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Ingestion of difructose anhydride III partially suppresses the deconjugation and 7 α -dehydroxylation of bile acids in rats fed with a cholic acid-supplemented diet

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Abbreviation

BA, bile acid; BSH, bile salt hydrolase; CA, cholic acid; DCA, deoxycholic acid; DFAIII; difructose anhydride III; MCA, muricholic acid; MS, mass spectrometry; NCDs, non-communicable diseases; LC; liquid chromatography; SCFA, short-chain fatty acid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid;

TUDCA, tauroursodeoxycholic acid; T α MCA, tauro- α -muricholic acid; T β MCA, tauro- β -muricholic acid; T ω MCA, tauro- ω -muricholic acid;

Abstract

Difructose anhydride III (DFAIII) is a prebiotic involved in the reduction of secondary bile acids (BAs). We investigated whether DFAIII modulates BA metabolism, including enterohepatic circulation, in the rats fed with a diet supplemented with cholic acid (CA), one of the 12 α -hydroxylated BAs. After acclimation, the rats were fed with a control diet or a diet supplemented with DFAIII. After 2 weeks, each group was further divided into two groups and was fed diet with or without CA supplementation at 0.5 g/kg diet. BA levels were analyzed in aortic and portal plasma, liver, intestinal content, and feces. As a result, DFAIII ingestion reduced the fecal deoxycholic acid level via the partial suppression of deconjugation and 7 α -dehydroxylation of BAs following CA supplementation. These results suggest that DFAIII suppresses production of deoxycholic acid in conditions of high concentrations of 12 α -hydroxylated BAs in enterohepatic circulation, such as obesity or excess energy intake.

Keywords:

Difructose anhydride III

Bile acid

Deconjugation

7 α -Dehydroxylation

Introduction

According to the World Health Organization, non-communicable diseases (NCDs) are responsible for almost 70% of deaths reported worldwide [1]. The number of deaths is expected to increase worldwide from 38 million in 2012 to 52 million by 2030 [2]. Unhealthy diets, including those with high portion of sugars or saturated fats (30%), drinking alcohol (30%), physical inactivity (10%), and smoking (10%) are the primary factors that raise the risk of NCDs. Dietary intervention is proposed as one of the suitable strategies to prevent NCDs [3]. Epidemiologic studies have revealed that the consumption of an energy-dense diet increases obesity rate as well as the risk of diseases, including insulin resistance and metabolic syndrome [4, 5].

One of prominent changes induced by energy-dense diet is the alteration in bile acid (BA) metabolism. BA is a steroid synthesized from cholesterol in the liver and contributes to lipid absorption [6]. Primary BAs are conjugated with taurine or glycine in the liver and stored in the gallbladder. The conjugated BAs are secreted in response to diet consumption into the intestine through the bile duct to assist lipid absorption. The secreted BAs are reabsorbed through the portal vein into the liver and reused in the process called as enterohepatic circulation [7]. In the large intestine, some gut microbes produce bile salt hydrolase (BSH), which deconjugates BA and liberates a taurine or glycine moiety [8]. The production of BSH depends on the species of bacteria, intestinal environment, and BA concentration [9]. The “free BA” becomes weakly acidic and undergoes 7 α -dehydroxylation to produce secondary BAs [10].

A significant increase in 12 α -hydroxylated BAs, especially taurocholic acid (TCA) in the bile and deoxycholic acid (DCA) in the large intestine, is observed in the rats fed with a high-fat diet in our previous study [11]. This observation suggests that the increased TCA flows into the gastrointestinal tract and undergoes deconjugation and 7 α -dehydroxylation, resulting in an increase in the excretion of DCA in feces. Such an alteration in BA metabolism was

observed in the rats fed with CA and was accompanied with an alteration in the gut microbiota composition [12]. In the intestine, a variety of gut microbes are involved in BA metabolism [13]. Ingestion of a prebiotic may modulate the gut microbiota and possibly alter BA metabolism and host health because some of the secondary BAs are risk factors associated with colon [14] and liver cancer [15].

