Total saponins from *Discorea nipponica* makino ameliorate urate excretion in hyperuricemic rats

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Submitted: 14-04-2014 Revised: 04-06-2014 Published: 10-07-2015

**ABSTRACT**

**Objective:** The objective was to study the mechanism of reducing level of the uric acid by rhizoma dioscoreae nipponese. **Materials and Methods:** A total of 40 rats were divided into four groups: A normal group, hyperuricemia group, benzbromarone group (9 mg/kg) and total saponins from rhizoma dioscoreae nipponese (TDN) group (40 mg/kg). Adenine (100 mg/kg) and ethambutol (250 mg/kg) were used to induce hyperuricemic rats. Immunohistochemical and Western blotting methods were used to detect the mRNA and proteins expressions of rat organic anion transporter1 (rOAT1), rat organic anion transporter3 (rOAT3) and rat urate transporter1 (rURAT1) in the kidneys of different groups. **Results:** It was found that the reduced concentration of blood uric acid was due to the enhancement of renal uric acid excretion. It was realized by up-regulating proteins expressions of rOAT1 and rOAT3 and down-regulating of rURAT1. **Conclusion:** The findings suggested that there were uricosuric effects of TDN by regulating renal organic ion transporters in hyperuricemic animals. Altogether, TDN may be a good Chinese herb in treating hyperuricemia, even a potential drug for gouty arthritis.

**Key words:** *Discorea Nipponica* makino, gouty arthritis, hyperuricemia, rat organic anion transporter1, rat organic anion transporter3, rat urate transporter 1

**INTRODUCTION**

Gouty arthritis (GA) is also called arthritis. It is a kind of common metabolic disease in clinical that is caused by too much production or reduced excretion of purine as a result of the disorder of purine metabolism.\(^{[1,2]}\) It is a sort of rheumatism kind pathological change that is caused by uric acid deposition in joint tissues such as capsular ligament, synovium, cartilage and so on.\(^{[3]}\) With the development of quality of life, it was estimated that the incidence of GA is growing all the time.\(^{[4,5]}\)

Excessive taken of food and drink, trauma, surgery, low temperature, fat, hypertension and so forth will all lead to enhanced concentration of uric acid in the blood.\(^{[6,7]}\) Hyperuricemia is an important biological foundation of GA. The case rates of both hyperuricemia and GA are higher in men than they are in women. Hyperuricemia might lead not only GA but also cardiovascular disease,\(^{[8]}\) end-stage renal disease,\(^{[9]}\) hypertension\(^{[10,11]}\) and type 2 diabetes.\(^{[12]}\) Therefore, it makes senses for the health of people to keep a stable level of the uric acid in the blood. Among causes of diseases too much production of urate accounts for 15–20% of the patients and its reduced excretion is the major mechanism that accounts for 80–85% of the patients. Excretion of uric acid, a compound of considerable medical importance, is largely determined by the balance between the renal secretion and reabsorption.\(^{[13]}\) Urate transport in the kidney has become the major drug target in treating these diseases.\(^{[14]}\)

Urate transporter1 (URAT1) that is located in the brush border membrane of the renal tubular epithelial cell is responsible for reabsorption of urate.\(^{[15,16]}\) This protein encodes single nucleotide polymorphisms of *SLC22A12*, which is the risk factor in inducing hyperuricemia.\(^{[17]}\) *SLC22A12* is responsible for most renal hypouricemia and URAT1 is the primary reabsorptive urate transporter, targeted by pyrazinamide, benzbromarone and probenecid *in vivo*.\(^{[18]}\) Several mutations in *SLC22A12* gene is associated with primary gout.\(^{[19]}\) Organic anion transporters OAT1 (encoded by *SLC22A6*) and OAT3 (encoded by *SLC22A8*) are both proved to be involved in the transport...
of the uric acid in the kidney by different mechanisms. OAT1 and OAT3 are primarily expressed in the basolateral membrane close to the tubular epithelial cells.\textsuperscript{20} Human organic anion transporter1 (hOAT1) is a key substance in mediating the secretion of the uric acid in the kidney. It plays a key role in absorbing the urate from the peritubular tissue space into the renal tubular epithelial cells.\textsuperscript{21} Expressions of both rat organic anion transporter1 (rOAT1) and rat organic anion transporter3 (rOAT3) were reduced in hyperuricemic rats-induced by combined administration of uricase inhibitor oxonic acid and uric acid.\textsuperscript{22} All above suggest that URAT1, OAT1, and OAT3 might have important roles in regulating excretion of urate and influence hyperuricemia occurrence.

