Intestinal Urea Metabolism: Could the Bacteria Involved in Nitrogen Cycle Lead to Reutilization of Intestinal Urea Nitrogen in Uremic Rabbits?

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Summary We aimed to evaluate the effect of bacteria involved in the nitrogen cycle on the reutilization of intestinal urea nitrogen in uremic rabbits. New Zealand white rabbits were made uremic via bilateral nephrectomy. Study and control rabbits were given live and heat-inactivated bacteria through their jejunostomies. After they were injected with 99mTc biurea intravenously, serial serum and stool levels of labeled nitrogen were assessed by instant thin-layer chromatography, and the change in the labeled-nitrogen level was determined. The serum labeled-nitrogen level increased significantly in the study group (r=0.990); however, this level decreased in the control group (r=0.662). Furthermore, the labeled-nitrogen level in the stool samples increased throughout the study in the control rabbits, but it decreased after the 6th hour in the study group. In conclusion, the results of this study suggest that when the bacterial flora of the intestinal system is changed to include bacteria involved in the nitrogen cycle in uremic rabbits, the intraintestinal and systemic nitrogen metabolisms could both be altered in favor of positive nitrogen balance.

Key Words nitrogen cycle, nitrifying bacteria, nutrition, urea, uremia.

In mammals, urea nitrogen is converted to ammonia by enteric flora bacteria in enterohepatic circulation and excreted in the feces as illustrated in Fig. 1. In uremia, the intestinal urea level increases in parallel with the serum level (1). Enteral antibiotics lead to an approximation of intraluminal urea concentrations in the intestines to the plasma levels and to a decrease in fecal ammonia levels in healthy subjects (2). This is also true for uremic patients who have higher fecal ammonia levels than the healthy controls (3). The elimination of intestinal flora bacteria, which convert urea nitrogen to fecal nitrogen, has been proposed to increase the bioavailability of urea nitrogen. The oral administration of neomycin and kanamycin to uremic patients resulted in a decrease in endogenous fecal nitrogen and to improved nitrogen balance (4). Isotopic studies performed in the humans and animals have shown that the daily amount of urea metabolized by the enteric flora bacteria is about 7 g, which is more than 20% of daily urea production and 40% of the urea pool (5). Many studies are being conducted to improve the impaired protein metabolism in uremic children, such as the use of essential amino acids and the α-ketoacids of these amino acids (6). However, these efforts did not significantly improve protein metabolism, nutrition, and growth in these children. The reutilization of intestinal urea nitrogen, which is normally excreted in the feces, might be an alternative solution for the nutrition and growth problems in uremic or nonuremic children.

Some plants can use free nitrogen in the air. The most significant group of plants that have this property is the leguminosae. The Rhizobium species—a Gram negative, aerobic, and motile rod living in the roots of leguminosae—fix the free nitrogen of the air by oxidation (7). The first product of nitrogen fixation is ammonia (NH₃). The oxidation of NH₃ to nitrate (nitrification) is performed by obligatory aerobic chemoheterotrophic bacteria in two steps. First, NH₃ is converted to nitrite (NO₂); then NO₂ is converted to nitrate (NO₃). The prototype of bacteria synthesizing NO₂ from NH₃ is Nitrosomonas, and that of converting NO₂ to NO₃ is Nitrobacter. These nitrifying bacteria are small Gram-negative rods and are found extensively both in soil and water (8). Nitrogen, which is usually taken in the form of nitrates (NO₃) in the soil, is reduced by some microorganisms and higher plants to be incorporated into the structure of amino acids and proteins. When the dead plants and animals putrefy, nitrogen reenters the soil in the form of ammonia (NH₃). The nitrogen of ammonia is oxidased to form NO₂ by the nitrifying bacteria (7).

The maximal metabolism of dietary protein could be an important solution for the undernutrition and growth failure in uremic as well as in nonuremic children. The aim of this study was to evaluate the effect of the bacteria involved in the nitrogen cycle on the reutilization of intestinal urea nitrogen in uremic rabbits. Biurea was used as the nitrogen source.

