First Indications Demonstrating the Preventive Effects of NZ-419, a Novel Intrinsic Antioxidant, on the Initiation and/or Progression of Chronic Renal Failure in Rats

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The concentration of NZ-419 (5-hydroxy-1-methylimidazolidine-2,4-dione), an intrinsic antioxidant, has been shown to increase in the sera of animals and patients with chronic renal failure (CRF). This is the first report that orally administered exogenous NZ-419 prevents the initiation and/or progression of CRF in rats using an adenine-loaded model. After 24 d of adenine loading, there was a ca. 90% decrease in creatinine clearance ($C_{Cr}$) in the control rats. Treatment with NZ-419 from the beginning significantly inhibited the decrease in $C_{Cr}$ and also the increase in serum creatinine ($sCr$). Bio-markers for in vivo hydroxyl radicals, the serum methylguanidine ($sMG$) level, and $sMG/sCr$ molar ratio, not only in serum but also in the urine, kidney, liver, and muscle indicated that NZ-419 inhibited the increase in oxidative stress induced by CRF in rats. An increase of guanidinosuccinic acid, another bio-marker of oxidative stress, was also inhibited with NZ-419.

Key words: NZ-419; oxidative stress; methylguanidine; chronic renal failure

Although the number of patients with end-stage renal disease (ESRD) has increased rapidly over the last three decades and, prior to the advent of dialysis, some treatments including dietary control, drug therapy with a spherical carbonaceous absorbent, and anti-hypertensive drugs including ACEIs (angiotensin converting enzyme inhibitors) and ARBs (angiotensin receptor blockades) have shown some inhibition of the progression of chronic kidney disease (CKD) and chronic renal failure (CRF), these treatments are still not effective enough to curtail this increase. Therefore, the development of new effective drugs preventing the progression of CKD and CRF is still an urgent requirement. In the present article, we show the first indication that NZ-419 could be such a candidate.

NZ-419 (5-hydroxy-1-methylimidazolidine-2,4-dione) (Fig. 1) is a newly recognized intrinsic antioxidant,1–8 a creatinine ($Cr$) metabolite, which was first isolated from inflamed skin inoculated with vaccinia virus1 and subsequently from the urine of patients with CRF.9 The concentration of NZ-419 increases in the sera of mammals with CRF.9

Both animal models and patients with CRF have been shown to be under more oxidative stress than corresponding normal animals and subjects,6,7 and, in those studies, the level of methylguanidine (MG), which is a product of Cr with a hydroxyl radical, and the MG/Cr molar ratio in serum have been recognized to be useful as markers of oxidative stress in vivo.4,5,9–13 In addition to conventional markers such as 8-hydroxyguanine,14 8-hydroxy deoxyguanosine,14,15 and so on.

The importance of oxidative stress in renal failure development has also been shown in different ways when more than two intrinsic anti-oxidant systems are inhibited, renal failure is induced. Acute renal failure (ARF) has been induced by vitamin E deficiency and glutathione (GSH) depletion in rats: hydroxyl radicals and lipid peroxide have been indicated to play important roles in renal failure formation.16

In drug-induced ARF models, such as glycerol-induced ARF, a role of hydroxyl radicals in its initiation has been suggested, and the hydroxyl radical scavenger dimethyliourea (DMTU) inhibited the development of ARF.17

Also, in CRF models, some antioxidants which can scavenge hydroxyl radicals, such as tannins in plants, have been shown to inhibit the progression of CRF in several models,18–20 together with inhibiting the increase in the MG level and MG/Cr ratio in serum and urine. The role of active oxygen in the progression of another CRF, murine lupus nephritis, has been shown, because DMTU inhibited the progression.21

Therefore, safe antioxidants have been proposed to be candidates for anti-CRF agents, although hydroxyl radical scavengers with some side effects such as DMTU and dimethyl sulfoxide (DMSO) might not be suitable.

