Acta Med. Nagasaki 48 : 9－14

Ultrastructural and Analytical Study of Retinas of Rats with Excess Copper

Akinori TAKEHARA

Department of Ophthalmology and Visual Sciences, Graduate School of Biomedical Sciences Nagasaki University

Purpose: To examine the structure of and elements in the retina of Cu-injected rats with an electron microscope (TEM) and an energy dispersive X-ray analyzer (EDXA).

Methods: One-month-old and 10-month-old normal Wistar Kyoto rats were injected with 5 mg/ml of CuCl2/kg body weight once a week for four weeks. After 4 weeks the retinas were observed by TEM and analyzed with an EDXA.

Results: The 10-month-old Cu-injected rats showed a decreased number of cells in the inner nuclear layer, and the nuclei showed clumps of chromatin and pyknosis. High density deposits were present in the retinal pigment epithelium (RPE). These deposits were analyzed with EDXA and found to contain iron, but not copper.

Conclusions: Although rats treated with $CuCl_2$ had a high serum Cu level, EDXA detected Fe, not Cu, in the RPE. TEM showed apoptotic changes in the inner nuclear layer. Hypercupremia might induce hemolysis and lead to iron accumulation. The retinal changes in the present study might be due to iron accumulation, i.e. siderosis.

ACTA MEDICA NAGASAKIENSIA 48 : 9－14, 2003

Key Words: copper, iron, retina, electron microscopy, energy dispersive X-ray analysis, rat

Introduction

Copper is an essential trace element for metabolism in animals and humans. Important copper-containing enzymes are dopamine hydroxylase, cytochrome oxidase, and superoxide dismutase (1). Copper is a heavy metal whose unbound ions are toxic. Almost all of the copper in the body is present as a component of copper proteins, thereby reducing the in vivo concentration of unbound copper ions to almost zero (1). Genetic mechanisms control the processes by which copper is incorporated into apoproteins and toxic accumulations of copper are avoided (2).

Wilson's disease, intraocular foreign body, and dietary chronic copper poisoning cause excess copper in ocular tissues. Although there have been many reports on these diseases in recent years (3-5), the mechanism of homeostasis in conditions of copper excess is not clear.

Wilson's disease is known to be due to high copper levels and abnormal copper metabolism. Patients show the characteristic Kayser-Fleischer corneal ring and sunflower cataract. Although many pathological studies of the cornea and the lens in Wilson's disease have been reported (6-8), the effect of copper on the retina has not been studied well.

The purpose of this study is to examine the structure of and elements in the retinas of Cu-injected rats with an electron microscope (TEM) and an energy dispersive X-ray analyzer (EDXA).

Materials and Methods

One-month-old and 10-month-old normal Wistar Kyoto rats were injected intraperitoneally with 5 mg/ml of $CuCl_2$/kg body weight once a week for 4 weeks. Age-equivalent control rats were injected with 1 ml of distilled water/kg body weight once a week. The rats were weighed at the time of each injection. After 4 weeks the eyeballs were enucleated under pentobarbital anesthesia after blood had been drawn from the heart for the determination of serum copper and iron levels. All animals were treated according to the ARVO resolution on the Use of Animals in Research. The rats were housed in stainless steel wire-bottomed cages and maintained at a constant room temperature of 21℃ with a 12h light and dark cycles at the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine.

Retinal specimens were fixed with 4% glutaraldehyde in 0.05M cacodylate buffer for 1h and washed with

Address Correspondence: Akinori Takehara, M.D.
Department of Ophthalmology and Visual Sciences, Graduate School of Biomedical Sciences Nagasaki University, 1-7-1 Sakamoto, Nagasaki, 852-8501 Japan.
Tel: +81-95-849-7345 Fax: +81-95-849-7347
E-mail: a-tkhr@air.linkclub.or.jp

0.05M cacodylate buffer. Specimens for TEM were postfixed with 1% osmium tetroxide in veronal acetate buffer for 1h, while specimens for EDXA were not postfixed. All specimens were dehydrated in a series of graded ethanols and embedded in Luveak 812.

