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Efficacy of Leu-Ile-Lys tripeptide in the correction of experimental urate nephrolithiasis

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Abstract

Aim. To study the effect of the Leu-Ile-Lys (leucine-isoleucine-lysine) tripeptide on the course of experimental urate nephrolithiasis.

Methods. The experiment was carried out on 23 male Wistar rats weighing 200–220 grams that were divided into a control (n=8, modeling of urate nephrolithiasis) and an experimental (n=15, modeling of urate nephrolithiasis + administration of Leu-Ile-Lys tripeptide) groups. The tripeptide was administered intragastrically through a tube at a dose of 11.5 mg/kg. Daily urine was collected on a weekly basis, and the activity of lactate dehydrogenase and gamma-glutamyl transpeptidase was determined. After three weeks of the experiment, the animals were euthanized, and kidneys were removed to determine the parameters of free radical oxidation [concentration of thiobarbituric acid reactive substances (TARS), total pro-oxidant and total antioxidant activity, the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX)] and conduct a morphological study in which the number and area of calculi and the condition of the renal tissues were determined. Statistical data processing was performed using the Statistica 12.0 software. We used the Mann–Whitney U test, the Wilcoxon signed rank test. The results are presented as median with the 25th and 75th percentiles. The differences were considered significant at $p < 0.05$.

Results. In the kidneys of the rats of the experimental group, the number of deposits decreased twofold, as compared with the control group (1.6±0.2 and 3.2±0.4, respectively, p=0.001). Lactate dehydrogenase activity in the urine of rats increased from 0.10 (0.06; 0.21) units/mg of creatinine per day initially to 0.75 (0.44; 1.07) units/mg creatinine per day on day 21 (p=0.012) in the control group, and from 0.10 (0.06; 0.12) units/mg creatinine per day to 0.45 (0.34; 0.71) units/mg creatinine per day on 21st day (p=0.003) in the experimental group. The concentration of thiobarbituric acid reactive substances in the experimental group was 1.2 times lower than that of the control group: 4.2 (3.9; 4.6) μmol and 5.1 (4.7; 5.5) μmol, respectively ($p < 0.001$). In addition, in the kidneys of the rats of the experimental group, the total antioxidant activity was 1.3 times higher than in control: 70.4 (65.4; 74.1)% and 53.8 (33.3; 62.2)% respectively ($p < 0.001$). Superoxide dismutase activity in the experimental group was 1.5 times higher than in the control group: 12.4 (11.0; 13.2)% against 8.1 (6.4; 13.1)% (p=0.016). Catalase activity in the experimental group was 1.2 times higher than in the control group: 31.1 (26.4; 36.1)% against 25.1 (20.3; 27.1)% (p=0.005).

Conclusion. The Leu-Ile-Lys tripeptide has a litholytic effect, manifested in a statistically significant decrease in the activity of oxidative stress markers ($p < 0,001$) and a twofold decrease in the average amount of uric acid kidney stones ($p < 0,001$).

Keywords: urate nephrolithiasis, Leu-Ile-Lys tripeptide, pharmacological correction.

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Background. One of the most common forms of urolithiasis is urate nephrolithiasis (UN) [1]. The origin of urate lithogenesis is based on altered uric acid synthesis and excretion, triggering the development of hyperuricemia and hyperuricosuria, uric acid crystals deposition in the renal tubules and kidney stone formation [2].

Modern ideas about the UN etiology and pathogenesis are largely associated with the so-called metabolic syndrome, which is currently the most prevalent disorder among internal human diseases [3]. This is secondary to poor feeding habits, a sedentary lifestyle, bad habits, etc. In light of this, experts have predicted a rise in the UN cases [3].

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Consequently, the creation of new UN treatment methods, primarily pharmacological methods, will become increasingly more important. Generally, the so-called "citrate therapy" is currently the basic approach to dissolving urate stones [4].

Simultaneously, this method has significant drawbacks. For instance, i citrate mixtures can provoke the formation of oxalate crystals on urate calculi due to the fact that under the action of citric acid, the synthesis of oxaloacetate, fumarate, and α-ketoglutarate is activated, leading to a rise in the oxalate concentration in urine and the formation of insoluble calcium oxalate crystals[5]. In other words, citrate therapy can provoke the development of a mixed urate-oxalate form of urolithiasis, whose medical treatment is particularly difficult.