Rhizoma Dioscoreae nipponese (RDN), scientific name Dioscorea nipponica makina, belongs to Dioscoreaceae. Modern pharmacological research reveals that there are effects of anti-inflammation and easing pain of RDN. RDN is widely used in treating inflammation. Our previous studies had identified the main components of total saponins from rhizoma Dioscoreae nipponese (TDN) by high-performance liquid chromatography method even some of them had been quantified and revealed that they could reduce uric acid levels of hyperuricemic mice by inhibiting production of uric acid and might have some therapeutic effects on GA.\textsuperscript{23–27} However, its mechanism in reducing the concentration of uric acid is still poorly understood. Hyperuricemia is the important biochemical foundation of gout. Gout is acute rheumatism kind pathological changes, which are caused by urate deposit in capsular ligament, synovial joint and cartilaginous tissues. About 5–12\% of patients will develop hyperuricemia to gout. Adenine could promote the biosynthesis of uric acid and ethambutol could inhibit the excretion of uric acid. Adenine and ethambutol are widely used to induce hyperuricemic rat.\textsuperscript{28,29}

The present study tried to reveal the influence of TDN on mRNA and protein expressions of rat urate transporter1 (rURAT1), rOAT1 and rOAT3 from the kidney of hyperuricemic rats-induced with adenine and ethambutol. In this way, pharmacological action of TDN was verified in order to explore traditional Chinese medicine in treating hyperuricemia and its complications.

**MATERIALS AND METHODS**

**Reagents**

Adenine was purchased from Sigma (USA). Ethambutol was purchased from MinSheng Pharma, HangZhou (China). Benzbromarone (Purity >98\%) was purchased from ChangJiang Pharma, Yijiang (China). Uric acid kit was purchased from NanJingJianCheng Bioengineering Institute (China). DAB kit was purchased from ZhongShan Gold Bridge Biotechnology Company (China). Hematoxylin, OAT1, OAT3, URAT1 polyclonal antibodies originated from rabbit; HRP-goat-anti-rabbit-IgG antibody were all purchased from BeiJing Biosynthesis Biotechnology Co. LTD (China).

**Animals**

Male Wistar strain of rats (5-week old and 180–220 g weight), were purchased from the animal center of Heilongjiang University of Chinese Medicine. License No. was SCXK (Jing) 2012–0001. They were allowed at least 1 week to adapt to their environment before used for experiments where light and environment could be controlled for 12 h light and 12 h dark. Forage was purchased from experimental animal center of the Heilongjiang University of Chinese Medicine. The ethical approval for the experiment was followed by the Legislation on the Protection of Animals Used for Experiment Purposes (Directive 86/609/EEC) on the fifth of June, 2012.

**Plant material and extraction**

Rhizoma Dioscoreae nipponese was purchased from the Heilongjiang Province Drug Company (Harbin, China). The voucher specimens (hj-201104) of the herb were authenticated by Professor Ke Fu, Institute of Traditional Chinese Medicine. One gram of the crude drug was extracted three times with 6 mL 50\% ethanol for one point five, one point five and one point 5 h, and separated and purified by D-101 macroporous adsorption resin (Polarity: Nonpolarity, Particle diameter: Zero point three-one point two five mm, Surface area: 480–520 m\(^2\)/g, Average pore diameter: 25–28 nm, Moisture content: 60–75\%). The process for purification was as follows: The concentration of the sample solution was 500 g/L; the loading quantities were 1 g sample/2 g resin; the adsorption flow rate was two bed volume/hour (BV/h); the eluent was 50\% ethanol; the eluent volume was 14 BV; the elution flow rate was two BV/h. The ethanol phase was evaporated under vacuum, and an oven dried at 60°C to obtain the extracts. The drug-extract ratio was four point nine seven\% (w/w).\textsuperscript{23}

**Experimental design**

Wistar rats were adapted to the environment for a week before used for experiments. They were divided in to four groups. Normal group was given normal saline. Hyperuricemia group was intragastrically administrated with adenine (100 mg/kg) (Sigma USA) and ethambutol (250 mg/kg) (MinSheng Pharma, Hang Zhou, China).
each day.\[^{30}\] TDN group was given TDN (40 mg/kg) an hour after the model was induced. Positive group was given benzbromarone (9 mg/kg) (Chang Jiang Pharma, Yijiang, China) after the hyperuricemia was induced. All the drugs were given for successive 7 days before the experimental treatment.