Two micrometer sections were cut with a microtome (Ultracut UCT, Leica, Austria) and stained with toluidine blue for light microscopy. The cells in the inner nuclear layer in a 80μm×20μm field at the posterior pole of the retina were counted under ×400 magnification.

Ultrathin sections were cut and placed on a nickel mesh. The sections were observed with a TEM (JEM-1210, JEOL, Japan) and analyzed with an EDXA (EDAX DX-4, Philips, The Netherlands). The analytical conditions were as follows: magnification x15,000, accelerating voltage 100_keV, sample current 60μA, and time 200_sec.

The copper and iron concentrations in the serum were determined by a standard atomic absorption spectrophotometer.

The serum level of copper and iron and the number of cells were analyzed statistically with the unpaired t-test. $P<0.05$ was considered to be significant.

Results

Of the 1-month-old rats treated with $CuCl_2$ 29% died within 3 weeks. All the 10-month-old rats treated with $CuCl_2$ survived for 1 month. Figure 1 shows the body weight changes. There was a significant difference in weight gain between 1-month-old Cu-injected and 1-month-old control rats ($P<0.05$). There was no significant difference in weight gain between 10-month-old Cu-injected and control rats ($P=0.90$).

The serum copper and iron levels are shown in Table 1. In the 1-month-old rats, there was no detectable difference in the serum copper level. In the 10-month-old rats, the serum copper level was significantly higher in the Cu-injected rats than in the controls ($P<0.01$). There was no significant difference in the serum iron level between Cu-injected rats and control rats in both age groups.

Light Microscopic Findings

In the 1-month-old Cu-injected rats, there was no obvious abnormality (Figure 2.). The 10-month-old Cu-injected rats showed a decreased number of cells in the inner nuclear layer (Figure 3), and the difference between the 10-month-old Cu-injected and control rats was significant ($P<0.001$)(Table 2). In the 1-month-old Cu-injected rats, on the other hand, there was no

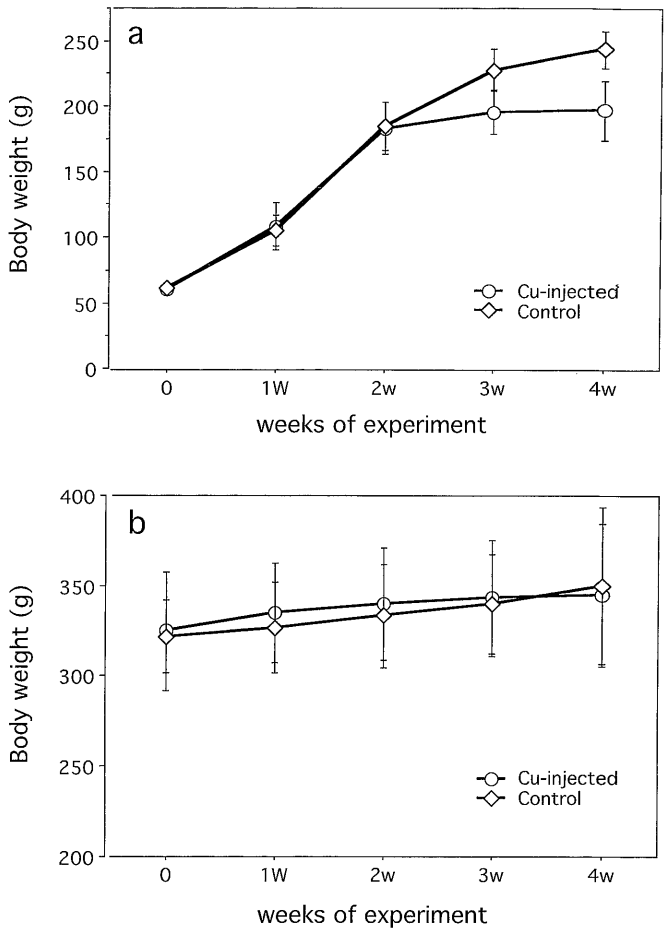

Figure 1 Body weight change of rats. There are significant differences in weight gain between 1-month-old Cu-injected and control rats (a) ($P<0.05$), but no differences in 10-month-old rats (b).

