Evaluation of Bioeffects of a Long-term Administering ChangLe on Rat by Proton NMR Method and Biochemical Examination

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High resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy has been employed to assess long-term toxicological effects of ChangLe (a kind of rare earth complex applied in agriculture). Male Wistar rats were administrated orally with ChangLe at doses of 0, 0.1, 0.2, 2.0, 10 and 20 mg/kg body weight daily, respectively, for 6 months. Urine was collected at day 30, 60, 90 and serum samples were taken after 6 months. Many low-molecular weight metabolites were identified by ^{1}H NMR spectra of rat urine. A decrease in citrate and an increase in ketone bodies, creatinine, DMA, DMG, TMAO, and taurine in the urine of the rats receiving high doses were found by ^{1}H NMR spectra. These may mean that high-dosage of ChangLe impairs the specific region of liver and kidney, such as renal tubule and mitochondria. The decrease in citrate and the increase in succinate and α-ketoglutarate were attributed to a combination of the inhibition of certain citric acid enzymes, renal tubular acidosis and the abnormal fatty acid catabolism. The information of the renal capillary necrosis could be derived from the increase in DMA, DMG and TMAO. The increase in taurine was due to hepatic mitochondria dysfunction. The conclusions were supported by the results of biochemical measurements and enzymatic assay. No obviously toxicological effects on metabolism were found for low-dosage ChangLe, but further studies on safety of ChangLe applied to agriculture are still required.

Keywords biofluids, NMR spectroscopy, metabolism, urinary analysis

Introduction

NMR spectroscopy of biofluids has brought new chemistry into life science and clinical medicine with the advent of high field Fourier transform NMR spectrometers with increased sensitivity, chemical shift dispersion, dynamic range and improved computing capabilities.[1] In recent years, high resolution NMR has been used for the rapid multicomponent analysis of low molecular weight compounds in biofluids including urine, blood plasma, bile, cerebrospinal fluid, saliva, milk and synovial fluid for potential clinical diagnosis of abnormal metabolism of the inborn disease and investigation of toxicological and pharmacological effects of drugs.[2-7] The major advantage[8,9] of using NMR to study biofluids is that little or no sample pretreatment is needed; the same sample may be used in subsequent assays by other methods as NMR is non-destructive. Moreover, since NMR is non-specific the simultaneous determination of a number of metabolites is possible, which promotes identifying important but unexpected or previously unknown metabolites. Therefore, NMR is particularly valuable for acquiring metabolite profile of a sample where prior knowledge is limited. A substantial reduction in labor and time can be achieved for multiple components analysis. In the present study, we applied high-resolution ^{1}H NMR and other methods to study toxicological effects of ChangLe (a kind of rare earth complex applied widespreadly in agriculture).

Since 1970's it was recognized in China that rare earths could not only increase the yields of various kinds of crops, but also improve the quality of tobacco, watermelon, beet, *etc*.[10] The progress of this investigation has opened vast vistas for using rare earth and also given a new subject of biological and toxicological effects of rare earths on human body.[11,12] During the last twenty years, results of toxicological effects of rare earths on human organs were reported.[13-17] However, their biological effects and mechanisms have not been throughly understood. The advantages afforded by NMR method should facilitate to gain insight into bioeffects and mechanism of rare earths and provide scientific evidence for the use of rare earths in agriculture.

Experimental

Sample collection and storage

Male Wistar rats (weight ranging 80—120 g, $n = 15$ per group) were treated orally with ChangLe [it is composed of La_2O_3(30.48%), CeO_2(54.67%), Pr_6O_{11}(6.05%) and Nd_2O_3(8.8%)] at doses of 0.1, 0.2, 2.0, 10 and 20 mg/

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kg body weight daily, respectively, and the controls (n = 15) received the same volume of 0.9% saline everyday. Animals were placed in metabolism cages and arranged for separate collection of urine and faeces. During experiments food and tap water were allowed *ad libitum*. Urine was collected at day 30, 60, 90 after ChangLe was given. After six months, serum samples of blood of the killed rats were separated by ultrafiltration and centrifugation.

The fresh urine samples of 2 mL each were lyophilized immediately and the fresh serum samples were stored frozen at −20 ℃ until measured.