Difructose anhydride III (di-D-fructofuranose-1,2':2,3'-dianhydride, DFAIII), a non-digestible disaccharide, is mainly derived from inulin [16]. DFAIII is highly fermentable and enhances short-chain fatty acid (SCFA) synthesis during microbial fermentation in the cecum [17]. Intestinal acidification after DFAIII supplementation [18,19] increases DFAIII-assimilating bacteria and decreases fecal secondary BA concentration [20]. However, no information is available on BA metabolism, including enterohepatic circulation. In this study, we investigated whether DFAIII ingestion affects BA metabolism in combination with CA supplementation.

Material & Methods

Animal and diets

The animal experiment was approved by the Institutional Animal Care and Use Committee of National University Corporation of Hokkaido University (14-0026). All animals were maintained according to the Hokkaido University Manual for Implementing Animal Experimentation. Male Wistar rats ($n = 32$; 3-week old; Japan SLC Inc, Hamamatsu, Japan) were individually housed in stainless steel cages in a controlled environment of temperature ($22 \pm 2^\circ\text{C}$), humidity ($55 \pm 5\%$), and light (from 8:00-20:00). Feeding schedule of the entire experimental period is presented in Fig. 1. Rats had free access to water and a control diet (C) based on AIN-93G [21] (Table 1) during the acclimation period for a week. The rats were then divided into two dietary groups, namely, C ($n = 16$) and D (DFAIII-supplemented diet)

($n = 16$). After feeding for 2 weeks, each group of rats was further divided into two groups and were fed diet with or without CA supplementation at 0.5 g/kg diet. Body weight and food intake were measured every 2 days. On day 33 of the test period, the rats were orally administered with a chromium (III) chloride hexahydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) [22] solution with ethylenediamine tetra acetate 2Na (Dojindo Laboratories, Kumamoto, Japan) for the determination of urinary chromium (Cr) excretion. Urine samples for 24 h were obtained after 24 h from oral administration for gut permeability test. Fecal samples were collected from day 34 to 35 and stored at -30°C for the evaluation of BAs. On day 35, the rats were sacrificed under anesthesia by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight, Somnopentyl, Kyoritsuseiyaku Corporation, Tokyo, Japan). Blood plasma samples were collected using syringe from both abdominal aorta and portal vein with aprotinin (final concentration of 500 KIU/mL) and heparin (final concentration of 50 IU/mL). The blood samples were centrifuged and the supernatant collected. Cecum was collected and the weights of whole cecum and cecal contents were measured. The cecal contents were diluted four times with deionized water and homogenized with Teflon homogenizer. The pH of homogenates was measured using a semiconducting electrode (ISFET pH sensor Argus; Sentron, Roden, Netherlands). Cecal contents were kept in liquid nitrogen and stored at -80°C for BA analysis. Epididymal adipose tissue was dissected and weighed. Liver tissue weight was measured and the tissues were kept in liquid nitrogen and stored at -80°C for BA analysis.

Gut permeability

The concentration of Cr in urine samples was measured using an atomic absorption spectrophotometer (Z-5310, Hitachi High-Tech., Tokyo, Japan). The proportion of urinary Cr

excretion following oral administration of Cr-ethylenediaminetetraacetic acid solution was calculated as an indicator of gut permeability [23].

