

## Original Article

## Intake of water with high levels of dissolved hydrogen (H<sub>2</sub>) suppresses ischemia-induced cardio-renal injury in Dahl salt-sensitive rats

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### Abstract

**Background.** Hydrogen (H<sub>2</sub>) reportedly produces an anti-oxidative effect by quenching cytotoxic oxygen radicals. We studied the biological effects of water with dissolved H<sub>2</sub> on ischemia-induced cardio-renal injury in a rat model of chronic kidney disease (CKD).

**Methods.** Dahl salt-sensitive rats (7 weeks old) were allowed *ad libitum* drinking of filtered water (FW: dissolved H<sub>2</sub>, 0.00 ± 0.00 mg/L) or water with dissolved H<sub>2</sub> produced by electrolysis (EW: dissolved H<sub>2</sub>, 0.35 ± 0.03 mg/L) for up to 6 weeks on a 0.5% salt diet. The rats then underwent ischemic reperfusion (I/R) of one kidney and were killed a week later for investigation of the contralateral kidney and the heart.

**Results.** In the rats given FW, unilateral kidney I/R induced significant increases in plasma monocyte chemoattractant protein-1, methylglyoxal and blood urea nitrogen. Histologically, significant increases were found in glomerular adhesion, cardiac fibrosis, number of ED-1 (CD68)-positive cells and nitrotyrosine staining in the contralateral kidney and the heart. In rats given EW, those findings were significantly ameliorated and there were significant histological differences between rats given FW and those given EW.

**Conclusion.** Consumption of EW by *ad libitum* drinking has the potential to ameliorate ischemia-induced cardio-renal injury in CKD model rats. This indicates a novel strategy of applying H<sub>2</sub> produced by water electrolysis technology for the prevention of CKD cardio-renal syndrome.

**Keywords:** chronic kidney disease; electrolyzed water; hydrogen water; inflammation; oxidative stress.

### Introduction

Chronic kidney disease (CKD) is a leading cause of end-stage renal disease. In addition, CKD constitutes an independent risk factor for cardiac events and has thus

become a serious public health concern [1]. In those cases, the kidney injury may induce pathological burdens to the heart and this in turn further exaggerates kidney dysfunction. The same cycle could start by heart injury. A common predisposed systemic pathology may damage the two organs simultaneously. Whichever the original process is, the pathological condition constitutes a vicious cycle of damage to two organ systems, the so-called cardio-renal syndrome [2, 3]. Among the causative factors associated with organ injury, it has been pointed out that oxidative stress and inflammation play crucial roles in the pathology [4, 5]. However, clinically available means to suppress these factors have been limited [6–8].

Recently, the novel role of H<sub>2</sub> as an antioxidant that reduces cytotoxic oxygen radicals has been revealed. Animal studies have demonstrated that the administration of H<sub>2</sub> suppresses ischemic reperfusion (I/R) injury in the brain [9] and liver [10], stress-induced oxidative injury to the hippocampus [11], drug-induced chronic inflammation in the colon [12] and inflammatory injury of transplanted intestinal grafts [13]. Notably, H<sub>2</sub> intake by drinking water may ameliorate chronic allograft nephropathy [14].

Regarding the method of H<sub>2</sub> administration in those studies, H<sub>2</sub> was directly inhaled [13] or dissolved in water by a bubbling technique [9, 11, 14]. Another way to supply H<sub>2</sub> water is to use water electrolysis technology. Water electrolysis gives rise to a unique property of dissolved H<sub>2</sub> at high levels under nanobubble conditions in cathode-side water [15] and chemically, it is shown to suppress oxygen radical generation similar to the action of H<sub>2</sub> water reported elsewhere [16]. This technology does not need processed H<sub>2</sub> gas for producing H<sub>2</sub> water, and therefore, it renders good applicability for clinical use [17–22].

Here, we used water with dissolved H<sub>2</sub> produced by electrolysis (EW) and studied its biological effect on cardio-renal injury in a rat model of CKD.

## Materials and methods

### Animals and protocols

Seven-week-old male Dahl salt-sensitive rats were allocated to two groups: one group was given filtered water (FW,  $n = 17$ ) and the other group was given water with dissolved  $H_2$  (EW,  $n = 18$ ). All rats were housed in a temperature- and humidity-controlled room with 12-h light/dark cycles. Rats were fed a 0.5% salt diet with *ad libitum* access to the group-specific water over a 6-week period. Water was changed twice a day, in the morning and afternoon, and was delivered by a metallic straw from a closed bottle. At Week 6, rats were subjected to unilateral clamping of the left kidney artery for 45 min and, thereafter, reperfused. One week after I/R, rats were killed for histological and plasma analyses.

