

# Suppressive Effect of High Hydrogen Generating High Amylose Cornstarch on Subacute Hepatic Ischemia-reperfusion Injury in Rats

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**We examined whether feeding high hydrogen generating resistant starch could suppress subacute hepatic ischemia-reperfusion injury. Rats were fed a control diet with or without 20% high amylose cornstarch (HAS) supplementation for 14 days. On day 12, rats were subject to ischemia-reperfusion treatment. Portal hydrogen concentration was higher in the HAS group compared with the control group. Increased plasma alanine and aspartate aminotransferase activities due to ischemia-reperfusion treatment tended to decrease, and a significant reduction was observed by HAS feeding when compared with the control group. In conclusion, HAS, which enhances hydrogen generation in the hindgut, alleviated subacute hepatic ischemia-reperfusion injury.**

**Key words:** hydrogen; high amylose cornstarch; large intestine; ischemia-reperfusion; antioxidant

Oxidative damage is a cause of various diseases, such as ischemia-reperfusion (IR) injury, atherosclerosis and diabetes [1, 2]. Alleviation of oxidative damage is therefore considered an effective measure in preventing these diseases. Many researchers have been investigating the effectiveness of various antioxidants, such as ascorbic acid, tocopherols, polyphenols, and carotenoids against such diseases. Recently, the antioxidative effects of H<sub>2</sub> on other organs have been reported in IR-treated rodents administered H<sub>2</sub> via inhalation of H<sub>2</sub> gas or administration of H<sub>2</sub> water [3–9]. It is clear from these studies that H<sub>2</sub> is an effective antioxidant *in vivo*. Previously, we found that H<sub>2</sub> generated from high amylose cornstarch (HAS) and pectin by fermentation in the large intestine alleviated initial acute hepatic IR injury, which was induced by 45 min of reperfusion following 30 min of ischemia [10]. Also, the portal H<sub>2</sub> concentration in rats fed HAS and pectin was comparable to or more than in those administered H<sub>2</sub> via inhalation of H<sub>2</sub> gas [3] or administration of H<sub>2</sub> water [11]. Therefore, H<sub>2</sub> production via large-bowel fermentation is a continuous and available means of supplying significant amounts of H<sub>2</sub> *in vivo*. However, the effect of H<sub>2</sub> generated in the large intestine on

subacute hepatic IR injury remains unclear.

Hepatic IR injury has been demonstrated to occur in a biphasic pattern: first, there is an initial acute phase characterized by hepatocellular damage and generation of reactive oxygen species at 1–6 hr, and then there is a subacute phase characterized by massive neutrophil infiltration and production of inflammatory mediators at 18–24 hr [12–14]. Many studies have demonstrated the effect of H<sub>2</sub> molecules on initial acute hepatic IR injury. However, to our knowledge, the effect of H<sub>2</sub> molecules, including H<sub>2</sub> generated by large intestinal fermentation, on oxidative stress in the subacute phase remains poorly understood. During the subacute phase of injury, the redox burden in damaged tissues is amplified by neutrophils, and then the injury worsens. Therefore, it is important to clarify the effect of H<sub>2</sub> generated in the large intestine on subacute hepatic IR injury. Increased urinary excretion of 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is an oxidation product of guanine, is observed due to oxidative damage of DNA [15–17]. We presumed that urinary 8-OHdG could be used as a marker of subacute hepatic IR injury. In the present study, to determine the suppressive effect of resistant starch (RS) on subacute hepatic IR injury, we examined the effects of HAS, which results in production of more H<sub>2</sub> in the large intestine than pectin, on liver damage in a rat model of subacute hepatic IR.