Since the concentration of endogenous NZ-419 in sera increases by CRF5,9 and NZ-419 has anti-oxidant activities,7,8 at least hydroxyl radical scavenging activity, we hypothesized that the endogenous NZ-419 increased under CRF conditions might act as a self-defense substance. In the present paper, we report the first results showing that NZ-419, exogenously administered perorally, through its antioxidant activities, may inhibit the initiation and/or progression of CRF.

MATERIALS AND METHODS

Animals and Chemicals The Guidelines for Animal Experimentation, approved by the University of Toyama, were followed in these experiments. Male Wistar rats (ca. 200—210 g), obtained from Japan SLC, Inc. (Hamamatsu, Japan), were used. They were maintained at a constant

![Fig. 1. Structures of NZ-419 and Guanidino Compounds, Cr, MG, and GSA](image)
humidity (ca. 60%) and temperature (ca. 23 °C) with a light/dark cycle of 12 h.

NZ-419 was prepared in the Institute of Bio-Active Science (IBAS: Nippon-Zoki). Other reagents used were of an analytically pure or HPLC grade.

**Experimental Design Using Adenine-Loaded CRF Rats**

(A) Rats underwent an adaptation period of several days, during which they were fed a commercial feed (type CE-2, CLEA Japan Inc., Tokyo, Japan). They were then fed by a pair feeding schedule on an 18% casein diet containing 0.75% adenine, which produced experimental renal failure in the animal. During the adenine-feeding period, an aqueous solution of NZ-419 (250, 500, 1000 mg/kg) was administered orally for 24 d as drinking water, while control rats received tap water. Twenty-four-hour urine was collected from the 23rd to 24th day. On day 24 of the administration period, serum was prepared. The urine and serum were used for the determination of biochemical parameters including three guanidino compounds, Cr, MG, and guanidinosuccinic acid (GSA) (Fig. 1). At the same time, the kidney, liver, and skeletal muscle were removed and used for the determination of guanidino compounds.

(B) Similarly, an aqueous solution of NZ-419 (25, 50, 100 mg/kg) was administered orally for 24 d as drinking water, and, on day 24, serum was prepared for the determination of Cr, MG, and GSA levels.

**Analyses** Blood urea nitrogen (BUN), calcium, and inorganic phosphate were determined using commercial reagents (BUN Kainos, from Kainos Laboratories, Inc., Tokyo, Japan; Calcium C-Test Wako and Phosphor B-Test Wako, from Wako Pure Chemical Industries, Ltd., Osaka, Japan). Cr, MG, and GSA were analyzed with a guanidino-compound analysing system (Japan Spectroscopic Co., Tokyo, Japan) by HPLC using a fluorogenic reagent, 9,10-phenanthrenequinone.

**Calculation of Creatinine Clearance** The creatinine clearance (C\(_{\text{Cr}}\)), an effective index for expressing the glomerular filtration rate (GFR), was calculated on the bases of urinary Cr (uCr), serum Cr (sCr), urine volume, and body weight using the following equation: C\(_{\text{Cr}}\) (ml/min/kg body weight) = uCr (mg/dl) × urine volume (ml)/sCr (mg/dl) × 1000/body weight (g) × 1000/1440 (min).

**Evaluation of Dose-Dependency of the Effect of NZ-419 on the Accumulation of Guanidino Compounds in Serum** After evaluating the effectiveness of NZ-419 on the serum levels of two guanidino compounds (Cr and MG) and molar ratio of MG/ Cr, each value/control value as a percentage was plotted versus its dose of NZ-419.

**Statistical Analysis** Data are expressed as means ± S.E. The significance of differences between each experimental group versus the control group was determined using Dunnett’s multiple comparison test. The level of significance was set at p<0.05.

**RESULTS**

**Adenine-Loaded Rats and Their Renal Function** Adenine causes renal disorders in rats, and the adenine-induced model has been recognized as a model with proximal tubular injury which exhibits degeneration of the proximal tubular epithelium and the infiltration of neutrophils and macrophages, forming foreign-body granulomas centering on an adenine metabolite, 2,8-dihydroxyadenine (DHOA), which is only slightly soluble in water and crystallizes and/or forms stones in the kidney, especially the proximal tubule. Although adenine does not have any direct effect on tubular cells, DHOA shows cytotoxicity. These rats have been indicated to be under a higher oxidative stress than glomerular injury model rats based on an electron spin resonance technique and analysis of MG, a biomarker of hydroxyl radicals. Since the activity of the radical scavenging enzyme has been shown to also decrease, the formation of excessive radicals and deterioration of defense mechanisms contribute to the development of oxidative stress, which could be a cause as well as a result of tubular cell injury.

