Irbesartan inhibits the formation of calcium oxalate stones in the kidney of diabetic rats

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Received October 21, 2016; Accepted November 14, 2017; Epub March 15, 2018; Published March 30, 2018

Abstract: Objective: To investigate the composition and formation mechanism of kidney stones and effect of irbesartan on the formation of nephrolithiasis in diabetic rats. Methods: Totally speaking, 60 Sprague & Dawley (SD) rats were selected (8 weeks-old, 240-280 g) and randomly divided into four groups as followings: control group, control-Irbesartan group, diabetic group, and diabetic-Irbesartan group. To each group, the oxalic acid was measured via potassium chromate oxidation of methyl red by catalytic spectrophotometry; the concentration of calcium ion (Ca²⁺) and uric acid were measured by the automatic biochemical analyzer 6 months later. Meanwhile, the physiological levels of angiotensin II and hyaluronic acid (HA), the expression of renal osteopontin (OPN), the pathological changes and the calcium oxalate crystals were detected under the help of ELISA assays, western blotting, HE staining, and environmental scanning electron microscopy one after another in kidney tissue. Results: As a result, the concentration of 24-hour uric acid in diabetic rats is significantly lower than that of normal rats (P < 0.05) and that in diabetic-Irbesartan rats is markedly lower than that of control-Irbesartan rats (P < 0.05). But to urine oxalate and calcium ion, compared with control-Irbesartan rats, the higher concentration can be detected in diabetic-Irbesartan rats (P < 0.05). Furthermore, the levels of hyaluronic acid and angiotensin II, the expression of OPN in the kidneys of diabetic rats are decreased by Irbesartan. At the same time, irbesartan ameliorated the abnormal pathological changes and hyaline degeneration of renal tubular epithelial cells in diabetic rats. Finally, the Irbesartan has an inhibitory effect on the formation of calcium oxalate crystals in diabetic rats. Conclusions: Irbesartan can protect diabetic kidney from abnormal pathological changes and calcium oxalate stones formation, which is related to preventing OPN expression.

Keywords: Irbesartan, diabetic, angiotensin II receptor antagonist, calcium oxalate kidney stones

Introduction

Urolithiasis is a growing health problem in industrialized countries and often correlated with habits, such as hypertension, high purine intake, diabetes, obesity and metabolic syndrome. Unexpectedly, 12% of the world’s population is affected by urinary system stone disease [1]. Urolithiasis is the formation of kidney stones in urine-collecting spaces of the kidneys. Under some certain conditions, substances normally dissolved in the urine can separate out as crystals and accumulate to form a solid mass called kidney stone. Stones could migrate into the ureters, the bladder and finally be evacuated in the urine [2].

With the rapid economic development and improvement of living standards, the prevalence of diabetes is rapidly increasing. Diabetes complicated with urinary tract stones in patients was common in clinical practice. Meydan N found that the incidence of urinary tract stones in diabetes was higher than that of urinary calculi incidence of ordinary people. It indicated that diabetes and urinary tract stones could have a certain relationship [3]. Lieske JC found that diabetes is a predisposing factor for kidney stones. They believed that there was a significant correlation in diabetes and kidney stones and further proved that the ratio of diabetic patients with uric acid stone formation is much higher than non-diabetic patients and
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diabetes was an independent risk factor for the formation of uric acid stones [4]. Daudon M showed that diabetes mellitus caused by the ability to damage the secretion of ammonia so as to reduce the pH value of urine. The reduction of urinary PH value is the main factor in the formation of uric acid stones, so diabetic patients are more likely to form uric acid stones [5].

The angiotensin-II-receptor antagonist Irbesartan was associated with better renal outcomes than the other agents (amlodipine, placebo, and antihypertensive agents) we used. In recent years, a large number of studies had shown that Irbesartan has the role of anti-diabetic renal fibrosis. Irbesartan can not only reduce the osteopontin (OPN) and HA expression in the kidney content [6], but also protect the renal cells by reducing the damage of the reactive oxygen species in the glomerular endothelial cells by reducing the content of Ang II in the kidney [7]. It can be seen that Irbesartan not only have the effect of protecting renal cell damage, but also has the effect of decreasing the expression of OPN and HA, which are not conducive to the formation of stones.

In this study, we established diabetic animal model to uncover the difference in the composition of the kidney stones compared with normal control, and the underlying mechanism. Then we further researched the act of Irbesartan on the formation of diabetic nephrolithiasis under hyperglycemia.

