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Age-related Copper, Zinc, and Iron Metabolism in Long-Evans Cinnamon Rats and Copper-Eliminating Effects of D-penicillamine and Trienthine-2HCl

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The Long-Evans cinnamon (LEC) rat is an animal model of human Wilson's disease. The hepatic copper content of LEC rats increased in an age-dependent manner from 4–5 days of age and was maintained at a high level from 15–16 weeks of age. The renal copper content of LEC rats showed a tendency to increase from 10 weeks of age and increased rapidly from 15 weeks of age. No difference in whole-brain copper concentration was observed between LEC and Long-Evans agouti (LEA) rats. LEC rats treated with D-penicillamine and trienthine-2HCl had reduced liver dysfunction. These agents reduced the hepatic copper content by ~1/2–2/3 and the renal copper content to within the normal range. They also decreased the copper content of hair and nails, and there was also a good correlation between the copper content of liver and white hair. J. Trace Elem. Exp. Med. 10:49–59, 1997.

Key words: Wilson disease; hepatitis; P-type ATPase

INTRODUCTION: AGE-RELATED CHANGES IN LEC RATS

The Long-Evans cinnamon (LEC) rats, which are a mutant inbred strain isolated from Long-Evans rats, develop hepatitis and hepatocellular carcinoma [1] in an autosomal recessive pattern of inheritance [2,3]. They also demonstrate many clinical and biochemical features of Wilson's disease, including a low serum copper level and ceruloplasmin oxidase activity, a high copper content in the liver, and increased urinary copper excretion [4,5]. LEC rats are considered a suitable animal model of Wilson's disease, which is an autosomal recessive disorder of copper metabolism. The major clinical signs and symptoms of the disease are liver dysfunction, extrapyramidal signs, and Kayser-Fleischer rings, due to copper deposition in various tissues, such as liver, brain, and cornea. The copper toxicity is due to loss of the ability to excrete copper from the liver to the bile and to incorporate copper into ceruloplasmin in the liver [6–9]. The gene for Wilson's disease was cloned in 1993 and encodes a

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putative copper-transporting P-type ATPase [10–13]. The rat gene homologous to the human Wilson's disease gene was cloned in 1994, and the LEC rat has a mutation in this gene [14,15].

In this study, we analyzed age-related changes of copper, zinc, and iron contents in the liver, kidney, and whole brain and the copper-eliminating effects of D-penicillamine and trienthine-2HCl for various tissues in LEC rats. The hepatic copper content of LEC rats were similar in an age-related manner to those of patients with Wilson's disease. These chelating agents also prevented the development of hepatitis and eliminated excessive tissue copper in LEC rats.

MATERIALS AND METHODS

Animals

Male and female LEC rats and Long-Evans agouti (LEA) rats as the control were maintained from day 0 to 58 weeks of age under the following conditions: lights on from 07:00 h to 19:00 h, temperature 23.0 ± 1.0°C, humidity 55 ± 5%. Water and food (CE-2, Clea, Tokyo, Japan) were given ad libitum. LEC and LEA rats were kindly provided by Dr. Noritoshi Takeichi (Hokkaido University, Sapporo, Japan) and were bred in the Department of Pharmacology, Toho University School of Medicine (Tokyo). This study was performed in accordance with the guidelines for animal experiments of the Toho University School of Medicine.

Contents of Copper in Various Tissues and Urine

Liver, kidney, whole brain, hair, and nails were wet ashed with nitric acid and exposed to a series of dilutions with distilled water. The above contents were analyzed using an inductively coupled plasma-mass spectrometer method (ELAN 5000, Perkin Elmer, Norwalk, CT).

Medication of Chelating Agents

LEC rats orally and spontaneously ingested solutions of D-penicillamine (~dose: 30, 60, 120 mg/kg/day) and trienthine-2HCl (~dose: 75, 150, 300 mg/kg/day) for 30, 90, and 180 days. The treatment was began at 6 weeks after birth. We used age-matched LEC rats that ingested distilled water as a control.

Activities of Serum AST, ALT, and LDH

Serum concentrations of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Lactate Dehydrogenase (LDH) were determined by the UV method with a Hitachi Autoanalyzer (736-60E, Hitachi Co, Tokyo, Japan).

Statistical Analysis

All values were expressed as the mean ± SEM. Differences between the means for all data were evaluated with the unpaired t = test. A P value of 0.05 or less was considered statistically significant.

