

# Molecular Hydrogen Suppresses Renal Injury in Chronic Kidney Disease Rats

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## Abstract

**Background:** Cathode side commercial hydrogen dissolved water (HW) exhibits low dissolved oxygen, high dissolved hydrogen and significant negative redox potential. Therapeutic applications of HW have recently been reported. Thus, the present study aimed to examine effects of HW consumption on renal injury in a rat model of chronic kidney disease.

**Methods:** Twenty Dahl S rats were given HW and tap water (TW), over a 4-week period. Thereafter, they were allocated to either group; non stressed (NS), and oxi-carbonyl stressed (OC: 5% salt diet and 1% methylglyoxal in drinking water) group, respectively (HW, TW n=5 each group). OS groups were subjected to unilateral kidney ischemic reperfusion (IR) in the final week.

**Results:** No differences were found in blood pressure and urinary parameters between HW and TW. Glomerular adhesion rates in the IR kidney and positive osteopontin of the non-IR kidney were significantly higher in OC rats on HW, respectively. ED-1 staining on HW was significantly lower than TW in both the IR and non-IR kidneys of the OC rats. Plasma MCP-1 was significantly lower on HW after IR.

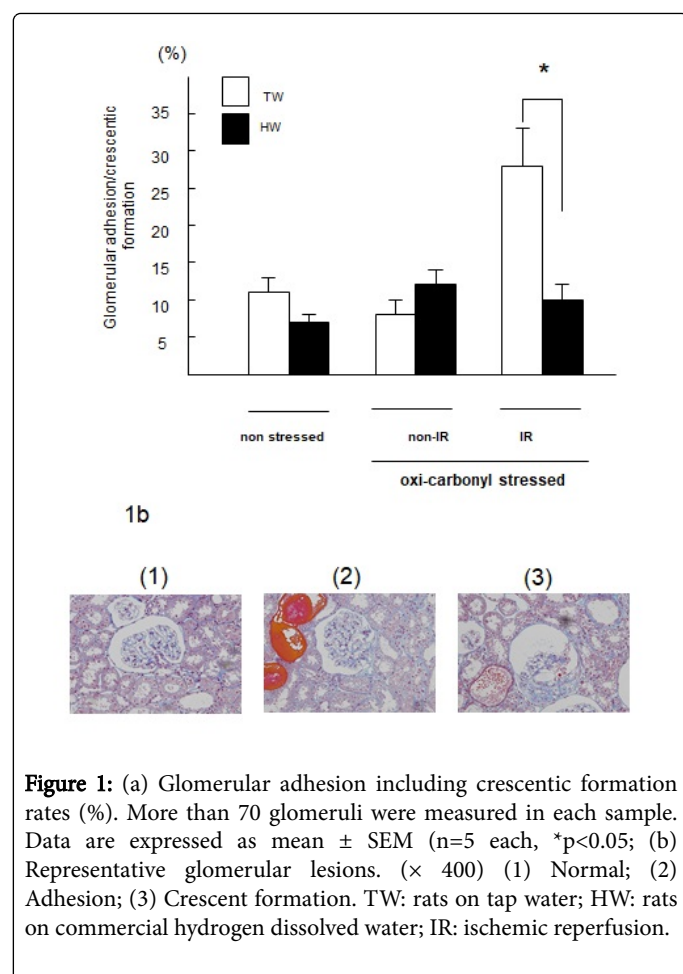
**Conclusion:** Drinking HW at least partly suppressed renal damage in rats with combination of oxidative, carbonyl and ischemic stimulus.

**Keywords:** Commercial hydrogen dissolved water; Dahl salt rat; Chronic kidney disease; Methylglyoxal; Ischemic reperfusion; Oxidative stress; Inflammation