Materials and methods

Animals and ethics

Sixty male Sprague-Dawley rats (240-280 g) were obtained from the Laboratory Animal Centre of Medical College of Nanchang University (Jiangxi, China). All animal procedures were approved and conducted in accordance with the guidelines for the care and use of animal of the ethics committee of Nanchang University.

General reagents and equipment

All the reagents and assay kits were from commercial sources. Streptozotocin (STZ) was purchased from Solarbio Life Science (Beijing, China). Irbesartan was purchased from Haiyan Pharmaceutical Co., Ltd (Beijing, China). Both rat hyaluronic acid (HA) ELISA kit and angiotensin II (ANG-II) ELISA kit came from Xinyu Biotech Co., Ltd (Shanghai, China). BCA Protein Assay Kit was purchased from Tiangen Biotech Co., Ltd (Beijing, China). Microscope (OLYMPUS, Germany) was used for section observation.

Grouping and surgical procedures

The rats were randomly divided into four groups (15 rats each): control group (normal rats), control-Irbesartan group (normal rats treated with 50 mg/kg Irbesartan), diabetic group (diabetic rats), and diabetic-Irbesartan group (diabetic rats treated with 50 mg/kg Irbesartan).

Establishment of diabetic rats

SD rats were fed with high fat diet for 2 months before STZ injection, and then intraperitoneally injected with a single dose of STZ (50 mg/kg body weight) that dissolved in 0.01 M citrate buffer immediately before used [8]. After injection, animals had free access to food and water. Tail-vein blood glucose was detected on the third day after STZ injection. SD rats with blood glucose values > 16.7 mmol/L indicated successful diabetic model.

Urine sample and kidney tissues collection

Rats in control group and diabetic group were orally administrated with 0.2% carboxy methyl cellulose sodium (CMC-Na) as a vehicle every day. Meanwhile, rats in control-Irbesartan group and diabetic-Irbesartan group were orally administrated with 50 mg/kg Irbesartan (dissolved in 0.2% CMC-Na) everyday. After 6 months of treatment that mention above, the animals were fasted overnight. Then 24 h urine samples were collected and centrifuged at 3000 rpm for 10 min, the supernatants were stored at -80°C for the measurement of oxalic acid, calcium ion (Ca^{2+}), and uric acid. The oxalic acid of in each group was measured via potassium chromate oxidation of methyl red by catalytic spectrophotometry. Calcium ion (Ca^{2+}) and uric acid were measured by the automatic biochemical analyzer.

After urine collection, all rats were killed under ether anesthesia. The left kidneys were immediately removed and rinsed several times in iced saline, then stored at -80°C for subsequent
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Figure 1. The results of urinary oxalate, calcium ion and uric acid in 24-hour urine of SD rats. A: normal rats, B: normal rats treated with 50 mg/kg Irbesartan, C: diabetic rats, D: diabetic rats treated with 50 mg/kg Irbesartan. *p < 0.05, compared with A; †p < 0.05, compared with B.

Figure 2. The results of hyaluronic acid and angiotensin II in renal tissue of rats. A. The results of hyaluronic acid in renal tissue; B. The results of angiotensin II in renal tissue. A: normal rats, B: normal rats treated with 50 mg/kg Irbesartan, C: diabetic rats, D: diabetic rats treated with 50 mg/kg Irbesartan. *p < 0.05.

detection. The right kidneys were cut into blocks and fixed by 10% neutral formalin for slice observation. The protein expression levels of osteopontin (OPN) of the kidney tissues were quantified by western blot. The HA and ANG-II were measured by enzyme-linked immunosorbent assays (ELISA) according to the instruction of the kits. The pathological changes and histologic evaluation were observed by microscope after hematoxylin and eosin (HE) staining and Von Kossa staining, respectively (GENMED, Shanghai, China). Calcium oxalate crystals accumulation in kidney cells were observed by environmental scanning electron microscopy (Quanta 200F, FEI, America).

Western blot analysis

Total proteins were separated by 12% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Immobilon-P, Bio-Rad, America). Blots were incubated with OPN antibody (1:200, Bioss, China) and thereafter with HRP-conjugated goat anti-rabbit antibody (1:4000, Boster, China). Immunoreactive protein was detected using the enhanced chemiluminescence method according to the manufacturer (Amersham, Aylesbury, UK).