During the course of the study, blood pressure, body weight, volume of drinking water, 24-h urinary volume, 24-h urinary excretion of protein and thiobarbituric acid reactive substances (TBARS) were regularly measured. Whole kidneys and heart for histological examination and blood samples from the aortic artery were collected at the end of the study.

During I/R, rats were anesthetized using intraperitoneal phenobarbital. All procedures were performed in accordance with institutional guidelines for the care and use of laboratory animals and all protocols were approved by the Animal Committee at Tohoku University School of Medicine.

### Generation and chemical properties of electrolyzed water

EW was generated using a TRIM ION TI-9000 system (Nihon Trim Co., Ltd, Osaka, Japan), which is equipped with a filtration system and electrolysis system connected to a tap. FW was generated by the TI-9000 system using only the filtration system. The properties of EW are shown in Table 1. EW showed negative oxidation–reduction potential and high levels of dissolved  $H_2$ .

### Measurements

Blood pressure was measured in the morning by the tail-cuff method using an MK2000A blood pressure monitor for mice and rats (Muromachi, Tokyo, Japan). Urinary protein was measured using a Quick Start bovine serum albumin standard set (Bio-Rad Laboratories, Hercules, CA). Urinary TBARS were measured by the lipid peroxidation assay method. Plasma creatinine and blood urea nitrogen (BUN) were measured using an auto-analyzer (Beckman Coulter, Fullerton, CA) and clearance of creatinine was calculated. Tumor necrosis factor  $\alpha$ , interleukin-6 and monocyte chemoattractant protein (MCP)-1 were measured using enzyme-linked immunosorbent assays (Invitrogen, Carlsbad, CA). Methylglyoxal and 3-deoxyglucosone were measured using liquid chromatography–mass spectrometry, as previously reported [23].

### Histological examinations

Kidney and heart sections were stained using the Elastica-Masson method for determining renal injury and cardiac fibrosis. Glomerular adhesion was determined from the findings of all cortical glomeruli in each rat ( $>70$ ). To semiquantify the glomerular matrix, 50 glomeruli were selected randomly. The percentage of each glomerulus occupied by mesangial matrix was estimated and given a score (0, normal; 1,  $<25\%$  of the glomerulus; 2, 25–50% of the glomerulus; 3, 50–75% of the glomerulus or 4,  $>75\%$  of the glomerulus). A glomerular injury score (GIS) was obtained using the following formula:  $[(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)]/50$ . To semiquantify the tubulointerstitial fibrosis area, five areas of renal medulla were randomly selected. The percentage of each area that showed sclerofibrotic changes (blue area in Masson staining) was measured by Image J software (National Institute of Health, Bethesda, MD). To semiquantify the tubular dilation area, five areas of renal medulla were randomly selected. The percentage of each area that showed dilated change (unstained area following Masson staining) was measured by Im-

age J software. To semiquantify the area of cardiac fibrosis, five areas of heart tissue were randomly selected. The percentage of each area that showed dilated change (blue area following Masson staining) was measured by Image J software. Cardiomyocyte size was determined according to the methods reported elsewhere [24]. Briefly, five areas of heart tissue from inner, medial and outer portions were randomly selected to measure the minimum length of sectioned myocardium in the short axis. The percentage of each area that showed dilated change (blue area in Masson staining) was measured by Image J software. Changes in the cardiac vascular wall from arteriole to small artery and post-capillary venule to venule were examined as previously reported [25]. Outer and inner diameters of respective vessels were measured to calculate the ratios of those two measurements. For immunohistochemical analysis, kidney and heart tissue was immediately fixed with 95% ethanol overnight and then again with 100% ethanol overnight. Tissue was embedded in paraffin and 3- $\mu$ m-thick sections were cut and mounted on slide glasses. Slides were deparaffinized with xylene and ethanol. Immunohistochemical staining was performed using monoclonal antibody against ED-1 (Serotec, Oxford, UK), polyclonal antibody against nitrotyrosine (Upstate, New York, NY) and monoclonal antibody against 4-hydroxy-2-nonenal (4-HNE) (NOF corporation, Tokyo, Japan). The slides were then incubated overnight at 4°C. Results were analyzed by counting the number of cells labeled positively for ED-1 in kidney and heart, nitrotyrosine-positive cells in heart and measuring the area of positive nitrotyrosine in kidney, 4-HNE staining in kidney and heart by Image J software.

### RNA preparation and quantitative reverse transcriptase-mediated polymerase chain reaction

Total RNA was isolated from the whole kidney by using the guanidine–isothiocyanate based-reagent Isogen (Nippon Gene, Tokyo, Japan) according to the instruction manual.

