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Possible contribution of rubiadin, a metabolite of madder color, to renal carcinogenesis in rats

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ABSTRACT

Madder color (MC) has been shown to exert carcinogenic potential in the rat kidney in association with degeneration, karyomegaly, increased cell proliferation of renal tubule cells and increased renal 8-OHdG levels. To clarify the causal relationship of components and metabolites of MC to renal carcinogenesis, male F344 rats were fed lucidin-3-*O*-primeveroside (LuP) or alizarin (Alz), and the genotoxic LuP metabolites lucidin (Luc) or rubiadin (Rub) for up to 26 weeks. After one week and four weeks, Luc did not induce any renal changes. In contrast, after one week, cortical tubule degeneration was apparent in the Alz and LuP groups, and cytoplasmic swelling with basophilic change and karyomegaly in the outer medulla was observed only in the Rub group. LuP and Rub increased the proliferative activity of tubule cells in the outer medulla, and Alz and LuP increased renal 8-OHdG levels. After 26 weeks, Rub but not Alz induced atypical tubules, a putative preneoplastic lesion, and karyomegaly in the outer medulla. These results indicate that Rub may be a potent carcinogenic metabolite of MC, targeting proximal tubule cells in the outer medulla, although oxidative stress increased by Alz or LuP might also be involved in renal carcinogenesis by MC.

1. Introduction

Madder color (MC) extracted from madder root, the root of *Rubia tinctorum* L., had long been accepted for use as a food additive in Japan (MHLW, 1995) and Korea, and widely applied for a variety of foods/drinks, such as confectionaries, processed meats (hams and sausages), boiled fish paste and beverages.

We found clear carcinogenicity of MC to the kidneys and liver in a two-year carcinogenicity study in F344 rats (Inoue et al., 2009). In addition to toxicity to the liver, kidney and blood cells, carcinogenic potential was predicted from evidence such as increased GST-P-positive hepatocellular altered foci, atypical renal tubules and renal cell adenomas in subchronic or chronic studies (Inoue et al., 2008a,b). Moreover, there have been reports of positive results in *in vitro* and *in vivo* genotoxicity studies of madder root, its components and metabolites such as lucidin (Luc) (Hachiya et al., 1985; Asanoma et al., 1984; Brown and Dietrich, 1979; Blömeke et al., 1992; Kawasaki et al., 1992; Yasui and Takeda, 1983; Westendorf et al., 1988, 1990, 1998; Poginsky et al., 1991). Therefore, the carcinogenicity of MC has been assumed to involve direct DNA damage.

Major constituents of MC used in Japan are anthraquinones, such as alizarin (Alz), lucidin-3-*O*-primeveroside (LuP) and ruberythric acid. They have been reported to have different genotoxicity profiles, LuP being positive, but Alz and ruberythric acid proving negative in mutation test using *Salmonella typhimurium* (Kawasaki et al., 1992), the *Drosophia* wing spot test (Marec et al., 2001) and an *in vivo* DNA adduct formation test (Poginsky et al., 1991). Oral administration of LuP to rats results in excretion of Luc and rubiadin (Rub) (Blömeke et al., 1992), both of which are strongly positive in *in vitro* mutation tests such as the Ames test and unscheduled DNA synthesis assay (Brown et al., 1979; Yasui et al., 1983; Kawasaki et al., 1992; Blömeke et al., 1992). Additionally, Luc forms DNA adducts *in vitro* and *in vivo* (Poginsky et al., 1991). Although there have been many reports on the genotoxocity of MC components and their metabolites, toxicological profiles of these anthraquinones *in vivo* and their linkage to renal carcinogenesis have hitherto remained unclear.

Abbreviations: MC, madder color; Alz, alizarin; LuP, lucidin-3-*O*-primeveroside; Luc, lucidin; Rub, rubiadin.

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Regarding the toxicological profile of MC, karyomegaly and increase of cell proliferation in proximal tubule cells in the outer medulla, as well as cortical degeneration including microvesicular vacuolar degeneration and increased levels of 8-OHdG have been observed (unpublished data). Therefore, we performed the present study to clarify which MC components and/or their metabolites play major roles in the induction of such toxicological changes possibly related to renal carcinogenicity of MC, using short- and medium-term assays in F344 rats.

2. Materials and methods

2.1. Chemicals

Alz was purchased from Sigma–Aldrich Japan (Tokyo, Japan). LuP was extracted from MC (powder of roots of *Rubia tinctorum L.*) used in Japan as a food coloring (San-Ei Gen. F.F.I., Inc., Osaka, Japan) and Luc and Rub were synthesized at ALPS pharmaceutical Ind. Co., Ltd. (Gifu, Japan). The purities of Alz, LuP, Luc and Rub were 97%, 90.5%, 93% and 99.9%, respectively. These compounds were well mixed with powdered basal diet CRF-1 (Oriental Yeast Co., Tokyo) at concentrations of 0.0016%, 0.008% and 0.04% for Alz, 0.06%, 0.3% and 1.5% for LuP and Rub, and 0.1%, 1.0% and 5.0% for Luc, respectively, for the short-term study. For the medium-term study, 0.04% Alz- or Rub-containing diet was prepared in the same way. Medium doses of Alz and LP were based on the concentrations of these chemicals in MC used in toxicity and carcinogenicity studies reported previously (Inoue et al., 2008a, b), and lower and higher doses were set with a common ratio of five. For Rub, because there was no data on the concentration in MC, selected doses were the same as for LuP which yields Rub on metabolism. Since no alterations were observed in the kidneys at the doses of LuP contained in MC in a preliminary study, the higher dose of Luc was set at 5.0%.

