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Combined effects of valproate and naringin on kidney antioxidative markers and serum parameters of kidney function in C57BL6 mice

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Valproate is known to disturb the kidney function, and high doses or prolonged intake may cause serum ion imbalance, kidney tubular acidosis, proteinuria, hyperuricosuria, polyuria, polydipsia, and dehydration. The aim of this *in vivo* study was to see whether naringin would counter the adverse effects of high-dose valproate in C57Bl/6 mice and to which extent. As expected, valproate (150 mg/kg bw a day for 10 days) caused serum hyperkalaemia, more in male than female mice. Naringin reversed (25 mg/kg bw a day for 10 days) the hyperkalaemia and activated antioxidative defence mechanisms (mainly catalase and glutathione), again more efficiently in females. In males naringin combined with valproate was not as effective and even showed some prooxidative effects.

KEY WORDS: calcium; catalase; hyperkalaemia; malondialdehyde; oxidative stress; potassium; sodium; superoxide dismutase

Sodium valproate and valproic acid (valproate further in the text) have long been indicated for the treatment of epilepsy and more recent prophylactic indications include bipolar disorder and migraine. Generally, adverse effects are rare and usually involve dermatological signs, pancytopaenia, an increase in blood ammonia or liver and pancreatic enzymes, or teratogenic effects (1, 2). Some epileptic patients receiving valproate over long time develop subclinical tubular injury with a number of symptoms, such as metabolic acidosis, aminoaciduria, decreased 25-OH vitamin D_3, and the Fanconi syndrome (1–3).

To address this problem, a number of researches have studied different supplements in combination with valproate to relieve valproate-associated adverse effects. Some supplementation experiments, such as the one with carnitine to compensate for the mitochondrial deficit, encountered challenges regarding the dose necessary to achieve protective effects (4). Research on animal models provided encouraging results for S-methyl methionine, L-cysteine, and quercetin (5–7), and recent reports of naringin (4',5,7-trihydroxyflavanone-7-rhamnoglucoside) as an agent protecting against hepatotoxicity in rats (8) or steatosis and dyslipidaemia in mice (9) show promise. Naringin belongs to the flavanone class of flavonoids, mainly present in citrus fruits. It has many beneficial biological functions, including anti-inflammatory and antioxidant, and regulates autophagy, apoptosis, and cell proliferation and differentiation (10). Several recent studies in rats have also evidenced naringin-associated nephroprotective effects (11–13).

The aim of our study was to continue our previous research (9) and to look deeper into the protective mechanisms of naringin in the kidney by comparing serum biomarkers of kidney function and tissue oxidative stress markers between mice treated with valproate, naringin, their combination, and controls.

MATERIALS AND METHODS

Experimental animals and husbandry

The study included 48 inbred C57BL/6 mice: 24 male (weighing 30±1.5 g), used in our previous study (9), and 24 female (weighing 25±1.5 g), added for the purpose of this research. The animals were obtained from the University of Zagreb Faculty of Science, Department of Animal Physiology, Zagreb, Croatia. They were kept under a conventional regime with 12:12 hours of light per day and had free access to standard laboratory diet (4 RF21, Mucedola, Settimo Milanese, Italy) and tap water in line with international standards on laboratory animal care (14). The experiments were approved by the Bioethics Committee of the Zagreb University

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Faculty of Science and Ministry of Agriculture Republic of Croatia Board on bioethics in laboratory animals (approval No: 251-58-10617-17-7).

Treatment groups and doses

The animals were randomly divided in four groups of six animals per group and per sex: control (C) receiving saline, valproate group (V) receiving 150 mg of valproate per kg of body weight (bw) a day, naringin group (N) receiving 25 mg/kg bw of naringin a day, and valproate plus naringin (V+N) group receiving combined treatment with the above doses of valproate and naringin. The doses were established in our previous study on liver (9) and correspond to a three-fold prescribed therapeutic dose of valproate (to simulate accidental overdose that could cause pathophysiological effects) and to the highest possible intake dose of naringin in concentrated food supplement preparations. Valproate was given in the form of a commercial mix of sodium valproate and valproic acid (Depakine®, Sanofi, Carbon Blanc, France), and naringin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Each compound was administered daily as a single oral dose of water solution by gavage in a volume of 0.2 mL per animal, between 8–10 a.m. to minimise circadian and metabolic differences. The animals were sacrificed after having received 10 daily doses of either compound.

