Regulatory effect of mangiferin on kidney function and in serum uric acid through hyperuricemia model mice experiment

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Abstract: Objective: It analyze the effect of magiferin on uric acid metabolism and interference on the urate re-absorption and delivery in vivo. It further study the role of three effector molecules, glucose transporter 9 (GLUT9), organic anion transporter 10 (OAT10) and urate anion exchanger 1 (URAT1) in these progress; Methods: An animal model was established by once a day intraperitoneal injection of uric acid with dose of 300 mg·kg⁻¹·d⁻¹ for 14 days, which suffer from hyperuricemia in long time. Meanwhile, it used benzbromarone as positive control, and normal saline as negative control. The expression of GLUT9, OAT10, and URAT1 was detected by RT-PCR and Western blot, respectively in gene and protein expression level; Results: Magiferin could significantly reduce the level of serum uric acid on model animals, which treated with mangiferin with dose of ≥ 3.0 mg·kg⁻¹·d⁻¹. But the benzbromarone almost needed more than dose of 25 mg·kg⁻¹·d⁻¹ to get the same effect in the parallel experiment. Furthermore, mangiferin gastric administration could inhibit the expression of GLUT9, but benzbromarone couldn’t; on the other hand, mangiferin did not interfere the expression of URAT1 and OAT10; Conclusion: Mangiferin could reduce GLUT9 expression both at gene and protein level on the hyperuricemic model mice, which result the reduction of re-absorption and delivery of urate.

Keywords: Mangiferin, hyperuricemia, GLUT9, animal model

Introduction

Uric acid is a product of the metabolic breakdown of purine nucleotides. High blood concentrations of uric acid can lead to gout, which associated with other disease including diabetes and the formation of ammonium acid urate kidney stones [1].

Hyperuricemia is an abnormally high level of uric acid in the blood. In the low pH conditions, uric acid exists largely as urate, as ion form [1, 2]. The amount of urate in the body depends on the changes of the amount of purines eaten, the amount of urate synthesized, and the amount of urate excretion [2]. In humans, the upper limit tolerance of the normal range is 360 µmol/L (6 mg/dL) for women and 400 µmol/L (6.8 mg/dL) for men [3].

Many factors contribute to hyperuricemia, including: genetics, insulin resistance, hypertension, hypothyroidism, renal insufficiency, obesity, diet, usage of diuretics, and alcoholic drinking [4, 5]. Causes of hyperuricemia can be classified into three types: [6] increased production of uric acid, decreased excretion of uric acid, and mixed type. Causes of increased production include high levels of purine in the diet and increased purine metabolism. Causes of decreased excretion include kidney disease, certain drugs, and competition for excretion between uric acid and other molecules. There are two contributing factors as uric acid excretion disorder and re-absorption of urate in the renal tubule for the mixed type [6]. It is a balance between them to maintain the uric acid at normal levels. Once that balance was broken, it caused hyperuricemia or other disease.

Solute carrier family 2, facilitated glucose transporter member 9 (GLUT9) is a protein that in humans is encoded by the SLC2A9 gene. This gene encodes a member of the SLC2A facilitate glucose transporter family. Members of this family play a significant role in maintaining glu-
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cose homeostasis. SLC2A9 has also recently been found in transporting uric acid process, and genetic variants of the transporter, which link to increasing risk of development of hyperuricemia, gout and Alzheimer’s disease.

Urate anion exchanger 1 (URAT1), encoded by the SLC22A12 gene, is a urate transporter and urate-anion exchanger which regulates the level of urate in the blood. This protein is an integral membrane protein primarily found in kidney tissue. Two transcript variants encoding different forms have been found for this gene, which altered re-absorption of uric acid [3, 4]. It expression change contributed to hyperuricemia.

An organic anion-transporting polypeptide (OATP) are present in the lipid bilayer of the cell membrane, acting as the cell’s gatekeepers. The organic anion transporter 10 (OAT10) has relationship with the uric acid metabolism. According to Le Chatelier’s principle, lower concentration of uric acid would interfere crystals of uric acid existing in the blood, if the dissolved uric acid can be excreted opportune. In contrast, the high concentration of uric acid crystals would accumulate in the joints and other tissues.

