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Relationship between body iron stores and diquat toxicity in male Fischer-344 rats

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Abstract

The effects of body iron stores on diquat (DQ)-induced toxicity were examined in male Fischer-344 rats, which are sensitive to this herbicide. The rats (5 weeks old) were fed diets containing 40 (lower iron storage [LIS] group) or 320 ppm iron (higher iron storage [HIS] group) for 5 weeks. The concentrations of non-heme iron and ferritin in the liver and kidney were significantly higher in the HIS group than in the LIS group ($P < 0.0001$), although there was no significant differences between the HIS and LIS groups in hematological parameters, including red blood cell count, hemoglobin concentration, and mean corpuscular volume. Three hours after administration of 0.1 mmol DQ/kg, serum alanine aminotransferase and urea nitrogen were significantly higher than in controls (saline injection) for both the LIS and HIS groups ($P < 0.01$), and, after DQ injection, these parameters were significantly higher in the HIS group than in the LIS group ($P < 0.01$). When the rats were injected with 0.075 or 0.1 mmol DQ/kg, the survival time was significantly shorter in the HIS group than in the LIS group ($P < 0.05$). These findings suggest that higher body iron stores result in more severe DQ toxicity in Fischer-344 rats.

Introduction

Diquat (DQ) is a bipyridyl herbicide that is known to induce hepatotoxicity and nephrotoxicity *in vivo* (Smith *et al.* 1985; Petry *et al.* 1992). DQ is reduced by NADPH-cytochrome P-450 reductase. The DQ radical formed transfers an electron to molecular oxygen to generate a superoxide anion radical (Thomas & Aust 1986; Jones & Vale 2000). Both the DQ radical and superoxide can reductively release iron from ferritin. The resulting free ferrous ion catalyzes the Fenton reaction to generate the even more potent hydroxyl radical, which can cause DNA strand breaks, inactivate enzymes, and initiate lipid peroxidation (Thomas & Aust 1986; Reif *et al.* 1988; McCord 1996; Jones & Vale 2000). Because DQ toxicity is thought to be due to iron-mediated oxidative stress, variations in the levels of stored iron in the body could affect the response to this chemical.

Rikans and Cai (1992, 1993) found an age-dependent increase in the sensitivity to DQ-induced cytotoxicity in rats and it was attributed to an age-associated increase in the content of liver ferritin (Rikans *et al.* 1997). Because aging seems to contribute anti-oxidant protection activity in the animals (Rikan *et al.* 1991), it is not clear whether DQ toxicity is directly related to the body iron status. Therefore, in the present study, we examined the effects of body iron stores on DQ-induced hepatotoxicity and nephrotoxicity as well as on survival after DQ administration in

male Fischer-344 rats, which are sensitive to this herbicide (Smith *et al*. 1985).

Materials and methods

Animals and treatments

These studies used 5-week-old male Fischer-344 rats (Clea Japan, Tokyo, Japan). Low iron powder diet No.A12501 (22% milk casein, 61% cornstarch, 5% crystalline cellulose, 4% purified soybean oil, 1% Vitamin Mix, and 7% Mineral Mix without iron) was obtained from Clea Japan. This diet contained 4–5 ppm iron. Ferric citrate (Kanto Chemical Co., Tokyo) was added to the diet to give total iron contents 40 and 320 ppm. The rats were split into lower (LIS) and higher iron storage (HIS) groups and were fed for 5 weeks the 40- and 320-ppm diets, respectively. Deionized distilled water was given to the rats in plastic bottles.

DQ dibromide was obtained from Labor Dr. Ehrenstorfer-Schafers (Augsburg, Germany). The rats were injected subcutaneously with DQ (0.05, 0.075, or 0.1 mmol/kg body weight) in 0.9% NaCl. Control rats were injected with an equivalent volume of 0.9% NaCl. Three hours after DQ administration, rats were anesthetized with pentobarbital, and blood samples were collected by cardiac puncture. The rats were then sacrificed, and livers and kidneys were removed. A portion of blood was heparinized for hematological analyses, and the remaining blood was coagulated to isolate serum. The serum samples and tissues were stored at −25 °C until use.