Table 1 Serum copper and iron levels (mean±SD).

	Serum copper (μg/100ml)	Serum iron (μg/100ml)
1-month-old rats		
Cu-injected (n=5)	107.6±8.7	157.0±1.7
Control (n=7)	98.3±3.8	194.7±30.1
10-month-old rats		
Cu-injected (n=5)	169.7±16.8*	265.5±72.2
Control (n=5)	114.7±9.1*	214.0±29.5

In 10-month-old rats the copper level in the serum is significantly higher in the Cu-injected rats than in the controls (* $P<0.01$).

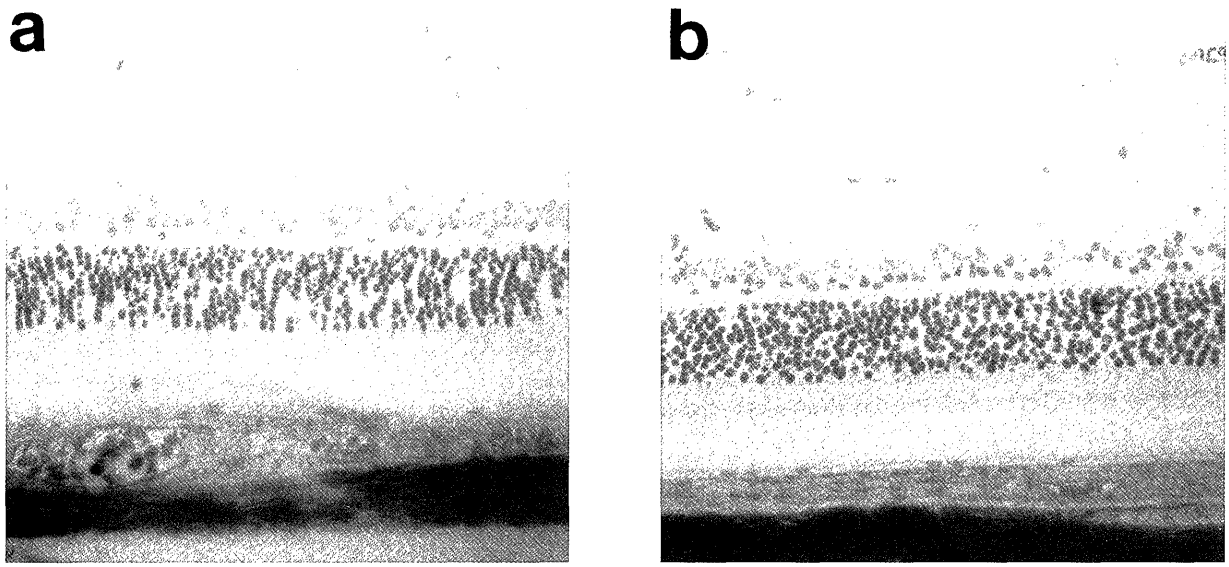

Figure 2 Light micrographs of retinas of 1-month-old rats. There is no obvious difference between a Cu-injected (a) and a control rat (b) (toluidine blue, ×400).