Medications most often used to treat hyperuricemia are of two kinds: xanthine oxidase inhibitors and uricosurics. Xanthine oxidase inhibitors decrease the production of uric acid, by interfering xanthine oxidase. Uricosurics increase the excretion of uric acid, by reducing the re-absorption of uric acid once the kidneys have filtered it out of the blood. Some of these medications are used as indicated. Several other kinds of medications have potential usage in treating hyperuricemia. Mangiferin is a xanthone molecule which is a glucoside of norathyril, finding in mangoes. Laboratory study has identified a variety of potential pharmacology associated with mangiferin, including antimicrobial and antioxidant activities, blocking the type II 5α-reductase in the body.

It has variety of potential effect, including scavenging oxides [9], reducing blood sugar levels [10], improving renal function [11], hepatoprotective action [12] and inhibits tumor [13].

However, whether mangiferin could decrease the level of uric acid in the blood by promoting uric acid excretion has not been established [14]. In this study, firstly, it constructed a hyperuricemia animal model by injection of large doses of uric acid. Then studied the effect of mangiferin on model animals. Furthermore, three genes (GLUT9, URAT1 and OAT10) as described above, related uric acid metabolism, were detected its expression level, and analyzed its relationship with intervention effect of mangiferin. All these findings might be useful for understanding the molecular mechanism of mangiferin effect clearly.

Materials and methods

Reagents

Uric acid was purchased from Sigma (St. Louis, MO, USA); mangiferin was extracted from the Biomedical Engineering Research Center, Kunming Medical University (Kunming, Yunnan, China); Benzbromarone was purchased from Herman Big Pharma (Germany).

Animal model experiment

70 Kunming strain male mice, with weights ranging from 18-22 g, were purchased from Sichuan Chengdu Biological Technology Co., Ltd. (Certificate No. SCXK2013-24, Chengdu, China). Animal model construction programs were carried out by intraperitoneal injection of mice with uric acid (pH 8.0) twice everyday at the dose of 150 mg·kg\(^{-1}\) for 14 days.

The intervention programs were as following: all the male mice (n=70) were randomly divided into three groups, as negative group treated with phosphate buffer saline (PBS, pH 8.0), positive group treated with benzbromarone (dose of 25 mg·kg\(^{-1}\)·d\(^{-1}\)) and experiment group with four ladder concentration gradients. Those different concentrations were with 1.5 mg·kg\(^{-1}\)·d\(^{-1}\), 3.0 mg·kg\(^{-1}\)·d\(^{-1}\), 6.0 mg·kg\(^{-1}\)·d\(^{-1}\) and 100.0 mg·kg\(^{-1}\)·d\(^{-1}\). In addition, it took 0.5% sodium carboxymethyl cellulose (CMC-Na) once a day for 14 days, in both experimental group and positive group. All animals were under intragastric administration.

Tested uric acid concentration in serum and liver

After different ways of intervention 14 days, all the mice were bled via the retroorbital plexus,
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then their blood and liver were collected to test the uric acid concentration by the phosphotungstic acid method [15]. A reagent kit using reduction phosphotungstic acid was employed to determine the serum uric acid levels according to the Carroll’s method [15]. As for testing for liver, it used 100 mg of homogenized frozen liver tissue samples. Additional, the 9-fold dilution was necessary, by double distilled water. Used the reduction phosphotungstic acid kit for this experiment, according to instruction manual.

Pathological examination of renal tissue

After mice dissection, it separated the renal tissue and fixed them in neutral formaldehyde solution (40 g/L) for 24 h. Then those samples were taken hematoxylin and eosin (HE) staining in Department of Pathology of Basic Medical College, Kunming Medical University. This Department taken photograph and made a pathological diagnosis for these samples.

Detected gene expression by RT-PCR

Separated the renal tissue and stored in liquid nitrogen. Grind these tissues and extracted the total RNA. The RNA was extracted following the instruction of the RNA Mini kit (Qiagene, Germany). The concentration and purity of RNA were measured as absorbance value at 280 nm and 260 nm and the ratio of A260/A280 ratios with value of between 1.8 to 2.1 was used for subsequent experiments.