Biochemical analyses

Each rat liver or kidney was homogenized in a Waring blender for 3 min with 9 volumes of 10 mM Tris containing 0.2 mM Pefabloc SC (Merck, Darmstadt, Germany) as a serine proteinase inhibitor. The homogenates were divided into two parts: one part was used for measuring nonheme iron; the second was centrifuged at 24,000 *g* for 20 min at 4 °C, after which the supernatant (tissue extract) was used for measuring ferritin protein. Total nonheme iron in tissue homogenates was measured according to the method of Bomford *et al*. (1981) except that ferrozine (Stookey 1970) was used as an indicator instead of bathophenanthroline. Ferritin in rat tissue extracts and sera was determined using a sandwich enzyme-linked immunosorbent assay as described previously (Watanabe *et al*. 2000). Serum alanine aminotransferase (ALT) and urea nitrogen (UN) were analyzed with an OLYMPUS AU400 Autoanalyzer (Olympus, Tokyo, Japan).

Statistical analyses

Correlation coefficients were determined by simple linear regression. Data from studies with only two groups were analyzed using the Student's *t*-test. The significance of differences in DQ-induced hepatotoxicity and nephrotoxicity was analyzed by two-way ANOVA followed by Tukey's test.

Results

Iron status in the liver and kidney and hematological parameters in the LIS and HIS groups

Table 1 shows ferritin and nonheme iron concentrations in the liver and kidney of the LIS and HIS groups, which were fed diets containing 40 and 320 ppm iron, respectively. Nonheme iron concentrations in the liver and kidney of the HIS group were 2.3- and 1.3-fold higher, respectively, than those in the LIS group. Ferritin concentrations in

Table 1. Comparison of nonheme iron and ferritin concentrations in the liver and kidney between LIS and HIS groups.

Parameters	LIS ($n=6$)	HIS ($n=6$)
Liver nonheme iron (μg/g wet weight)	14.5 ± 2.0	32.9 ± 4.4[a]
Liver ferritin (μg/g wet weight)	15.5 ± 6.9	82.0 ± 13.9[a]
Kidney nonheme iron (μg/g wet weight)	19.7 ± 1.2	25.3 ± 1.5[a]
Kidney ferritin (μg/g wet weight)	9.0 ± 3.1	30.8 ± 2.2[a]

Values are expressed as means ± SD.[a]Significantly different from the values of LIS group ($P < 0.0001$).

the liver and kidney of the HIS group were 5.3- and 3.4-fold higher, respectively, than those in the LIS group. As shown in Figure 1, there were very strong positive correlations between nonheme iron and ferritin concentrations in the liver ($R = 0.995$, $P < 0.0001$) and the kidney ($R = 0.956$, $P < 0.0001$). Table 2 shows that there were no significant differences in the white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), or mean corpuscular hemoglobin concentration (MCHC) between the LIS and HIS groups.

Effects of DQ administration on serum ALT and UN in the LIS and HIS groups

In both the LIS and HIS groups, administration of 0.1 but not 0.05 mmol DQ/kg significantly increased serum ALT activities compared with the saline control, and the more severe hepatotoxicity was found in the HIS group than in the LIS group (Figure 2). Serum UN values were significantly elevated after injection of either dose of DQ, and the more DQ injected, the higher the UN values (Figure 3). The more severe nephrotoxicity was found in the HIS group than in the LIS group.

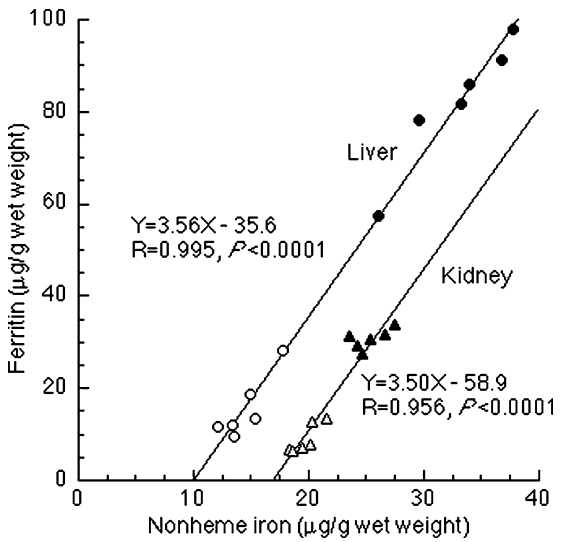

Figure 1. Relationship between nonheme iron and ferritin concentrations in the liver and kidney. Rats were fed a diet containing 40 (open symbols; $n = 6$) or 320 ppm Fe (closed symbols; $n = 6$). Circles and triangles represent liver and kidney, respectively.

Table 2. Comparison of hematological parameters between LIS and HIS groups.