Statistical analysis

Results were derived from three independent experiments and analyzed with SPSS 17.0 software (SPSS, Chicago, USA). Data was shown as mean ± SD. Statistical analyses were performed using one-way analysis of variance. The P value < 0.05 was considered to be statistically significant.
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Results

Uric acid, oxalate and calcium ions in the urine of diabetic rats with high blood glucose and normal rats were not affected by Irbesartan

As shown in Figure 1, the 24-hour urine uric acid levels in diabetic rats and diabetic-Irbesartan rats were significantly lower than that in normal rats and normal-Irbesartan rats respectively (P < 0.05). On the other hand, the 24-hour urine oxalate and calcium ion concentration of diabetic rats were significantly higher than that in normal rats (P < 0.05). However, there was no significant difference between diabetic rats and diabetic-Irbesartan rats in urine uric acid levels, urine oxalate, and calcium ion concentration (P > 0.05).

Irbesartan decreased levels of hyaluronic acid and angiotensin II in renal tissues of rats

The levels of hyaluronic acid and angiotensin II in renal tissues of SD rats were determined. As shown in Figure 2A and 2B, angiotensin II and hyaluronic acid content in renal tissue of diabetic rats were significantly higher than that in normal rats (P < 0.05). Irbesartan could significantly inhibit the levels of hyaluronic acid and angiotensin II, indicating that Irbesartan played an important role in lowering the risk factors of kidney-stone formation in hyperglycemia and diabetes.

Irbesartan decreased OPN protein in renal tissues of rats

The content of OPN and β-actin in the kidneys were analyzed quantitatively by ImageJ 1.48u software. The percentage of gray bands were calculated and analyzed by LSD-t test. As shown in Figure 3A and 3B, compared with normal kidney, the expression of osteopontin in diabetic kidney was increased obviously. However, Irbesartan can decrease the expression of osteopontin in both normal and diabetic kidney interestingly. As a result, we can conclude that OPN expression in diabetic kidney was much higher than that of normal kidney and Irbesartan fights against the OPN expression in both normal and diabetic rats.
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The buildup of calcium oxalate crystals in kidney tissue was observed by Von-Kossa staining and environmental scanning electron microscopy. As shown in Figures 5 and 6, there were no calcium oxalate crystals in the kidney tissues of normal rats but opposite consequences in kidney tissue of diabetic rats. After treated with Irbesartan, the amount of crystals was reduced in diabetic kidneys. Consequences above indicated that Irbesartan can inhibit or delay the formation of calcium oxalate kidney stones in diabetic rats.

**Discussion**

24 hours Uric acid concentration detection showed that urinary uric acid concentration in diabetic rats was lower than that in normal rats. Also, uric acid concentration in normal rats treated with Irbesartan was lower than that in diabetic rats treated with Irbesartan. So urinary uric acid concentration in diabetic rats with high blood glucose was decreased and lower than that in normal rats. The causes may be as follows: first, elevated blood glucose level in diabetes rats causes increased insulin resistance [9], which could reduce uric acid clearance, leading to decreased insulin resistance [10]. Second, sharply and frequently fluctuated blood glucose level of day and night was more prone to damage the renal tubular cells [11]. With high blood glucose, accelerated renal dysfunction could lead to decreasing renal tubular excretion of uric acid, leading to decreased urinary uric acid. It’s well known that the reduction of urinary uric acid was not conducive to the formation of uric acid stones. So diabetes with high blood glucose was not conducive to the formation of uric acid stones.
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24 hours urinary oxalate, calcium ion concentrations detection showed that urinary oxalate and calcium ion concentrations in diabetic rats were higher than that of normal rats. On the other hand, urinary oxalate and calcium ion concentrations in normal rats treated with Irbesartan was higher than that in diabetic rats treated with Irbesartan. The reason for increased urinary oxalate in diabetes with high blood glucose may be as follows: lack of intestinal formic acid oxalic acid bacteria in diabetes reduced the decomposition of intestinal oxalic acid, leading to increased intestinal absorption of oxalate and increased urinary oxalate excretion [12]. Brian H Eisner and CT Lee have reported that urinary oxalate and calcium concentrations in the urine were supersaturated and were higher than those in normal urine [13, 14], which is consistent with our experiment results.