Based on the instruction of reverse transcription kit (TAKARA), reverse transcription reaction mixture was prepared (2 × miRNA RT buffer 10 μL; 0.1% BSA 2 μL; miRNA reverse transcriptase mixture 2 μL; RNA quantification to 0.5 μg, plus Rnase-free water to the total volume of reaction solution 20 μL) with reaction at 37°C for 60 min, at 85°C for 5 s. The synthesized cDNA were stored at -20°C. All the used primers were list in Table 1. Internal control gene was used the GAPDH.

The PCR reaction conditions were as following: denatured at 94°C for 30 sec, annealed at 57°C for 35 sec, and extended at 72°C for 2 min. There were 30 cycles for mGLUT9 and mURAT1, but 29 cycles for mOAT10. The PCR product were electrophoresed on 1.5% agarose gels, visualized with Bio-Rad Gel Doc XR+, and then quantified using Bio-Rad Quantity One 1-D analysis software.

Tested the protein expression by western blot

Freeze the samples of renal tissue -80°C and thawed them at room temperature, for two times. The mixture was centrifuged at 10000 g for 30 min at 4°C (Thermo, USA). The supernatant was collected and analyzed using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then transferred the proteins onto a polyvinylidene difluoride (PVDF) membrane (Millipore; California). The membrane was blocked with 5% BSA Tris-HCl buffered saline and 0.05% Tween-20 for 2 h at 25°C. It incubated with primary antibodies of mouse anti target protein monoclonal antibody and secondary antibodies of rabbit anti mouse monoclonal antibodies. The primary antibody for GLUT9, URAT1 and OAT10 were got from Sigma (St. Louis, MO, USA). The secondary antibodies were got from Abmart, Inc. (Shanghai, China).

It observed the gray bands and calculated the protein expression, according to value of Western Blot bands gray, using an enhanced chemiluminescence system (ECL, Bestbio, China), following the manufacturer’s protocol. The β-actin was used as internal quality control.

Statistical analysis of data

All data were represented as means ± SD (± s) of three or more independent experiments. If the Data are homogenous, analysis of variance, Student-Newman-Keuls and Pearson’s correlation will be used. If the data are not homogenous, Kruskal-Wallis, Games-Howell test, as well as Spearman Rank correlation analysis will

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
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<tbody>
<tr>
<td>URAT1</td>
<td>F 5’-GCTACCAGAATCGGCACGCT-3’; R 5’-CACGGAAATCCCAAATTC-3’</td>
</tr>
<tr>
<td>GLUT9</td>
<td>F 5’-GAGATGTCATTGAGGGAGC-3’; R 5’-GTGCACTTCTGCAGCGG-3’</td>
</tr>
<tr>
<td>OAT10</td>
<td>F 5’-GAAAGAACCTGAAGAACCTC-3’; R 5’-TCTGCACCAAATCTCGC-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F 5’-CAAGGATCCATGACAAACTTG-3’; R 5’-GTCACACCCCTGTCTGTAG-3’</td>
</tr>
</tbody>
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![Figure 1](image1)

**Figure 1.** Effects of mangiferin on serum uric acid level in the hyperuricemic mice. (means ± SD) (means ± S.E.M). ***P > 0.001 vs. normal group, #P < 0.01, ###P < 0.001 vs. model group, &&&P < 0.001 vs. Benzbromarone (t-test).

![Figure 2](image2)

**Figure 2.** Effects of mangiferin on hepatic homogenate uric acid levels in hyperuricemic mice (P > 0.05) (means ± S.E.M).

14 days. It detected their uric acid level in serum and calculated the changes trend. **Figure 1** illustrated that mangiferin could reduce the serum uric acid in some ways.

The benzbromarone used for positive group, PBS for negative group. It found that mangiferin intragastric administration can decrease the serum uric acid level in experimental group with dose of 3.0 and 6.0 mg/kg once a day in hyperuricemic mice. There was significant differences between experimental group with dose ≥ 3.0 mg/kg and negative control group in decreasing the uric acid level (**P < 0.01).