**Quantitative reverse transcription–polymerase chain reaction.** For cDNA synthesis by Invitrogen Superscript III First-strand Synthesis SuperMix (Invitrogen) according to the instruction manual, 2  $\mu$ g of total RNA was used as a template. We performed real-time polymerase chain reaction analysis with probe sets from Applied Biosystems (Foster City, CA). The gene-specific primers of NADPH oxidase 4: NM\_053524.1—forward: ACTGCCTCCATCAAGCCAAGA and reverse: CTCCAAATGGGCC-ATCAATGTA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): NM\_017008.3—forward: GGCACAGTCAAGGCTGAGAATG and reverse: ATGGTGGTGAAGACGCCAGTA were used for the amplification of certain cDNAs using the Syber Premix Ex Taq solution (Takara Bio, Shiga, Japan). The relative expression level of each messenger RNA (mRNA) was normalized by using the glyceraldehyde-3-phosphate dehydrogenase mRNA level.

### Statistical analysis

Data are expressed as mean  $\pm$  standard error of the mean and were analyzed using the independent *t*-test or two-way repeated measure analysis of variance. Differences between groups were considered significant for values of  $P < 0.05$ . All analyses were performed using Sigmasat 3.5 software (Systat Software, Chicago, IL).

## Results

Changes in mean body weight, blood pressure, food consumption, water consumption, urinary protein and albumin during the course of 6-week feeding before I/R are shown in Table 2. No differences were found in the changes of these parameters between the FW and EW groups.

Plasma and urinary parameters in sham and I/R rats at 1 week after I/R are shown in Table 3. Significantly higher levels were seen in plasma MCP-1, methylglyoxal and BUN and in urinary MCP-1 in rats given FW with I/R as compared to sham. Whereas no differences were found in those plasma parameters in rats given EW, although no statistical differences were found between FW and EW of I/R rats.

**Table 1.** Chemical properties of test water<sup>a</sup>

Water type	pH	Dissolved hydrogen (mg/L)	Redox potential (mV)
FW	8.33 $\pm$ 0.02	0.00 $\pm$ 0.00	+140.1 $\pm$ 4.2
EW	10.43 $\pm$ 0.01	0.35 $\pm$ 0.03	–148.0 $\pm$ 4.0

<sup>a</sup>Values represent mean  $\pm$  SEM.

Representative histological findings of kidney and heart are shown in Figure 1.

Figure 2 shows the histological comparisons of kidney between the two groups (FW versus EW) in sham and I/R rats, respectively. In rats given FW, there were significant increases in adhesive glomeruli (Sham versus I/R:  $3.6 \pm 0.1\%$  versus  $9.2 \pm 0.8\%$ ,  $P < 0.05$ , Figure 2a), GIS ( $0.09 \pm 0.02$  versus  $0.43 \pm 0.05$ ,  $P < 0.05$ , Figure 2b) and nitrotyrosine staining area ( $1.46 \pm 0.37\%$  versus  $5.51 \pm 1.74\%$  per slice;  $P < 0.05$ , Figure 2e), while no differences were found in interstitial fibrosis ( $10.0 \pm 2.1\%$  versus  $11.9 \pm 1.8\%$  per slice, Figure 2c), number of ED-1 cells ( $64.7 \pm 16.6$  versus  $75.3 \pm 14.6$ , Figure 2d) and 4-HNE staining area in kidney ( $56.0 \pm 3.2\%$  versus  $57.6 \pm 2.7\%$  per slice, Figure 2f). In the rats given EW, there were significant increases in GIS (Sham versus I/R:  $0.09 \pm 0.03$  versus  $0.36 \pm 0.05$ , per slice;  $P < 0.05$ , Figure 2b), number of ED-1 cells ( $18.0 \pm 4.9$  versus  $41.0 \pm 6.9$  per slice;  $P < 0.05$ , Figure 2d) and nitrotyrosine staining area ( $1.58 \pm 0.01\%$  versus  $2.93 \pm 1.01\%$ ,  $P < 0.05$ , Figure 2e), while no differences were found in interstitial fibrosis ( $8.9 \pm 1.2\%$  versus  $9.3 \pm 0.5\%$ , Figure 2c), adhesive glomeruli ( $3.1 \pm 0.3\%$  versus  $4.7 \pm 0.8\%$ , Figure 2a), 4-HNE staining area ( $55.7 \pm 6.1\%$  versus  $55.0 \pm 3.8\%$  per slice, Figure 2f). In comparison of the rats on FW and EW, the levels were significantly less in the glomerular adhesion rate in I/R rats on EW ( $P < 0.05$ , Figure 2a), ED-1 staining in sham and I/R rats on EW ( $P < 0.05$ ; Figure 2d), nitrotyrosine staining in I/R rats on EW ( $P < 0.05$ ; Figure 2e), as compared to the counterparts, respectively.