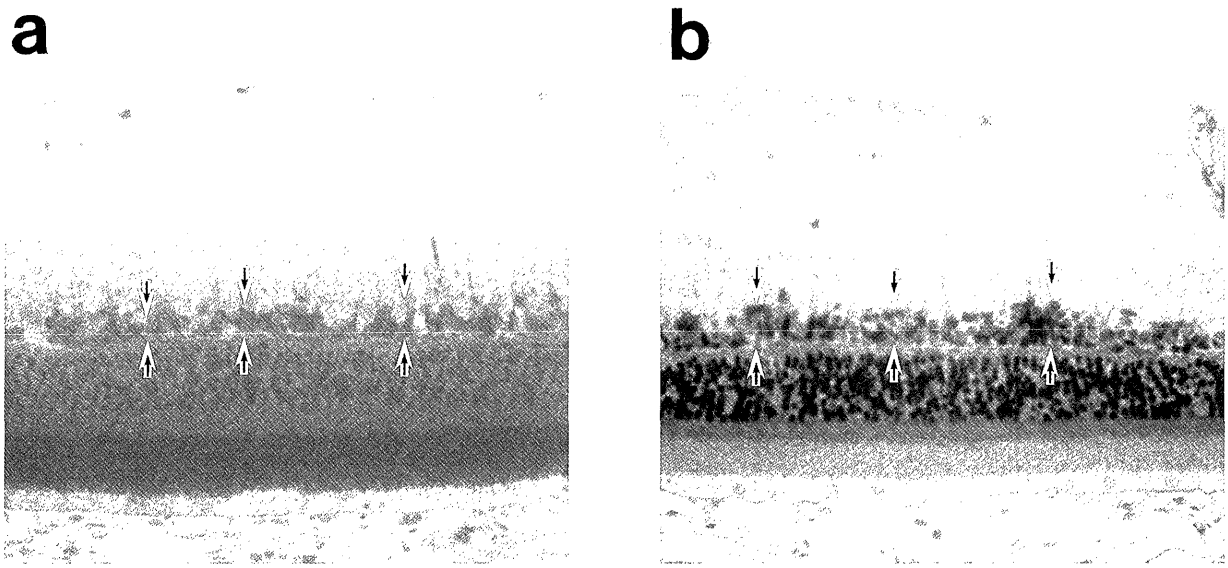

Figure 3 Light micrographs of retinas of 10-month-old rats. (a) The retina of a Cu-injected rat shows a decrease of cells in the inner nuclear layer (arrows). (b) The retina of a control rat is thicker than that of a Cu-injected rat (arrows) (toluidine blue, ×400).

Table 2 Number of cells in the inner nuclear layer per 1600μm^2 field at the posterior pole of the retina (mean±SD).

	Cu-injected	Control
1-month-old rats (n=5)	75.0±8.2	79.6±5.0
10-month-old rats (n=7)	45.0±7.2*	73.6±4.5*

There is a significant difference between 10-month-old Cu-injected and control rats (*P<0.001).

significant difference in the number of cells in the inner nuclear layer between Cu-injected and control rats.

Electron Microscopic Findings

In the inner nuclear layer of the 10-month-old Cu-injected rats, TEM showed clumps of chromatin and pyknosis in the nuclei (Figure 4). High density deposits were present in secondary lysosomes in the apical portion of the retinal pigment epithelium (RPE) (Figure 5). These deposits in the 10-month-old Cu-injected rats were analyzed with EDXA and found to contain iron,

