**MAJOR PAPER**

**Protective Effect of Hydrogen-rich Water against Gentamicin-induced Nephrotoxicity in Rats using Blood Oxygenation Level-dependent MR Imaging**

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Purpose: We assessed intrarenal oxygenation in gentamicin-induced nephrotoxicity (GIN) and the protective effect of hydrogen-rich water (HW) against GIN using blood oxygenation level-dependent magnetic resonance (MR) imaging.

Materials and Methods: We acquired T2*-weighted images (T2*WI) of 21 rats on Days 0, 2, 4, and 7 using a 1.5-tesla MR imaging system. The rats were divided into 3 groups of seven each: control rats had free access to standard water and no gentamicin (GM) injection; rats designated the GM group had free access to standard water and were injected with GM (80 mg/kg/day) subcutaneously for 7 days; and the third group, designated the GM + HW group, had free access to HW and were injected with GM. R2* (= 1/T2*) was estimated from T2*WI.

Results: R2* values in the cortex were significantly decreased on Days 2, 4, and 7 compared with those on Day 0 in the GM group but not significantly changed in the control and GM + HW groups. R2* values in the medulla did not change significantly in any group.

Conclusions: Our findings suggested reduced oxygen utility, mainly in the cortex, in gentamicin-induced nephrotoxicity and an ameliorative effect of hydrogen-rich water against GIN.

Keywords: blood oxygenation level-dependent MRI, gentamicin-induced nephrotoxicity, hydrogen-rich water, oxygen consumption, rat

**Introduction**

Gentamicin (GM) is an important aminoglycoside antibiotic widely used to treat life-threatening gram-negative bacterial infections,1,2 but its clinical usefulness is limited by the associated risk of development of nephrotoxicity, characterized by direct tubular necrosis.2,3

Oxidative stress can result from increased generation of reactive oxygen species (ROS), depression of antioxidant systems, or both.4 Particularly, hydroxyl radical is highly reactive and a strong mediator of tissue injury.2,4 Although the exact mechanism of GM-induced nephrotoxicity is unclear, ROS are considered important mediators.2,3,5–11

Recent studies have shown molecular hydrogen as an efficient antioxidant, owing to its ability to diffuse rapidly into tissues and cells across membranes.12 It has also been reported that water dissolving molecular hydrogen (hydrogen-rich water [HW]) improved metamorphosis accompanying decreased apoptosis in the kidney and nephrotoxicity induced by cisplatin, a widely used drug for cancer therapy.13 Using dynamic contrast-enhanced computed tomography, Kitamura and colleagues reported the protective effect of HW against cisplatin-induced nephrotoxicity in rats.14 Therefore, we hypothesized that hydrogen-rich water might be useful to alleviate GM-induced nephrotoxicity.

Blood oxygenation level-dependent magnetic resonance imaging (BOLD MRI) provides images of renal function that are useful for evaluating in-
trarenal oxygenation, an important factor responsible for the onset and progression of renal failure. To the best of our knowledge, no reports have investigated intrarenal oxygenation in GM-induced nephrotoxicity and the protective effect of HW against this nephrotoxicity, so we undertook this investigation in rats using BOLD MRI.

**Materials and Methods**

**Animals**

We used 21 8-week-old male Sprague-Dawley rats weighing 284±23 g (mean±standard deviation [SD]) purchased from Charles River Japan (Yokohama, Japan) and housed in clean plastic cages in a temperature- and humidity-controlled facility with a constant 12-hour light/dark cycle. The animal ethics committee of Osaka University School of Medicine approved animal use and the experimental protocol.

**Hydrogen-rich water**

We purchased hydrogen-rich water sealed in a 200-mL aluminum pouch from I’rom Pharmaceutical Co., Ltd. (Tokyo, Japan). The concentration of dissolved hydrogen was 1.2±0.1 mg/L (mean±SD) in HW and 0.06±0.01 mg/L in standard water, as measured using a dissolved hydrogen analyzer (DH-35A, DKK-TOA Co., Tokyo, Japan). For feeding, HW from the aluminum pouch was placed into a closed glass vessel equipped with a metal tube containing 2 ball bearings to minimize hydrogen loss during feeding (Fig. 1). Twice a day, we freshly prepared the vessels containing hydrogen-rich water given to the rats.

