SOD), **(B)** Catalase (CAT), **(C)** Glutathione Peroxidase (GPx) and **(D)** Reduced Glutathione (GSH)-to-Oxidized Glutathione (GSSG) in kidney of Streptozotocin (STZ)-induced diabetic rats. Results are expressed as mean ± S.E.M. for n=8 per group. The results were subjected to 1-way ANOVA with Bonferroni's multiple comparison post hoc test using Graph Pad Prism 5 and were considered significant at $p \leq 0.05$; *vs. control, #vs. STZ.

Discussion

Rats treated with STZ showed typical responses of diabetes such as hyperphagia, hypoinsulinemia, reduced weight gain, persistent hyperglycemia, severe glucose intolerance and renal dysfunction. To the best of our knowledge, this preliminary chronic study is the first to demonstrate the beneficial effects of UA in STZ-induced rats with hyperglycemia and renal dysfunction. In this study the glucose lowering effect of UA was not associated with normalization of circulating insulin. This suggests it may have a potential role as an insulin-independent co-treatment with insulin secretagogues or insulin to lower diabetic hyperglycemia at any stage of diabetes. Such an insulin-independent approach possesses minimal risk of hypoglycemia with UA treatment. These results suggest that UA may have improved glucose metabolism since UA-treated diabetic rats also exhibited reduced glucose intolerance. It is likely that changes in appetite or energy expenditure with UA treatment may have occurred over time which contributed to changes in blood glucose. Although, further studies are needed to clarify such mechanisms.

Diabetic rats as expected showed a glucuretic response. Concurrent UA treatment further enhanced this response and may have added benefits in reducing extracellular volume and circulating glucose load. Results suggest, this benefit may not be due to a decreased number of SGLT-2 since renal expression of this isoform remained unchanged in study animals. Thus, it is likely that UA may either have reduced the activity of SGLT-2 or modulated the expression and/or activity of GLUT-2. These require further detailed investigation. Moreover, SGLT-2 inhibition has been shown to unmask SGLT-1 significantly (~40%) [35]. In agreement with this notion, untreated diabetic rats showed an increase in renal SGLT-1 expression that was reduced significantly by UA. So, it is likely that UA's glucuretic effects were also due to a reduced number of renal SGLT-1 isoforms. Vidotti, et al. [36] also have reported increased renal cortical expression of SGLT-1 protein after long term type 1 diabetes which was normalized with insulin therapy. In untreated diabetic kidneys, oxidative stress seemed to favor formation of hydrogen peroxide as activity of SOD was increased while CAT activity was decreased in our study. A compensatory detoxification of hydrogen peroxide may have led to a decrease in GSH: GSSG and increase in GPx activity. Yet, UA normalized such modest enzymic changes which is in agreement with past reports [17,20]. Oxidative stress in diabetes has been associated with increase in renal threshold of glucosuria and facilitated renal reabsorption of glucose via both SGLT isoforms. Based on preliminary results, it may be considered that chronic treatment of UA may have beneficial glucosuric effects that are partially dependent on its ability to mitigate oxidative changes.

While studying renal regulation of blood glucose it is important to determine renal function changes. Although glomerular hyperfiltration is an early reliable predictor of renal dysfunction, significant challenges exist for the field of hyperfiltration. A pathological decline in Glomerular Filtration Rate (GFR) might be indistinguishable from a 'beneficial' resolution of hyperfiltration over a short follow up. Moreover, renal function varies considerably when diuresis occurs, e.g. during diabetes. Therefore, analysis of multiple sensitive and specific indices would satisfactorily predict progression of renal function. Appearance of protein in urine as a function of glomerular filtration is a very sensitive marker of renal function [37]. Diabetic rats treated with UA showed a reduced rate of protein excretion which suggests UA may have protected the glomerular leakage of protein. Tubular epithelium is a barrier that regulates the composition of the glomerular filtrate. Urea Nitrogen (UN) and Creatinine (Cr) are filtered through the glomerulus. UN is reabsorbed, while Cr is neither secreted nor reabsorbed in renal tubules. Thus, Cr clearance does not indicate tubular function. Also, glomerular filtration of Cr is one of the factors that determines Cr concentration in serum [38]. Conversely, FE_{UN}, a function of urea and Cr clearance with glomerular filtration, is a sensitive and reliable predictor of renal excretory and reabsorptive capacity [39,40]. In diabetic rats, hyperfiltration with an increase in FE_{UN} indicates reabsorption uremia. BUN-to-Cr is also used in conjunction with FE_{UN} for gross determination of uremic abnormalities. Preliminary results suggest that chronic treatment with UA normalized reabsorption of urea and may have protected kidney against uremic toxicity in diabetic rats. Likewise, urine-to-plasma creatinine (UP_{cr}) ratio is a measure of urinary concentrating ability of distal tubules. Usually, increases in FE_{UN} is associated with a decline in UP_{cr} under severe renal damage. In the present study, UP_{cr} is partially depressed in untreated and UA-treated diabetic rats therefore we speculate that diabetes did not affect distal tubules. Additionally, histological analysis would support our finding and we do agree that absence of histological result is a severe limitation of our study.

Conclusions

Preliminary results of our chronic study show that UA may act in an insulin-independent manner to lower diabetic hyperglycemia [41] as well as may protect kidney against uremic abnormalities [42]. Based on results, it may appear that UA-mediated lowering of glucose is not sufficient to meet daily anti-hyperglycemic target, but this does not rule out the possible beneficial actions of UA in diabetic kidney diseases. Adjunct therapy of UA with insulin secretagogues or insulin may provide greater benefits, however further studies are needed to confirm the glucoregulatory and renoprotective mechanism(s) of UA in diabetes [43].

Consent for Publication: None.

Competing Interests: None

Funding: None

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