In addition, the efficiency threshold dose of mangiferin was 6.0 mg/kg. Even using the dose of 100 mg/kg in the experimental group, it can’t get the more effects. Furthermore, after compared with the positive control group with benzbromarone (dose of 25 mg/kg), treatment effect with mangiferin was more obvious.

The change of uric acid level in liver tissues

It separated the liver of mice after intervention, and detected the uric acid in liver tissues. However, after calculation, there was no significant difference between control group and experimental group with four different doses of mangiferin in the change of serum uric acid levels. The benzbromarone couldn’t cause any change of that level too. The result was showed in **Figure 2**.

Histopathological test for kidney

To investigate the improvement effect of mangiferin in kidney, renal tissues were separated and stained to be made pathological observation. After detecting, the 5-10% thickening and volume hypertrophy of glomerular mesangial cells could be observed. It found that 1-5% glomerular capillary expansion, renal tubular epithelial cell vacuoles degeneration, and renal

be used. All the analyses were carried out using the SPSS19.0 software (SPSS Inc, Chicago, IL, USA). Values less than 0.05 were considered to be statistically significant.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Kunming Medical University (Permit Number: KMUA201319-337851).

**Results**

**Successful modeling**

Under intraperitoneal injection of uric acid twice daily with dose of 150 mg·kg⁻¹ for 14 days, the uric acid levels of model mice serum increased enough higher (P < 0.001) than before injection. It indicated that intraperitoneal injection of uric acid could increase its concentration in serum, and the program of construction hyperuricemic animal model was success.

**Effective Intervention by mangiferin**

The intervention of different dose of mangiferin was carried out on the hyperuricemic mice for
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Figure 3. Renal function was improved via gavage of mangiferin and benzbromarone to hyperuricemic mice. A: Normal renal function presented in normal group mice. B: Damaged renal tissue presented in the model group: The black arrow represents renal tubular necrosis and the white arrow indicates interstitial inflammatory cell infiltration. B*: Enlargement of it. C-F: Renal function improved via gavage mangiferin at a dose of 1.5, 3.0, 6.0, and 100 mg/kg. G: Renal function improved via gavage benzbromarone at a dose of 25 mg/kg.

Figure 4. Effect of mangiferin on the levels of gene expression of URAT1, GLUT9, and OAT10 by RT-PCR testing. A: URAT1 expression in the Model vs. Normal P > 0.05, Mangiferin vs. Model P > 0.05 (t-test). B: GLUT9 expression in the Model group *P < 0.05 vs. Normal group, mangiferin treatment group ##P < 0.05 vs. Model group, (t-test). C: OAT10 expression in the Model group P > 0.05 vs. Normal, P > 0.05 Mangiferin vs. Model (t-test). Bars represent the mean ± S.E.M. of three independent experiments.

Mangiferin interfered target gene expression

After intervention by mangiferin, it detected the target gene expression, which was including URAT1, GLUT9 and OAT10. The expressions of their RNA were analyzed by RT-PCR. A dose-dependent trend was found in the experimental group with different mangiferin dose for the GLUT9 gene, but not for URAT1 and OAT10 gene. And there was a significant difference between experimental group and other groups in GLUT9 gene expression (P < 0.05) (Figure 4).

Western blot for target protein

To investigate the effects of mangiferin on the levels of target protein expression with URAT1, GLUT9, and OAT10, respectively. It taken west-
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Western blot assay for animal model after mangiferin intervention. The result was showed in Figure 5. URAT1 and OAT10 protein expression did not differ from normal mouse renal tissues between after and before intervention (P > 0.05).

However, OAT10 was lower expression in the negative intervention group, compared with the normal health mice (P < 0.05). Just by the opposite, GLUT9 protein was higher expression (P < 0.01). As for GLUT9 protein, mangiferin could reduce its expression in animal model, compared with normal level (P < 0.05).

Discussion and conclusions

There are many treatment research about hyperuricemia models animal using mangiferin. Hu has investigated the effects of mangiferin on the decrease in uric acid levels in hyperuricemic mice induced by intragastric administration once daily with potassium oxonate for seven days, and their found that allopurinol decreased the levels of uric acid, suggested that mangiferin might enhance uric acid excretion [16]. However, the effect of mangiferin on uric acid production can’t be excluded.