The level of renal impairment becomes aggravated as the period of adenine feeding increases. It was previously confirmed that renal failure was already present after 6 d of adenine ingestion: GFR at this period is 1/2—1/3 of the normal level. Also, on the 24th day, renal functions such as GFR, renal plasma flow, and so on decreased to less than 10% of normal level. Thus, rats can be used as an animal model with ESRD showing uremia.

The sCr and BUN levels, which are also diagnostic indices of renal failure, indicated that renal function of the control group at 24 d was severe, showing azotemia. The sCr level in rats at 24 d was about 2.5—4.0 mg/dl, and their renal function was estimated to be similar to that of patients with ESRD whose sCr levels are 7.0—11.0 mg/dl just before introduction to hemodialysis.

**Effects of NZ-419 on C\(_{\text{Cr}}\) in Adenine-Loaded Rats**
The normal level of C\(_{\text{Cr}}\) is a conventional marker for GFR, in normal rats has been reported to be about 5.7 ml/min/kg (n = 6), and has been also shown to decrease to 11% of its normal level after 24 d of adenine loading. As expected, the C\(_{\text{Cr}}\) value in control rats decreased gradually to reach 0.65 ml/min/kg by adenine loading. In contrast, as shown in Fig. 2A, the C\(_{\text{Cr}}\) values in the rats orally given 250, 500, and 1000 mg/kg of NZ-419 were significantly higher than control level: 1.01 ml/min/kg, 1.13 ml/min/kg, and 1.15 ml/min/kg, respectively: ca. 18, 20, and 20% of the normal level, respectively. Since NZ-419 was administered from the beginning, this result indicates that NZ-419 has a protecting effect(s) on kidney function during the initiation and/or progression of CRF. This effect might seem weak, but we should remember that this model is so severe that the progression of CRF is much more rapid than that in human CRF patients: in the latter case, it takes years and not weeks of CRF progression before ESRD. Even compounds which are useful for the treatment of patients with renal insufficiency have not yet been reported to be effective in this model.

**Effects of NZ-419 on sCr and BUN in Adenine-Loaded Rats**
The normal levels of sCr and BUN have been reported to be 0.45 and 12.0 mg/dl (n = 6), respectively. The sCr level in the control rats markedly increased beyond the normal level, showing azotemia. The administration of NZ-419 at doses of more than 250 mg/kg significantly reduced the sCr level (Fig. 2B). Similarly, the BUN level of the control group increased to ca. 100 mg/dl. The administration of NZ-419 at all doses above 250 mg/kg significantly reduced the BUN levels (Fig. 2B).

NZ-419 significantly decreased both sCr and BUN levels,
which increased in CCr. This result also suggested that NZ-419 has a protecting effect(s) on kidney function impaired by CRF. This consideration was also supported by the results of serum phosphate and calcium.

**Effects of NZ-419 on Serum Calcium and Inorganic Phosphate Levels in Adenine-Loaded Rats** The serum calcium and inorganic phosphate levels in the control rats decreased and increased on adenine loading, respectively. The administration of NZ-419 at all doses above 250 mg/kg significantly maintained both nearly at reported normal levels (Ca: 10.23, and P: 8.25 mg/dl; \( n = 6 \)) (Fig. 2C).

**Effects of NZ-419 on Serum and Urinary Levels of Two Uremic Toxins, MG and GSA, in Adenine-Loaded Rats** In sera of normal rats, neither MG nor GSA is detectable.\(^{23,27}\) Urinary MG of normal rats has been reported to be about 2 \( \mu g/\)d.\(^{27}\) Not only the serum but also urinary MG level in control rats markedly increased from the normal level to reach 7.27 \( \mu g/\)d and 51.5 \( \mu g/\)d, respectively, on adenine loading. This indicated the hyperproduction of MG. The administration of NZ-419 significantly reduced both levels in a dose-dependent manner (Fig. 3A).