Figure 3 shows the histological comparisons of heart between the two groups (FW versus EW) in sham and I/R rats, respectively. In rats given FW, there were significant increases in fibrosis area (Sham versus I/R:  $3.0 \pm 0.2$  versus  $3.9 \pm 0.3$ ,  $P < 0.05$ , Figure 3a), number of ED-1 cells ( $60.4 \pm 10.4$  versus  $99.9 \pm 10.7$  per slice;  $P < 0.05$ , Figure 3b), number of positive nitrotyrosine cells ( $41.3 \pm 7.6$  versus  $70.3 \pm 8.2$  per slice;  $P < 0.05$ , Figure 3c), 4-HNE staining area in heart ( $46.1 \pm 8.0\%$  versus  $63.0 \pm 4.4\%$  per slice;  $P < 0.05$ , Figure 3d), while no difference was found in heart/body weight ratio ( $0.32 \pm 0.01\%$  versus  $0.33 \pm 0.01\%$ ), left ventricular wall thickness ( $1.90 \pm 0.09$  mm

versus  $1.90 \pm 0.04$  mm), arterial or venous wall thickness ratios ( $51.7 \pm 2.5\%$  versus  $52.5 \pm 1.3\%$ ), cardiomyocyte size ( $41.3 \pm 1.6$   $\mu$ m versus  $41.7 \pm 0.8$   $\mu$ m). In rats given EW, there were significant increases in fibrosis area (Sham versus I/R:  $2.0 \pm 0.2\%$  versus  $2.9 \pm 0.3\%$ ,  $P < 0.05$ , Figure 3a), number of ED-1 cells ( $29.5 \pm 4.2$  versus  $40.9 \pm 4.9$ , per slice;  $P < 0.05$ , Figure 3b), while no difference was found in 4-HNE staining area ( $48.5 \pm 7.4$  versus  $47.8 \pm 5.7$ , per slice, Figure 3d) and number of nitrotyrosine cells ( $28.6 \pm 5.0$  versus  $30.2 \pm 1.9$ , per slice, Figure 3c). There were no differences in cardiomyocyte size ( $48.0 \pm 1.6$   $\mu$ m versus  $49.0 \pm 1.6$   $\mu$ m), heart/body weight ratio ( $0.32 \pm 0.01\%$  versus  $0.33 \pm 0.01\%$ ), left ventricular wall thickness ( $1.95 \pm 0.06$  mm versus  $1.97 \pm 0.04$  mm) and arterial or venous wall thickness ratios ( $47.5 \pm 2.1\%$  versus  $51.1 \pm 1.9\%$ ). In comparison of the rats on FW and EW, the significantly lower levels were found in cardiac fibrosis in sham and I/R rats given EW ( $P < 0.05$ ; Figure 3a), ED-1-positive cells in sham and I/R rats given EW ( $P < 0.05$ ; Figure 3b), nitrotyrosine-positive cells in I/R rats given EW ( $P < 0.05$ ; Figure 3c) and 4-HNE staining area in I/R rats given EW ( $P < 0.05$ ; Figure 3d), respectively as compared to the counterparts. Cardiomyocyte size was smaller in sham and I/R rats given FW (data not shown,  $P < 0.05$ ).

There were no differences in mRNA levels (per GAPDH ratio) between sham and I/R rats given FW or EW (Table 4), but there was a significant difference in heart NOX4 expressions in I/R rats between FW and EW.

## Discussion

A growing amount of evidence suggest that renal ischemic insults induce immune activation which plays a crucial role for distant organ injury [26]. Notably, unilateral kidney I/R induces an inflammatory reaction in the contralateral kidney [27, 28]. In the present study, CKD model rats were subjected to unilateral kidney I/R to induce injury in the contralateral kidney and heart (cardio-renal injury model). Rats were allowed *ad libitum* drinking of group-specific water for 6 weeks. Findings at 1 week after I/R are summarized as follows. Firstly, significant increases were seen