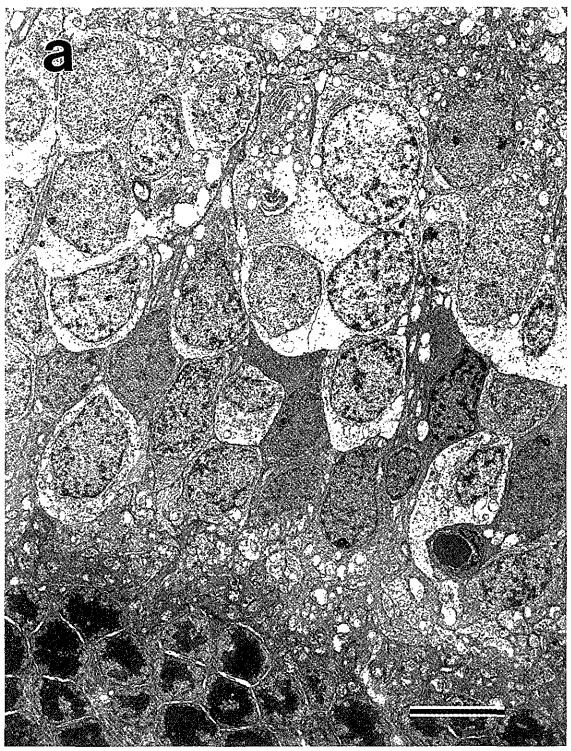

Figure 4a TEM of inner nuclear layer of a 1-month-old Cu-injected rat. There are no abnormal changes in the nuclei in the inner nuclear layer (Bar=5μm, ×1500).

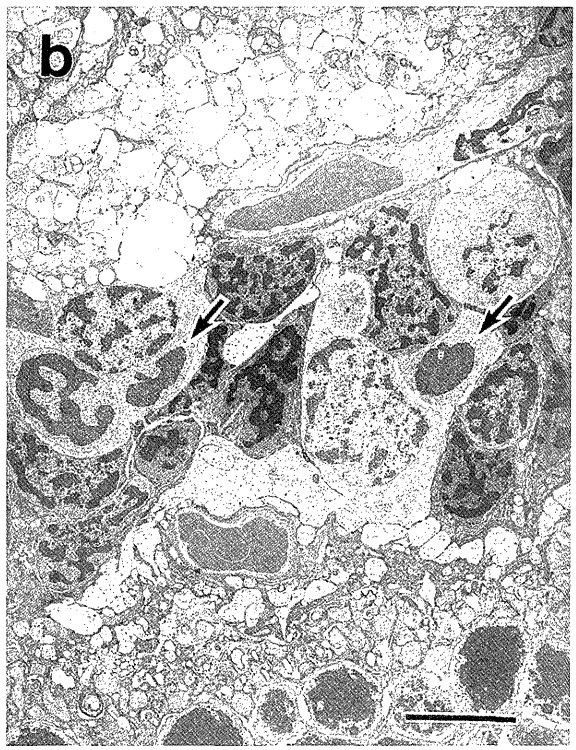

Figure 4b TEM of inner nuclear layer of a 10-month-old Cu-injected rat. The number of cells is definitely decreased and the nuclei show irregularly shaped clumps of chromatin and pyknosis (arrows) (Bar=5μm, ×2000).

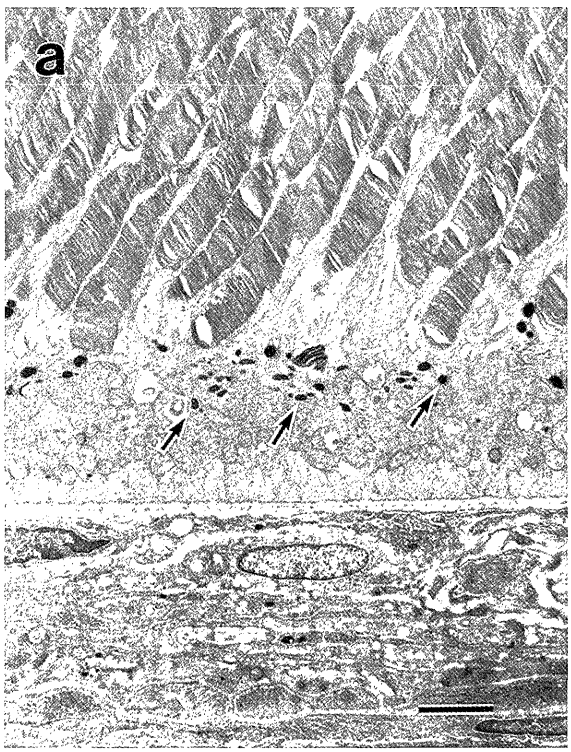

Figure 5a TEM of the RPE of a 1-month-old Cu-injected rat. Although high density secondary lysosomes (arrows) are seen in the RPE, no iron peaks were detected with EDXA (Figure 6a) (Bar=2μm, ×3000).