^{1}H NMR *measurement*

Urine samples were redissolved in 0.5 mL of D_2O before measurements. Proton NMR measurements were carried out at 399.94 MHz on a Varian Unity-400 spectrometer at 300 K. Sixty-four free induction decays (FIDS) were collected into 7680 computer points with a spectral width of 3895.6 Hz using 90° (24 μs pulses) and an acquisition time of 1.5 s. A further delay of 3.0 s between pulses was added to ensure that the spectra were fully T_1-relaxed, *i.e.*, the total accumulation time for each spectrum was 288 s. A pre-saturation sequence was used to minimize the water resonance during the pre-acquisition delay. To test precision and reproducibility, more than three ^{1}H NMR spectra of urine sample for each dosage group were recorded under the same experimental conditions.

The creatinine singlet at δ 3.05 was taken as the reference of ^{1}H chemical shift for urine spectra. Resonance assignments of metabolites were based on chemical shift, spin-spin coupling patterns and coupling constants. Although the concentrations of endogenous metabolites were determined from the relative peak height of the creatinine whose concentration was independently determined in the same samples by the standard Jaffee reaction, no NMR quantitation was attempted in this study because of, for example, line-width effects, pH effects of urine, and overlap of peaks.

Conventional biochemical measurements

Levels of growth hormone (GH), cortisol and insulin in the Wistar rat serum were measured by radioimmunoassay. The other biochemical indices in serum were assayed by conventional methods: glucose (GLC), toluidine method;[18,19] blood urea nitrogen (BUN), diacetylmonoxime colorimetry;[20,21] creatinine (Crea), Jaffee reaction.[21,22] The assay for glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), γ-glutamyl transpeptidase (γ-GT), urine acid (UA) and alkaline phosphatase (ALP) in serum was based on routine enzymatic methods: a volume of 10 mL of plasma was kept at 37 ℃ for an hour, and serum was obtained from plasma by centrifugation (2500 r/min, 20 min). The enzymes were assayed on a Hitachi biochemistry-7250 auto-analyzer by using 100 μL serum.

Results

High-resolution ^{1}H NMR *spectra of rats urine samples*

The types and amounts of low M_r compounds detected in control rat urine by ^{1}H NMR in our work were similar to those which have been reported previously for normal rat urine.[1,3,23,24] We did not find the obvious effects of rare earth on the chemical shift and line shape of NMR spectra, and a little change in chemical shift for some metabolites such as α-ketoglutarate is due to pH variation in rat urine.[25] ChangLe treatment of rats caused some alterations in intensity of the ^{1}H NMR spectra of certain metabolites compared with those of the control ones. Fig. 1 shows ^{1}H NMR spectra of rat urine after oral administration of 0.2 and 10 mg of ChangLe/kg body weight and several common endogenous metabolites detectable in rat urine were marked in the spectra.[26] The metabolites obviously affected by ChangLe dosing are tabulated in Table 1.

The peaks corresponding to the ketone bodies (3-hydroxybutyrate, acetone and acetoacetate) increased in amplitude in the two higher ChangLe doses of 10 and 20 mg/kg. The urinary excretions of succinate and α-ketoglutarate were elevated in higher dosage groups and the reverse is for citrate.

The intensity of dimethylamine (DMA), dimethylglycine (DMG) and trimethylamine-*N*-oxide (TMAO) as well as creatinine peaks increased in almost all of the ChangLe dose groups with the exception of the lowest dosage group (0.1 mg/kg). It could also be found from the ^{1}H NMR experiment that the amount of taurine was boosted in high dosage groups.

The excretion of phenylalanine, glycine and glutamine was grossly elevated with the increase of ChangLe dose. Peak intensity of several other amino acids including tyrosine and histidine varied remarkably but the variance did not show explicit correlation with ChangLe dosage.

No attempt was made to integrate the urea signal which might be affected by the irradiation of H_2O due to exchanging of NH protons with those of water.

In similar studies using urine samples from the same rats receiving ChangLe doses for thirty and sixty days, ^{1}H NMR results did not show obvious differences from those treated for ninety days.

Assessment of biochemical indices in serum

After administration of ChangLe for 6 months, the level of GH in the serum reduced for the 20 mg/kg group, however it increased for the rats at the dose of 0.1 mg/kg and 0.2 mg/kg. Cortisol levels tended to rise in most of the groups, but only for rats receiving the highest doses which were significantly higher than those of the controls statistically. The dosage of groups with elevated insulin levels were 10 mg/kg and 20 mg/kg (Table 2). After normal food without ChangLe was given for one month, the hormone levels in the rat serum returned to control levels with the exception of the level of GH in the 0.1 mg/kg group (data not shown).