Serum and kidney tissue preparation and measurements

On day 11, 24 h after having received the last dose, the mice were anaesthetised with isoflurane (3 % in O_2 flow 0.8–1.5 L/min) and received 100 mg/kg ketamine and 10 mg/kg xylazine.

After the cardiac puncture to obtain the blood, the animals were sacrificed by cervical dislocation. Followed centrifugation at 1,500×g for 15 min to obtain serum, which was aliquoted and stored at -80 °C until potassium, sodium, and total calcium were measured using corresponding VetScan™ commercial kits for the VetScan™ analyser (Abaxis, Inc., Union City, CA 94587) according to manufacturer's instructions.

Kidney tissue samples were first homogenised with 50 mmol/L phosphate-buffered saline (PBS, pH=7.4) in an ultrasonic homogeniser (SONOPLUS Bandelin HD2070, Bandelin Electronic GmbH & Co KG, Germany) with an MS73 probe (Bandelin, Electronic GmbH & Co KG Germany) to obtain a 10 % tissue mass homogenate. The next step was sonification over three 10-second intervals, with the samples kept on ice. The last phase was centrifugation (at 20,000×g and 4 °C for 15 min), and storing at -80 °C until thawing (+6 °C) and repeated centrifugation (20,000×g, 4 °C, 15 min) to obtain supernatants for analysis (15). Kidney protein concentrations were measured to express the levels of redox parameters per mg/protein using the Lowry method with bovine serum albumin as the standard solution as described elsewhere (9, 15, 16).

Kidney lipid peroxidation was assessed through malondialdehyde (MDA) levels determined as described elsewhere (9, 15). After incubating 200 μL of the sample with the reagent mixture that contained 200 μL of sodium dodecyl sulphate (8.1 %), 1.5 mL of acetic acid (20 %, pH 3.5), and 1.5 mL of thiobarbituric acid (TBA, 0.81 %) at 95 °C for 60 min, the reaction was stopped by ice cooling, and the coloured MDA-TBA complex was measured at 532 nm and 600 nm on Libro S22 spectrophotometer (Biochrom Ltd., Cambridge, UK). MDA concentration was calculated from the molar absorption coefficient of the MDA-TBA complex (1.56×10^5 mol/L cm) and then divided by the protein tissue concentration.

To determine kidney superoxide dismutase (SOD) activity, we followed a procedure described in more detail earlier (9, 15, 17) by adding 25 μL of the sample to 1.45 mL of a reaction mixture containing cytochrome C (0.05 mmol/L) and xanthine (1 mmol/L) mixed with 5-5'-dithiobis [2-nitrobenzoic acid] (DTNB) in a 10:1 (v/v) ratio. The reaction was started by pipetting 20 μL of xanthine oxidase solution (0.4 U/mL) to the reaction mixture. This reaction forms coloured cytochrome C product, whose absorbance was measured at 550 nm with a Libro S22 spectrophotometer (Biochrom Ltd.) for 3 min. When tissue homogenate is added, the rate of reaction (superoxide anion generation) over 30 min is partly inhibited by SOD in the tissue sample, and one SOD unit corresponds to 50 % inhibition of on the calibration curve. The obtained SOD units were then divided by protein concentration.

The determination of catalase activity (CAT) in kidney homogenates relied on the H_2O_2 degradation rate as described elsewhere (9, 15, 19). The reaction was initiated by adding 100 μL of sample to 900 μL of the reaction mixture containing 33 mmol/L H_2O_2 in 50 mmol/L phosphate buffer (pH 7.0). The absorbance at 240 nm (Libro S22 spectrophotometer Biochrom Ltd. Cambridge, UK) was recorded for 3 min, and CAT activity calculated from mean absorbance change per minute and molar absorption coefficient for H_2O_2 (43.6 mol/L cm). The results are expressed as U/mg protein (9, 15, 19).

Reduced glutathione (GSH) concentrations were determined following the Ellman's method as described elsewhere (9, 15, 20). Briefly, 20 μL of sample was incubated with 40 μL of 35 mmol/L HCL for 10 min. At the same time, we prepared the enzyme working solution by adding 20 μL of glutathione reductase (0.2 U/mL) to 9.98 mL NADPH (0.8 mmol/L). The reaction mixture was prepared in a 96-well plate by pipetting 40 μL of 10 mmol/L DTNB, pretreated sample, and100 μL of enzyme working solution. The absorbance at 412 nm was monitored for 5 min (ELISA plate reader, BIORAD Laboratories, Hercules CA, USA) to obtain the mean change per minute. GSH concentration was calculated using the calibration curve, and the results are reported as nmol/L per mg of proteins.