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MATERIALS AND METHODS

Experimental animals. Twenty New Zealand white rabbits (Orytolagus cuniculus) were enrolled in the study. All the rabbits were fed with standard pellets for 3 wk in laboratory conditions. They were kept at room temperature and at 12-h light-dark cycles with free access to water. Their weights were determined at the onset of the study.

Uremia model. All the animals were anesthetized by intraperitoneal ketamin (80 mg/kg) and inhalational ether. After that, a midline incision in the abdomen was performed to reach the peritoneal space. Both renal pedicles (including the renal vein, artery, and ureter) were then ligated with 5/0 silk, and blanching of the kidneys were noted.

Jejunostomy operation. At the same session, an 8 Fr feeding tube was inserted into the proximal jejunum and exteriorized through skin on the back of each rabbit (Stamm-type tube jejunustomy) (9).

Preparations of the bacterial solutions

Nitrosomonas: One milliliter of Nitrosomonas solution was inoculated into the tubes containing 4 mL mixture of macroelements (K₂HPO₄, MgSO₄, CaCl₂) + microelements (H₃BO₃, Na₂MoO₄, CoCl₂, MnCl₂, CuSO₄, ZnSO₄, NiSO₄) + iron chelate + (NH₄)₂SO₄ + bromothymol blue + sterile water. After 28 d of incubation at 28°C, when the color of the solution turned to yellow, the bacteria count was calculated by the method of MPN (most probable number) (10, 11). Each milliliter of the solution included about 10³ bacteria.

Nitrobacter: One milliliter of Nitrobacter solution was inoculated into the tubes containing 4 mL mixture of macroelements + microelements + iron chelate + KNO₃ + sterile water. After 28 d of incubation at 28°C, the bacteria count was calculated by the method of MPN (most probable number) (10, 11). Each milliliter of the solution included about 10³ bacteria.

Rhizobium: Clover roots were used as the source of Rhizobium, since this bacterium could not be grown at in vitro conditions to form specific solution. Thus 500 g of ground clover roots was suspended in 1.000 mL of sterile water, and this solution was used as the source of Rhizobium. The presence of Rhizobium bacteria in this suspension was confirmed by an inoculation of 1 mL of suspension into the petri dishes containing Yeast Extract Mannitol Agar solid medium and incubation for 5 d at 28°C. After the incubation period, Rhizobium colonies grown in the solid medium were counted (12).

Labeling of biurea with ⁹⁹ᵐTc. Urea and SnCl₂·2H₂O were purchased from Merck. Na⁹⁹ᵐTcO₄ was obtained from Department of Nuclear Medicine of Ege University.

Biurea (Fig. 2) was prepared as follows: Dry urea of 10 mg was heated for 2 h at 200°C. ⁹⁹ᵐTc-biurea: Freshly prepared SnCl₂·2H₂O solution (1 mg/1 mL 0.1 N HCl) was added to freshly prepared biuret (2.5 mg/mL) solution in a tube. Then approximately 0.3–0.5 mCi (11.1–18.5 MBq) Na⁹⁹ᵐTcO₄ was added and allowed to settle for 20–25 min at room temperature. Labeling yield was checked by radio-TLC, radio-HPLC, and radio-paper electrophoresis. Labeling yield and specific activity exceeded 90% and 3.1×₁₀¹⁰ Bq/mmol, respectively.

Radio-TLC conditions: ⁹⁹ᵐTc-biurea was analyzed by the use of TLC supply using cellulose-coated plastic sheets (Merck 5565). These sheets were cut into 1×10 cm strips. A point was marked at 1 cm from one end as a marker for the origin. The samples of 5 μL were applied here, and without drying the spots were developed immediately. Two solvent systems were used: 20% dextrose solution and 20% citrate acid as eluant. The solvents were allowed to reach about 8 cm from the origin. The strips were dried and cut into 0.5 cm pieces. The radioactivity of each piece was determined in a gamma well-type NaI(Tl) scintillation detector of a multichannel analyzer. The Rf values and labeling yield of ⁹⁹ᵐTc-biurea were calculated from the strip developed in both solvent systems.