## Introduction

Hydrogen molecules are scavengers of hydroxyl radicals. *In vitro*, H<sub>2</sub> can selectively reduce ROS. It only reacts with the strongest oxidants, which means that the use of H<sub>2</sub> is mild and has no serious side effects [1]. Molecular hydrogen can inhibit the release of cell adhesion molecules and proinflammatory cytokines. H<sub>2</sub> can increase the level of anti-inflammatory cytokines. H<sub>2</sub> enhances HO-1 expression and activity, suggesting that H<sub>2</sub> can inhibit excessive inflammation and endothelial injury through Nrf2 (nuclear factor RBC 2 p45 related factor 2)/ HO-1 pathway [2]. Water electrolysis gives rise to unique properties in cathode-side water (HW), such as increased alkalinity, low dissolved oxygen, high dissolved hydrogen, and negative oxidation-reduction potential [3]. In the Lipopolysaccharide (LPS) or cecitis group, the serums Cr were much higher than in the sham group. The increases of serum Cr were significantly reduced in the H<sub>2</sub> inhalation group [4-7]. But one study found no significant difference in BUN/Cr rates across all groups. Molecular hydrogen therapy may not be as effective as we thought when the BUN/Cr ratio was used to determine whether azotemia or tubular ischemia predated the presence of AKI [7]. In addition, someone suggested that hydrogen

molecules have the ability to influence a variety of ways, and help the MPO (myeloperoxidase), MCP, Caspase 3, Caspase-12, TNF (tumor necrosis factor), interleukin, Bcl-2, Bax and cox-2 (as shown in Figure 1) [8] gene regulation or protein expression. In chemical terms, HW is known to suppress generation of superoxide anions and hydrogen peroxide during the oxidative process [3], and decreases oxidative injury to DNA *in vitro* [3,9]. Biologically, HW protects islet cells from oxidative injury induced by high glucose levels [10].



**Figure 1:** (a) Glomerular adhesion including crescentic formation rates (%). More than 70 glomeruli were measured in each sample. Data are expressed as mean  $\pm$  SEM (n=5 each, \*p<0.05; (b) Representative glomerular lesions. ( $\times$  400) (1) Normal; (2) Adhesion; (3) Crescent formation. TW: rats on tap water; HW: rats on commercial hydrogen dissolved water; IR: ischemic reperfusion.

Furthermore, drinking HW reportedly ameliorates hyperglycemia in streptozotocine-induced diabetes rats [11] and renal ischemia reperfusion induced remote organ damage. The renal histological score of cecitis group increased significantly. It was remarkably relieved in the H<sub>2</sub> inhalation group which was no different from the sham group [4,5]. Acute renal injury (AKI) is a common disease in sepsis patients, which can aggravate the condition of septic shock patients and lead to higher mortality. In addition to the degree of oxidative stress and inflammation, serum urea nitrogen (steamed bread) and creatinine (Cr) also showed liver function. H and E staining of renal tissue revealed renal tubular epithelial cell edema, damaged brush margins, and hemorrhagic stromal edema in septicemic mice. Tubular epithelial cell injury was improved in the H<sub>2</sub> inhalation group. Similar results were shown in tem analysis of glomerular filtration membranes [7].

Oxidative stress and kidney inflammation are similar to those of the lungs and liver. Inflammatory cytokines (TNF-IL-6 and HMGB1) increased, while anti-inflammatory cytokines IL-10) decreased in sepsis. The activity of antioxidant enzymes (SOD and CAT) decreased, and the oxidation products (MDA and 8-iso-PGF2) increased. H<sub>2</sub> inhalation alleviates these changes [4,5,7]. However, in Liu et al. study, IL-10 levels did not change among all groups [7]. Enhanced oxidation and carbonyl stress are characteristic features of chronic kidney disease (CKD) [12,13]. These stressors supposedly play a crucial role in progressive renal deterioration. Kidney injury in the Dahl salt-sensitive (SS) rat, a model of CKD, has been shown to be ameliorated by antioxidants such as vitamins C and E [14], the superoxide dismutase-

mimetic tempol [15], and N-acetylcysteine [16]. However, no promising results have been confirmed in the clinical application of antioxidants [17]. Despite some controversy, molecular hydrogen therapy is considered an effective way to reduce kidney structural damage, protect kidney function, and resist inflammation and oxidation. All studies have shown that molecular hydrogen therapy improves the survival rate of septic animals regardless of the effects of drug administration and sepsis [18]. In this context, the application of HW for CKD could offer an innovative treatment. The present study examined the effects of HW drinking on kidney damage in CKD model rats.