In the present study, consecutive intraperitoneal injection of uric acid at a dose of 150 mg·kg⁻¹ twice daily was used to construct hyperuricemic model mice, which had more advantage.

On the other hand, the increase of blood uric acid could result in the increase of uric acid excretion under the condition of the same production rate of uric acid. These results showed that intragastric treatment with mangiferin significantly decreased the serum urate levels of hyperuricemic mice with different dosage. The effects of mangiferin with a dose of 3.0 and 6.0 mg·kg⁻¹·d⁻¹ were as good as the effects of benz bromarone.

The mangiferin could improve excretion and decrease serum uric acid levels through preventing renal tubular re-absorption uric acid. To our knowledge, this might be a novel report that mangiferin can significantly decrease serum uric acid levels in long term hyperuricemic exposure mice.

In addition, our findings offer insights into the beneficial effects of mangiferin on pathological changes in hyperuricemic mice kidneys, and serious renal tubular necrotic lesions could be repaired by using mangiferin at a dose of 6.0 and 100 mg·kg⁻¹·d⁻¹.

The kidney plays an important role in uric acid metabolism [6]. Abnormal uric acid excretion in the kidney results in clinical hyperuricemia. The
excretion of uric acid was mediated by renal urate transporters such as URAT1. The first report on the relation of urate transporters to renal excretion showed that it was located in the branches of proximal tubules and is mainly responsible for uric acid re-absorption [17]. However, the function of URAT1 was controversial. Urate and creatinine concentrations in URAT1 knockout mice were significantly higher than the normal mice, which suggest that URAT1 plays an important role in uric acid re-absorption. On the other hand, the URAT1 was not the only transporter for urate re-absorption [18]. Unlike mice, the presence of uricase in human result in relatively weak urate re-absorption via URAT1 [19]. There was increasing evidence that URAT1 might not be as the predominant urate transporter occurring in nature [17].

This study showed that intraperitoneal injection of uric acid 300 mg·kg⁻¹·d⁻¹ and intragastric administration of mangiferin once daily for 14 days did not induce changes of URAT1 gene and protein expression. These results suggest that other urate transporters might be more efficient in urate re-absorption, which was similar to the results of previous investigation [17].

Several common important urate re-absorption transporters other than URAT1 have been reported, including GLUT9 [7, 20-22]. Serum uric acid level was closely related to the GLUT9 gene expression [20]. This conclusion was supported by other research studies, which suggest that GLUT9 was a highly efficient urate transporter [21]. The efficiency of GLUT9 transporter was higher than URAT1 with eight-fold [7], which played a key role in uric acid re-absorption at the proximal renal tubule. GLUT9 transporter could maintain the balance between serum uric acid levels and uric acid transfers [20-24].

In this present study, GLUT9 gene and protein expression were up-regulated (P < 0.05, P < 0.01) in mice after intraperitoneal injection of uric acid with dose of 300 mg·kg⁻¹·d⁻¹ for 14 days. GLUT9 gene expression was effectively down-regulated by each dose of mangiferin and its effect of regulation was better than benzbro bromarone (25 mg·kg⁻¹·d⁻¹). These results suggested that GLUT9 could be used as a target of mangiferin to improve uric acid excretion and to decrease uric acid levels in the body.

OAT10 is a new urate transporter as confirmed by RT-PCR and immunohistochemical analyses, which is located at the top cell membrane of renal proximal tubule cells [25]. Our results showed that OAT10 gene expression showed no obvious changes among all of the experimental groups. OAT10 protein expression was down-regulated in the hyperuricemia mouse model group (P < 0.05), while it was up-regulated in mangiferin intragastric administration groups (P > 0.05). However, the relationship of URAT1, GLUT9, and OAT10 transporters remains elusive and thus required further study.

Conclusions

The effects of mangiferin about regulation for serum uric acid and improvement renal pathological changes were better than using benzbro bromarone. Mangiferin can decrease uric acid levels by regulating the gene and protein expression of the urate re-absorption transporter GLUT9, but no effects for URAT1 and OAT10.

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Disclosure of conflict of interest

None.

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