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High amylose cornstarch (Hi-maize 1043) was kindly supplied from Nippon NSC Ltd. (Tokyo, Japan). The study was approved by the Nayoro City University Animal Use Committee, and animals were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals, Nayoro City University. Seven-week-old male Sprague-Dawley rats were obtained from Japan SLC (Shizuoka, Japan). They were housed in individual cages with screen bottoms made of stainless steel in a room maintained at  $23 \pm 1^\circ\text{C}$  with humidity ranging from 50% to 70% under lighting conditions with 12 hr of light and 12 hr of darkness, daily. The rats were acclimated by feeding a laboratory chow diet (CE-2, CLEA Japan, Inc., Tokyo) for 5–6 days in order to select high  $\text{H}_2$ -generating (HG) rats before they were subjected to the experiments. After the acclimation period, net  $\text{H}_2$  excretion from expired air and flatus per 5 minutes was measured using a GC (Biogas analyzer BAS-1000, Mitleben, Osaka, Japan). Forty-nine rats were assigned into 4 groups ( $n = 12\text{--}13$ ) so as to balance the net  $\text{H}_2$  excretion and body weight. Two groups were administered the C diet, and the remaining group was administered the 20% HAS diet for 14 days. The C diet was composed of 250 g/kg casein, 482.5 g/kg cornstarch, 100 g/kg sucrose, 70 g/kg soybean oil, 35 g/kg AIN-93G mineral mix [18], 10 g/kg AIN-93 vitamin mix [18], 2.5 g/kg choline bitartrate and 50 g/kg cellulose. On day 10, net  $\text{H}_2$  excretion was measured, and urine was collected for 2 days. On day 12, one C group and one HAS group underwent IR treatment. The remaining C and HAS groups were sham operated. Furthermore, urine was collected for 2 days after IR treatment and sham operation. Under pentobarbital sodium anesthesia (70 mg/kg ip), the hepatic artery and portal vein to the left lateral and median lobes were occluded to interrupt blood supply to the liver for 30 min, and then reperfusion was initiated at  $37^\circ\text{C}$ . Rats were euthanized at 48 hr after reperfusion for sampling. Sham-operated rats were prepared in a similar manner except without vascular occlusion. One-milliliter samples of blood from the portal vein were successively collected into sealed heparin vials and microtubes for  $\text{H}_2$  analysis and plasma preparation. A 1 ml sample of the gaseous phase was withdrawn using a gas-tight syringe, and the  $\text{H}_2$  concentration was determined using GC (Biogas analyzer BAS-1000). The remaining blood was separated by centrifugation ( $1,200 \times g$  for 20 minutes at  $4^\circ\text{C}$ ), and plasma samples were stored at  $-80^\circ\text{C}$ . The liver was perfused immediately after blood withdrawal with cold saline. Immediately after perfusion, the median lobe (ischemic area) was removed, and a portion was

rapidly frozen in liquid nitrogen; the samples were then stored at  $-80^\circ\text{C}$  until oxidative stress analyses. Activities of alanine and aspartate aminotransferase (ALT and AST) in plasma were measured using a commercial kit (Transaminase CII-test Wako, Wako, Tokyo, Japan). Reduced hepatic glutathione (GSH) and oxidized glutathione (GSSG) levels were determined using the method of Anderson [19]. The malondialdehyde (MDA) concentration in the liver was measured according to the procedure of Ohkawa et al. [20]. Hepatic activity of superoxide dismutase (SOD) and myeloperoxidase (MPO) was determined by the method of Sun et al. [21] and Krawisz et al. [22], respectively. Urine 8-OHdG content was measured using a commercial ELISA kit (Highly Sensitive 8-OHdG Check ELISA; Nikken SEIL Co., Ltd, Fukuroi, Japan). Allantoin and creatinine excretion in urine were determined by the methods of Young and Conway [23] and Bonsnes and Taussky [24], respectively. Values obtained from the experiments were expressed as means  $\pm$  SEM. Data were subjected to Bartlett's test for homogeneity of variances. For samples with equal variances, one-way ANOVA was used, followed by the Tukey-Kramer post hoc test for multiple comparisons between individual group means. If sample variances were unequal, we used the Steel-Dwass test [plasma ALT and AST activities, net  $\text{H}_2$  excretion and portal  $\text{H}_2$  concentration]. Furthermore, to examine the possible role of cecal  $\text{H}_2$  in alleviating oxidative stress, we used the Mann-Whitney U test using only IR rat data for plasma ALT and AST activity analyses. The Tukey-Kramer test and Mann-Whitney U test were performed using the SAS JMP software (version 8.0.1; Tokyo, Japan), and the Steel-Dwass test was performed using KyPlot (version 5.0; KyensLab Inc, Tokyo, Japan). Significance was defined as a  $p < 0.05$ .