Renal Ang II examination showed that the renal Ang II content in diabetic rats was significantly higher than that in normal rats. On the other hand, Ang II in normal rats treated with Irbesartan was higher than that in diabetic rats treated with Irbesartan. Avidotti DB had put forward that in vivo Ang II under high blood glucose was increased in the study of angiotensin II metabolism mechanism of diabetic rats [15]. The conclusion was the same as our experiment result. As the increase in Ang II could cause renal cell damage by free radicals [16], our results indirectly indicated that renal cells were impaired in diabetes with high blood glucose. The renal paraffin section HE staining results showed that the renal cells in diabetic rats with high blood glucose were significantly impaired. Damaged renal cells may be caused by excessive free radicals under high blood glucose which has exceeded their own free radical scavenging rate [17], leading to directly damaging the renal cells [18]. From the above, we could see that renal cells were damaged in diabetes with high blood glucose. Damaged renal cells were conducive to the formation of calcium oxalate kidney stones. So diabetes with high blood glucose was conducive to the formation of calcium oxalate kidney stones.

OPN is a phosphate protein with a relative molecular mass of 44 Kda [19]. Approximately half of the amino acids of the protein are aspartic acids and glutamic acids. OPN have multiple binding sites including “integrin sites” and “cal-

Figure 6. The environmental scanning electron microscopy picture of Kidney tissues (x6000). A. Normal rats, B. Normal rats treated with 50 mg/kg Irbesartan, C. Diabetic rats, D. Diabetic rats treated with 50 mg/kg Irbesartan. Arrows: calcium oxalate crystal.
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cium ion binding sites” [20, 21]. The integrin sites of OPN could be attached to cells by binding to multiple integrin receptors on the cell surface [22]. The calcium binding sites of OPN were prone to bind calcium ions in calcium oxalate. OPN is not only related to bone metabolism, cardiovascular system, and tumor, but also related to the urinary system. Free-state normal-structured OPN is an inhibitor of calcium oxalate crystal growth and aggregation [23]. However, renal epithelial cells that can’t be attached with calcium oxalate crystals can be converted to renal epithelial cells to promote the formation of calcium oxalate stones [24]. Thus, OPN can promote the formation of calcium oxalate kidney stones instead of inhibiting it. According to the results of HA content in the kidney, we knew that the HA content in the kidney of diabetic rats was higher than that of normal rats. Meanwhile, HA content in normal rats treated with Irbesartan was higher than that in diabetic rats treated with Irbesartan. Study by Yevdokimova N Y has confirmed that in the high-glucose environment, renal synthesis of HA was elevated through increasing in renal HA content and prostaglandins [25]. HA cannot only moisturize, but also prevent arteriosclerosis and cancer. Also, HA is closely related to the formation of urinary tract stones. A large number of studies have shown that HA was one of the main adhesion molecules of calcium oxalate crystals, promoting the formation of calcium oxalate stones [26]. In the diabetic kidney under high blood glucose, OPN and HA contents promoted the formation of calcium oxalate kidney stones.

We know that urinary oxalate and calcium ions in the urine are super-saturate in diabetes. At the same time, damaged kidney cells, high-expressed OPN and HA were risk factors to promote the formation of calcium oxalate stones [27]. Therefore, calcium oxalate kidney stones are possibly formed in diabetes.

With von Kusa staining, calcium oxalate kidney stones in diabetes were observed [28]. We observed black oxalate crystal deposition in diabetic rats. Although von Kusa staining can detect calcium oxalate, it still has defects. Thus we used environmental electron microscopy to further confirm the calcium oxalate crystal accumulation on renal cell surface. We found calcium oxalate crystal adhesion on diabetic kidney cell surface. But Irbesartan could effectively prevent calcium oxalate crystal adhesion on renal cell surface, indicating that Irbesartan could inhibit or delay the formation of calcium oxalate kidney stones in diabetes.

According to the detection of related factors in the formation of calcium oxalate kidney stones and renal pathological changes, we found that uric acid, oxalate and calcium ions in the urine of diabetic rats with high blood glucose and normal rats were not affected by Irbesartan. But Irbesartan could reduce OPN, HA, and Ang II contents in normal rats and diabetic kidneys and weaken renal cell damage. In conclusion, Irbesartan inhibited or delayed calcium oxalate kidney stone formation in diabetes was related to reduced OPN and HA contents and weaken the renal cell oxidative damage, which finally prevented the aggregation and adhesion of calcium oxalate crystals.

Disclosure of conflict of interest

None.

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