Statistical analysis

The results are given as medians and ranges. They were compared between the groups using the Kruskal-Wallis and Tukey's test in GraphPad Prism 17 (GraphPad Software, San Diego, CA,

USA). The difference was significant if the p-value was less than 0.05.

RESULTS

Serum sodium and calcium did not change significantly in any of the treatment groups, but valproate significantly increased potassium ($p<0.05$) in male mice (Table 1). Combined treatment restored potassium levels to near control.

In the kidney tissue, MDA concentrations significantly ($p<0.05$) increased in all groups compared to control (Figure 1A). However, separate analysis by sex (Figure 1B) shows that female mice were more susceptible to valproate alone than males, whereas naringin countered its effects and lowered MDA to near control levels. In male mice, however, combined valproate plus naringin treatment caused even higher lipid peroxidation than valproate alone (Figure 1B).

CAT activity, in turn, significantly rose only in the valproate alone group in mice of both sexes, and combined treatment lowered it to control levels (Figure 2).

SOD activity significantly ($p<0.05$) increased in female mice treated with valproate alone, while combined treatment reduced it to near control levels. No changes were observed in male mice, regardless of treatment.

In contrast, all groups showed significantly ($p<0.05$) lower GSH levels compared to control, regardless of sex (Figure 4).

Table 1 Serum sodium, potassium, and calcium levels in mice by groups and sex (medians and ranges)

Group	Sex	Sodium (mmol/L)	Potassium (mmol/L)	Calcium (mmol/L)
C	Both	153 (145–161)	4.8 (4.3–4.9)	1.75 (1.65–1.90)
V		152 (140–160)	**6.3 (4.6–8.5)***	1.82 (1.80–1.92)
N		148 (147–165)	5.5 (5.3–5.9)	1.81 (1.70–1.90)
V+N		154 (141–161)	5.1 (4.2–5.8)	1.80 (1.60–1.90)
C	Male	155 (153–161)	4.8 (4.3–4.9)	1.84 (1.80–1.90)
	Female	153 (145–159)	4.8 (4.4–4.8)	1.75 (1.65–1.82)
V	Male	151 (140–158)	**7.2 (5.6–8.5)***	1.82 (1.80–1.91)
	Female	152 (149–160)	**5.0 (4.6–7.0)#**	1.83 (1.80–1.92)
N	Male	158 (147–165)	5.6 (5.3–5.9)	1.81 (1.75–1.90)
	Female	145 (142–152)	5.4 (5.3–5.6)	1.82 (1.70–1.89)
V+N	Male	157 (152–161)	5.1 (4.5–5.3)	1.80 (1.62–1.90)
	Female	150 (141–159)	5.2 (4.2–5.8)	1.81 (1.60–1.90)

C – control group; N – group receiving naringin (25 mg/kg); V – group receiving valproate (150 mg/kg), V+N – group receiving valproate (150 mg/kg) + naringin (25 mg/kg); *significant difference ($p<0.05$) from control (C); # significant difference from males

DISCUSSION

The valproate-induced significant increase in serum urea and creatinine in our previous study (9) points to early disturbances in kidney function after prolonged valproate overdose. In this study we wanted to see if this was owed to an ion imbalance in blood and changes in kidney tissue antioxidative defences. Sodium and calcium levels remained unchanged, and potassium levels only changed slightly in males. Males were more sensitive to valproate kidney damage than female mice.