**Table 2.** Comparison of parameters between rats on FW and EW<sup>a</sup>

	Group	Baseline	2 weeks	4 weeks	6 weeks
Body weight (g)	FW	99.9 $\pm$ 0.7	196.6 $\pm$ 1.2	308.8 $\pm$ 1.7	363.3 $\pm$ 2.1
	EW	101.2 $\pm$ 0.9	196.6 $\pm$ 1.6	307.7 $\pm$ 1.9	361.5 $\pm$ 2.7
Blood pressure (mmHg)	FW	111.1 $\pm$ 2.0	115.9 $\pm$ 2.1	143.7 $\pm$ 4.3	162.2 $\pm$ 3.0
	EW	117.3 $\pm$ 1.8	115.4 $\pm$ 2.4	148.7 $\pm$ 1.7	162.5 $\pm$ 3.3
Food consumption (g/day)	FW	23.4 $\pm$ 0.9	24.0 $\pm$ 1.5	26.2 $\pm$ 1.0	28.6 $\pm$ 1.3
	EW	22.5 $\pm$ 0.7	24.9 $\pm$ 1.4	25.9 $\pm$ 0.9	27.8 $\pm$ 1.9
Water consumption (g/day)	FW	28.0 $\pm$ 1.3	31.3 $\pm$ 0.9	35.5 $\pm$ 1.2	37.3 $\pm$ 1.1
	EW	27.4 $\pm$ 0.8	30.8 $\pm$ 1.0	35.1 $\pm$ 0.8	36.0 $\pm$ 2.2
Urinary protein (mg/mg Cr)	FW	11.2 $\pm$ 1.5	38.5 $\pm$ 2.8	85.8 $\pm$ 5.4	89.8 $\pm$ 6.6
	EW	12.0 $\pm$ 2.0	37.8 $\pm$ 2.8	92.5 $\pm$ 3.8	98.1 $\pm$ 7.9
Urinary albumin (mg/mg Cr)	FW	0.30 $\pm$ 0.04	0.80 $\pm$ 0.06	1.30 $\pm$ 0.10	1.30 $\pm$ 0.07
	EW	0.30 $\pm$ 0.06	0.90 $\pm$ 0.08	1.20 $\pm$ 0.11	1.30 $\pm$ 0.07
Urinary TBARS ( $\mu$ M MDA/mg Cr)	FW	ND	ND	ND	0.12 $\pm$ 0.10
	EW	ND	ND	ND	0.12 $\pm$ 0.07

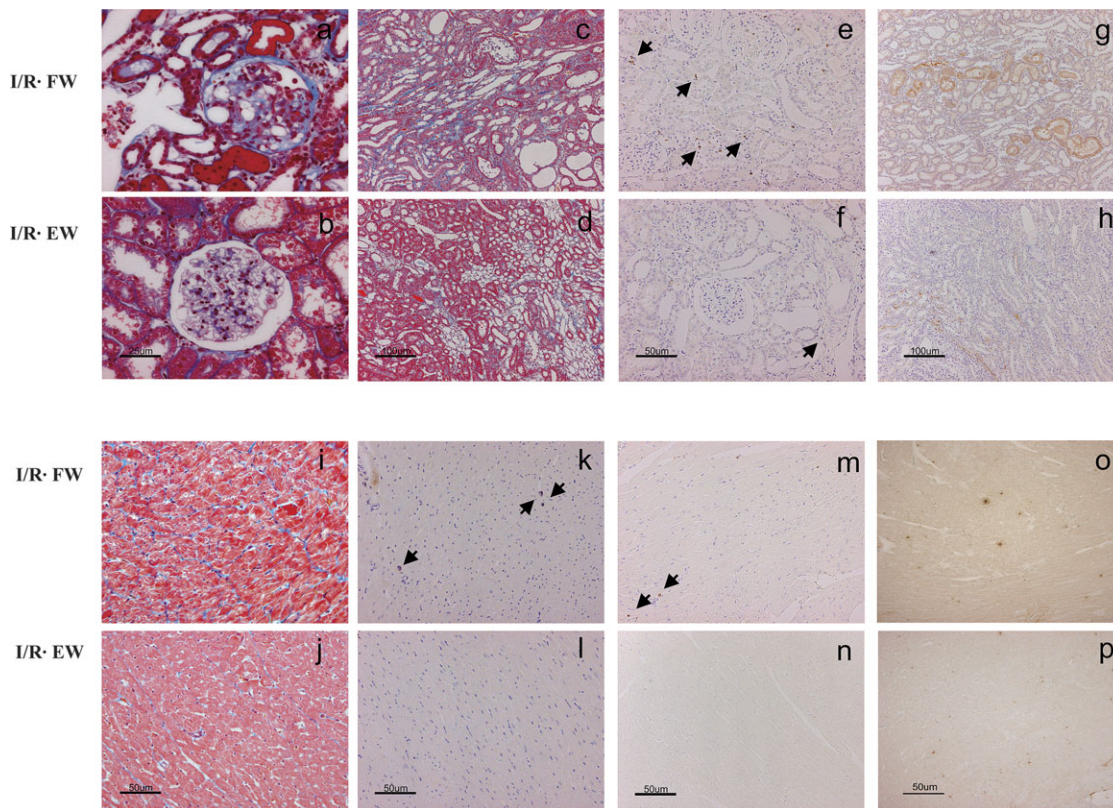
<sup>a</sup>Values represent mean  $\pm$  SEM. ND, not determined.