**Animal experimental protocol**

We divided the rats into 3 groups of seven each. A control group (body weight [BW], 304±25 g, mean±SD) had free access to standard laboratory food and water and no GM injection; a second GM group (BW, 271±9 g) had free access to standard laboratory food and water and were subcutaneously injected with GM (80 mg/kg BW/day) for 7 consecutive days; and a third GM plus hydrogen-rich water (GM+HW) group (BW, 281±18 g) had free access to standard laboratory food and HW and were subcutaneously injected with GM (80 mg/kg BW/day) for 7 consecutive days. GM was purchased from Nichi-Iko Pharmaceutical Co., Ltd. (Toyama, Japan).

We performed MR imaging studies on Days 0, 2, 4, and 7 after rats were anesthetized by intraperitoneal injection of chloral hydrate solution (4%, 400 mg/kg BW; Sigma Aldrich, St. Louis, MO, USA). At the end of experiments on Day 7, blood samples were taken by cardiac puncture and used to determine levels of serum creatinine (Cr) and blood urea nitrogen (BUN). The blood samples were tested by SRL Inc. (Tokyo, Japan).

**MR imaging protocol**

MR imaging studies were performed with a 1.5-tesla system for animal experiments (MRmini, DS Pharma Biomedical Co., Ltd., Osaka, Japan), in which a solenoid coil of 38.5-mm diameter was used as the radiofrequency coil. To avoid movement and artifacts during experiments, we immobilized the left kidney with a plastic holder of our own manufacture.

We used a spin-echo pulse sequence to obtain transverse relaxation time (T2)-weighted images (T2WI) as anatomical images: repetition time (TR), 2000 ms; echo time (TE), 90 ms; excitation pulse flip angle (FA), 90°; field of view (FOV), 63×31.5 mm2; matrix size, 256×128; 4 slices of 3.2-mm thickness and without slice gap; number of excitations (NEX), 2; and total scan time, 512 s. After obtaining T2WI, we used a single gradient-echo pulse sequence to acquire apparent transverse relaxation time (T2*)-weighted images (T2*WI): TR, 75 ms; TEs, 6.5, 10, 20, and 30 ms; FA, 36°; FOV, 63×31.5 mm2; matrix size, 256×128; 4 slices with 3.2-mm thickness and without slice gap; NEX, 6; and total scan time, 230.4 s.

**Histopathological observation**

After completion of MR imaging studies on Day 7, we sacrificed rats by overdose administration of chloral hydrate solution.
anesthetic agent and removed their kidneys for histopathological observation. The resected kidney was sectioned in blocks, fixed in 20% formalin, dehydrated in graded concentrations of alcohol, and embedded in paraffin. The kidney block was cut into 2-μm sections and stained with periodic acid Schiff (PAS) reagents to demonstrate polysaccharides, neutral mucopolysaccharides, and glycoproteins from epithelial tubular membranes. Slices were incubated with periodic acid for 10 min, washed with distilled water, incubated with Schiff’s reagent for 20 min, and counterstained with hematoxylin for 3 min. We used light microscopy for histopathological observation.

Data analysis

We generated maps of apparent transverse relaxation rate ($R_2^*$) by fitting a regression line to the signal intensity of $T_2^*$WI transformed into logarithm versus TE on a pixel-by-pixel basis. We used $T_2$WI as a reference to place regions of interest (ROIs) in the renal cortex and medulla on the $R_2^*$ map. Figure 2 shows a typical example of the ROIs drawn on $T_2$WI. Unless specifically stated, the term “medulla” means the outer stripe of the outer medulla in this study. To correct for variation in MR imaging sensitivity, we also imaged a water-filled phantom placed near rats as an external standard substance and used it to normalize the signal intensities of $T_2$WI in rats.

Statistical analysis

Data were represented as mean ± standard error (SE); differences among groups were analyzed by one-way analysis of variance (ANOVA); and statistical significance was determined by Tukey’s multiple comparison test. $P<0.05$ was considered statistically significant.

Results

BOLD MRI study

We performed BOLD MRI studies to obtain $R_2^*$ values on Days 0, 2, and 7. Figure 3 shows typical examples of $R_2^*$ maps superimposed on corresponding $T_2^*$WI in all groups. $R_2^*$ values did not change significantly in the medulla compared with those on Day 0 in all groups, but they decreased significantly in the cortex compared with those on Day 0 in the GM group (Fig. 4).

Normalized $R_2^*$ values in the cortex differed significantly on Day 2 among the control, GM, and GM + HW groups (Fig. 5a) and on Day 7 between the control and GM groups (Fig. 5a). Normalized $R_2^*$ values in the medulla did not differ significantly among the control, GM, and GM + HW groups (Fig. 5b).