RESULTS

Age-related copper contents in various tissues (Fig. 1). The copper content in the liver of LEC rats was 4.5 ± 0.8 μg/g of wet tissue in 0-day-old animals. It was significantly higher than that of LEA rats, which had hepatic copper contents of 2.0 ± 0.1 μg/g of wet tissue. Then, the copper content in the LEC rat liver markedly increased, becoming >50 times higher than in the liver of age-matched LEA rats of 15 weeks.

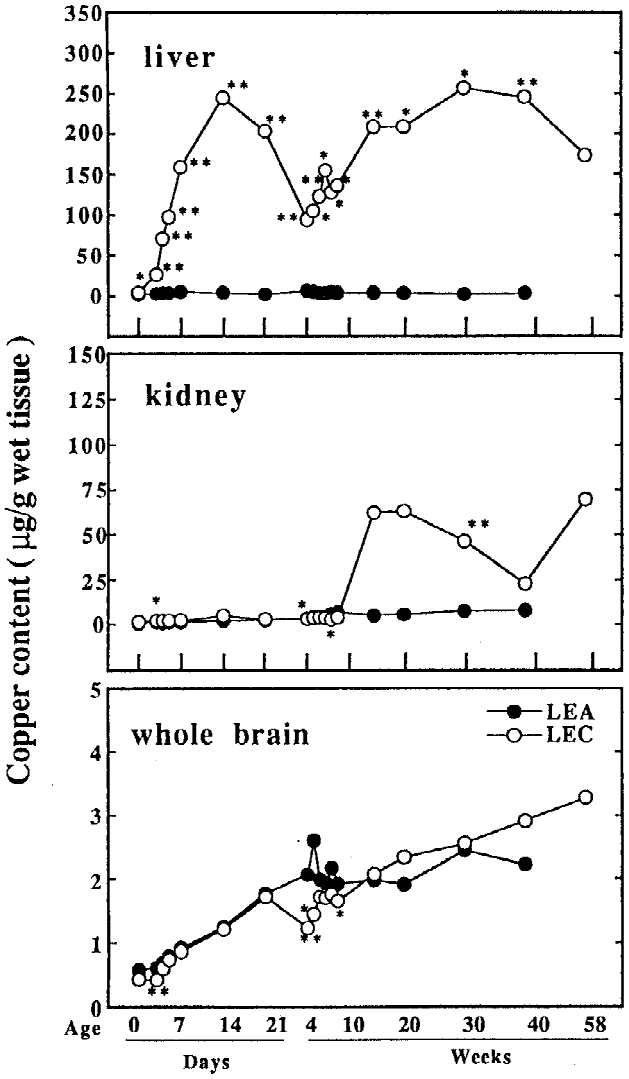

Fig. 1. Age-related changes of copper content in the liver, kidney, and whole brain of LEC and LEA rats during development. Open circles are LEC rats, and closed circles are LEA rats. Standard error bars of mean are shown unless smaller than the symbols. n = 3–6. Significantly different from LEA: $*P < 0.05$, $**P < 0.01$.

In the kidney, the copper concentration of LEC rats was significantly higher than of LEA rats on the 3rd day, and in the 4th and 8th weeks. After the 15th week, the copper accumulation in the kidney of LEC rats rapidly increased. The copper content of whole brain increased with age in both the LEC and the LEA rats, and there was no significant difference between these groups.

Age-related zinc contents in various tissues (Fig. 2). In liver, zinc concentration of LEC rats were significantly higher than LEA rats at any period. In kidney and brain, LEC rats showed definite high zinc concentration rather than LEA rats at some period.