BA analysis

BA extraction and liquid chromatography (LC) separation were performed with Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific corporation, San Jose, CA, USA) according to our previous study [24]. Mass spectrometry (MS) was performed using Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization probe under negative-ion mode. Full-scan MS spectra (from m/z 200–700) were acquired with Orbitrap analyzer after accumulation to a target value of $1e6$ in the linear ion trap. Resolution in Orbitrap system was set to $r = 17,500$. Standard mass spectrometric conditions for all experiments were as follows: spray voltage, 3.8 kV; sheath gas flow rate, 35 L/h; aux gas flow rate, 10 L/h; heated capillary temperature, 250°C; aux gas heater temperature, 450°C; s-lens RF level, 100%. Individual BA concentration was measured using nordeoxycholic acid (23-nor-5 β -cholanolic acid-3 α ,12 α -diol) as an internal standard. BAs were measured as follows: 5 β -cholanolic acid-3 α ,7 α ,12 α -triol (CA); 5 β -cholanolic acid-3 α ,6 β ,7 α -triol; 5 β -cholanolic acid-3 α ,6 β ,7 β -triol (β -muricholic acid, β MCA); 5 β -cholanolic acid-3 α ,6 α ,7 β -triol (ω -muricholic acid, ω MCA); 5 β -cholanolic acid-3 α ,6 α -diol; 5 β -cholanolic acid-3 α ,7 β -diol; 5 β -cholanolic acid-3 α ,7 α -diol; 5 β -cholanolic acid-3 α ,12 α -diol (DCA); 5 β -cholanolic acid-3 α -ol; 5 β -cholanolic acid-3 α ,6 α ,7 α -triol; 5 β -cholanolic acid-3 α ,7 β ,12 α -triol; 5 β -cholanolic acid-3 α ,12 α -diol-7-one; 5 β -cholanolic acid-3 α -ol-7-one (7-oxo-LCA); 5 β -cholanolic acid-3 α -ol-12-one; 5 β -cholanolic acid-12 α -ol-3-one (3 α 12 α); 5 β -cholanolic-3 α ,7 α ,12 α -triol-*N*-(2-sulphoethyl)-amide; 5 β -cholanolic-3 α ,6 β ,7 α -triol-*N*-(2-sulphoethyl)-amide (tauro- α -muricholic acid, T α MCA); 5 β -cholanolic-3 α ,6 β ,7 β -triol-*N*-(2-sulphoethyl)-amide (tauro- β -muricholic acid, T β MCA); 5 β -cholanolic-3 α ,6 α ,7 β -triol-*N*-(2-sulphoethyl)-amide (tauro- ω -muricholic acid, T ω MCA); 5 β -

cholanic-3 α ,6 α -diol-*N*-(2-sulphoethyl)-amide; 5 β -cholanic-3 α ,7 β -diol-*N*-(2-sulphoethyl)-amide (tauroursodeoxycholic acid, TUDCA); 5 β -cholanic-3 α ,7 α -diol-*N*-(2-sulphoethyl)-amide (taurochenodeoxycholic acid, TCDCA); 5 β -cholanic-3 α ,12 α -diol-*N*-(2-sulphoethyl)-amide (taurodeoxycholic acid, TDCA); 5 β -cholanic-3 α -ol-*N*-(2-sulphoethyl)-amide; 5 β -cholanic-3 α ,7 α ,12 α -triol-*N*-(carboxymethyl)-amide; 5 β -cholanic-3 α ,6 α -diol-*N*-(carboxymethyl)-amide; 5 β -cholanic-3 α ,7 β -diol-*N*-(carboxymethyl)-amide; 5 β -cholanic-3 α ,7 α -diol-*N*-(carboxymethyl)-amide; 5 β -cholanic-3 α ,12 α -diol-*N*-(carboxymethyl)-amide; and 5 β -cholanic-3 α -ol-*N*-(carboxymethyl)-amide.

Fecal DFAIII excretion

Fecal DFAIII excretion in the DFAIII-fed rats was measured based on the method as previously described [20]. Briefly, homogenized fecal samples were added to deionized water and sonicated. After centrifugation, the supernatant was collected and degreased with chloroform. The amount of DFAIII was analyzed using high-performance LC (Hitachi High-Tech Science Co., Tokyo) with a TSKgel Amide-80 column (4.6 $\times$ 250 mm).

Statistical analysis

All data are presented as the mean $\pm$ standard error of means (SEM). Two-way analysis of variance (ANOVA) test was used to represent significant interaction between two factors, BA and DFAIII. Student's *t*-test was used for comparison of DFAIII excretion between two groups of rats fed diet with or without CA supplementation. All statistical analysis was performed using JMP software version 13.0 (SAS Institute Inc., Cary, NC, USA).

Results

Reduction of fecal DFAIII concentration

To investigate whether the ingested DFAIII was assimilated by the intestinal microbes, the fecal DFAIII excretion was determined as shown in Fig. 2. Fecal excretion of DFAIII was the highest on day 8 and gradually decreased thereafter. On day 21, the rats were divided into two groups and fed with or without CA-supplemented diet to evaluate the effect of CA supplementation on fecal DFAIII excretion. As a result, the fecal DFAIII was detected at trace levels under CA supplementation condition. A significant difference was observed between the groups on day 27, but only a trace level was detected.