## Methods

### Animals and protocols

Seven-week-old male Dahl SS rats were housed in a temperature and humidity-controlled room with 12-h light/dark cycles. A 0.5% salt diet was provided, and rats were allocated to HW and tap water (TW) groups (n=10 each) with ad libitum access to water over a 4-week period. Thereafter, rats were reallocated as follows: 10 rats (n=5 each from HW and TW groups) remained in the same groups for an additional 16 weeks (non-stressed group), while the remaining rats (n=5 each from HW and TW groups) were given a 5% salt diet and 1% methylglyoxal (MGO) in drinking water for 16 weeks (oxi-carbonyl stressed rats). Further, rats of the latter group were subjected to a unilateral kidney artery clamp (left kidney, for 45 min), followed by ischemic reperfusion (IR) in week 20. Water was changed twice a day, in the morning and afternoon, and was delivered by metallic straw from a closed bottle.

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 measured every 4-weeks regularly. Whole kidneys for histological examination and blood samples from the aortic artery were collected at the end of the study in the non-stressed group, and 3 days after unilateral IR in the oxi-carbonyl stressed group.

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

### Generation and chemical properties of HW

What we used was one of the commercial products. Most of the hydrogen water's character is pH>8.5, hydrogen concentration>1 ppm, ORP<-600 mV, the properties of HW are shown in Table 1.

Water type	pH	Dissolved oxygen	Dissolved hydrogen	Redox potential (mV)
		(mg/l)	(mg/l)	
TW	7.0~7.2	2.6~7.0	0	+200 ~ +350
HW	10~10.5	1.3~3.5	0.3~0.6	-200 ~ -120

**Table 1:** Chemical properties of commercial hydrogen dissolved water (HW).

## Measurements

Blood pressure was measured by the tail-cuff method using Blood pressure monitor for mice and rats MK2000A (Muromachi, Tokyo, Japan) in the morning. Urinary protein was measured using a Quick Start bovine serum albumin standard set (Bio-Rad Laboratories, Hercules, and CA). Urinary TBARS were measured by lipid peroxidation assay method. Plasma creatinine and BUN (Blood urea nitrogen) were measured using an auto-analyzer (Beckman Coulter, Fullerton, CA) and MCP-1 was measured using an ELISA (Enzyme linked Immuno Sorbent Assay) kit (Invitrogen, Carlsbad, CA). MGO was measured by a LC/MS (Liquid chromatography-mass spectrometry) method, as previously reported (6) at Trim Medical institute Co. Ltd.

## Histological examinations

Kidney sections were stained using the Elastica-Masson method for determining renal injury and cardio-injury, then examined using light microscopy. Glomerular adhesion was determined from the findings of all cortical glomeruli in each rat (>70). Cardio-fibrosis area was measured by using Image J software (NIH public domain for experimental analyze). For immunohistochemical analysis, kidney tissue was immediately fixed with 95% ethanol overnight, then 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 analysis was performed using monoclonal antibodies against osteopontin (OPN; Santa Cruz Inc, Santa Cruz, CA) and ED-1 (Serotec, Oxford, UK), then incubated overnight at 4°C. Results were analyzed using Image J software.

## Statistical analysis

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

## Results

Changes in body weight, volume of drinking water ingested, mean blood pressure (BP) urinary protein and TBARS are shown in Tables 2 and 3. No differences were found in the change of those parameters between TW and HW subgroups in both non-stressed and oxo-carbonyl stressed groups.

		Baseline	4 weeks	8 weeks	16 weeks	20 weeks	P
Body weight	TW	163 $\pm$ 2	259 $\pm$ 2	340 $\pm$ 5	440 $\pm$ 7	469 $\pm$ 9	
(g)	HW	164 $\pm$ 1	266 $\pm$ 1	328 $\pm$ 3	434 $\pm$ 7	451 $\pm$ 6	NS
Water intake	TW	25.2 $\pm$ 1.4	38.8 $\pm$ 1.8	30.8 $\pm$ 2.6	40.4 $\pm$ 0.2	40.6 $\pm$ 12.2	
(g/day)	HW	26.8 $\pm$ 1.8	42.3 $\pm$ 1.8	33.6 $\pm$ 2.8	40.3 $\pm$ 0.2	58.6 $\pm$ 4.6	NS
Mean BP	TW	107 $\pm$ 2	103 $\pm$ 4	166 $\pm$ 5	178 $\pm$ 11	184 $\pm$ 14	
(mmHg)	HW	110 $\pm$ 1	116 $\pm$ 5	162 $\pm$ 11	161 $\pm$ 14	190 $\pm$ 8	NS
Urine-protein	TW	23.7 $\pm$ 6.9	107.3 $\pm$ 12.4	158.9 $\pm$ 17.4	206.9 $\pm$ 16.1	250.5 $\pm$ 43.0	
(mg/day)	HW	23.2 $\pm$ 6.3	110.8 $\pm$ 13.7	155.3 $\pm$ 18.6	181.0 $\pm$ 40.7	267.1 $\pm$ 38.4	NS
Urine-TBARS	TW	74.7 $\pm$ 12.8	170.9 $\pm$ 17.7	156.1 $\pm$ 10.1	145.2 $\pm$ 9.6	139.6 $\pm$ 15.1	
( $\mu$ M MDA/day)	HW	79.5 $\pm$ 10.6	152.9 $\pm$ 26.3	147.8 $\pm$ 5.6	101.5 $\pm$ 13.0	127.9 $\pm$ 10.6	NS