For the two higher ChangLe dose groups, GLC concen-

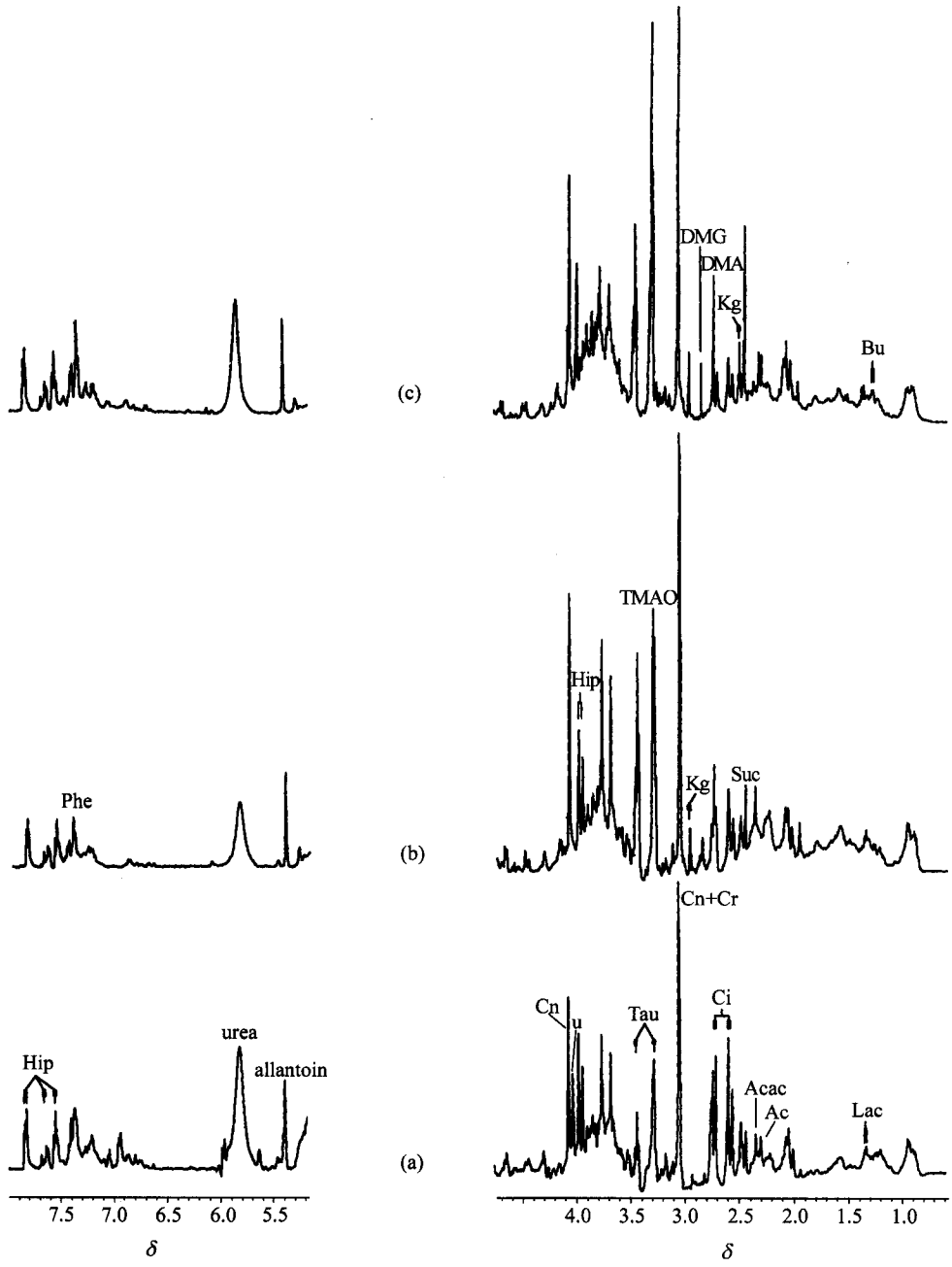


Fig. 1 ^{1}H NMR spectra (400 MHz) of urine from rats at the ninetieth day after dosing. (a) control rat; (b) rat receiving a dose of 0.2 mg of ChangLe/kg body weight; (c) rat receiving a dose of 10 mg of ChangLe/kg body weight. Abbreviations for peak assignments: Gly, glucine; Glu, glutamine; Bu, 3-hydroxybutyrate; Lac, lactate; Ac, acetone; Acac, acetonacetate; Suc, succinate; Kg, α-ketoglutarate; Ci, citrate; DMA, dimethylamine; DMG, dimethylglycine; Cn, creatinine; Cr, creatine; TMAO, trimethylamine-*N*-oxide; Tau, taurine; Hip, hippurate; His, histidine; Phe, phenylalamine; u, unassigned resonances.