Alteration of BA metabolism in rats fed with the DFAIII diet

Thirty molecular species of BAs were analyzed using LC-MS at the end of the experimental period, as shown in Fig. 3. TCA was the most abundant BA in the liver, intestinal contents, and portal plasma. CA supplementation increased the concentration of TCA at these sites. Two-way ANOVA results revealed the synergetic effect of CA and DFAIII supplementation on TCA concentration in the portal plasma. A similar tendency was observed in the ileal contents. CA supplementation increased the concentration of TDCA in the liver; a significant interaction was found between CA and DFAIII. Similar changes were observed in the cecal and fecal DCA concentrations, as shown in the liver TDCA. A synergetic effect of CA and DFAIII supplementation was observed on both CA and TCA concentrations in the cecal contents and feces. The trend in fecal 3 α 12 α excretion resembled that observed for DCA. On the other hand, the concentration of ω MCA decreased after CA and DFAIII supplementation. Similar tendency was also observed in the feces.

Biochemical parameters in the rats fed the DFAIII diet

Growth and biochemical parameters are shown in Table 2. DFAIII-supplemented diets decreased the total food intake, final body weight, and epididymal adipose tissue weight. The

weights of whole cecum and its contents were higher in the rats fed with DFAIII-supplemented diets than in those from the other groups and a reduction in the pH of the cecal content was observed. The pH reduction was significantly affected by CA supplementation. CA supplementation enhanced gut permeability, as observed with urinary Cr excretion, but no difference was reported in the rats fed with DFAIII-supplemented diet.

Discussion

Gas chromatography/MS mainly used for the determination of BA profiles necessitates the derivatization step. This derivatization process breaks taurine or glycine conjugation of BAs and poses difficulty in the discrimination between conjugated and unconjugated BAs [25]. In the present study, we analyzed not only unconjugated BAs but also the conjugated forms of BAs using LC/MS [24] that enabled us to elucidate the effect of DFAIII ingestion on BA metabolism, especially in the organs related with enterohepatic circulation.

In the rats fed control diet, the proportion of 12 α -hydroxylated BAs was almost comparable that of non-12 α -hydroxylated BAs in liver, ileal contents, and portal plasma (Fig. 3). On the other hand, an increase in the proportion of non-12 α -hydroxylated BAs was observed in large intestine and in feces such as β MCA and ω MCA. Those results suggest that 12 α -hydroxylated BAs are selectively reabsorbed in ileal epithelial cells. This notion is supported by observations in mice [26] that show enormous excretion of ω MCA in feces rather than 12 α -hydroxylated BAs. As expected, the CA-supplemented diet raised the concentration of 12 α -hydroxylated BAs, for example TCA in enterohepatic circulation and DCA in cecal contents (Fig. 3). The DFAIII-supplemented diet reduced cecal and fecal ω MCA concentrations. β MCA is converted into ω MCA in the large intestine by anaerobic bacteria, such as *Eubacterium lentum* strain [27], suggesting that the ingestion of DFAIII reduces conversion of β MCA into ω MCA by the gut microbes. Interestingly, in combination

of CA with DFAIII, massive increase of TCA concentration was observed in the organs related with enterohepatic circulation accompanied by decreased fecal DCA excretion. These results suggest that 12 α -hydroxylated BAs were selectively reused in enterohepatic circulation.

Simple feeding with CA-supplemented diet resulted in an increase in BA concentration mainly in the organs associated with enterohepatic circulation. CA supplementation increased DCA concentration in the large intestine and feces, indicating that the ingested CA was completely dehydroxylated into DCA by the gut microbes. On the other hand, the elevated DCA concentration was normalized in the rats fed with DFAIII that resulted in the reduction in the pH of the cecal contents. Minamida and colleagues [25] measured the conversion of CA to DCA using thin-layer chromatography and found that DCA production was significantly suppressed at pH 5.8 as compared to that observed at pH 7.5, suggestive of the connection between low pH and reduction in 7 α -dehydroxylation under DFAIII-fed condition. The decrease in pH of the large intestine is thought to inhibit 7 α -dehydroxylase activities in the luminal contents, contributing to the reduction in DCA concentration. In addition, DFAIII ingestion increased acetate and other organic acid levels in the cecal contents that may serve as a contributing factor for the reduced pH of the cecal contents [20]. A significant increase in the population of *Ruminococcus productus* was observed in the cecal contents of the rats fed with DFAIII that negatively correlated with the reduction in DFAIII excretion in the feces [20]. On the other hand, *R. productus* was undetected in control rats. These results suggest that DFAIII is consumed by, and contributes to the growth of, *R. productus*. *R. productus* was shown to assimilate DFAIII under in vitro conditions. Almost all BAs of the feces in control rats were secondary BAs but those in DFAIII-fed rats were primary BAs [20], suggesting that the altered gut microbiota by DFAIII ingestion had less ability to perform 7 α -dehydroxylation.