In the first 12 days of the experimental period, body weight gain and food intake did not differ among the groups (Table 1). However, body weight gain and food intake from days 13–14 of the experimental period were lower in the IR rats compared with the sham-operated rats. Net  $\text{H}_2$  excretion on day 10 and portal  $\text{H}_2$  concentration on day 14 were about 10-fold and 5-fold higher in rats from the HAS group compared with those in the C group, respectively. Plasma ALT and AST activities were significantly higher in the IR-C group compared with the sham group. Plasma ALT and AST activities in the IR-HAS group tended to decrease, and a significant reduction was observed in the IR-HAS group compared with the IR-C group ( $p = 0.0725$  and  $0.0167$ ), respectively. Urinary excretion of 8-OHdG corrected with creatinine on days 11–12 and days 13–14 did not

Table 1. Effect of high amylose cornstarch on body weight gain, food intake and net H<sub>2</sub> excretion in IR-treated and sham-operated rats

	Sham		IR		Two-way ANOVA		
	C	HAS	C	HAS	Diet	Treatment	Interaction
Body weight gain							
Start-day 12 (g/12 days)	71 ± 3	72 ± 3	71 ± 3	70 ± 2	0.9606	0.6134	0.7514
Days 13–14 (g/2 days)	3.7 ± 1.1	3.9 ± 0.5	-3.3 ± 1.4	-3.1 ± 1.8	0.8598	<0.0001	0.9961
Food intake							
Start-day 12 (g/12 days)	272 ± 4	270 ± 5	275 ± 8	274 ± 6	0.7942	0.6065	0.9485
Days 13–14 (g/2 days)	32 ± 1	33 ± 1	27 ± 1	28 ± 1	0.2106	<0.0001	0.7163
Net H <sub>2</sub> excretion (μmol/5 min)							
Start	0.327 ± 0.153	0.469 ± 0.180	0.450 ± 0.174	0.345 ± 0.152	0.9121	0.9936	0.4594
After 10 days	0.130 <sup>a</sup> (0.065–0.420)	0.823 <sup>b</sup> (0.142–0.548)	0.127 <sup>a</sup> (0.055–0.548)	0.603 <sup>b</sup> (0.107–9.88)			

C, control; HAS, high amylose cornstarch; IR, ischemia-reperfusion; H<sub>2</sub>, hydrogen. High H<sub>2</sub> generating rats, which we selected, were used. After 30 min of ischemia, 45 min of reperfusion was performed in IR rats. Values are expressed as means ± SEM or medians (minimum value–maximum value), n=12–13. Data were analyzed with two-way ANOVA and Tukey-Kramer's post hoc test or nonparametric multiple test (Steel-Dwass test). Mean values within a row with different superscript letters are significantly different (p < 0.05).

Table 2. Effect of high amylose cornstarch on H<sub>2</sub> concentration and aminotransferase activities in the portal vein, and urinary 8-OHdG and allantoin concentrations in IR-treated and sham-operated rats

	Sham		IR		Two-way ANOVA		
	C	HAS	C	HAS	Diet	Treatment	Interaction
Portal							
H <sub>2</sub> (μmol/L)	0.780 <sup>ab</sup> (0.197–5.70)	3.16 <sup>b</sup> (0.374–17.7)	0.353 <sup>a</sup> (0.087–2.35)	2.57 <sup>b</sup> (0.345–16.8)			
Plasma ALT (μkat/L)	0.159 <sup>a</sup> (0.082–0.352)	0.175 <sup>a</sup> (0.090–0.243)	0.384 <sup>b</sup> (0.096–2.68)	0.229 <sup>ab</sup> (0.046–0.992)			
Plasma AST (μkat/L)	1.46 <sup>ab</sup> (1.14–2.43)	1.50 <sup>a</sup> (1.32–1.82)	1.84 <sup>b</sup> (1.45–9.10)	1.39 <sup>ab,*</sup> (0.95–4.96)			
Urine							
8-OHdG (μmol/mol creatinine)							
Days 10–12	8.02 ± 1.18	10.4 ± 2.4	8.71 ± 1.35	12.5 ± 2.6	0.1218	0.4775	0.7190
Days 12–14	11.2 ± 1.9	9.70 ± 2.30	11.6 ± 2.6	13.8 ± 4.4	0.9075	0.4510	0.5390
Allantoin (mol/mol creatinine)							
Days 10–12	3.56 ± 0.17	3.60 ± 0.17	3.54 ± 0.09	3.60 ± 0.11	0.7223	0.9616	0.9416
Days 12–14	4.47 ± 0.12	4.83 ± 0.24	4.66 ± 0.11	4.85 ± 0.13	0.0943	0.5175	0.5901