This justifies the relevance of novel pharmacologic agents for the management of UN.

It was revealed that the individual tripeptide Leu-Ile-Lys exhibits pronounced anti-lithogenic activity in 6-week and chronic 16-week experimental oxalate nephrolithiasis [6, 7]. Taking this into account, we decided to test the potential efficacy of the Leu-Ile-Lys tripeptide in relation to experimental UN in order to assess the prospects of its use both for the correction of urolithiasis monoforms and mixed urate-oxalate nephrolithiasis management.

The study aimed to study the effect of the Leu-Ile-Lys tripeptide on the course of experimental UN.

Materials and methods. This study was performed in the Research Center for Shared Use and the Department of Pharmacology, Altai State Medical University (Barnaul). For the biological system, 23 male Wistar rats aged 2 to 3 months with a body weight of 200 to 220 g were used. These rats were obtained from the cytology and genetics research institute vivarium (Novosibirsk). The rodents were placed in individual cages whose design permits urine collection. Throughout the experiment, a standard laboratory diet was used in feeding these rats. The study was approved by the local ethics committee of the Altai State Medical University of the Ministry of Health of Russia (extract from minutes No. 9 of 23.10.2020).

Concerning the experimental design, the mouses were separated into two groups, namely a control group (simulation of UN, n = 8) and experimental group (simulation of UN + administration of the Leu-Ile-Lys tripeptide, n = 15).

Formation of experimental UN, by the generally accepted model, entailed a 500 mg/kg mixture of oxonic acid and a 1000 mg/kg uric acid administered intragastrically to the mouses daily for 3 weeks [8]. From the11th day to the end of the experiment, the Leu-Ile-Lys tripeptide was administered daily to rats of the experimental group intragastrically via a tube at a dose of 11.5 mg/kg, correspondeding to the anti-lithogenic dose of the tripeptide as previously revealed in the experimental oxalate nephrolithiasis [6]. This substance was obtained through chemical synthesis at the Shanghai Apeptide CO., LTD (Shanghai, China) by the mediation of Evalar (Biysk, Russia).

Prior to the experiment, and then on the days 7, 11, 14, 17, and 21, a 24-hour urine sample was obtained, in which the excretion of uric acid and creatinine was determined, as well as the activity of lactate dehydrogenase and γ-glutamyl transpeptidase which are enzymes considered as biochemical markers of urothelial damage [9]. The analysis was performed by a laboratory biochemical analyzer CS-T240 (Dirui Industrial Co., Ltd., China) using reagent kits from Diacon-DS, Russia.

After 3 weeks of the experiment, the rats of each group were euthanized using ether anesthesia [10], after which both kidneys were surgically excised to determine the level of oxidative stress in the renal tissue as well as morphological study of the structural and functional state of the renal tissue, and to determine the amount and size of urate deposits.

The parameters of the activity of the free radical oxidation process, namely the concentration of thiobarbiturate-reactive products, the total prooxidant activity, the total antioxidant activity, the activity of catalase, superoxide dismutase, and glutathione peroxidase, were assessed in accordance with generally accepted methods [11].

For histological examination, the test material was assessed using the TISSUE-TEK VIPTM 6 apparatus (Sakkura, Japan), followed by obtaining 5–7 μm thick sections with the obligatory capture of the renal papilla using the Accu-Cut SRM apparatus (Sakkura, Japan). Then the specimens were stained using hematoxylin and eosin studied at a × 400 magnification on a Nikon Eclipse E200 microscope (China) to identify the number and area of urate deposits in the renal tissue, as well as assess the microscopic presentation of the kidneys. Morphometric studies were performed using the Axio Vision 3.1 software packages from Carl Zeiss, as well as Image J 1.43 from Wayne Rasband.

Data analysis was performed using the Statistica 12.0 electronic kit Windows. As regards biochemical analysis, the median and the interquartile range (25%; 75%) were calculated; and regarding morphometric parameters, the mean and standard error of the mean were calculated. For statistically significant differences, the normal distribution of the data obtained was tested.Given that the dataset did not follow a normal distribution, nonparametric tests like the Wilcoxon test (to assess differen-

Table 1. Dynamics of activity of marker enzymes of renal tissue damage in urine of experimental rats.