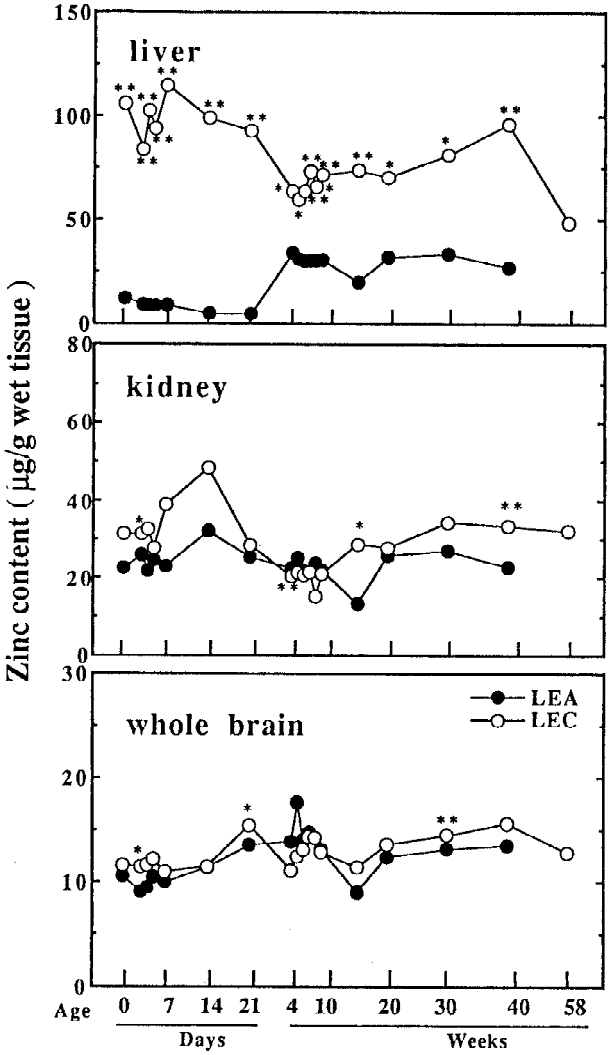

Fig. 2. Age-related changes of zinc content in the liver, kidney, and whole brain of LEC and LEA rats during development. Open circles are LEC rats, and closed circles are LEA rats. Standard error bars of mean are shown unless smaller than the symbols. n = 3–6. Significantly different from LEA: $*P < 0.05$, $**P < 0.01$.

Age-related iron content in various tissues (Fig. 3). The hepatic iron concentration in LEC rats was significantly higher than in LEA rats that were 0–21 days old. After this, a similar tendency continued to be observed. There was a significant difference between the iron content of kidney and brain in LEC rats and LEA rats at some periods.

Serum copper content and liver-derived enzymes of LEC rats treated with chelating agents. The LEC rats treated with chelating agents for 30 days (10 weeks old) were aged before the onset of hepatitis. They had significant differences in their serum copper concentration and their liver-derived enzymes compared with age-matched control LEC rats (Table I). On the 90th and 180th days of treatment with chelating agents, the serum copper concentration, ALT, AST, and LDH levels of

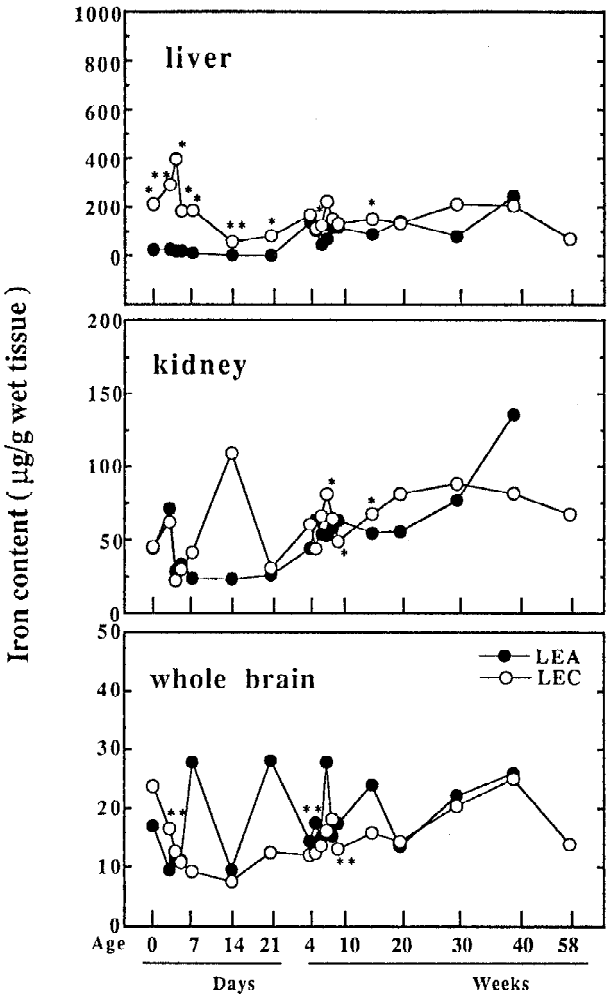

Fig. 3. Age-related changes of iron content in the liver, kidney, and whole brain of LEC and LEA rats during development. Open circles are LEC rats, and closed circles are LEA rats. Standard error bars of mean are shown unless smaller than the symbols. n = 3–6. Significantly different from LEA: $*P < 0.05$, $**P < 0.01$.