Deconjugation of BA is catalyzed by BSHs in the intestinal bacteria that hydrolyze the amino bond and liberate taurine or glycine moiety from the steroid ring of BA [8]. In general, an enormous decrease in conjugated BA levels was observed in the large intestine after deconjugation; these deconjugated BAs were converted into secondary BAs in the subsequent dehydroxylation step [24]. High concentration of TCA was observed in the large intestine of the rats fed with CA and DFAIII, wherein BAs underwent 7 α -dehydroxylation by the gut microbiota. The suppression of BA deconjugation reaction by DFAIII ingestion was responsible for the subsequent reduction in secondary BA production.

A decrease in body weight was observed in the rats fed with DFAIII supplementation as compared to those fed with DFAIII-free diets. As mentioned before, an increase in acetic acid level was observed in the gut that served as a potent stimulator to secrete anorexic gut hormones such as glucagon-like peptide-1 and peptide YY [28]. It was recently revealed that acetic acid reduces appetite via direct influence on hypothalamus in the brain without induction of these gut hormones [29]. In both cases, acetic acid is one of the candidates that suppressed appetite in the rats fed with DFAIII, resulting in the reduction in body weight. Furthermore, we observed a different influence of CA supplementation on BA concentrations in portal and aorta blood. CA supplementation increased portal BAs but not aorta BAs. The absorbed BAs in the ileal epithelia entered the enterohepatic circulation, as reflected in the BA profiles of the portal blood. The supplemented CA also enters this route rather than the systemic blood flow (aortic blood). This observation suggests that the effect of CA supplementation on the host may be restricted in the organs related to enterohepatic circulation. We observed that CA supplementation increased the gut permeability, but DFAIII intervention had no significant influence on permeability (Table 2). DCA is reported to disrupt the gut barrier in the large intestine [30] and cultured epithelial cells [31]. Regardless of the significant reduction in DCA concentration observed in the cecal contents

and feces following DFAIII supplementation in the present study, no influence was observed on gut permeability following the CA supplementation (Table 2). In the rats fed with CA-supplemented diet, an increase in DCA or TCA was found in cecal contents or ileal contents, respectively (Fig. 3). TCA might induce leaky gut in small intestine, not in the large intestine.

In our previous study, we found that BA metabolism especially in the gut induced after CA supplementation (0.5 g/kg) resembles that observed in the rats fed with a high-fat diet, suggesting that the consumption of energy-dense diet may selectively increase DCA levels in the large intestine [11]. Such increases in DCA levels in the stool were reported in the patient with colorectal cancer, suggestive of the link between DCA production in the large intestine and cancer development in the colon [32]. Only limited data are available on conjugated BAs in feces. Under normal conditions, almost no conjugated BAs were observed in the stool of humans and feces in experimental animals [33,34]. In our previous study, we measured the levels of conjugated BAs in the feces of rats fed with soy pulp and *Bacillus coagulans* and found that conjugated BAs were absent in the feces [35]. It may be rare to observe fecal-conjugated BAs following dietary intervention, as observed in the present study. Therefore, dietary DFAIII may be a prebiotic that reduces secondary BA concentration via suppression of 7 α -dehydroxylation and deconjugation.

In conclusion, DFAIII altered BA metabolism, especially in the gut, through the suppression of deconjugation and 7 α -dehydroxylation, which results in the reduction in DCA level in the large intestine probably through the modulation of gut microbiota. Hence, DFAIII may serve as a prebiotic source that reduces secondary BA production.