Term	Lactate dehydrogenase, units/mg creatinine per day		γ-Glutamyl transpeptidase, units/mg creatinine per day	
	Control	Experiment	Control	Experiment
Baseline level	0.10 (0.06; 0.21)	0.10 (0.06; 0.12)	0.74 (0.48; 1.12)	0.54 (0.43; 0.62)
Day 7	0.13 (0.11; 0.19)	0.16 (0.10; 0.19). +60%. $p_{b/l} = 0.018$	0.34 (0.24; 0.45). –54%. $p_{b/l} = 0.017$	0.23 (0.19; 0.45). –57%. $p_{b/l} = 0.011$
Start of Leu-Ile-Lys tripeptide administration				
Day 11	0.19 (0.16; 0.22)	0.19 (0.15; 0.22). +90%. $p_{b/l} < 0.001$	0.49 (0.29; 0.62)	0.36 (0.23; 0.45). –33%. $p_{b/l} = 0.004$
Day 14	0.23 (0.22; 0.29). +130%. $p_{b/l} = 0.049$	0.23 (0.17; 0.32). +130%. $p_{b/l} = 0.003$	0.29 (0.20; 0.45). –61%. $p_{b/l} = 0.014$	0.23 (0.16; 0.32). –57%. $p_{b/l} < 0.001$
Day 17	0.32 (0.26; 0.42). +220%. $p_{b/l} = 0.017$	0.47 (0.26; 0.50). +370%. $p_{b/l} < 0.001$	0.30 (0.22; 0.48). –59%. $p_{b/l} = 0.017$	0.22 (0.16; 0.36). –59%. $p_{b/l} = 0.009$
Day 21	0.75 (0.44; 1.07). +650%. $p_{b/l} = 0.012$	0.45 (0.34; 0.71). +350%. $p_{b/l} = 0.003$	0.25 (0.21; 0.38). –66%. $p_{b/l} = 0.017$	0.27 (0.25; 0.31). –50%. $p_{b/l} = 0.003$

Note: Data variability is represented by the median, showing the 25th and 75th percentiles; $p_{b/l}$ is the level of statistical significance of the change in the indicator within the group relative to the initial level.

ces within a group) and the Mann – Whitney test (to assess intergroup differences) [12].

Results and discussion. The lactate dehydrogenase activity rose consistently in the control group increased consistently during the entire 3 weeks. As a consequence, on day 21, it exceeded the initial level by 7.5 times ($p = 0.012$) (Table 1). Similarly, in the experimental group, the activity of lactate dehydrogenase increased within the first 17 days of the experiment, however, by the end of the week 3, this growth stopped, and the value of the indicator was 4.5 folds higher than the initial level ($p = 0.003$). The γ-glutamyl transpeptidase activity against it during the experiment in both groups consistently experienced a 3-fold decreased in the control group ($p = 0.017$), and by 2 folds in the experimental group ($p = 0.003$). During this course, indicators of the level of diuresis and excretion of creatinine did not reveal statistically significant intergroup differences throughout the experiment (not presented in Table 1).

Study of the prooxidant status indicators in rat kidneys of the group, in which the Leu-Ile-Lys tripeptide was administered to correct the experimental UN, disclosed a thiobarbiturate concentration 1.2-fold less than in the control group (4.2 (3.9; 4.6) μmol and 5.1 (4.7; 5.5) μmol, respectively; $p < 0.001$) after the third week. Figure 1 indicates the statistical significance level. There was no statistically significant prooxidant activity differences between the groups ($p = 0.615$).