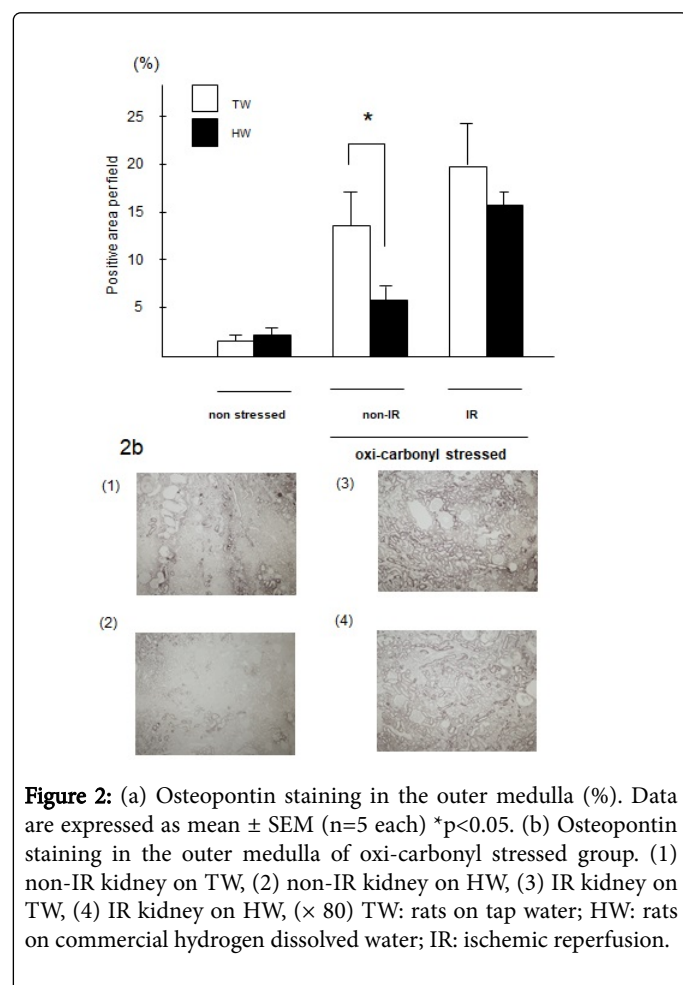
**Table 2:** Comparison of parameters between rats on tap water (TW) and commercial hydrogen dissolved water (HW) in the non-stressed group.

		Baseline	4 weeks	8 weeks	16 weeks	20 weeks	P
Body weight	TW	160 $\pm$ 3	254 $\pm$ 1	324 $\pm$ 3	344 $\pm$ 5	307 $\pm$ 5	
(g)	HW	157 $\pm$ 3	253 $\pm$ 3	319 $\pm$ 3	334 $\pm$ 11	301 $\pm$ 11	NS
Water intake	TW	30.9 $\pm$ 1.3	45.2 $\pm$ 1.7	38.3 $\pm$ 2.2	47.6 $\pm$ 4.7	58.6 $\pm$ 2.2	
(g/day)	HW	28.4 $\pm$ 2.9	41.8 $\pm$ 1.9	38.7 $\pm$ 2.3	53.2 $\pm$ 5.7	56.3 $\pm$ 1.8	NS
Mean BP	TW	120 $\pm$ 2	123 $\pm$ 2	177 $\pm$ 8	191 $\pm$ 9	198 $\pm$ 8	
(mmHg)	HW	124 $\pm$ 2	121 $\pm$ 3	156 $\pm$ 6	173 $\pm$ 13	152 $\pm$ 10	NS
Urine-protein	TW	21.7 $\pm$ 3.6	88.1 $\pm$ 14.5	135.7 $\pm$ 5.8	161.6 $\pm$ 25.0	217.2 $\pm$ 40.9	
(mg/day)	HW	20.3 $\pm$ 2.4	61.3 $\pm$ 6.3	136.7 $\pm$ 5.4	159.4 $\pm$ 10.0	153.7 $\pm$ 20.6	NS