2.2. Animals

Male F344/DuCrj rats at 5 weeks of age, purchased from Charles River Japan Inc. (Kanagawa, Japan), were used in the present study. They received basal dietary pellet CRF-1 and tap water *ad libitum* during the 1 week acclimation period, housed 3 per polycarbonate cage with sterilized softwood chips as bedding in a barrier-sustained animal room conditioned at 23–25 °C and 50–60% humidity, and on a 12-h light/dark cycle.

2.3. Experimental design

In the short-term assay, groups of six rats were fed Alz, LuP, Rub or Luc containing diets for one week. Luc containing diets were also administered to animals for four weeks because of the lack of remarkable changes with one-week treatment. In the medium-term assay, groups of 15 animals were fed Alz- or Rub-containing diet at 0.04% for 26 weeks in order to further clarify renal changes found in the one-week study. Control rats were fed basal diet alone. The diet and drinking water were available *ad libitum* and the animals were observed daily for clinical signs and mortality. At weeks 1, 4 and 26, subgroups were anesthetized with ether, weighed and euthanized by exsanguination from the abdominal aorta. Left and right kidneys were removed, weighed and cut to provide central slices including the pelvis for histopathological examination. The renal papilla was then removed from the remainder to provide mainly cortex and outer medulla tissues for rapid freezing with liquid nitrogen.

The animal protocols were reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

2.4. Histopathological assessment

The slices of both kidneys fixed in 10% buffered formalin for two days were routinely processed for paraffin embedding and production of sections was stained with hematoxylin and eosin. Evaluation was performed to give degrees of distribution of each lesion as follows: ±, <20%; +, 20–50%; ++, 50–70%; +++, >70% in the renal cortex or outer medulla.

2.5. Immunohistochemical assessment of PCNA-positive renal tubule cells

Because increased PCNA-immunoreactive cells and tumor development were observed in the proximal tubular epithelium of the outer medulla in MC-treated animals in previous toxicity and carcinogenicity studies (Inoue et al., 2008a, b), nuclear immunoreactivity for PCNA was assessed. Deparaffinized and hydrated kidney sections were treated with 0.3% H_2O_2 in absolute methanol for 15 min for blockage of endogenous peroxidase activity, and then incubated with a mouse anti-PCNA monoclonal antibody (clone PC-10, ×100 dilution; Dako Japan, Kyoto, Japan) at 4 °C overnight. Immunodetection was carried out with the horseradish peroxidase-avidin-biotin complex method using a VECTASTAIN® Elite ABC KIT (Vector Laboratories Inc., Burlingame, CA, USA) following the manufacturers protocol and 3,3′-diaminobenzidine as the chromogen. Numbers of immunoreactive nuclei per 1000 proximal tubule cells were counted under a light microscope in five randomly selected fields of the outer medulla, sized 250 × 250 μm for each kidney, at 200× magnification under a microscope (total of five fields per animal).

Table 1
Final body, absolute and relative kidney weights of male F344 rats given alizarin, lucidin-3-*O* -primeveroside, rubiadin and lucidin for one week.

Group	Final BW (g)	Kidneys	
		Absolute (g)	Relative (g/100 g BW)
Control[a] (for Alz and LP)	146.8 ± 6.4[b]	1.24 ± 0.09	0.843 ± 0.023
Alz (% in diet)			
0.0016	146.5 ± 7.5	1.21 ± 0.07	0.826 ± 0.013
0.008	144.9 ± 8.1	1.21 ± 0.07	0.834 ± 0.011
0.04	142.2 ± 7.4	1.24 ± 0.08	0.871 ± 0.016*
LuP (% in diet)			
0.06	143.3 ± 9.8	1.23 ± 0.08	0.857 ± 0.017
0.3	144.3 ± 11.8	1.21 ± 0.10	0.839 ± 0.009
1.5	141.5 ± 7.2	1.18 ± 0.06	0.834 ± 0.014
Control (for Rub)	143.3 ± 6.5	1.15 ± 0.07	0.802 ± 0.020
Rub(% in diet)			
0.06	134.7 ± 4.8*	1.14 ± 0.05	0.846 ± 0.024
0.3	105.1 ± 4.6**	0.90 ± 0.04**	0.855 ± 0.028
1.5	75.5 ± 3.4**	0.78 ± 0.04**	1.035 ± 0.075**
Control (for Luc)	149.7 ± 11.6	1.24 ± 0.09	0.83 ± 0.02
Luc (% in diet)			
0.1	154.0 ± 7.5	1.27 ± 0.08	0.82 ± 0.02
1.0	152.0 ± 6.9	1.23 ± 0.08	0.81 ± 0.02
5.0	147.3 ± 2.0	1.20 ± 0.05	0.81 ± 0.03

Abbreviations: Alz, alizarin; BW, body weight; LuP, lucidin-3-*O*-primeveroside; Rub, rubiadin; Luc, lucidin.
* $p < 0.05$ vs. controls.
** $p < 0.01$ vs. controls.
[a] Number of animals examined was six in all groups.
[b] Means ± SD.