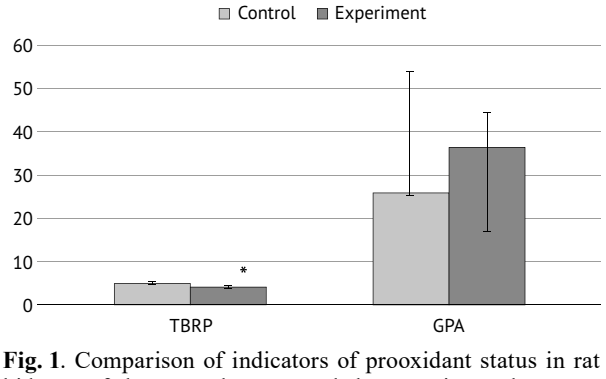

Fig. 1. Comparison of indicators of prooxidant status in rat kidneys of the control group and the experimental groups; TBRP — thiobarbiturate-reactive products; GPA — general prooxidant activity. The horizontal axis presents the name of the indicator and the unit of measurement. The vertical axis presents the indicator value. The values are presented as median and interquartile range. An asterisk denotes statistically significant differences in the experimental group compared to the control group

Determination of the antioxidant indicator status in rat kidneys of the experimental groups revealed that the total antioxidant activity in the experimental group with the use of the Leu-Ile-Lys tripeptide was 1.3 times higher than in the control group, namely 70.4 (65.4; 74.1) % and 53.8 (33.3; 62.2)%, respectively ($p < 0.001$) (Fig. 2). Superoxide dismutase activity in the experimental group was 12.4 (11.0; 13.2) %, exceeding the level of rats in the control group by 1.5 times ($p = 0.016$). Catalase activity in the experimental group exceeded the performance of rats in the control group by 1.2 times,

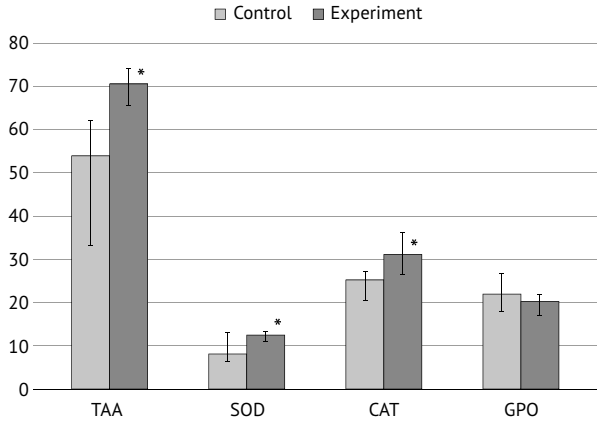

Fig. 2. Comparison of total antioxidant activity (TAA) indicators and the antioxidant enzymes activity in rat kidneys of the control group and the experimental group. The horizontal axis presents the name of the indicator and the measurement unit. The vertical axis is the indicator value. The values are presented as median and interquartile range. An asterisk denotes statistically significant differences in the experimental group compared to the control group. SOD — superoxide dismutase; CAT — catalase; GPO — glutathione peroxidase

namely 31.1 (26.4; 36.1) % versus 25.1 (20.3; 27.1) %, respectively (p = 0.005). The activity of glutathione peroxidase between the groups almost did not differ (21.8 (17.7; 26.6) % in the control group and 20.1 (17.0; 23.9) % in the experimental group).

When simulating UN in the control group, on the 21st day of the experiment, urate deposits were detected in different amounts in every case 8 (100%). Microliths of various shapes, blue appearance were revealed in the tubules of the cortical layer and the kidney papilla. They were located in the cystic-dilated renal tubules, among cell detritus and inflammatory infiltrate, consisting mainly of leukocytes (Fig. 3, *a*). The epithelium of such cystic-dilated tubules looked flattened, and the cell nuclei were reduced in size, in a state of pycnosis. In the stroma, a pronounced inflammation was noted. The number of deposits in the tubules averaged 3.2 ± 0.4 in the visual field with a × 400 magnification. The average area of deposits was 432.8 ± 41.8 μm^2.