**Effect of total saponins from rhizoma dioscoreae nipponese on uric acid concentration in the blood serum and the total amount of the uric acid within 24 h**

Wistar rats were randomly divided in to four groups: A normal group, hyperuricemia group, TDN group and benzbromarone group. Each group was consisted of 10 rats. All the groups were removed of eye balls an hour after the drugs were given on the 7\(^{th}\) day of the experiment to obtain the blood.\[^{28}\] At the same time, all the groups were collected of the urine within 24 h. The blood was agglutinated an hour at room temperature. They were centrifuged at the speed of 4000 rpm for 20 min to obtain the serum. The serum was used for testing level of the uric acid. The concentrations of the uric acid in the urine were tested using uric acid kit by automatic biochemistry analyzer. Total amount of uric acid excreted were calculated.

**Immunohistochemistry and Western blot analysis of rat organic anion transporter1, rat organic anion transporter3, and urate transporter1**

Wistar rats were randomly divided into three groups: A normal group, hyperuricemia group, and TDN group. Each group was consisted of 10 rats. An hour after the model was induced. Positive group was given on the 7\(^{th}\) day of the experiment; all the groups were given anesthesia to obtain the kidneys. Part of the kidneys were preserved in 4\% formaldehyde stationary liquid for 24 h and embedded in the paraffin. Part of the kidneys were put into −80°C until analysis.

Serial sections (5 \(\mu\m\)) were collected and stained with immunohistochemistry according to the instruction of Elivsion two-step method for rOAT1, rOAT3, and rURAT1. Primary antibodies were anti-rat rOAT1 antibody (raised in rabbit, one: 500), anti-rat rOAT3 antibody (raised in rabbit, one: 500) and anti-rat rURAT1 antibody (raised in rabbit, one: 800). Each slide was examined at a magnification of 400 times in an Olympus BX 60 Microscope (Japan), and all images were the same size. Image-proplusversion six image analysis system (Media Cybernetics, America) was used to analyze the areal densities of rOAT1, rOAT3 and rURAT1 in the full image.

A total of 200 \(\mu\g\) tissues were grinded, and 100 \(\mu\l\) cooled protein lysate was added. The mixture was centrifuged at the speed of 14,000 rpm for 10 min at 4°C. The supernatant was collected and tested for the protein concentration.

40 \(\mu\g\) protein was mixed with buffer solution and was boiled for 5 min. The mixture was loaded into SDS degeneration polyacrylamide gel electrophoresis page. The gel electrophoresis was electrotransferred to NC membrane and blocked out with 1\% dried skimmed milk. Relative antibodies were incubated staying over at 4°C and second antibodies were added and incubated for 60 min at room temperature. The integral optical density of bands were determined by Gel-Pro four software (Media Cybernetics, Inc., US). The protein levels of rOAT1, rOAT3 and rURAT1 were expressed as the OD value.

**Statistical analysis**

All data were expressed as the mean ± standard error of the mean (S.E.M.), and statistical analysis was performed using independent samples t-test to determine levels of the significance. A value of \(P < 0.05\) was considered to be statistically significant.

**RESULT**

**Effects of total saponins from rhizoma dioscoreae nipponese on uric acid level of blood, the total amount of urine within 24 h and the excretion amount of uric acid of hyperuricemic rats**

As it was shown in Table 1, the concentrations of the uric acid in the blood serum (SUA) in the hyperuricemia group was higher than that is in the normal group significantly (\(P < 0.01\)), indicated that the hyperuricemia was built successfully. The SUA concentrations in TDN group were reduced significantly when compared with the hyperuricemia group (\(P < 0.01\)). When compared with the normal group, there was no significant difference between the normal group and the hyperuricemia group of the total amount of urine (TU). TDN and benzbromarone could enhance the TU significantly (\(P < 0.05\); \(P < 0.01\)). The total excretion of uric acid (TEU) in the hyperuricemia group was decreased significantly when compared with the normal group (\(P < 0.01\)). Both TDN and benzbromarone could enhance the TEU when compared with the hyperuricemia group significantly (\(P < 0.05\), \(P < 0.05\)).