TABLE I. Activities of Serum Liver-derived Enzyme in Chelator-treated LEC Rats

	Dose (mg/kg/day)	Day	AST (IU/l)			ALT (IU/l)			LDH (IU/l)		
			30th	90th	180th	30th	90th	180th	30th	90th	180th
Control		Mean	161	555	588	74	555	674	3,572	4,037	3,115
n = 3		SE	4.8	142.9	83.4	3.5	154.2	74.2	740.9	510.8	243.3
Trienthine-2HCl	75	Mean	145	320	394	87*	313	432	1,725	4,163	2,929
n = 3		SE	9.1	1.5	61	2.5	27	25.5	740.9	1,163.50	782
	150	Mean	198	529	128**	95	353	85**	2,677	6,197	1,000**
		SE	9.8	184.7	16.6	21	103.9	26.1	333.1	903.2	75.7
	300	Mean	149	176*	94*	83	125*	71*	2,496	2,909	496**
		SE	9.4	27.7	4.9	7.5	18	5.8	291.3	1,010.80	214.7
D-penicillamine	30	Mean	138	271	146*	60	293	78*	2,560	4,913	3,293
n = 3		SE	14.7	55.8	9.9	3.9	135.9	3.8	463.2	1,476.90	632.8
	60	Mean	160	317	198*	103	353	80*	1,654	4,075	3,639
		SE	33.8	151	33.4	42.5	245.4	11	489.3	743.3	606.9
	120	Mean	137	189*	119*	62	126*	81**	2,355	1,697*	2,623
		SE	16.1	19	12.3	5.6	14.5	16	742.9	540.1	547.3

Significantly different from control: *$P < 0.05$, **$P < 0.01$.

treated LEC rats were dose-dependently lower than for control LEC rats manner (Table I).

Copper concentration of various tissues in LEC rats treated with chelating agents. The hepatic copper concentration of LEC rats treated with 300 mg/kg/day of trienthine-2HCl on the 90th day and with 120 mg/kg/day of D-penicillamine at any period was lower than for control LEC rats (Table II). However, it was still about 30 times higher than for age-matched LEA rats. The copper content in the kidneys of LEC rats that were treated by chelating agents was significantly lower in comparison with control LEC rats (Table II). Treatment with 300 mg/kg/day trienthine-2HCl and any dose of D-penicillamine could maintain the renal copper content within the normal range.

The copper content of hairs and nails of LEC rats that were given 300 mg/kg/day of trienthine-2HCl, or 120 mg/kg/day of D-penicillamine was measured. In control LEC rats, the copper concentration in cinnamon-colored hair on the 180th day was higher than in white hair (Table III). The copper content of hair was significantly lower on the 180th day in trienthine-2HCl-treated LEC rats and on both the 90th and the 180th day in given D-penicillamine in comparison with control LEC rats (Table III). The copper content in nails of the treated LEC rats showed a significantly lower level on the 180th day of treatment (Table III).

DISCUSSION

Between the ages of 16 and 23 weeks, 90% of LEC rats have acute hepatitis. The serum AST level rises to >1,200 IU, the ALT level reaches ~500 IU, and ~30% of these animals die with acute hepatic failure [3]. The hepatic copper content of LEC rats increases to >50 times higher than that of LEA rats. The renal copper content of LEC rats markedly increases during this period (Fig. 1). The copper concentration in the LEC rat liver reaches a toxic level, and the hepatocytes become necrotic. As a result, nonceruloplasmin-binding copper overflows into the peripheral blood and begins to accumulate in extrahepatic organs, including the kidneys.

LEC rats show no neurological symptoms even in the long surviving cases. The copper concentration of whole brain in LEC rats was not significantly different from that of LEA rats (Fig. 1). Wilson's disease patients with neurological symptoms accumulate excess copper in the brain, especially in the basal ganglia [16,17]. Differences exist between LEC rats and human Wilson's disease patients. Although differences may be due to the differences in species, the details remain unknown. The distribution of copper content in the brain of LEC rats should be examined.

The zinc concentration in the liver of LEC rats was higher than in LEA rats (Fig. 2). Most of the accumulated copper and zinc in the hepatocytes of LEC rats binds with metallothioneine [19]. The copper accumulation in hepatocytes induces a hyperexpression of metallothioneine, and the zinc concentration also increasingly binds with metallothioneine [18]. Both the copper and zinc contents in the liver of LEC rats decreased temporarily from 21 days to 4 weeks of age (Figs. 1, 2). We speculate that this decrease depends on a change of metallothioneine expression.