Signal intensity of $T_2$WI

Figure 6 shows typical examples of $T_2$WI on Days 0, 2, 4, and 7 in all groups. Figure 7 shows the signal intensities of $T_2$WI in the cortex and medulla as a function of days after first GM injection in the control (a), GM (b), and GM + HW (c) groups. They were normalized by the signal intensity of the water-filled phantom placed near rats as an external standard substance to correct for variation in MR imaging sensitivity.

The signal intensities of the phantom normalized by that on Day 0 were $0.987 ± 0.200$ (mean ± SD) on Day 2; $0.929 ± 0.259$ on Day 4; and $1.03 ± 0.264$ on Day 7. The signal intensity of $T_2$WI in both the control and GM groups did not differ significantly in the cortex and medulla on Days 2, 4, and 7 compared with Day 0 but increased significantly in the cortex on Day 7 compared with Day 0 in the GM + HW group (Fig. 7).

Blood test

Levels of Cr and BUN were significantly higher in the GM than control group (Fig. 8a, 8b), indicating that GM administration reduced renal function in the GM group. The Cr level tended to be lower in the GM + HW group than the GM group but did not differ significantly (Fig. 8a). The BUN level did not differ significantly between the GM and GM + HW groups (Fig. 8b).

Histopathological observation

Histopathological examination of the kidney

Fig. 2. Example of regions of interest drawn in the cortex (solid line) and medulla (dotted line) on the transverse relaxation time ($T_2$)-weighted image ($T_2$WI).
Fig. 3. Typical examples of apparent transverse relaxation rate ($R_2^*$) maps on Days 0, 2, 4, and 7 in control (a), gentamicin (GM) (b), and GM + hydrogen-rich water (HW) groups (c). The $R_2^*$ maps in the kidney are displayed in color and superimposed on the apparent $T_2$ ($T_2^*$)-weighted images ($T_2^*$WI) on the same day.

Fig. 4. Apparent transverse relaxation rate ($R_2^*$) values in the cortex (open circles) and medulla (closed squares) as a function of days after first gentamicin (GM) injection in control (a), GM (b), and GM + hydrogen-rich water (HW) groups (c). Bars represent mean ± standard error (SE). *$P < 0.05$ compared with $R_2^*$ value on Day 0.

showed some tubular dilatation and desquamation of the tubular epithelium in the GM group (Fig. 9b), which was less severe in the GM + HW group (Fig. 9c).

Discussion

In this study, we quantitatively evaluated intrarenal oxygenation in GM-induced nephrotoxicity and the effect of hydrogen-rich water on this nephrotoxicity in rats by measuring $R_2^*$ value in the kidney using BOLD MRI and the signal intensity of $T_2$WI. Our results suggested an ameliorative effect of HW against GM-induced nephrotoxicity.

Recently, BOLD MRI has been utilized to assess intrarenal oxygenation in humans and experimental models of kidney disease.15–19 As previously described, BOLD MRI can provide images of renal function with high spatial resolution without the use of ionizing radiation and injection of contrast agents that may aggravate acute renal failure.21 Furthermore, BOLD MRI allows repeated measurements in a single animal and simultaneous evaluation of individual renal morphology and...
function. Therefore, this method appears useful for evaluating the time courses of intrarenal oxygenation as we have done.

GM is known to cause renal cortical mitochondria to release iron and enhances the generation of superoxide anion and hydrogen peroxide in them. The interaction of the anion and peroxide in the presence of a metal catalyst leads to the generation of toxic hydroxyl radicals that oxidize membrane lipids or bind to macromolecules, and tissue injury results. Fukuda and associates reported that 7-day treatment with GM had no effect on Na⁺–K⁺-
Fig. 7. Signal intensities of transverse relaxation time ($T_2$)-weighted image ($T_2$WI) in the cortex (open circles) and medulla (closed squares) as a function of days after the first gentamicin (GM) injection in the control (a), GM (b), and GM + hydrogen-rich water (HW) groups (c). Bars represent mean ± standard error (SE). *$P<0.05$ compared with the signal intensity of $T_2$WI on Day 0.

Fig. 8. Serum creatinine (a) and blood urea nitrogen levels (b) in the control, gentamicin (GM), and GM + hydrogen-rich water (HW) groups on Day 7. Error bars represent standard error (SE). *$P<0.05$.

Fig. 9. Light micrographs of renal cortices from the control (a), gentamicin (GM) (b), and GM + hydrogen-rich water (HW) groups (c) (periodic acid Schiff [PAS] stain × 200). Calibration bar (100 μm) is also shown. Note that tubular dilatation with desquamation of tubular epithelium was observed in the GM group (arrows).

adenosine triphosphatase (ATPase) in microdissected medullary thick ascending limbs but markedly reduced enzyme activity in the proximal tubule cells. Therefore, the renal cortex appears to be the site of the active effect of GM and an important target in the kidney for assessing GM-induced nephrotoxicity. Our results shown in Figs. 4 and 5 demonstrated that though the $R_{2}^*$ value in the
medulla did not significantly change in the GM group, that in the cortex was significantly decreased after GM administration, which suggested reduced oxygen utility in the cortex from GM-induced nephrotoxicity and the greater susceptibility of the cortex than the medulla to GM.