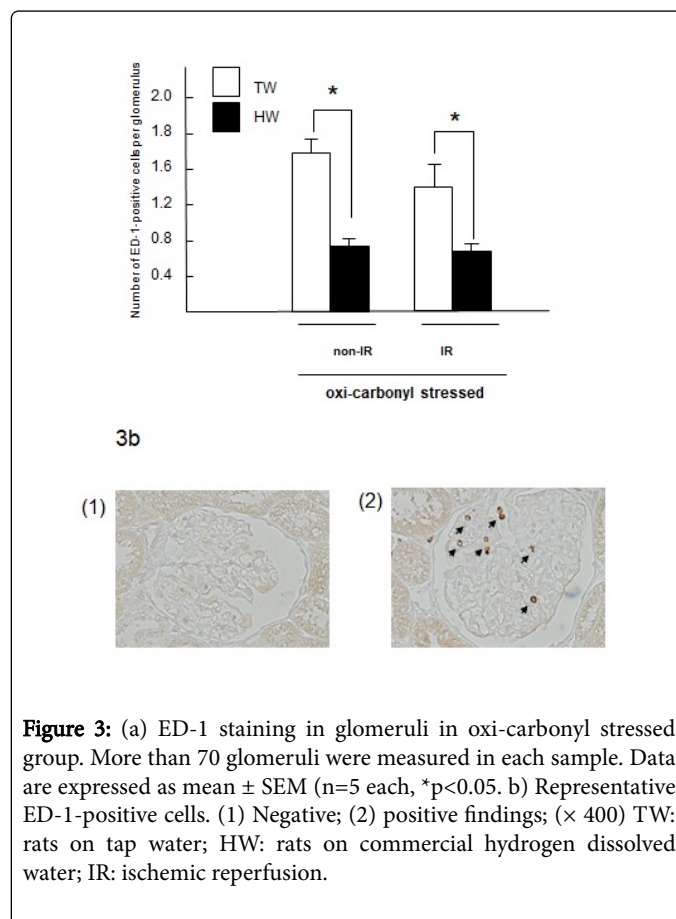
Urine-TBARS	TW	136.8 ± 14.8	171.1 ± 25.3	154.5 ± 6.5	189.9 ± 9.1	159.3 ± 17.2	
(μM MDA/day)	HW	128.4 ± 7.3	145.5 ± 15.4	157.1 ± 9.2	167.4 ± 8.5	133.0 ± 14.9	NS

**Table 3:** Comparison of parameters between rats on tap water (TW) and commercial hydrogen dissolved water (HW) in the oxi-carbonyl stressed group.

Representative histological findings are shown in Figures 1b, 2b, 3b and 4b. Histologically, no differences were found in glomerular adhesion rate between rats on TW or HW in the non-stressed group and the non-IR kidney of the oxi-carbonyl stressed groups. However, a significantly lower rate was found in the IR kidney of rats on HW ( $28.5 \pm 2.3\%$  in TW vs.  $11.7 \pm 1.8\%$  in HW per slice;  $p < 0.05$ ) (Figure 1a). No differences were found in OPN staining area between rats on TW and HW in the non-stressed group and the IR kidney of the oxi-carbonyl stressed groups. While a significantly lower level was found in the non-IR kidney of rats on HW of the oxi-carbonyl stressed groups ( $14.0 \pm 3.8$  in TW vs.  $5.0 \pm 0.6\%$  in HW;  $p < 0.05$ ) (Figure 2), with ED-1 staining, significantly fHWR positive cells were seen in rats on HW as compared to those on TW in the non-IR and IR kidneys of the oxi-carbonyl stressed group ( $1.6 \pm 0.4$  vs.  $0.7 \pm 0.1$  cells/glomerulus; and  $1.1 \pm 0.2$  vs.  $0.6 \pm 0.0$  cells/glomerulus, respectively,  $p < 0.05$ ) (Figure 3a).