The oral administration of copper-chelating agents inhibits the elevation of serum liver-derived enzyme levels and serum copper concentration (Table I). These agents reduced the hepatic copper concentration, but it was still remarkably higher than in

TABLE II. Copper Content of Various Tissues in Chelator-treated LEC Rats

	Dose (mg/kg/day)	Day	Liver (μg/g wet tissue)			Kidney (μg/g wet tissue)			Whole brain (μg/g wet tissue)		
			30th	90th	180th	30th	90th	180th	30th	90th	180th
Control		Mean	158.9	236.5	232.6	9.8	116.7	25.4	1.9	2.1	2.6
n = 3–4		SE	4.6	28.9	15	1.7	34.9	6.7	0.1	0.1	0.1
	75	Mean	163.8	163.8	168	4.9*	44.9	29.9	2.1	2.1	2.4
		SE	19.5	15	34.2	0.5	4.5	9.2	0.2	0.1	0.1
Trienthine-2HCl	150	Mean	161.5	219.5	217	4.5*	5.7*	15.5	2	1.9	2.4
n = 2–4		SE	15.4	26.1	29.7	0.2	1.4	2.3	0.1	0.1	0.2
	300	Mean	154.3	133.3*	213.6	4.1*	4.2*	5.2*	2	1.8**	2.2*
		SE	37.6	15.8	27	0.2	0.3	0.6	0.1	0	0.1
	30	Mean	140.5	189.5	223.2	6.8	6.9*	8.2*	1.9	2.2	2.4*
		SE	14.2	13	22.1	0.9	0.7	1.4	0	0.1	0.1
D-penicillamine	60	Mean	138.8	158.3	143**	6.2	4.4*	5.2*	1.9	2.1	2.2**
n = 3–4		SE	20	28.1	9.2	1	0.4	0.6	0	0.1	0.1
	120	Mean	103.5**	113.8*	101.3**	4.6*	4.6*	3.9*	1.9	2.1	2.1**
		SE	9.3	10.3	8.6	0.6	0.3	0.4	0.1	0.1	0.1

Significantly different from control: *$P < 0.05$, **$P < 0.01$.

TABLE III. Copper Content of Hair and Nail in Chelator-treated LEC Rats

	Dose (mg/kg/day)	Day	White hair 90th	White hair 180th	Cinnamon hair 90th	Cinnamon hair 180th	Nail 90th	Nail 180th
Control		Mean	11.9	12.9	8.9	14.7	19.4	37.5
n = 3–4		SE	0.8	0.5	0.7	0.5	2.9	3.6
Trienthine-2HCl	300	Mean	7.7**	10.6	6.9	11.4	18.3	20.6*
n = 4		SE	0.6	1.1	0.7	0.8	4.2	3.5
D-penicillamine	120	Mean	9.4	7.7**	7.7	8.7*	12.6	15.9*
n = 4		SE	1.9	0.3	2.1	1.6	2.8	3.1

Significantly different from control: *$P < 0.05$, **$P < 0.01$.

LEA rats (Table II). Togashi et al. [20] reported similar results for D-penicillamine treatment. The copper-chelating ability of D-penicillamine is ~2.5 times stronger than trienthine-2HCl [21]. However, trienthine-2HCl has enough chelating efficiency to prevent the onset of hepatitis in LEC rats. The copper that is bound with metallothioneine is not toxic for hepatocytes, and copper-metallothioneine is not reduced by D-penicillamine and trienthine-2HCl. In the kidneys, the copper concentration was kept in the normal range with chelating agents (Table II). We administered chelating agents before the onset of hepatitis. Because the nonceruloplasmin copper that overflows from necrosed hepatocytes is chelated and excreted in urine, copper does not accumulate in extrahepatic organs.

The copper content of hair and nails in LEC rats was no different in comparison with age-matched LEA rats [22]. However, the copper concentration of chelator-treated LEC rats was lower than for untreated rats (Table III) and correlated with the hepatic copper content. We speculate that measurement of the copper content of hair and nails is an indication of the copper concentration of liver.

The gene mutation of LEC rats is the partial deletion of the rat Wilson's disease gene [15]. The deletion is at least 900 bp from the 3′-end coding region, and it removes the information encoding the conserved ATP binding domain. Therefore, it inevitably inactivates the function of the gene as a copper transporter [15]. Human patients with Wilson's disease have some frequent mutations and many rare defects [23]. The correlation between genotype and phenotype has been investigated and discussed, although the details remain unclear [23,24]. However, patients who have mutations and make truncated protein indicate a hepatic phenotype [24]. LEC rats also make truncated protein if their Wilson's disease gene expresses protein. The truncated protein causes severe impairment of the Wilson disease gene and leads to early-onset liver dysfunction. The LEC rat is a good animal model of the hepatic type, especially the fulminant type, of human Wilson's disease, both clinically and genetically.

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