**Table 1: SUA, TU within 24 h and TEU in the urine of hyperuricemic rats**

| Group       | Dose (mg/kg) | SUA (μmol/L) | TU (ml) | TEU (μmol) |
|-------------|-------------|--------------|---------|------------|
| Normal      | -           | 205.81±54.90 | 12.41±2.32 | 8.97±2.41  |
| Model       | -           | 280.82±51.85 | 12.03±3.12 | 6.88±1.94  |
| TDN         | 40          | 220.24±40.52 | 16.07±3.92 | 9.14±2.87  |
| Positive    | 9           | 210.79±55.31 | 17.38±4.54 | 9.73±3.13  |

The data are expressed as the means±SEM (n=10). \(^* P<0.05\); \(^** P<0.01\); versus normal group (independent samples t-test); \(^* P<0.05\); \(^** P<0.01\); versus model group (one-way ANOVA followed by Dunnett’s t-test). SEM: Standard error of mean; SUA: Uric acid level of blood serum; TU: Total amount of urine; TDN: Total saponins from rhizoma dioscoreae nipponese; TEU: Total excretion of uric acid.
Effects of total saponins from rhizoma Dioscoreae nipponese on the expressions of rat organic anion transporter1, rat organic anion transporter3 and rat urate transporter 1 proteins in rat kidney by Immunohistochemistry

As it was shown in Table 2, [Figure 1a, d and g], the levels of the rOAT1, rOAT3 and rURAT1 proteins in the normal group were 0.014 ± 0.002, 0.053 ± 0.018 and 0.002 ± 0.000, respectively. Those in hyperuricemia group were 0.005 ± 0.001, 0.007 ± 0.003 and 0.029 ± 0.011, respectively [Figure 1b, e and h]. rOAT1 and rOAT3 were decreased by 64% and 87%, respectively, compared with the normal group (P < 0.01, P < 0.01). rURAT1 was increased by 1350% compared with the normal group (P < 0.01). Those in TDN group were 0.018 ± 0.011, 0.033 ± 0.004, 0.012 ± 0.002, respectively [Figure 1c, f and i]. Compared with the hyperuricemia group, the levels of the rOAT1 and rOAT3 increased by 29% and 38%, respectively (P < 0.01, P < 0.01). Compared with the hyperuricemia group, the level of the rURAT1 decreased by 59% (P < 0.05).

Effects of total saponins from rhizoma Dioscoreae nipponese on the expressions of rat organic anion transporter 1, rat organic anion transporter3 and rat urate transporter 1 proteins in rat kidney by Western blot method

As it was shown in Table 3, [Figures 2 and 3], the level of the rOAT1 protein was 0.61 ± 0.04, 0.39 ± 0.05 and 0.56 ± 0.04 in the normal group, hyperuricemia group and TDN group, respectively. Compared with the normal group (P < 0.01), the level of rOAT1 protein in the hyperuricemia group was decreased by 36%. The level of the rOAT1 protein was increased by 44% in the TDN group, compared with the hyperuricemia group (P < 0.01). The level of rOAT3 protein was 0.60 ± 0.04, 0.39 ± 0.02 and 0.52 ± 0.03 in the normal group, hyperuricemia group and TDN group.

Table 2: Effects of TDN on the expressions of rOAT1, rOAT3 and rURAT1 proteins in rat kidney by immunohistochemistry

| Group          | Dose (mg/kg) | rOAT1       | rOAT3       | rURAT1      |
|----------------|--------------|-------------|-------------|-------------|
| Normal         | -            | 0.014±0.002 | 0.053±0.018 | 0.002±0.000 |
| Hyperuricemia  | -            | 0.005±0.001**| 0.007±0.003**| 0.029±0.011**|
| TDN            | 40           | 0.018±0.011**| 0.033±0.004**| 0.012±0.002**|

The data are expressed as the mean±SEM (n=10). *P<0.05, **P<0.01 versus normal group (independent samples t-test); [26] #P<0.05, ##P<0.01 versus hyperuricemia group (independent samples t-test). SEM: Standard error of mean; TDN: Total saponins from rhizoma Dioscoreae nipponese; rOAT1: Rat organic anion transporter 1; rOAT3: Rat organic anion transporter 3; rURAT1: Rat urate transporter 1.
respectively. Compared with the normal group (P < 0.01), the level of the rOAT3 protein in the hyperuricemia group was decreased by 35%. The level of the rOAT3 protein was increased by 33% in the TDN group, compared with the hyperuricemia group (P < 0.01). The level of the rURAT1 protein was 0.30 ± 0.04, 0.52 ± 0.07 and 0.34 ± 0.03 in the normal group, hyperuricemia group and TDN group, respectively. Compared with the normal group (P < 0.01), the level of the rOAT3 protein in the hyperuricemia group was increased by 73%. The level of the rURAT1 protein was decreased by 35% in the TDN group, compared with the hyperuricemia group (P < 0.01).