Judging by high levels of lipid peroxidation (MDA), especially in males, as well as higher CAT and lower glutathione and SOD (in females), it seems that the oxidative imbalance occurred within the cytoplasm of kidney cells. The literature shows that valproate metabolises into valproyl-CoA, 4-ene-valproate, 2,4 diene valproate, and other intermediates (20–22), which can weaken mitochondrial β-oxidation and inhibit CYP450 enzymes (6). This, in turn, causes the accumulation of reactive oxygen species (8) and triggers

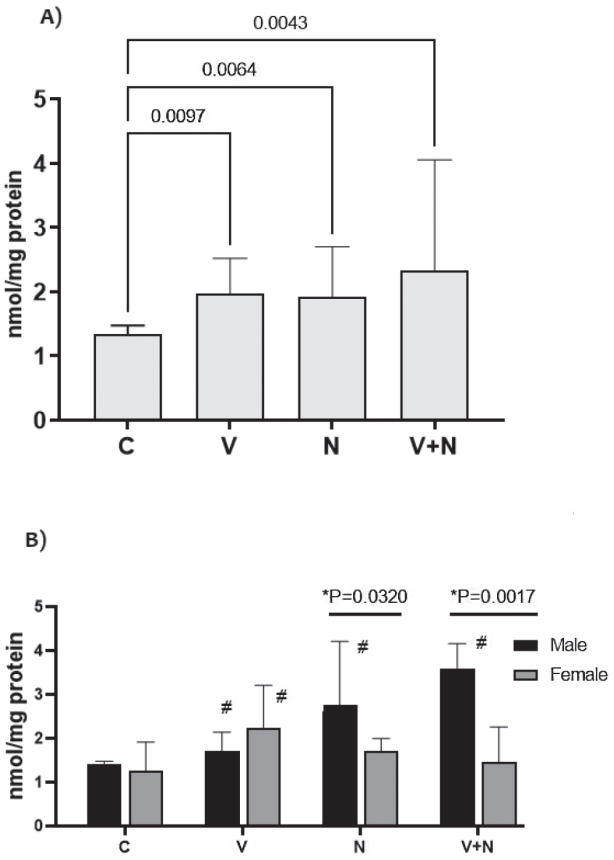

Figure 1 Kidney tissue MDA levels in mice by groups (A) and by sex and groups (B). C – control group; N – group receiving naringin (25 mg/kg); V – group receiving valproate (150 mg/kg), V+N – group receiving valproate (150 mg/kg) + naringin (25 mg/kg). Connecting lines – significant differences ($p<0.05$) between the groups. # significant differences ($p<0.05$) from control

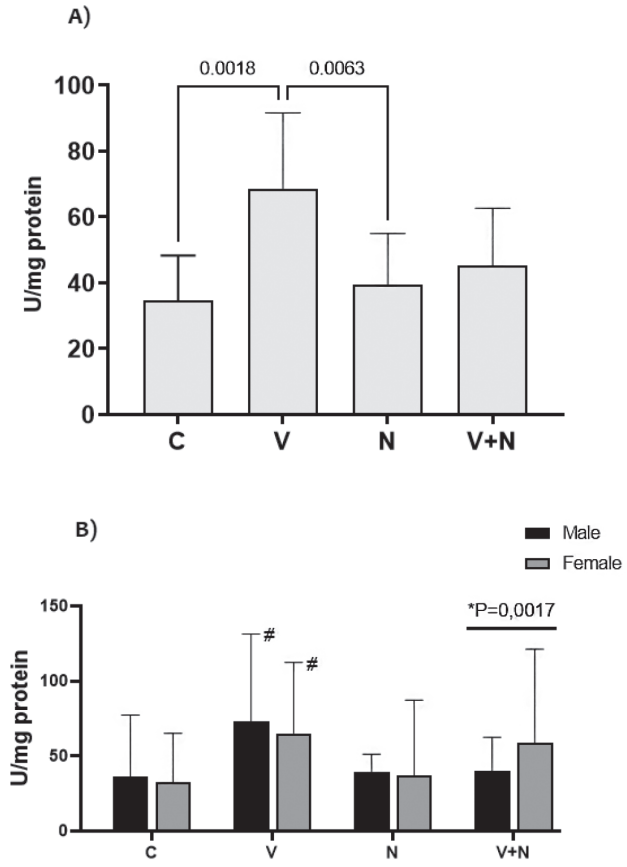

Figure 2 Kidney tissue catalase activity in mice by groups (A) and by sex and groups (B). C – control group; N – group receiving naringin (25 mg/kg); V – group receiving valproate (150 mg/kg), V+N – group receiving valproate (150 mg/kg) + naringin (25 mg/kg). Connecting lines – significant differences ($p<0.05$) between the groups. # significant differences ($p<0.05$) from control

antioxidant defence mechanisms (CAT, SOD, GSH), which was also observed in our experiment.