**Figure 2:** (a) Osteopontin staining in the outer medulla (%). Data are expressed as mean  $\pm$  SEM ( $n=5$  each)  $*p < 0.05$ . (b) Osteopontin staining in the outer medulla of oxi-carbonyl stressed group. (1) non-IR kidney on TW, (2) non-IR kidney on HW, (3) IR kidney on TW, (4) IR kidney on HW, ( $\times 80$ ) TW: rats on tap water; HW: rats on commercial hydrogen dissolved water; IR: ischemic reperfusion.



**Figure 3:** (a) ED-1 staining in glomeruli in oxi-carbonyl stressed group. More than 70 glomeruli were measured in each sample. Data are expressed as mean  $\pm$  SEM ( $n=5$  each,  $*p < 0.05$ ). (b) Representative ED-1-positive cells. (1) Negative; (2) positive findings; ( $\times 400$ ) TW: rats on tap water; HW: rats on commercial hydrogen dissolved water; IR: ischemic reperfusion.

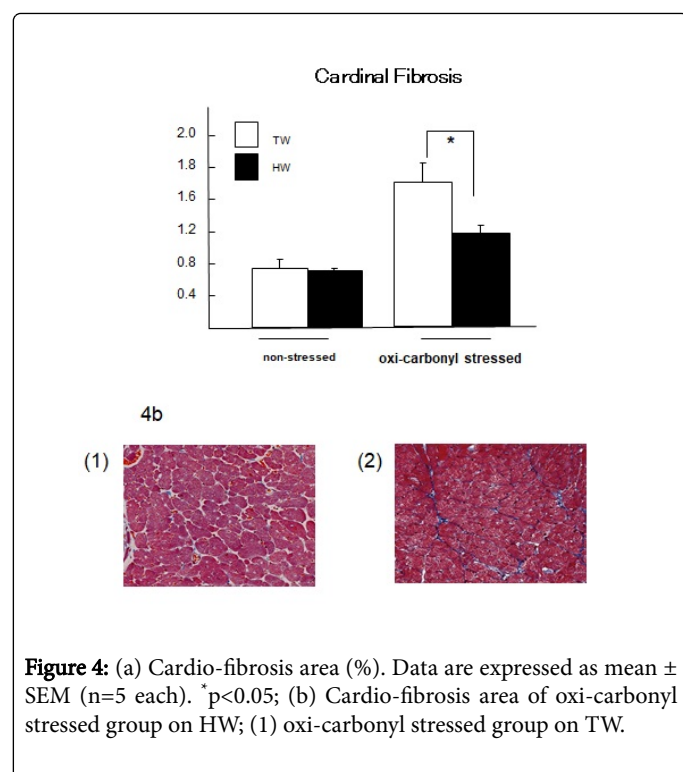
With cardio-fibrosis significantly higher fibrosis area was seen in rats on HW as compared to those on TW in oxi-carbonyl stressed groups ( $5.0 \pm 0.63$  in TW vs.  $3.2 \pm 0.30\%$  in HW;  $p < 0.05$ ), not in rats of no stressed groups.

Plasma levels of creatinine, BUN and MCP-1 in the oxi-carbonyl stressed group are shown in Table 4. No significant differences were found in creatinine or BUN levels between TW and HW groups, whereas significantly lower MCP-1 values were found in rats on HW ( $61.5 \pm 12.9$  vs.  $19.4 \pm 1.0$  pg/ml;  $p < 0.05$ ).

Parameter	TW	HW	P
Creatinine (mg/dl)	38.0 $\pm$ 2.7	29.3 $\pm$ 1.4	NS
Blood urea nitrogen (mg/dl)	0.6 $\pm$ 0.1	0.6 $\pm$ 0.1	NS
MCP-1 (pg/ml)	61.5 $\pm$ 12.9	19.4 $\pm$ 1.0	<0.05