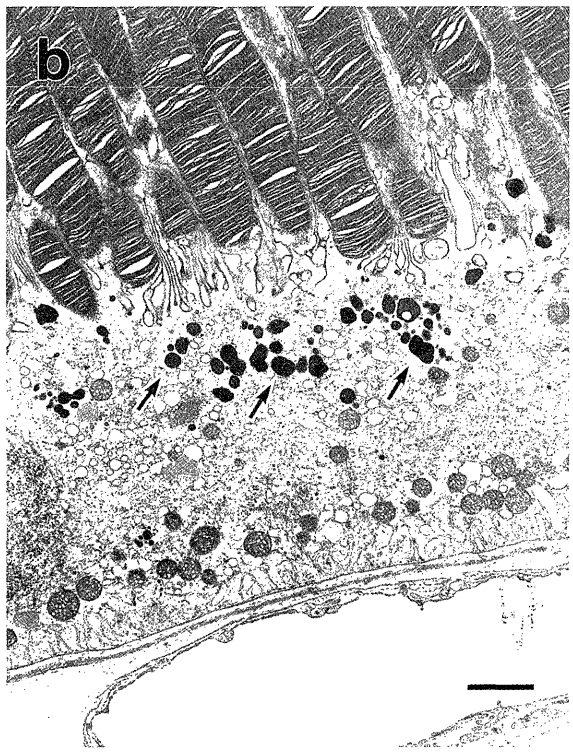

Figure 5b TEM of the RPE of a 10-month-old Cu-injected rat. High density deposits (arrows) are seen in secondary lysosomes in the apical portion of the RPE and show iron peaks with EDXA (Figure 6c) (Bar=2μm, ×3000).

but not copper (Figure 6-c). No significantly high copper and iron peaks were detected in the 1-month-old Cu-injected rats (Figure 6-a,b,d). Photoreceptor cells showed no abnormal ultrastructural changes in either of the Cu-injected groups.