**Table 3.** Comparison of parameters between rats on FW and EW<sup>a</sup>

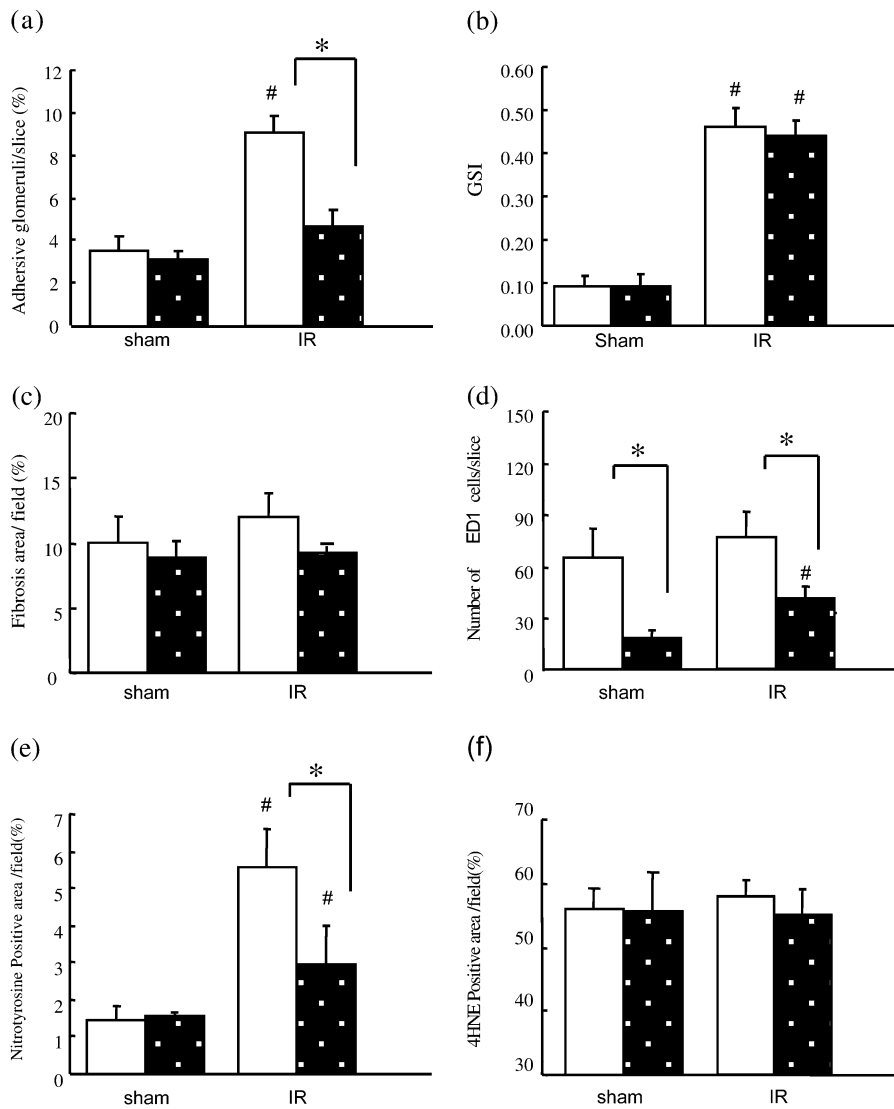
	Group	Sham rat	I/R rat	P
Plasma MCP-1 (pg/mL)	FW	22.1 ± 1.6	28.4 ± 1.5	<0.05
	EW	27.1 ± 3.3	32.7 ± 1.6	NS
Plasma IL-6 (pg/mL)	FW	15.2 ± 2.6	13.6 ± 1.4	NS
	EW	13.0 ± 1.8	14.3 ± 1.2	NS
Plasma TNF- $\alpha$ (pg/mL)	FW	6.5 ± 0.8	10.0 ± 3.5	NS
	EW	6.9 ± 0.9	8.4 ± 1.1	NS
Plasma methylglyoxal (nM)	FW	92.8 ± 5.1	106.4 ± 3.8	<0.05
	EW	94.5 ± 4.1	104.8 ± 3.8	NS
Plasma 3-deoxyglucosone (nM)	FW	651.6 ± 63.2	673.7 ± 26.0	NS
	EW	666.5 ± 61.4	674.4 ± 25.0	NS
Plasma creatinine (mg/dL)	FW	0.52 ± 0.06	0.36 ± 0.03	NS
	EW	0.42 ± 0.02	0.45 ± 0.02	NS
BUN (mg/dL)	FW	19.0 ± 0.5	23.0 ± 0.8	<0.05
	EW	18.7 ± 1.2	21.2 ± 0.8	NS
Urinary TBARS excretion ( $\mu$ M MDA/mg Cr)	FW	0.13 ± 0.01 <sup>b</sup>	0.13 ± 0.01	NS
	EW	0.10 ± 0.01	0.13 ± 0.02	NS
Urinary albumin excretion (mg/mg Cr)	FW	1.58 ± 0.36	1.87 ± 0.17	NS
	EW	1.12 ± 0.27	1.96 ± 0.20	<0.05
Urinary MCP-1 excretion (pg/mg Cr)	FW	0.26 ± 0.03	0.39 ± 0.03	<0.05
	EW	0.19 ± 0.01	0.38 ± 0.02	<0.05

<sup>a</sup>Values represent mean  $\pm$  SEM. NS, no significance; IL-6, interleukin 6; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ .

<sup>b</sup>FW versus EW, P < 0.05.



**Fig. 1.** Representative histological findings of kidneys (the contralateral side) and hearts of rats with I/R. Glomerular adhesion with crescent formation in a rat with I/R given FW (**a**) and EW (**b**). Tubular dilation and fibrosis in a rat with I/R given FW (**c**) and EW (**d**). ED-1 staining in the renal cortex of a rat with I/R given FW (**e**) and EW (**f**). Nitrotyrosine staining in the renal cortex of a rat with I/R given FW (**g**) and EW (**h**). Myocardium fibrosis of a rat with I/R given FW (**i**) and EW (**j**). ED-1 staining in the heart of a rat with I/R given FW (**k**) and EW (**l**). Nitrotyrosine staining in the heart of a rat with I/R given FW (**m**) and EW (**n**). 4-HNE staining in the heart of a rat with I/R given FW (**o**) and EW (**p**).



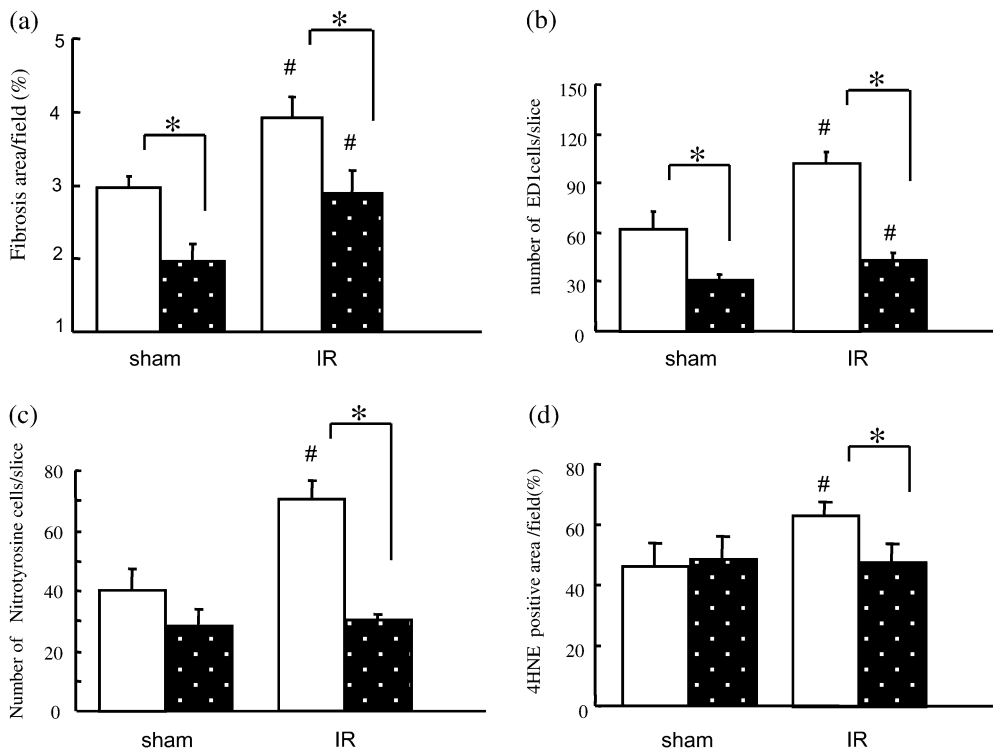
**Fig. 2.** Histological findings of kidney in rats  $\pm$  I/R given FW or EW. (a) Glomerular adhesion ratios per slice (%) in sham rats ( $\square$ FW:  $n = 7$ ,  $\blacksquare$ EW:  $n = 5$ ) and I/R rats (contralateral kidney,  $\square$ FW:  $n = 11$ ,  $\blacksquare$ EW:  $n = 12$ ). (b) Glomerular sclerosis index in sham rats and I/R rats. (c) Tubular fibrosis area per field (%) in sham rats and I/R rats. (d) Number of ED-1-labeled cells per slice in sham rats and I/R rats. (e) Nitrotyrosine-positive area per field (%) in sham rats and I/R rats. (f) 4-HNE-positive area per field (%) in sham rats and I/R rats. #P < 0.05 versus sham; \*P < 0.05 versus FW.

in plasma MCP-1, methylglyoxal and BUN in the FW group, whereas no changes were found in the EW group. Secondly, in histological analysis, significant increases in glomerular adhesion, cardiac fibrosis, ED-1-positive cells and nitrotyrosine staining were seen in the contralateral kidney and the heart of rats given FW. In rats given EW, those findings were significantly ameliorated and there were significant differences in histological data between rats given FW or EW not only in the sham group (EW: fewer ED-1 cell numbers in kidney and heart, less fibrosis in the heart) but also in the I/R group (less glomerular adhesion, fewer ED-1 cells and less nitrotyrosine staining in the kidney and heart).