Author contribution

S.I., T.N., H.S., S.F, and A.Y. designed the experiments. DG.L., S.H., O.K., S.K., R.Y., Y.T., K.T., S.F. and S.I. performed experiments. All authors discussed the data. DG.L. and S.I. wrote the paper.

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Figure captions

Fig. 1. Feeding schedule

After acclimation for 1 week, the rats were divided into two groups, including those fed with control diet (C) and DFAIII-supplemented diet (D) for 2 weeks. Rats from each group were further divided into two groups and fed the diet supplemented with CA (CB and DB) or without CA (C and D) (n = 8 each) for another 2 weeks.

Fig. 2. Changes in fecal DFAIII excretion in the rats fed with DFAIII with or without CA supplementation

The proportion of fecal DFAIII following DFAIII supplementation was calculated in D (open circle, n = 8) and DB (closed circle, n = 8) groups. Asterisk represents significant differences in the values between groups at the same time point ($P < 0.05$).

Fig. 3. Distribution of BAs in the rats fed with four different diets at the end of the experimental period

BA concentrations were measured in the aorta plasma, portal plasma, liver, ileal contents, cecal contents, and feces of the rats fed with four different diets. Values are the mean $\pm$ SEM (n = 7 or 8 rats per group). Two-way ANOVA was used to determine significant differences ($P < 0.05$).

Table 1. Diet compositions

	C	CB	D	DB
	g/kg diet			
Corn starch ^a	397.5	397.5	367.5	367.5
Casein ^b	200	200	200	200
Dextrin ^c	132	132	132	132
Sucrose ^d	100	99.5	100	99.5
Soybean oil ^e	70	70	70	70
Crystalline cellulose ^f	50	50	50	50
Mineral mixture ^g	35	35	35	35
Vitamin mixture ^h	10	10	10	10
L-Cystine	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5
Cholic acid	-	0.5	-	0.5
DFAIII	-	-	30	30

C, control diet; D, DFAIII-supplemented diet; CB, control diet with CA; DB, DFAIII-supplemented diet with CA.

^a Amylalpha, Chuoshokuryo, Japan

^b NZMP Acid Casein (Fonterra Co-Operative Group Limited, Auckland, New Zealand),

^c TK-16 (Matsutani Chemical Industry Co., Ltd., Hyogo, Japan)

^d Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan

^e J-Oil Mills, Inc., Tokyo, Japan

^f Crystalline cellulose (Ceolus PH-102, Asahi Kasei Chemicals Corp., Tokyo, Japan)

^g AIN93G mineral mixture [21]

^h AIN93 vitamin mixture (MP Biomedicals, USA) [21]

Table 2. Growth and other parameters in the rats

	Two-way ANOVA <i>P</i> -value						
	C	CB	D	DB	BA (B)	DFAIII (D)	B × D
Growth parameters (g)							
Total food intake	423 ± 8	407 ± 9	390 ± 4	386 ± 9	NS	0.0050	NS
Final body weight	221 ± 5	217 ± 6	200 ± 7	201 ± 3	NS	0.0003	NS
Tissue weight (g/100g body weight)							
Liver	4.28 ± 0.07	4.36 ± 0.09	4.39 ± 0.14	4.72 ± 0.14	0.0490	NS	NS
Epididymal adipose	2.01 ± 0.09	2.10 ± 0.11	1.80 ± 0.11	1.59 ± 0.11	NS	<0.0001	NS
Whole cecum	1.34 ± 0.04	1.50 ± 0.08	2.68 ± 0.19	2.55 ± 0.13	NS	<0.0001	NS
Cecal contents	1.12 ± 0.04	1.25 ± 0.08	2.26 ± 0.17	2.12 ± 0.12	NS	<0.0001	NS
Other parameters							
pH in cecal contents	6.65 ± 0.22	7.06 ± 0.16	6.23 ± 0.20	5.78 ± 0.09	NS	<0.0001	0.0224
Urinary Cr excretion (%)	2.54 ± 0.23	4.51 ± 0.53	2.71 ± 0.39	3.65 ± 0.28	0.0006	NS	NS

Values are shown as mean with SEM (n=8).

Fig. 1.

Fig. 2.

Fig. 3.