The mechanisms of valproate-associated tubular damage are still a matter of investigation. Our findings point to a possible damage of mitochondria, resulting in low levels of adenosine triphosphate (ATP), especially in the proximal tubules, since ATP-ase is a sodium-potassium protein pump (Na^+/K^+ ATP-ase) and one of the most important mechanisms of potassium-sodium transport through the cell membrane. This is in line with reports of several mechanisms underlying valproate mitochondrial toxicity (20, 23–26). Valproate can directly impede β-oxidation of fatty acids by inhibiting the participating enzymes and reducing coenzyme A and carnitine depots. Consequently, ATP levels drop and so does Na^+/K^+ ATP-ase activity, which mediates the reabsorption of electrolytes in the proximal tubule. An additional complication is the accumulation of reactive oxygen species. We therefore believe that accumulation of potassium in the blood, noted in male mice may be owed to ATP depletion and impaired transport of the protein pump.

In addition, valproate increases fatty acid accumulation in its own right by interfering with genes involved in fatty acid transport and synthesis. For example, it lowers the expression of the peroxisome proliferator-activated receptor α (PPARα) in male mice, and PPARα is the main transcription factor for oxidising enzymes that drive fatty acid oxidation in cells (9, 27). It also inhibits mitochondrial oxygen consumption and damages mitochondrial membrane (5).

Naringin showed limited protective effects in the kidney, but was not as effective when co-administered with valproate as we expected. The protective effects were more prominent in female mice. Serum potassium levels were reversed to near control levels (Table 1). Similar results in literature are scarce, save for Oyagbemi et al. (28), who have reported protective effects in naringin co-administration with the hypertension inducer L-NG-nitro arginine methyl ester (L-NAME). As for the observed increase in superoxide dismutase (SOD) activity in our study, other authors (10–12, 27–31) report similar behaviour of SOD activity and significant nephroprotective effects. Elsawy et al. (13) also report lower GSH

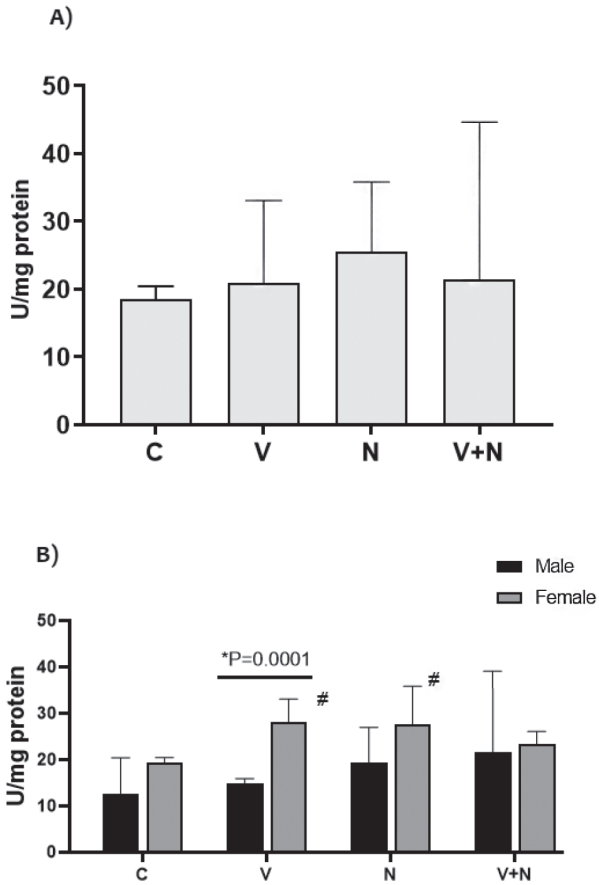

Figure 3 Kidney tissue superoxide dismutase activity levels in mice by groups (A) and by sex and groups (B). C – control group; N – group receiving naringin (25 mg/kg); V – group receiving valproate (150 mg/kg), V+N – group receiving valproate (150 mg/kg) + naringin (25 mg/kg). # significant differences ($p<0.05$) from control