Parameters	LIS ($n = 6$)	HIS ($n = 6$)
WBC ($\times 10^2/\mu l$)	64 ± 15	49 ± 9
RBC ($\times 10^4/\mu l$)	864 ± 38	851 ± 41
HGB (g/dl)	15.7 ± 0.7	15.4 ± 0.5
HCT (%)	46.0 ± 1.8	45.2 ± 1.1
MCV (fl)	53.2 ± 1.1	53.2 ± 3.5
MCH (pg)	18.2 ± 0.4	18.2 ± 0.6
MCHC (%)	34.2 ± 0.3	34.2 ± 1.8

Values are expressed as means ± SD.

Effects of DQ administration on serum ferritin in the LIS and HIS groups

In the LIS group, administration of neither 0.05 nor 0.1 mmol DQ/kg significantly increased serum ferritin concentrations compared with saline (Figure 4). On the contrary, in the HIS group, the serum levels of ferritin were significantly elevated after injection of 0.1 but not 0.05 mmol DQ/kg.

Comparison of survival time between the LIS and HIS groups after DQ administration

Following administration of 0.075 mmol DQ/kg, the mean survival time of the HIS group was significantly shorter than that of the LIS group (3.31 ± 0.44 *vs.* 5.30 ± 1.72 h, respectively; $P < 0.05$; Figure 5A). There was also a significant difference in the survival times between the HIS and LIS groups injected with 0.1 mmol DQ/kg (2.91 ± 0.43 *vs.* 4.30 ± 1.25 h; $P < 0.01$; Figure 5B). None of the control rats injected with saline died or showed abnormal symptoms.

Discussion

Both DQ radical produced by NADPH-cytochrome P-450 reductase and a superoxide anion radical formed by the DQ radical can reductively release iron from ferritin (Thomas & Aust 1986; Reif 1992; Jones & Vale 2000). In fact, Reif *et al.* (1988) have reported that DQ treatment of rats increases levels of hepatic low molecular weight chelatable iron. The increased biliary excretion of nonheme iron observed in Fischer-344 rats after administration of hepatotoxic doses of DQ may

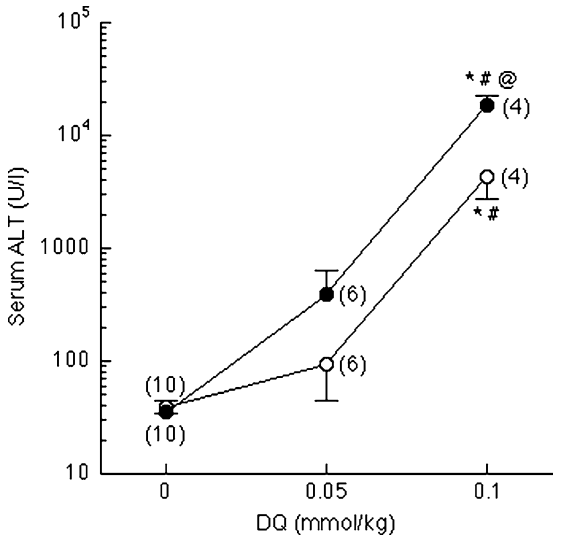

Figure 2. Comparison of serum ALT between the LIS and HIS groups injected with DQ. Rats of the LIS (open circles) and HIS (closed circles) groups were treated with 0 (saline), 0.05, or 0.1 mmol DQ/kg. Data are the means ± SD with the numbers of animals in parentheses. *,# Different from saline- and DQ (0.05 mmol/kg)-treated groups, respectively, fed a diet containing the same iron content, $P < 0.01$. @ Different from the LIS group given the same dose of DQ, $P < 0.01$.

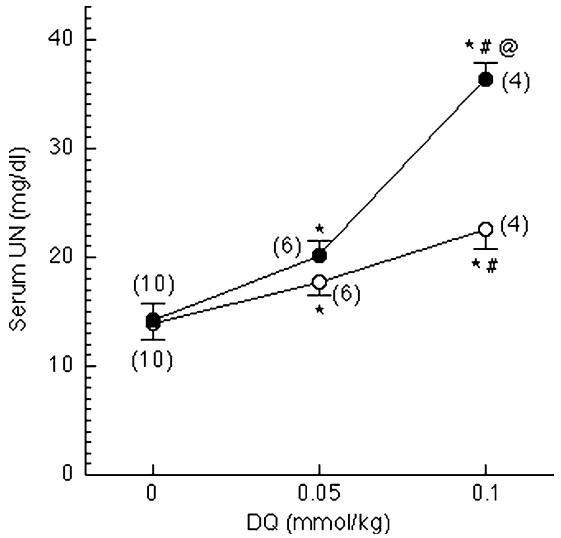

Figure 3. Comparison of serum UN between the LIS and HIS groups injected with DQ. Rats of the LIS (open circles) and HIS (closed circles) groups were treated with 0 (saline), 0.05, or 0.1 mmol DQ/kg. Data are the means ± SD with the numbers of animals in parentheses. *,# Different from saline- and DQ (0.05 mmol/kg)-treated groups, respectively, fed a diet containing the same iron content, $P < 0.01$. @ Different from the LIS group given the same dose of DQ, $P < 0.01$.