ALT, alanine aminotransferase; AST, aspartate aminotransferase; C, control; HAS, high amylose cornstarch; IR, ischemia-reperfusion; H<sub>2</sub>, hydrogen; 8-OHdG, 8-hydroxy-2'-deoxyguanosine. High H<sub>2</sub> generating rats, which we selected, were used. After 30 min of ischemia, 45 min of reperfusion was performed in IR rats. Values are expressed as means ± SEM or medians (minimum value–maximum value), n=12–13. Data were analyzed with two-way ANOVA and Tukey-Kramer's post hoc test or nonparametric multiple test (Steel-Dwass test). Mean values within a row with different superscript letters are significantly different (p < 0.05).

\* p < 0.05 compared with the IR-C group. Values of plasma ALT and AST activities in both IR groups were analyzed by Mann-Whitney's U test in case of the variances that were too different between the sham-operated and IR treated groups.

differ among the groups (Table 2). Urinary excretion of allantoin, which is a metabolite of purine bodies, corrected with creatinine also did not change among the groups. No significant differences in SOD activity, glutathione concentration and MDA concentration were observed. Significant differences were observed in hepatic MPO activity between the groups (Table 3). These parameters are related to oxidative stress and inflammation. Hepatic MPO activity was higher in IR rats compared with sham-operated rats, but no significant effect was observed by HAS feeding.

Plasma ALT and AST activities are used clinically as a marker of hepatic injury. In the present study, low plasma ALT and AST activities in IR rats fed the HAS diet were observed compared with those fed the control

diet. Therefore, it is possible that hepatic injury was alleviated by H<sub>2</sub> molecules generated in the large intestine. H<sub>2</sub> generated in vivo should relieve hepatic IR injury in the subacute phase as well as the initial acute phase. We previously found that pectin and HAS increase cecal H<sub>2</sub> production and relieve initial acute hepatic IR injury in rats [10]. Recently, many studies have reported that H<sub>2</sub> molecules exert an antioxidant effect in vivo. Ohsawa and his collaborator demonstrated the alleviation effect of H<sub>2</sub> gas on IR injury of the brain [3], liver [5] and heart [4]. Furthermore, a similar effect was shown for the myocardium and intestine [25, 26] via administration of H<sub>2</sub>-rich saline. In the liver, IR injury has been demonstrated to occur in a biphasic pattern: an initial acute phase characterized by hepatocellular

Table 3. Effect of high amylose cornstarch on hepatic glutathione and MDA levels and SOD and MPO activities in IR-treated and sham-operated rats

	Sham		IR		Two-way ANOVA		
	C	HAS	C	HAS	Diet	Treatment	Interaction
<b>Glutathione</b>							
Total ( $\mu\text{mol/g}$ tissue)	9.78 $\pm$ 0.19	10.0 $\pm$ 0.3	9.96 $\pm$ 0.30	10.5 $\pm$ 0.3	0.1664	0.2441	0.6182
GSH ( $\mu\text{mol/g}$ tissue)	9.45 $\pm$ 0.20	9.62 $\pm$ 0.25	9.64 $\pm$ 0.31	10.1 $\pm$ 0.3	0.2217	0.1893	0.5356
GSSG ( $\mu\text{mol/g}$ tissue)	0.326 $\pm$ 0.027	0.405 $\pm$ 0.047	0.318 $\pm$ 0.026	0.336 $\pm$ 0.036	0.1701	0.2749	0.3756
GSH/GSSG	30.9 $\pm$ 2.4	26.7 $\pm$ 2.4	32.2 $\pm$ 2.3	32.6 $\pm$ 2.3	0.4174	0.1306	0.3396
<b>SOD activity</b>							
Total (U/g tissue)	8660 $\pm$ 340	7160 $\pm$ 820	8400 $\pm$ 840	7350 $\pm$ 670	0.0752	0.9575	0.7488
Mn type (U/g tissue)	417 $\pm$ 66	262 $\pm$ 38	370 $\pm$ 120	279 $\pm$ 38	0.8435	0.1124	0.6759
Mn type/Total	0.0488 $\pm$ 0.0080	0.0378 $\pm$ 0.0046	0.0464 $\pm$ 0.0153	0.0384 $\pm$ 0.0047	0.3281	0.9213	0.8775
MDA (nmol/g tissue)	135 $\pm$ 11	152 $\pm$ 9	148 $\pm$ 8	151 $\pm$ 10	0.3219	0.5575	0.4786
MPO (U/g tissue)	0.0247 $\pm$ 0.0042	0.0326 $\pm$ 0.0069	0.112 $\pm$ 0.064	0.153 $\pm$ 0.048	0.5625	0.0167	0.6954