When simulating UN using a 10-day administration of the Leu-Ile-Lys tripeptide, on the day 21 of the experiment, urate deposits were detected in all 15 (100%) cases. They had varying shapes, appeared blue and were located in the cortex and the renal papilla, in the cystic-dilated renal tubules, among cellular detritus and inflammatory infiltrate consisting of leukocytes (Fig. 3, *b*). The inflammatory reaction in the stroma was moderately pronounced. Epithelial changesof the tubules were less distinct, the cellular nuclei were of different sizes, and there were some hypertrophic nuclei. The tubular deposits in comparison with the control de-

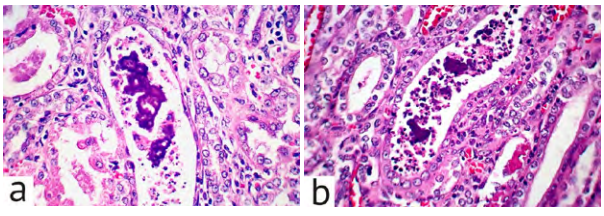

Fig. 3. Histological presentation of urate nephrolithiasis in the kidneys of experimental rats. Coss's staining. Magnification × 400: a — urate deposits in the cystic-altered tubule of the kidney in a control group rat; b — urate deposits in the kidney tubule on the day 21 of the experiment when using the Leu-Ile-Lys tripeptide

creased by 2-folds and averaged 1.6 ± 0.2 in the visual field using a × 400 magnification (p = 0.001). The average area of deposits was less relative to the control and amounted to 371.5 ± 112.3 μm^2 (p = 0.615).

This study reported that the use of the Leu-Ile-Lys tripeptide against the 3-week experimental UN was accompanied by characteristic biochemical and morphological signs of alleviation of the pathological course.

Thus, by the end of the course of administration of the Leu-Ile-Lys tripeptide, an increase in the activity of lactate dehydrogenase, a marker enzyme of nephrocyte cytolysis, stopped [9]. On the 21st of the experiment, the value of this indicator in the experimental group which received the treatment was 4.5 times higher than the initial value (p = 0.003), while in the control group, it was 7.5 times higher (p = 0.012). The indicator of the statistical significance of the intergroup difference was p = 0.051.

Simultaneously, in rat kidneys that received the tripeptide, there was a reduction of oxidative stress, which is an important pathogenetic factor in the development of nephrolithiasis [13]. There was consequently a decrease in the concentration of thiobarbiturate-reactive products and an increase in the overall antioxidant activity.

Thiobarbiturate-reactive products represent an indicator of membrane phospholipid peroxidation [14]. Its decrease hypothetically indicates a decrease in the degree of cell membrane damage during treatment [14]. In turn, it is assumed this could be due to an increase in the general antioxidant activity, which is an integrative indicator of non-enzymatic and enzymatic mechanisms of antioxidant cell protection.

Exploring of literature data on possible mechanisms of the antioxidant action of short peptides, including those containing the amino acids lysine, leucine, and isoleucine, three most probable causes of oxidative stress weakening under the influence of the Leu-Ile-Lys tripeptide should be distinguished [15, 16].

1. Free radical binding due to the presence of terminal lysine.

2. Chelation of metal ions with potential destructive property attributed to the amino group of the terminal lysine.

3. Strengthening of these properties in the lipid membrane of cells due to leucine and isoleucine hydrophobic properties.

The results of earlier experiments can be regarded as an additional indirect confirmation of the peptide ability to chelate metal ions [7]. It is revealed that long-term use of Leu-Ile-Lys tripeptide in presence of 16-week oxalate nephrolithiasis resulted in a 4.4-fold decrease in urinary calcium ion concentration compared to the control group ($p < 0.001$).

Evidence of the registered anti-lithogenic activity of the Leu-Ile-Lys tripeptide in experimental UN were the results of morphological studies, according to which the average number of urate deposits after 10-day treatment decreased by 2 times ($p = 0.001$) with an incidental weakening of oxidative and inflammatory processes in the kidneys.

CONCLUSION

Ten-day use of the Leu-Ile-Lys tripeptide in experimental UN in rats resulted in a 2-fold decrease in the average number of urate kidney stones with an adjoining decrease in renal oxidative stress.

Author contributions. A.Yu.Zh. was the research supervisor, created the study design, collected and performed statistical processing of the data, and wrote the text of the article; O.N.M. and O.G.M. analyzed the biochemical parameters; I.P.B. performed histological studies, and took part in writing the article; A.S.K. wrote the article, conducted and processed the results; G.V.Zh. simulated the urate nephrolithiasis, and conducted the biochemical studies; N.N.Ya. and I.E.G. created the study design, and interpreted the experimental results.
Conflict of interest. The authors declare no conflict of interest.
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