trations in serum were significantly decreased; however, BUN contents were obviously elevated. UA levels in the serum of the rats receiving the highest doses of ChangLe were significantly high. Creatinine in the serum was less decreased (Table 2).

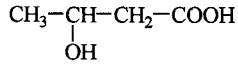
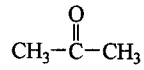
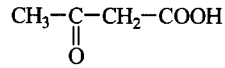
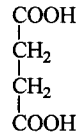
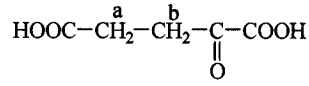
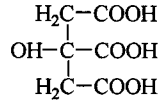
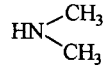
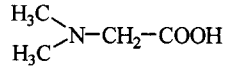
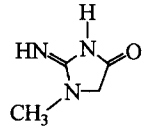
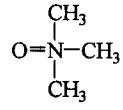
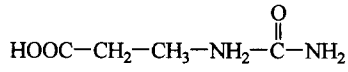
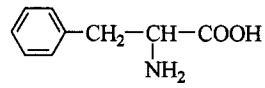
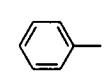
Assessment for the enzymes in serum

ALP concentrations in serum were elevated for almost all of the experimental groups, but several other enzymes including γ-GT, GOT and GPT for experimental rats were of no obvious difference compared with those of the controls except the higher levels of GPT for the 20 mg/kg group (Table 3).

Discussion

Urine is the end product of metabolism discharged from the body by glomerular filtration and reabsorption of renal tubule. The different variances and levels of metabolites in urine were related to the different physiological states and pathological conditions. The changes in composition of urine, indicated by NMR, will reflect not only the disease states of

Table 1 Assessment of the urinary metabolites by ChangLe-dosed rats for three months

Metabolite	Structure	^{1}H shift (δ) & multiplicity	Group	ChangLe dose (mg/kg)				
				0.1	0.2	2.0	10	20
3-*D*-hydroxybutyrate		1.24 (d)	CH_3	—	—	—	↑	↑
Acetone		2.28 (s)	$(CH_3)_2$	—	—	—	↑	↑
Acetoacetate		2.32 (s)	CH_3	—	—	—	↑	↑
Succinate		2.43 (s)	$(CH_2)_2$	—	↑	↑	↑↑	↑↑
α-ketoglutarate		2.46 (t) 2.97 (t)	a: CH_2 b: CH_2	— ↓	— ↑	— ↑	↑ ↑	↑ ↑
Citrate		AB 2.52 (d) 2.69 (d)	$(CH_2)_2$	—	↓	↓	↓	↓
Dimethylamine		2.72 (s)		—	↑	↑	↑	↑
Dimethylglycine		2.81 (s)	$(CH_3)_2$	—	—	↑	↑	↑
Creatinine		3.05 (s) 4.06 (s)	N-CH_3 N-CH_2	↑ —	↑ ↑	↑ ↑	↑↑ ↑	↑↑ ↑
Trimethylamine-*N*-oxide		3.27 (s)	N-CH_3	↑	↑	↑	↑↑	↑
Taurine	$H_2N-CH_2-CH_2-SO_3H$	3.27 (t) 3.43 (t)	S-CH_2 N-CH_2	— —	↑ ↑	↑ ↑	↑ ↑	↑ ↑
Glycine	H_2N-CH_2-COOH	3.57 (s)	N-CH_2	—	—	↑	↑	↑
Glutamine		3.77 (t)	N-CH_3	—	—	↑	↑	↑
Phenylalanine		7.34 (m) 7.38 (m)		—	—	—	↑	↑

Abbreviations and keys: s, singlet; t, triplet; AB, second order; —, not detectably different from control level; ↑, a detectable but minor elevation in concentration, of up to 2 times control; ↑↑, an elevation in concentration corresponding to 2 to 3 times control levels; ↓, a minor decrease (20%—50%) from control levels; ↓↓, a moderate decrease (50%—90%) from control levels.

urinary system but also the metabolic state of other systems in the body.