Electrophoresis was done with a Gelman electrophoresis chamber supply using cellulose acetate strips (25x1 cm). After the strips were moisturized by buffer solution (0.8 mL glacial acetic acid and 10 mL pyridine made up to 250 mL with distilled water, pH 6.1), a sample was applied on a marked strip. This strip
was settled in the electrophoresis chamber. Applied voltage was 250 V, and standing time was 2 h. The developed strip was then dried and cut into 1 cm pieces, and each piece was counted by a NaI(Tl) scintillation detector.

HPLC (Shimadzu LC-10 TvP) with a UV detector (Shimadzu SPD-10 AvP) and equipped with a Cd(Te) detector (RAD 501) was used to check the $^{99m}$Tc-biurea structure. HPLC conditions: the UV detector was settled to 195 nm. Column: 250/4.6 Nucleosil 100-5 C-18. Eluent: 0.02 M Na$_2$HPO$_4$ buffer and 0.005 M dodecylbenzene sulfonate were mixed and adjusted to pH 5.5 with orthophosphoric acid. The flow rate of eluent was 1.0 mL/min. The obtained chromatograms for biuret, urea, and $^{99m}$Tc-biurea at UV and radioactivity detectors were shown in Fig. 3.

Administration of bacterial solutions. After bilateral ligation of renal pedicles and jejunostomy, 10 rabbits (study group) were given 10 mL of each bacterial solution four times at 3-h periods (0, 3, 6, and 9 postoperative hours) through jejunostomies. The remaining 10 rabbits (control group) were given the same amount of heat-inactivated (at 100°C for 30 min) bacterial solutions by the same route.

Administration of $^{99m}$Tc-biurea to the rabbits and obtaining blood and stool samples. At the postoperative 12th hour, an intravenous access through an ear vein was performed for each rabbit. First, 1 mL of blood was obtained for baseline analyses; then 5 mCi/kg of $^{99m}$Tc-biurea solution was injected into each rabbit. After that, at the 5th minute (0 h) and every 2 h thereafter, 1 mL of blood samples was obtained for 8 h (2nd, 4th, 6th, and 8th hours). Further, each stool sample of each rabbit was obtained, and the time of defecation was noted during this 8-h period.

Analysis of $^{99m}$Tc-biurea levels in the blood and stool samples. Each timely obtained blood and stool sample was analyzed for change in the level of $^{99m}$Tc-labeled nitrogen by ITLC. The change in the level of the labeled-nitrogen with respect to time was calculated for each rabbit, and the mean values for the study and control groups were determined. A calculation of the radioactivity of samples was performed by a well-hole-type scintillation detector with multichanneled NaI(Tl) analyzer, through setting for a 141 keV γ-photon peak of $^{99m}$Tc. The results obtained by radio-TLC were controlled by radio-HPLC and radioelectrophoresis.

Further, blood urea nitrogen (BUN) level was determined in each blood sample by the routine spectrophotometric method (DACOS XL autoanalyser).

Bacterial culture of the stool samples
Nitrosomonas and Nitrobacter: After the stool samples were weighed and homogenized in sterile water, they were inoculated into the culture mediums defined previously (10, 11). At the end of an incubation period, bacteria count was determined by the MPN method and expressed as “number of bacteria per one gram of stool.”

Rhizobium: The presence of Rhizobium in stool homogenates was confirmed by an inoculation of 1 mL of suspension into the petri dishes containing yeast extract mannitol agar solid medium and incubation for 5 d at 28°C. After the incubation period, Rhizobium colonies grown in the solid medium were counted (12).

Statistical analyses. A statistical evaluation of the results was performed via an SPSS computer program using Friedman, Wilcoxon signed rank and Mann-Whitney U tests.
RESULTS

The mean weights of the study and control rabbits were 2,003±137 and 1,985±168 g, respectively (p>0.05). Because two of the study and one of the control rabbits died during the early postoperative period, the study was completed with 17 rabbits.

BUN levels at the onset and throughout the study did not differ between the groups. On the other hand, the increase of BUN with respect to time was significant in both groups (Table 1, p<0.005). Labeled-biurea solutions given at the onset of the study did not significantly affect the BUN levels of the rabbits. This is verified by similar BUN levels measured before and 5 min after the injection of 99mTc-biurea (Table 1, p>0.05).