C, control; HAS, high amylose cornstarch; IR, ischemia-reperfusion; GSH, reduced glutathione; GSSG, oxidized glutathione; SOD, superoxide dismutase; MDA, malondialdehyde; MPO, myeloperoxidase. High  $\text{H}_2$  generating rats, which we selected, were used. After 30 min of ischemia, 45 min of reperfusion was performed in IR rats. Values are expressed as means  $\pm$  SEM,  $n=12-13$ . Data were analyzed with two-way ANOVA. Total glutathione and GSSG concentrations were expressed as GSH equivalent.

damage at 3–6 hr and a subacute phase characterized by massive neutrophil infiltration at 18–24 hr, [12, 13], which is similar to the conditions used in our present experiment. However, the effect of  $\text{H}_2$  molecules, including  $\text{H}_2$  generated by large intestinal fermentation on oxidative stress in a subacute phase, remains poorly understood. Our present study is the first to demonstrate the suppressive effect of  $\text{H}_2$  on hepatic IR injury in the subacute phase.

Plasma ALT and AST activities after 48 hr of reperfusion in the present study were lower than after 24 h of reperfusion in our previous study [10]. This may indicate that the liver had recovered from injury due to relieved oxidative stress during the 24–48 hr period of reperfusion. The production of reactive oxygen species by neutrophils exacerbates inflammation [27]. Hepatic MPO activity, which indicates the presence of neutrophils, was higher after 48 hr of reperfusion due to the IR treatment. This result was consistent with the report of Kawaguchi et al., in which the hepatic MPO content after 48 hr of reperfusion was shown to be high due to IR treatment [28]. However, in the present study, HAS feeding had no significant effect on hepatic MPO activity. Because  $\text{H}_2$  exerts an antioxidant effect in the initial acute phase, the effect of  $\text{H}_2$  generated from HAS in the subacute phase may be dependent on low inflammation in the initial acute phase. That is, the migration and infiltration of neutrophils may occur less frequently during low inflammation in the initial acute phase. A few studies have reported the relationship between large intestinal fermentation and liver MPO activity; however, further study, including histological evaluation, is required to evaluate the effect of  $\text{H}_2$

derived from dietary fiber and HAS in the subacute phase on recruitment of neutrophils. Also, oxidative stress markers (hepatic glutathione, MDA concentration and activities of SOD) were not affected by HAS feeding. When rats were subjected to IR treatment (reperfusion time, 15 min–24 hr), the oxidative stress markers were altered, as an oxidative status was caused. This discrepancy is likely to be caused by the different reperfusion times. HAS might have no effect on these markers in this study because the rats had enough time to recover from oxidative stress. In this study, we also examined whether HAS feeding inhibited urinary excretion of 8-OHdG, which is an oxidation product of guanine, in IR rats. Increased urinary excretion of 8-OHdG is observed due to oxidative damage of DNA, caused by various factors such as brain IR [15], diabetes [16, 17] and atherosclerosis [17]. Unfortunately, no change in the urinary oxidation product could be detected as a result of IR and HAS treatment in the present study. We used urine collected during the 0–48 hr period of reperfusion, and it included urine samples collected during the 0–24 hr period of reperfusion. Urine collected between 0–24 h may exhibit a change in 8-OHdG excretion due to IR treatment and HAS.

In conclusion, we found that high  $\text{H}_2$  generating HAS alleviated subacute hepatic IR injury as well as initial acute IR injury. However, various substances such as short-chain fatty acids as well as  $\text{H}_2$  are produced by fermentation in the large intestine. Therefore, further study is required to evaluate the effect of  $\text{H}_2$  derived from dietary fiber and RS in the large intestine on the subacute phase.

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