The dose-dependent changes in the excretion of three Krebs cycle intermediates were observed: the decrease in urinary citrate and the increase in excretion of succinate and *α*-ketoglutarate at the higher ChangLe dose levels. It has been known that the urinary excretion of citrate is decreased during renal tubular acidosis (RTA).[27] The intracellular pH and the intracellular bicarbonate concentration control the rates of transport of citrate across the inner mitochondrial membrane,

Table 2 Assessment of some biochemical indexes in the serum of rats receiving ChangLe for six months

Index	Control group	0.1 (mg/kg) group	0.2 (mg/kg) group	2.0 (mg/kg) group	10 (mg/kg) group	20 (mg/kg) group
GH (μg/L)	2.26 ± 0.22[a]	2.61 ± 0.17	2.60 ± 0.08	2.22 ± 0.28	2.06 ± 0.26	1.84 ± 0.26
cortisol (μg/L)	20.60 ± 2.23	22.40 ± 2.58	22.66 ± 2.40	21.05 ± 1.69	21.15 ± 2.32	23.82 ± 2.17[b]
insulin (mIU/L)	17.90 ± 1.10	19.02 ± 2.98	22.22 ± 2.30[b]	22.43 ± 2.54[b]	27.36 ± 1.64[b]	25.57 ± 2.27[b]
GLC (mg/100 mL)	93.78 ± 11.21	99.90 ± 10.80[c]	88.56 ± 16.56	83.88 ± 18.36	50.76 ± 19.98	36.10 ± 6.66
BUN (mmol/L)	8.35 ± 0.86	7.30 ± 0.63	8.65 ± 0.70	7.84 ± 0.68	9.36 ± 1.68	9.78 ± 0.67
Creatinine (μmol/L)	67.60 ± 3.13	60.63 ± 5.50	58.71 ± 6.55	63.56 ± 5.34	58.86 ± 6.78	57.86 ± 10.61
UA (μmol/L)	238.83 ± 32.33	252.36 ± 25.50	252.44 ± 34.79	250.04 ± 19.06	243.61 ± 26.97	274.43 ± 33.28

[a] Mean ± SE, $n = 13$ for each group; [b] $P < 0.05$; [c] $P < 0.001$.

Table 3 Assessment of enzymes in the serum of rats receiving ChangLe for six months

Enzyme	Control group	0.1 (mg/kg) group	0.2 (mg/kg) group	2.0 (mg/kg) group	10 (mg/kg) group	20 (mg/kg) group
GOT (IU/L)	185.88 ± 19.86	190.86 ± 30.30	206.02 ± 32.27	193.71 ± 31.26	190.51 ± 53.52	193.15 ± 23.83
GPT (IU/L)	35.90 ± 5.17	35.24 ± 6.28	35.80 ± 12.24	34.99 ± 5.86	36.01 ± 7.82	43.52 ± 5.16[a]
GO/GP	5.03 ± 0.95	3.61 ± 0.32[a]	4.92 ± 0.84	4.69 ± 0.67[b]	4.33 ± 0.45[b]	4.28 ± 0.42[b]
γ-GT (IU/L)	1.39 ± 0.63	1.00 ± 0.12	1.40 ± 0.16	1.00 ± 0.21	1.00 ± 0.47	1.41 ± 0.34
ALP (IU/L)	99.13 ± 27.51	122.45 ± 29.74	129.95 ± 93.77	163.17 ± 22.23[c]	194.84 ± 49.78[c]	233.70 ± 45.17[c]

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

the activity of the citric acid cycle enzyme aconitase and the degree of utilization of citrate by the renal tubular cells.[28] In this study, urinary citrate levels are lower and the urinary pH is high for rats receiving the higher doses, indicative of RTA. But the effects of rare earth on the urinary excretion of citric acid cycle intermediates can not be explained simply in terms of RTA. As for the simple RTA caused by acetazolamide injection, the excretion of both citrate and α-ketoglutarate is reduced.[29] It is possible that certain kinds of rare earth in ChangLe have a high affinity for thiolates and sulfide like as Hg^{2+},[30] and could inhibit certain Krebs cycle enzymes, such as the malate dehydrogenase (contains about 12 SH/mol) and succinate dehydrogenase (contains one iron thiolate-sulfide cluster). The inhibition of these enzymes could explain the increased excretion of α-ketoglutarate and succinates, found in this study, as the renal tubular cell would be less able to utilize these substrates.