reflect the increased intrahepatocellular transit pool of iron deriving from DQ-stimulated release from ferritin (Gupta *et al.* 1994). The redox-active iron in this pool and/or hydroxyl radical formed by Fenton chemistry are involved in the lipid peroxidation and DNA fragmentation, which are associated with DQ-induced hepatic necrosis (Smith *et al.* 1985; Smith 1987; Gupta *et al.* 2000). This is supported by the fact that pretreatments with desferrioxamine and ferrous sulfate attenuated and potentiated the hepatic damage produced by DQ, respectively (Smith 1987). The rate of iron release from ferritin by paraquat, which is the closely related chemical to DQ, was found to be directly proportional to the ferritin concentration used *in vitro* (Thomas & Aust 1986). Therefore, it is reasonable to consider that the higher the tissue ferritin content (or iron stores), the more severe the DQ toxicity *in vivo*.

The American Institute of Nutrition (1977) recommends at least 35 ppm iron in the diet for the normal growth of rats. In the present study, rats in the LIS and HIS groups were fed diets containing 40 and 320 ppm iron, respectively. The rats in the LIS group did not show anemic symptoms, indicating that 40 ppm iron in the diet was sufficient for hemoglobin synthesis. The ferritin levels in the liver and kidney of the HIS group were considerably higher than those of the LIS group, indicating that these two groups had remarkably different body iron stores.

To determine whether variations in the levels of body iron storage could confer differing responses to DQ, we examined the effect of DQ on these two groups. We found increased hepatotoxicity and nephrotoxicity in rats with higher iron stores in the liver and kidney. Furthermore, the survival after DQ administration correlated inversely with body iron content. These results indicate that higher body iron stores result in more severe DQ toxicity in Fischer-344 rats. However, it remains to be determined whether this iron stores-dependent DQ toxicity is paralleled by the differences in the levels of redox-active iron released from ferritin during the redox cycling of DQ.

It is surprising that 100% mortality was observed by 4 h in the HIS group rats injected with 0.1 mmol DQ/kg in the present study, because Smith *et al.* (1985) have reported that animal mortality is very low even 24 h after injection of the same doses of DQ into Fischer-344 rats. We found that the mortality of Fischer rats fed CE-2 standard

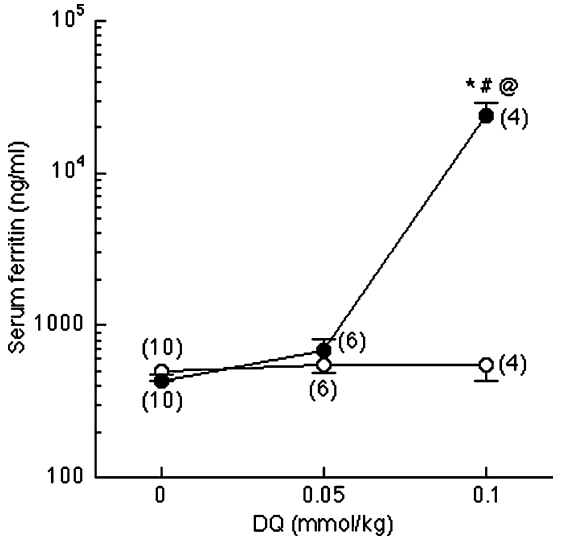

Figure 4. Comparison of serum ferritin between the LIS and HIS groups injected with DQ. Rats of the LIS (open circles) and HIS (closed circles) groups were treated with 0 (saline), 0.05, or 0.1 mmol DQ/kg. Data are the means ± SD with the numbers of animals in parentheses. *,# Different from saline- and DQ (0.05 mmol/kg)-treated groups, respectively, fed a diet containing the same iron content, $P < 0.01$. @ Different from the LIS group given the same dose of DQ, $P < 0.01$.

chow (Clea Japan), which contained 320 ppm iron, was 30% 24 h after administration of 0.1 mmol DQ/kg (our unpublished observation), which is comparable with the data of Smith *et al.* Such differences in the survival are considered to be attributable to the differences in the components between the purified diet used in the present study and the standard diet. These two diets contain almost the same compositions and contents of vitamins and minerals. The components that have protective effects on DQ- induced oxidative stress remain to be identified and characterized.