Table 1. The mean blood urea nitrogen levels of the study and control rabbits (mean±SD).

| Time      | Study group (n=8) | Control group (n=9) | p    |
|-----------|------------------|---------------------|------|
| Onset     | 83.33±22.12      | 85.14±34.04         | 0.952|
| 0 hours   | 83.38±22.15      | 89.44±19.20         | 0.736|
| 2nd hour  | 91.63±15.68      | 101.11±26.07        | 0.630|
| 4th hour  | 101.71±15.90     | 111.67±24.12        | 0.525|
| 6th hour  | 107.63±18.57     | 113.44±28.72        | 0.847|
| 8th hour  | 115.90±20.25     | 120.56±31.33        | 1.000|
| p         | 0.0002           | 0.0000              |      |

1 Blood urea nitrogen levels increased significantly with time (p=0.0002).
2 Blood urea nitrogen levels increased significantly with time (p=0.0000).
3 The values measured before (onset) and after 5 min (0 hours) of injection of the radiolabeled-nitrogen.

The level of radioactivity because of labeled-nitrogen in the blood samples of study and control groups at the 5th minute of injection was 45.63 and 37.22%, respectively, of the radioactivity of the original biurea solution (Table 2). Radioactivity decreased at the 2nd hour, remained stable at the 4th hour, and tended to increase at the 6th and 8th hours in the study group (Table 2). The changes in the level of labeled-nitrogen with time, however, was not significant in this group (p=0.080). The levels in the control group decreased progressively at the 2nd and 4th hours, tended to increase at the 6th hour (though it was lower than at the 2nd hour), and again decreased at the 8th hour. The changes in the level of labeled nitrogen with time was also not significant in the control group (p=0.097). The results obtained by radio-TLC were supported by radio-HPLC and radioelectrophoresis methods.

When the changes in the level of labeled-nitrogen in the study and control groups were compared, the levels at the onset and 2nd hour were not different. On the other hand, the study rabbits were determined to have significantly higher levels at the 4th, 6th, and 8th hours (p=0.015, p=0.050, and p=0.001, respectively) (Table 2).

When the relation of the BUN level to time was analyzed, the study and the control groups both showed a significant positive correlation (r=0.394, p<0.05, and r=0.571, p<0.05, respectively).

When the relation of blood labeled-nitrogen level to time was analyzed, there was a rapid decline during the first 30 min after the initial peak level in the study group. After that, the labeled-nitrogen level increased in time (r=0.990). The control group, however, showed a progressive decline (r=0.662) after an initial peak. The stool labeled-nitrogen level in the study group reached a peak at the 6th hour, then declined again.

The stool samples of both the study and control rabbits were cultured for all three types of bacteria. *Rhizobium* could not be grown in either group. The numbers of *Nitrosomonas* and *Nitrobacter* grown in 1 g of stool in the study group were 98.75±106.09 and 2.00±2.30, respectively. The corresponding values for the control group were 21.56±48.30 and 12.56±12.13, respectively. Although the study group had a significantly higher number of *Nitrosomonas* colonies (p=0.019), the number of *Nitrobacter* did not differ between the groups (p=0.190).

DISCUSSION

A probiotic is a viable microbial dietary supplement that beneficially affects the host through its effects in the intestinal tract. Probiotics are widely used to prepare fermented dairy. In the future they may also be found in fermented vegetables and meats. Several health-related effects associated with the intake of probiotics, including an alleviation of lactose intolerance and immune enhancement, have been reported in human studies (13). Probiotics are also used in veterinary medicine to decrease the elimination of food with feces, increase the availability of undigested food, and

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eliminate harmful microorganisms from the intestinal flora. Cattle administered probiotics are reported to gain normal weight with lower energy intake (14). In this study, the probiotic effect of the bacteria involved in the nitrogen cycle, when inoculated into the intestinal flora of uremic rabbits, was evaluated in promoting the use of intestinal urea nitrogen in protein synthesis.