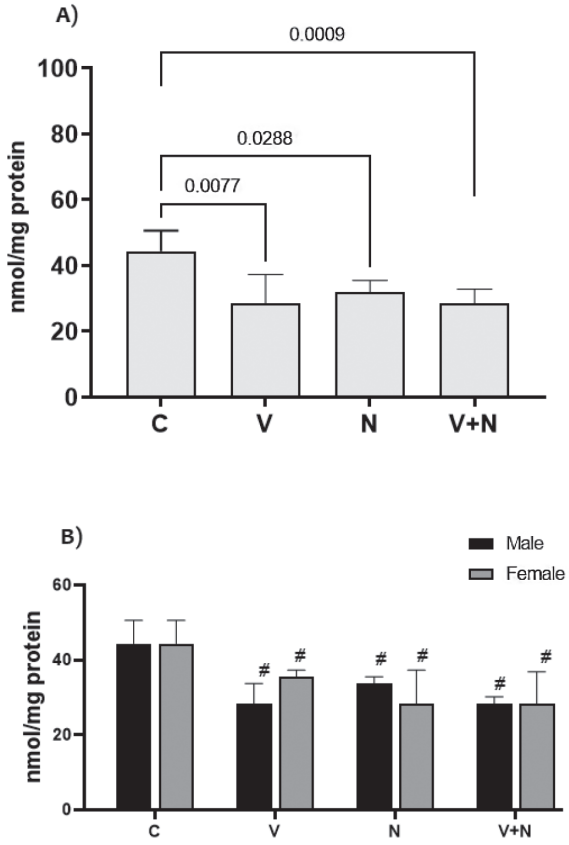

Figure 4 Kidney tissue glutathione levels in mice by groups (A) and by sex and groups (B). C – control group; N – group receiving naringin (25 mg/kg); V – group receiving valproate (150 mg/kg), V+N – group receiving valproate (150 mg/kg) + naringin (25 mg/kg). Connecting lines – significant differences ($p<0.05$) between the groups. # significant differences ($p<0.05$) from control

(suggesting the activation of antioxidant defences) as in our work, and lower MDA, inflammatory cytokines (such as IL-6 and TNF-α), serum urea, and creatinine after naringin co-administration with methotrexate. Amini et al. (32) report a similar increase in antioxidant activity and lower oxidative activity in kidney reperfusion injury after administration of naringin.

However, in this study the combination of naringin and valproate showed weaker effects in male than female mice. In fact, in males we even see stronger expression of lipid peroxidation in the kidney (MDA is very high compared to all other groups and 2.5 times higher than control). It seems that, at least in this experiment, these two substances potentiate prooxidative action in male kidney tissue, quite likely due to sex difference in biotransformation enzyme levels. Besides, Galati et al. (33) report that naringin or naringenin may act as prooxidants at certain doses, which may also apply to ours. All this calls for further investigation that should focus on sex differences in biotransformation mediated by the P450 enzymes and on the interaction between naringin and valproate metabolites that could affect kidney function.

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Združeni učinci valproata i naringina na antioksidacijske i serumske pokazatelje bubrežne funkcije u miševa soja C57BL6

Valproat je jedan od najčešće primjenjivanih antiepileptika, a poznato je da prouzročuje poremećenu funkciju proksimalnih bubrežnih tubula. Fiziološki poremećaji i nefrotoksični učinci u nekih bolesnika nakon visokih doza ili produljenog uzimanja valproata uključuju disbalans iona u serumu, bubrežnu tubularnu acidozu, proteinuriju, hiperurikozuriju, poliuriju, polidipsiju, dehidraciju i druge poremećaje. U okviru ovog eksperimentalnog rada primijenili smo visoke doze valproata i združeni tretman valproata i naringina u C57Bl/6 miševa. Naringin je poznati antioksidans i protuupalna flavonoidna molekula iz citrusnog voća. Cilj rada bio je utvrditi mogu li biološka svojstva naringina umanjiti štetne učinke na bubrege nakon tretmana valproatom. Valproat je *in vivo* prouzročio serumsku hiperkalijemiju, izraženiju u mužjaka nego u ženki miševa. Hiperkalijemija prouzročena valproatom bila je ublažena naringinom, a antioksidacijski obrambeni mehanizmi (uglavnom katalaza i smanjena glutationacija) bili su aktivirani, više u ženki. U mužjaka, zajednički tretman valproatom i naringinom nije bio tako učinkovit, a rezultati upućuju na moguće prooksidacijsko djelovanje u bubrežnom tkivu kada se obje tvari primjenjuju zajedno.

KLJUČNE RIJEČI: hiperkalijemija; kalcij, kalij; katalaza; malondialdehid; oksidacijski stres; superoksid dismutaza