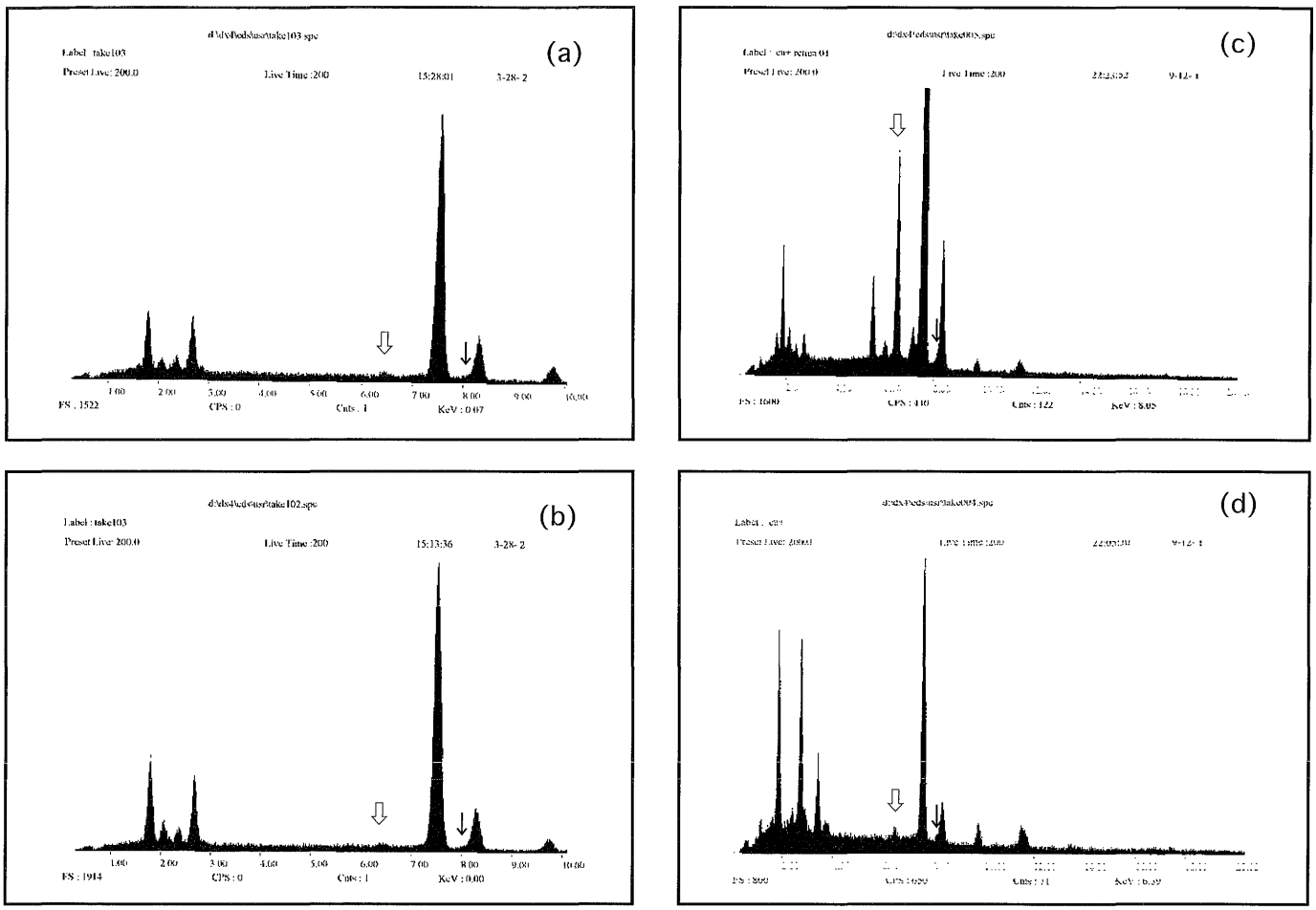

Figure 6 Representative spectrum of deposits in the RPE of a 1-month-old Cu-injected rat(a), a 1-month-old control rat(b), a 10-month-old Cu-injected rat(c), and a 10-month-old control rat(d). Arrows show the iron and copper peaks (⇩: Fe ⬇: Cu).

Discussion

In this experiment, regardless of the dose of copper administered to rats for 4 weeks, copper accumulation was not evident in the retina. Normal hepatocytes eliminate the extra copper in the bile and can store the copper with metallothionein if the dose is reasonable (9,10). Although the Cu-injected rats in the present study seemed clearly to have copper poisoning because of weight loss and a high death rate at 1 month of age, copper was not detected in the retina with EDXA but iron was evident. We cannot explain this discrepancy, but we can present a hypothesis.

In this study EDXA detected iron, not copper, in the RPE. It is known that copper catalyzes a free-radical reaction which leads to the decomposition of fatty acids of membrane phospholipids and thus to oxidative damage to red blood cells (11-14). Hypercupremia may induce hemolysis and lead to iron accumulation. Toyokuni (15) reported hemolysis and liver damage in rats treated with copper doses equivalent to ours. The loss of ceruloplasmin synthesized in the liver may have influenced iron accumulation. Ceruloplasmin contains more than 95% of the copper in human plasma. It is synthesized mainly in the liver. Ceruloplasmin can oxidize ferrous iron to ferric iron. In some experiments, ceruloplasmin was shown to play a role in the mobilization and oxidation of iron from tissue stores with subsequent incorporation of ferric iron into transferrin (16). Recent studies have shown that aceruloplasminemia characterized by mutations in the ceruloplasmin gene and iron accumulation in the basal ganglia as well as in parenchymal tissues in the central nervous system is caused by a total deficiency of ceruloplasmin ferroxidase activity (17-21). In our experiment the loss of ceruloplasmin might have been caused by liver damage which

prevented iron transfer and led to iron accumulation, because the retina is part of the central nervous system.