Regarding the pathological characteristics of rats given FW in the present study, the model indicates enhanced oxidative stress and inflammation in remote organs by unilateral kidney I/R. There were findings of enhanced glomerular adhesion and cardiac fibrosis, with accompany-

ing increased ED-1 cells and nitrotyrosine staining, which reflects nitric oxide deactivation by oxygen radicals. Those findings may well support our hypothesis. In the findings of rats given EW, there were the same changes in the contralateral kidney as those of rats given FW, but the levels were significantly less than those of rats given FW. In the heart, no increases were found in cardiac fibrosis, ED-1 cells and nitrotyrosine staining with I/R, and the levels were significantly less than those of FW. This indicates the bioaction of drinking EW for ameliorating inflammatory and oxidative stress and protecting histological derangement due to those injuries, partly by suppressing nitric oxide inactivation.

In a comparison of rats given EW or FW without I/R, the number of ED-1 cells in the kidney was lower in rats given EW, with accompanying lower levels of TBARS and MCP-1 in the urine. Furthermore, less cardiac fibrosis and fewer ED-1 cells were found in rats given EW. Therefore, it is



**Fig. 3.** Histological findings of heart in rats  $\pm$  I/R given FW or EW. (a) Cardiac fibrosis per field (%) in sham rats ( $\square$ FW:  $n = 7$ ,  $\blacksquare$ EW:  $n = 5$ ) and I/R rats ( $\square$ FW:  $n = 11$ ,  $\blacksquare$ EW:  $n = 12$ ). (b) Number of ED-1-labeled cells per slice in sham rats and I/R rats. (c) Number of nitrotyrosine-labeled cells per slice in sham rats and I/R rats. (d) 4-HNE-positive area per field (%) in sham rats and I/R rats. # $P < 0.05$  versus sham; \* $P < 0.05$  versus FW.

**Table 4.** Comparison of mRNA level of NADPH oxidase 4 between rats on FW and EW<sup>a</sup>

	Group	Sham rat	I/R rat	P
Heart tissue	FW	0.008 $\pm$ 0.005	0.028 $\pm$ 0.011 <sup>b</sup>	NS
(NOX4/GAPDH)	EW	0.005 $\pm$ 0.003	0.012 $\pm$ 0.017	NS
Kidney tissue	FW	1.599 $\pm$ 1.053	1.701 $\pm$ 0.789	NS
(NOX4/GAPDH)	EW	1.442 $\pm$ 0.790	0.986 $\pm$ 0.464	NS

<sup>a</sup>NOX4, NADPH oxidase 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Values represent mean  $\pm$  SEM. NS, no significance.

<sup>b</sup>FW versus EW,  $P < 0.05$ .

speculated that as a result of the preceding drinking for 6 weeks before I/R, less inflammatory status may be preconditioned, which could contribute to less histological damage.

Special mention should be made of the fact that these differences were achieved simply by changing the daily drinking water to EW, without the use of any supplements such as antioxidants. The findings of the present study thus suggest that daily EW drinking may offer an innovative approach to preventing cardio-renal injury and ischemic stress.

Regarding the primary biological mechanisms by which EW suppresses the histological changes, it is speculated that H<sub>2</sub>, which quenches oxygen radicals such as superoxide anions or hydroxyl radicals, may play a crucial role. Previous studies employed water with dissolved H<sub>2</sub> levels  $>0.4$  mM [9], approximately equivalent to the level in the EW employed in the present study. In addition, EW is known to suppress the generation of hydrogen peroxide during the oxidative stress. This chemical character may

benefit the rats given EW with I/R. It is known that plasma methylglyoxal, which was increased with I/R in rats given FW, reacts with hydrogen peroxide to generate carbon radicals, which could be involved with progressive cellular injury. Whether there are differences in hydrogenated water produced by bubbling or electrolysis could be an interesting issue to be clarified for clinical use.

In conclusion, EW at least partially suppressed cardio-renal syndrome in rats with the combination of oxidative and ischemic insults. The leading mechanism by which EW exerts effects may involve the inhibition of inflammation and oxidative stress. This indicates a novel strategy for applying H<sub>2</sub> produced by water electrolysis for the prevention of CKD cardio-renal syndrome.

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