**DISCUSSION**

Rhizoma dioscoreae nipponese is the root of *D. nipponica* makina that belongs to *Dioscoreaceae* family. RDN was traditionally used to treat rheumatic arthritis, injury from falling down and so on.[31] Modern pharmacological research revealed that there were effects of anti-inflammation, regulation of the immune system, and easing pain by RDN.[32] Our previous studies showed that RDN could treat GA.[23-26] However, the treating mechanism is still not completely understood. The purpose of the study was to investigate whether the effect of TDN on reducing uric acid level was realized by promoting the excretion of uric acid. As ethambutol could inhibit the excretion of uric acid, it was combined with traditional used adenine to induce the hyperuricemic rats.[33] Benz bromarone was chosen as the positive drug because it could promote the excretion of uric acid.[34]

The classic model of excretion of the uric acid in the kidney is consisted of four parts: The glomerular filtration (100%),...
the reabsorption of kidney tubules (98%-100%), the re-excretion of kidney tubules (50%) and the reabsorption after excretion (40%). Finally, approximately 8.12% of the uric acid is excreted via glomerular filtration. Organic anion transporters OAT1 and OAT3 are expressed at basement membrane of the epithelium in the kidney tubules. They are responsible for transporting of the uric acid from the blood capillary around the tubule to the epithelium in the kidney tubules. It is the first step of excretion of the uric acid. Onion exchanger of uric acid URAT1 is expressed at peniculus of epithelium in the kidney tubules. It is responsible for the reabsorption of the uric acid. The effects of these proteins in regulating the level of the uric acid make them the important targets for drugs that are used for treating hyperuricemia.\textsuperscript{18,22}

In the present study, we demonstrated that TDN could reduce serum urate levels and enhance urate excretion via regulating the abnormal expressions of renal rOAT1, rOAT3 and rURAT1 in hyperuricemic rats. At present, drugs that are used for promoting the excretion of uric acid are mainly Probenecid, Sulfispyrazone, and Benzbramaron.\textsuperscript{33,34} They function mainly by inhibiting near-end kidney tubule from absorbing uric acid. Few of the patients will suffer from erythra, fever, and other side effects. The greatest shortcoming in using these drugs is that they might cause deposition of urate crystals in the urinary passage. It will lead to renal colic and renal injury. As a result, their application is restricted.\textsuperscript{35,36} RDN is a kind of medical plant that possesses great economic value, extensive of vegetation resources and little side effects. The price of RDN is low. There are extensive exploiting values of RDN.

It is the first time that RDN was found to regulate the serum urate levels by influencing the expressions of rOAT1, rOAT3, and rURAT1. It makes senses to further explore RDN to be used to treat hyperuricemia and other complications such as GA. Although the mechanism of TDN in treating hyperuricemic rats had been identified, concrete components of TDN responsible for the regulation of transporters still need to be studied. In conclusion, the present study demonstrated that there was potent uricosuric effect of TDN on hyperuricemic rats by decreasing the expressions of renal rURAT1 while increasing the expressions of renal rOAT1 and rOAT3. They were all attributable to the enhancement of the uric acid excretion.

**ACKNOWLEDGMENTS**

The work was supported by National Natural Science Fund projects (81173618, 81403156), The Youth Science Foundation of Heilongjiang Province, China (QC2009C46), Innovation talent research Special Fund of Harbin city (QC2009C46), Innovation talent research Special Fund of Harbin city (2012RFQXS039), Scientific Fund of Heilongjiang University of Chinese Medicine (2013bs05), The post-doctoral assisted Fund of Heilongjiang Province (LBH-Z13191, LBH-TZ0520) and the outstanding innovative talent support programs of Heilongjiang University of Chinese Medicine.

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Cite this article as: Zhou Q, Yu D, Liu S, Liu Y. Total saponins from Dioscorea nipponica makino ameliorate urate excretion in hyperuricemic rats. Phcog Mag 2015;11:567-73.

Source of Support: National Natural Science Fund projects (81173618, 81403156), The Youth Science Foundation of Heilongjiang Province, China (QC2009C46), Innovation talent research Special Fund of Harbin city (QC2009C46), Innovation talent research Special Fund of Harbin city (2012RFQX0309), Scientific Fund of Heilongjiang University of Chinese Medicine (2013bs05), The post-doctoral assisted Fund of Heilongjiang Province (LBH-Z13191, LBH-TZ0520) and the outstanding innovative talent support programs of Heilongjiang University of Chinese Medicine.

Conflict of Interest: None declared.