Biurea was chosen as the nitrogen source in this experiment. It has been demonstrated previously that biurea stabilized by isobutylidene (isobutylidene diurea) is equal to or even better than urea as a nitrogen supply for rumen microbes (15, 16).

BUN levels increased throughout the study period in both the study and control groups and did not differ between them. The blood labeled-nitrogen level, however, decreased progressively in the control group, but increased after a period of fall in the study group. This indicates that labeled-nitrogen entering the intestinal lumen of these rabbits is reutilized by the effect of live-inoculated bacteria. A decline of labeled-nitrogen after 6 h in the stool samples of this group of rabbits also supports the previous finding.

The ITLC method used in this study enabled us to discriminate $^{99m}$Tc bound to nitrogen from free $^{99m}$Tc detached from nitrogen (17). Thus only the $^{99m}$Tc bound to nitrogen was used in the measurements, and free $^{99m}$Tc did not effect the results. However, increased labeled-nitrogen levels detected after the fourth hour of study in the study group might reflect the presence of the $^{99m}$Tc-biurea or other molecule(s) binding labeled-nitrogen, such as amino acids and peptides. The results obtained by ITLC were supported by radio-HPLC and radioelectrophoresis. However, these methods are unable to discriminate the nature of molecule(s) binding labeled-nitrogen. Thus other techniques are needed to evaluate the nature of molecule(s) binding labeled-nitrogen in this group of rabbits.

Although the study and control groups differed significantly with respect to the blood labeled-nitrogen level, we could not compare them with respect to change in the level of $^{99m}$Tc-labeled-nitrogen in the stool. This was because of the discordance in time in obtaining the stool samples from the animals.

We used enterostomy to inoculate the bacteria into the intestinal lumen (9). We did this because bacteria administered orally might be inactivated by oral or gastric secretions. In the future it may be possible to prepare oral formulations of these bacteria.

The growth of *Rhizobium*, *Nitrosomonas*, and *Nitrobacter* in stool cultures is the gold standard for demonstrating the colonization of these bacteria in the intestinal lumen of the rabbits. Although *Nitrosomonas* and *Nitrobacter* were cultured in the stool samples of both groups, *Rhizobium* could not be grown in either. On the other hand, the role of *Rhizobium* in the nitrogen cycle is to fix free nitrogen in the air (7). Thus *Nitrosomonas* and *Nitrobacter* are probably more important as far as the use of nitrogen incorporated into the structure of ammonia in the intestinal lumen is considered. In other words, we believe that the failure of colonization of *Rhizobium* in the intestinal lumen of the rabbits did not affect the kinetics of this study. Although both *Nitrosomonas* and *Nitrobacter* were supposed to be grown in higher numbers in the study rabbits, only the number of *Nitrosomonas* was higher significantly in this group. This finding suggests that either *Nitrobacter* is found naturally in the intestines of rabbits or that heat-inactivation was not completely successful for *Nitrobacter*. In either case, presence of *Nitrosomonas* in the same medium seems to complete the cycle.

The compatibility of these bacteria with the normal intestinal flora of the rabbits was not known before the study. To promote the colonization of these bacteria in the intestinal lumen, enteral antibiotic administration could be considered (6). However, because these bacteria require nitrogen in the form of ammonia, the presence of normal intestinal flora was required (1–3).

Hemorrhage, especially gastrointestinal, developing during the operations could lead to major alterations in BUN levels of the animals. But use of the same procedures for both groups, control and study, and attention to good hemostasis during the operations eliminated such a complication. This is verified by the parallel course of BUN levels in the two groups.

How the reabsorbed labeled-nitrogen was metabolized or into which molecules it was incorporated could not be determined in this study.

Long-term follow-up was not possible because of the short half-life of the radiopharmaceutical and the short life expectancy of the nephrectomized animals.

In conclusion, the results of this study suggest that when the bacterial flora of the intestinal system is changed to include bacteria involved in the nitrogen cycle in uremic rabbits, both intraintestinal and systemic nitrogen metabolism could be altered in favor of positive nitrogen balance. However, more detailed studies should be performed to determine how the metabolism of urea nitrogen occurs and what kind of organic compounds are formed by these bacteria.

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