**Table 4:** Comparison of blood parameters between rats on TW and HW in the oxi-carbonyl stressed group after IR.





## Discussion

The present study aimed to examine the effects of ad libitum access to HW drinking on kidney damage in the combination of oxidative and carbonyl stress model rats, compared to those on TW. We employed Dahl SS rats classified into two groups: a non-stressed group with an absence of extrinsic renal insults; and an oxi-carbonyl stressed group with 3 types of insult (high salt, MGO and unilateral IR). In both non-stressed and oxi-carbonyl stressed groups, no differences were found in body weight, water intake, BP or urinary parameters between rats on HW and TW (Figure 4). However, in the non-IR kidneys of oxi-carbonyl stressed group, glomerular adhesions and OPN-positive ratios were significantly lower with HW as compared to TW. Furthermore, in oxi-carbonyl stressed group, significantly fHwer ED1-positive cells were seen in glomeruli with HW as compared to TW in both of non-IR and IR kidneys. Plasma MCP-1 and cardio-fibrosis area was significantly lower with HW compared to TW after IR. Special mention should be made of the fact that these differences were achieved simply by changing daily drinking water to HW, without any supplementation such as vitamin C, vitamin E, tempol or N-acetylcysteine. Thus, the finding of the present study suggests that daily HW drinking may offer an innovative approach to preventing oxi-carbonyl and ischemic stress.

In the present study, the oxi-carbonyl stressed group received MGO in the drinking water, and this was expected to result in high plasma MGO levels equivalent to the uremic milieu, 10 times higher than normal (unpublished data). Accordingly, oxi-carbonyl stressed rats in the present study were exposed to the same level of carbonyl stress observed in patients with CKD stage 5 [13]. The main difference between the kidney of non-stressed and the non-IR kidney of oxi-carbonyl stressed group was significantly higher OPN-positive ratios in the oxi-carbonyl stressed group as compared to the non-stressed

group. The results may suggest that HW could suppress enhancement of medullary OPN expression in the non-IR kidney of oxi-carbonyl stressed rats. The medulla is highly sensitive to oxidative stress [14], carbonyl stress and ischemic insult [13], and protection of the medulla could play a key role in preserving kidney function. OPN is expressed in the loop of Henle and distal nephron, and expression is known to be closely related to renal fibrosis [19]. Renal ischemia reperfusion induced remote organ damage, like cardio-fibrosis involved oxidative stress and inflammatory system, and drinking commercial hydrogen dissolved water with high concentration hydrogen protect this organ damage. In this context, it seems that HW drinking showed a protective effect on the medullary portion under enhanced oxidative and carbonyl stress. Furthermore, we performed IR in oxi-carbonyl stressed group to test the influence of ischemic insult in CKD condition. As a result, glomerular adhesion rates, ED-1 staining in glomeruli, and plasma MCP-1 levels were significantly lower in rats on HW as compared to those on TW, respectively. Thus, it was indicated that HW drinking inhibits inflammatory conditions leading to glomerular adhesions by suppressing macrophage activations.

Taken together the results, it was indicated that HW drinking showed a protective effect on the medullary as well as glomerular portions against the enhanced oxidative and carbonyl stress and inflammatory condition, and this may indicate a therapeutic potential of HW for CKD management. Nevertheless, no effect of HW was observed in terms of BP or urinary parameters during the course of the study, and the histological differences were limited. Several mechanisms may have contributed to this finding. First, the chemical properties of HW may not have been preserved throughout the day. For example, oxidation-reduction potential level was shown to be elevated up to 60% at 24 h (data not shown). Second, the anti-oxidative capacity of HW administered in the present study, may not have been sufficient to counteract the oxidative stressors in oxi-carbonyl stressed rats. This needs further investigations.

Characteristic chemical features of HW are the high dissolved hydrogen and low dissolved oxygen. The biological mechanism of HW to suppress oxidative stress has not clearly elucidated so far [3]. Recently, a series of studies on inhalation of hydrogen gas have revealed that hydrogen application has suppressive effects on brain infarct lesions by cerebral artery occlusion [1] and liver injury by ischemic-reperfusion [20]. Furthermore, drinking hydrogen-saturated water could ameliorate brain oxidation [21], and recent functional memory disturbance caused by oxidative stress [13]. Drinking HW can suppress oxidative stress and inflammation induced by ischemia reperfusion. Thus, the biological effects of HW may, at least partly, involve dissolved hydrogen levels of water. Those studies employed water with dissolved hydrogen levels >0.4 mM [21], approximately equivalent to the water employed in the present study. The role of hydrogen of HW needs to be addressed in future studies.

In conclusion, HW at least partially suppressed renal damage in rats with the combination of oxidative, carbonyl stress and ischemic insult. The leading mechanism by which HW exerts its effects may involve the inhibition of oxidative-carbonyl- inflammatory conditions. HW consumption may open a novel approach to managing CKD patients.

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