As Fig. 5a shows, R2* values varied widely in the control group. Because we allowed the rats free access to tap water before experiments, their individual states of hydration may have differed widely. The large variation observed in the control group (Fig. 5a) may be due to their various states of hydration and/or the heterogeneity of R2* in the background.

Jeong and colleagues reported that human patients with renal cortical necrosis showed higher signal intensity on T2WI than in the normal cortex and explained that delayed filtration in the glomeruli and congestion of the renal cortex increase cortical intensity.22 As Fig. 7b shows, signal intensity on T2WI in the cortex in the GM group tended to be higher on Day 7 than Day 0, though not statistically different. In light of Jeong’s findings,22 our results (Fig. 7b) may suggest that GM-induced nephrotoxicity produced some necroses in the cortex on Day 7, which is also confirmed by the histopathological data shown in Fig. 9b. It should be noted that the signal intensity of T2WI in the cortex in the GM+HW group was significantly increased on Day 7 compared to that on Day 0 (Fig. 7c), although the histopathological data showed less severe extent of necrosis than that in the GM group (Fig. 9c). Thus, this increase may be due to the variation in the state of hydration rather than the cortical necrosis. As previously described, the change of R2* reflects tissue oxygen consumption,15 whereas the increase in signal intensity of T2WI in the cortex reflects some cortical necrosis.22 These findings suggest the potential use of BOLD MRI combined with T2WI for evaluating both functional and morphological changes in the kidney caused by GM-induced nephrotoxicity. However, further studies are needed to establish the usefulness of this method in clinical settings.

It is well known that ROS, such as superoxide anion and hydrogen peroxide, react with polyunsaturated fatty acids and cause tissue damage,24 but they also act as signaling molecules and regulate apoptosis, cell proliferation, and differentiation.25 Thus, excessive scavenging of these ROS by antioxidants will induce unexpected side effects. For example, Ben Ismail and associates5 reported that ascorbic acid acts as an antioxidant at moderate dose and as a prooxidant at higher dose. A wide variety of antioxidants have been reported to reveal protective effects against GM-induced nephrotoxicity,2,3,5-11 but they generally require high doses to exhibit a significant protective effect, which may cause various side effects. On the other hand, Ohsawa’s group12 found that molecular hydrogen selectively reduced hydroxyl radicals, one of the most cytotoxic ROS. Furthermore, hydrogen did not react with other ROS, such as superoxide anion and hydrogen peroxide, which possess physiological roles as mentioned above. Thus, hydrogen or HW appears to be much safer and easier to apply than other antioxidants. Excess hydrogen taken in would be expired via the lungs.13 Therefore, hydrogen-rich water has great potential for clinical use.

Association of GM-induced nephrotoxicity with marked increase in levels of Cr and BUN has been reported,7,26 and we observed significant increases of both Cr and BUN levels in the GM group compared with the control group (Fig. 8). On the other hand, the Cr level tended to be lower in the GM+HW group than the GM group, though not statistically different (Fig. 8a), and the BUN level was significantly lower in the GM+HW group than the GM group (Fig. 8b). These results suggest that hydrogen-rich water ameliorates renal dysfunction caused by GM-induced nephrotoxicity. As previously described, although R2* values in the cortex were significantly decreased on Days 2, 4, and 7 compared with Day 0 in the GM group (Fig. 4b), they did not significantly change in the GM+HW group (Fig. 4c). These results also appear to suggest that HW has an ameliorative effect against GM-induced nephrotoxicity.

As previously described, the study dose of GM was 80 mg/kg/day, much higher than the 4 mg/kg/day used in clinical settings.27 In addition, rats were given hydrogen as drinking water ad libitum in this study. Further studies using different doses of GM and/or administration routes of hydrogen-rich water, such as intravenous or intraperitoneal injection, will be needed to elucidate the protective effect of HW against GM-induced nephrotoxicity and to establish its usefulness. We believe that BOLD MRI will play an important role in such studies.

Conclusion

This study suggested reduced oxygen utility, mainly in the cortex, in GM-induced nephrotoxicity and an ameliorative effect of hydrogen-rich water against this nephrotoxicity in rats.
References