Low citrate levels and high succinate levels could also happen as a result of increased fatty acid catabolism.[31] Toxin induced in fatty acid oxidation would result in the increased amounts of propionyl-CoA generated, which would lead to the increased amounts of succiny-CoA (and therefore succinate) and inhibition of citroyl synthetase (decreased citrate) and acetyl CoA carboxylase. Ketone bodies would then be primary products of acetyl CoA. When ketone bodies in the liver become saturated at high levels, as any reabsorptive process, they would overflow into urine.

An elevation in urinary creatinine may be related to the higher rates of glomerular filtration which might be expected due to perturbation of the renin-angiotension system.[32,33] It implied that the administration of ChangLe to rats resulted in a selective necrosis at some extent at the tip of the renal papilla, similar to that described in rats after the injection of propylene imine.[30] This, in turn, is probably related to the less decreased levels of serum creatinine observed in rats receiving the dose of ChangLe.

The intensity of the peaks for DMA, DMG and TMAO increased with the increase in dose of ChangLe. It was reported that renal capillary necrosis led to the increase in TMAO, DMA and DMG excretion.[34,35] It seems reasonable to speculate that similar process occurs for ChangLe, and this possibility is supported by the increase in UA and BUN concentrations of the serum for rats receiving the higher rare earth doses.

In the present study, the only amino acids with elevated levels in urine after ChangLe dosing were phenylalanine, glycine and glutamine. This increase is a common phenomenon observed in patients with hepatic failure and is regarded as a distinctive feature of hepatic coma.[24] It may be the result of the high doses of ChangLe impairing organs and tissues, including kidneys and livers of the rats and thus creating an obstacle to the reabsorption of such amino acids and consequently leading to aminoaciduria. These amino acids seem to be good candidates as NMR markers for ChangLe-induced organism dysfunction.

The elevation of taurine levels is the most important NMR marker for liver damage,[36] which could also lead to the elevated levels of BUN in serum. From the increase of ALP concentration in the serum, which is one of the important indices reflecting pathological state of the liver (Table 3), the indications of liver dysfunction due to a long-term high dosage ChangLe could also be seen.

Low GLC levels in the rat serum (Table 2) could occur due to elevated insulin levels at the two higher ChangLe dosage groups. It is likely that certain dosage of rare earth can exert effects on endocrine system,[37,38] and cause hormones change in the rat serum including insulin, GH and cortisol. They gradually return back to normal levels if rare earth dosing was withdrawn.

Compared with $La(NO_3)_3$,[39] the toxicological effects of ChangLe were weaker at the same dose, which probably indicated that different rare-earth ions might behave in different manner in the body. The possibility is supported by the results of Gao *et al*.[40] They found that chromosome damage due to ChangLe may not be attributed to cerium nitrate; however, lanthanum, praseodymium and neodymium nitrate can induce chromosome damage.[41] It has been found that different rare-earth ions in ChangLe had different accumulation rates and metabolic rates in the body where the order for the accumulation rate was La > Ce > Nd > Pr and for the metabolic rate was Nd > Ce > La, Pr.[42] In a practical application in agriculture, it is possible to adjust the proportion of different rare earths to reduce toxicity at the same effectiveness.

Conclusions

NMR analysis is an efficient and sensitive means of achieving important information on toxicological and pharmacological effects of drugs, which can provide new insight into the biochemical changes associated with physiological states and pathological conditions. Our initial results from the ^1H NMR analyses and biochemical assessments suggest that high-dosage ChangLe impairs the specific region of liver and kidney. The decrease in urinary citrate and the increase in urinary succinate and α-ketoglutarate could be attributed to tubular acidosis, the inhibition of Krebs cycle enzymes and the abnormal fatty acid catabolism. The information of the renal capillary necrosis and hepatic mitochondria damage can be derived from the increase in DMA, DMG, TMAO and taurine. It is very likely that different rare-earth ions might behave in different manners and cause different effects in the body. Obviously toxicological effects on organs and tissues in the body have not been found from the NMR spectra of rat urine for low-dosage experimental group. But further studies on the safety in the application of rare earths in agriculture are still opened.

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