Similarly, both serum and urinary GSA levels in the control rats markedly increased on adenine loading to reach 115 \( \mu g/\)d and 112.9 \( \mu g/\)d, respectively, and the administration of NZ-419 significantly reduced the value in a dose dependent manner (Fig. 4).

**Effects of NZ-419 on Tissue Levels of MG in Adenine-Loaded Rats** In normal rats, MG levels in the kidney and liver have been reported to be undetectable, although a small amount of MG has been detected in muscle (ca. 0.1 \( \mu g/g \) tissue).\(^{27}\) As shown in Fig. 3C, MG levels in tissues (kidney, liver and muscle) increased markedly on adenine loading to 0.41, 0.19, and 0.32 \( \mu g/g \), respectively. The administration of NZ-419 significantly reduced levels in both the liver and muscle at all dosages tested. Although its only significant effect was at the highest dosage because of the high standard errors in the control group, the MG levels in the kidney of NZ-419 groups also decreased in a dose-dependent manner.

**Effects of NZ-419 on Serum MG/Cr Molar Ratios in Adenine-Loaded Rats** The molar ratio of MG/Cr, the conversion ratio from Cr into MG, has been thought to be one of the bio-markers for oxidative stress in vivo, because the hydroxyl radical plays an important role in the conversion.\(^{28,29}\) The serum value for intact rats was nearly zero, and increased on adenine loading. These facts indicated that oxidative stress increases in their bodies. The administration of NZ-419 significantly reduced the value in a dose dependent manner (Fig. 4).
Detecting renal effect, other independent reno-protective effects might indicate the possibility that, in addition to a GFR-pro-

The NZ-419-administered groups maintained almost consistent with the results of CCr: CCr/CCr0 just before the introduction of hemodialysis, and this was likely by decreasing enhanced hydroxyl radical levels there.

Fig. 4. The Effect of NZ-419 on the Molar Ratio of sMG/sCr in Rats with CRF

![Image](50x492 to 288x570)

Fig. 5. Dose-Dependency of the Effect of NZ-419 on the Serum Accumulation of Cr, MG, and GSA in Rats with CRF

- groups in high-dose experiment (250, 500, 1000 mg/kg/d); : groups in low-dose experiment (25, 50, 100 mg/kg). n=6—8, *p<0.01 vs. control group.

The Threshold of the Effective Dose of NZ-419 Regarding Its Inhibitory Effect on the Accumulation of Three Guanidino Compounds, Cr, MG, and GSA, in Serum

As shown in Fig. 5, the inhibitory effect of NZ-419 on each compound occurred in a dose-dependent manner. The threshold for each effective dose of NZ-419 was about 50 or 100 mg/kg/d.

DISCUSSION

The sCr and BUN levels, which are also diagnostic indices of renal failure, indicated that the renal function of the control group at 24 d was severely impaired, showing azotemia. The sCr level of rats with an equivalent relative GFR (C<sub>G</sub>/C<sub>G0</sub>, C<sub>G0</sub> indicates a normal level) is 1/2—1/3 of that in human subjects. The level in rats at 24 d was about 3.66 mg/dl, and their renal function was estimated to be similar to that of patients whose sCr levels are 7.0—11.0 mg/dl just before the introduction of hemodialysis, and this was consistent with the results of C<sub>C</sub>/C<sub>C0</sub> = 1/10. NZ-419 treatment of adenine-CRF rats significantly inhibited the increase in both sCr and BUN levels as well as the decrease in C<sub>C</sub>. These reno-protective effects against glomerular injury, however, could not be complete: up to C<sub>C</sub>/C<sub>C0</sub> = 1/2. Other reno-protective effects of NZ-419 could be shown, but sometimes varied.