In rats, the plasma ferritin concentration has been reported to parallel plasma ALT activity during acute D-galactosamine-HCl-induced hepatitis (Zuyderhoudt *et al.* 1980). In the present study, two markers of hepatic injury, serum ferritin and ALT, showed the same increase after administration of 0.1 mmol DQ/kg in the HIS group. In the LIS group, however, administration of the same dose of DQ did not significantly increase serum ferritin. This may be due to the extremely low liver ferritin content of the LIS group.

Clothier *et al.* (2000) found that SWR mice were considerably more sensitive to DQ than C57BL/10ScSn mice and suggested that this is due to the higher hepatic iron status of the SWR mice. Although the liver and kidney iron levels of Fischer-344 rats are significantly lower than in Wistar rats under the same feeding conditions, the Fischer-344 rats are more sensitive to DQ (our unpublished observations). Therefore, the differences in the sensitivity to DQ cannot be explained by the differences in the body iron status between the strains. Also, Smith *et al.* (1985) have reported the similar strain differences in susceptibility to DQ-induced hepatotoxicity between Fischer-344 and Sprague–Dawley rats.

Gupta *et al.* (2000) found that male Fischer-344 rats are more sensitive than females to hepatic damage by DQ. This does not seem to be due to differences in the liver iron status because the hepatic iron levels are considerably lower in the male rats (Linder *et al.* 1973). DQ caused more DNA fragmentation in male rats given 0.15 or

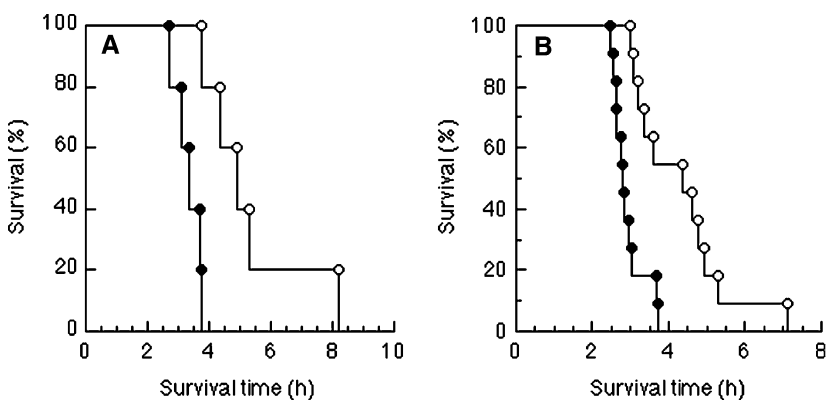

Figure 5. Survival of rats treated with DQ. Rats in the HIS (closed circles) and LIS groups (open circles) were injected with 0.075 mmol DQ/kg (A; $n = 5$ in each group) or 0.1 mmol DQ/kg (B; $n = 11$ in each group).

0.2 mmol DQ/kg than in similarly treated female rats (Gupta *et al*. 2000), which may be associated with the gender differences in the sensitivity to DQ.

As described in the present study, body iron status affects the sensitivity to DQ. Because other redox cyclers, such as paraquat, adriamycin, and alloxan, also release iron from ferritin and induce oxidative damage (Reif 1992), it is possible that their toxicities are also influenced by body iron stores.

Epidemiological studies have shown a relationship between body iron status and disease in humans. For example, liver iron is predictor of death in patients with alcoholic cirrhosis (Ganne-Carrié *et al*. 2000). In addition, a moderately elevated iron level corresponds to an increased risk of cancer and death (Stevens *et al*. 1994). Likewise, reduction in iron stores due to repeated blood donation is associated with a lower incidence of cancer (Merk *et al*. 1990). Finally, higher body iron stores are found to be associated with an increased risk of type 2 diabetes in healthy women (Jiang *et al*. 2004).

Studies using experimental animals also show the involvement of iron in the development of diseases. For example, apolipoprotein E-deficient mice fed an iron-deficient diet have reduced atherosclerotic lesions (Lee *et al*. 1999). Also, hepatic iron deprivation can prevent the spontaneous development of fulminant hepatitis and liver cancer in Long-Evans Cinnamon rats (Kato *et al*. 1996). Therefore, reducing iron stores may be considered to lead to a decreased risk of many diseases resulting from iron-mediated oxidative stress. Further studies are required to establish a link between excess iron, which promotes the generation of free radicals, and diseases induced by oxidant stress. However, iron deficiency as well as iron excess must be recognized as a human health problem, because iron deficiency is the most widespread nutritional deficiency throughout the world and impairs resistance to infection, cognitive performance, physical capacity, work output, and possibly maintenance of body temperature (Crichton 1990).

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