Thus, the analysis of serum calcium and inorganic phosphate indicated their abnormalities in this model, as shown before, with decreasing and increasing levels, respectively. The NZ-419-administered groups maintained almost normal levels, and this inhibition was nearly complete. This might indicate the possibility that, in addition to a GFR-protecting renal effect, other independent reno-protective effects of NZ-419, especially in proximal tubules via its anti-oxidant effects, seem realistic.

Since not only the serum but also urinary MG levels in the control rats markedly increased on adenine loading, as shown previously, the production of MG was shown to be enhanced. The administration of NZ-419 significantly reduced both levels in a dose-dependent manner, and so NZ-419 was thought to directly inhibit production of the guanidino uremic toxin. Similarly, the production of GSA, the other guanidino uremic toxin whose production was also enhanced in CRF, was inhibited in a dose-dependent manner on treatment with NZ-419.

As reported previously, MG was markedly accumulated in kidney, liver, and muscle tissues after adenine loading, and the administration of NZ-419 significantly reduced those levels (Fig. 3C). This indicated that MG also accumulates in tissues when accumulation in blood can be observed, and that NZ-419 decreases MG levels in all areas of the body. In the kidney, the main MG production site in adenine-loaded rats has been shown to be the proximal tubules; thus, NZ-419 might inhibit MG production in the proximal tubules, most likely by decreasing enhanced hydroxyl radical levels there.

The contributions of reactive oxygen species in the production of these two guanidino uremic toxins, MG and GSA, were previously reported, and so the decrease in both levels meant that not only their production but also oxidative stress in rats decreases on the administration of NZ-419. Such guanidino-uremic toxins and oxidative stress are thought to be factors leading to the “worsening” of CRF. If we support this hypothesis, then NZ-419 could be “a double blocker” against the progression of CRF.

The inhibitory effects of NZ-419 against an increase in biomarkers, MG and GSA, of oxidative stress, seem to be able to reach near normal levels, although high doses would be required under severe conditions like in the adenine-induced rat model. As shown in Fig. 5, even at low doses (50, 100 mg/kg) where no effect on GFR was recognized, significant inhibitory effects against an increase in MG and GSA were indicated. This might indicate that the reno-protective effects of NZ-419 via its anti-oxidant effects might include some other than the GFR-protecting one.

The electron spin resonance trapping technique using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) has shown that NZ-419 competitively inhibited the formation of DMPO-OH (·OH adduct) but not DMPO-OOH (superoxide adduct) in the Fenton reaction mixture. In addition to the cell-free system, an anti-oxidant activity has also been observed in isolated rat glomeruli: luminal chemiluminescence stimulated by phorbol-12-myristate-13-acetate (PMA) was inhibited by NZ-419. Furthermore, glycerol-induced ARF has been effectively prevented by NZ-419: both pathological indices such as tubular degeneration and biochemical parameters such as the sCr level have been significantly restored. Therefore, our results regarding MG and GSA indicated that NZ-419 might also exhibit anti-oxidant effects in CRF model rats.

Figure 5 suggests that the threshold effective dose is less than 250 and probably around 50 or 100 mg/kg/d, and more precise experiments using rather low dosages are now in progress on the basis of this indication.

Since, in this experiment, NZ-419 was given from the be-
beganning when the renal function was normal, at least a preventative effect on the initiation and/or progression of CRF was indicated. In order to evaluate the possibility of using NZ-419 for clinical trials, we need to know whether NZ-419 can prevent the progression of CRF in rats which have already suffered from CRF. The preliminary results of works in progress show that NZ-419 also prevents the progression.

Further discussion on the precise mechanism whereby NZ-419 inhibits the progression of CRF will be reported with more data.

Even at high NZ-419 doses, no side effects were observed, agreeing with the toxicological data (not shown) that NZ-419 is a safe drug.

In conclusion, the present results indicate that if an intrinsic antioxidant, NZ-419, is administered as an extrinsic drug to rats with progressive CRF, the initiation and/or progression of CRF can be inhibited. If we take its low toxicity into account, then NZ-419 may be a very promising therapeutic agent against progressive CRF.

REFERENCES