Iron accumulation only in the RPE may be due to the following: TEM studies (22-24) on ocular siderosis in animals and humans have demonstrated iron accumulation in the secondary lysosome as a siderosome. This is in accord with our findings. According to these reports, histochemistry showed diffuse iron in the sensory retina as well as in the RPE. EDXA can detect elements only in high electron dense deposits. Thus, fine iron accumulations may be present in other layers of the retina. TEM showed deformed nuclei in the inner nuclear layer. We speculate that the free radical reaction induced by iron may provoke such a nuclear change. Ferrous iron is the electron donor in the presence of hydrogen peroxide and generates a hydroxyl radical. The retinal damage of siderosis and aceruloplasminemia have been considered to share the same mechanism. Thus the retinal changes may be due to secondary iron accumulation induced by Cu excess.

It is not clear why the retinal changes occurred only in 10-month-old Cu-injected rats, but not in 1-month-old rats receiving the same dose. We speculate that young rats died prior to a great amount of iron accumulation in the RPE because of the lower 50% lethal dose in young rats. In spite of $CuCl_2$ injection, survived one-month-old rats might be resistant to Cu toxicity. In addition, Cu intoxication occurs suddenly after long-term administration of Cu and the effects of Cu exposure might be indirect rather than direct, due to Cu accumulation.

Although free radicals tend to impair first the photoreceptor outer segment which is rich in unsaturated fatty acids, the inner nuclear layer receives the greater damage, so the number of cells in this experiment was decreased. Generally speaking, toxic effects appear first at the photoreceptor outer segment discs. For instance, siderosis (23) and aluminum toxicity (25) destroy the outer segment disc severely. However, secondary siderosis due to Cu excess represents another aspect of morphological damage. Although we cannot describe the mechanism completely, we assume that the retinal damage in the present study is due to iron accumulation, probably affected in part by long-term Cu excess. Further studies are required.

Experimental hypercupremia may induce secondary siderosis in the rat retina.

Acknowledgment

The author wishes to thank Professor Tsugio Amemiya, Department of Ophthalmology and Visual Sciences, Graduate School of Biomedical Sciences Nagasaki University, for his valuable suggestions and advices.

References

(1) O'Dell BL, Sunde RA: Handbook of nutritionally essential mineral elements. (Marcel Dekker Inc., New York) pp. 231-241, 1997
(2) Sternlieb I, Morell AG, Scheinberg IH: The remarkable selectivity of hepatocytes in the uptake of glycoproteins. *Gastroenterology* 64:1049-1052, 1973
(3) Nittis T, Gitlin JD: The copper-iron connection: Hereditary aceruloplasminemia. *Semin Hematol* 39 : 282-289, 2002
(4) Ogra Y, Suzuki K: Intracellular trafficking and regulation of copper in mammalian cells. *Biomed Res Trace Elements* 13:3-10, 2002
(5) Ozawa M, Aoki T: Inherited neurological disease-relationship between an essential trace elements and Wilson/Menkes disease. *Nippon Rinsho* 54:117-122, 1996
(6) Tso MO, Fine BS, Thorpe HE: Kayser-Fleischer ring and associated cataract in Wilson's disease. *Am J Ophthalmol* 79:479-488, 1975
(7) Johnson RE, Campbell RJ: Wilson's disease. Electron microscopic, x-ray energy spectroscopic, and atomic absorption spectroscopic studies of corneal copper deposition and distribution. *Lab Invest* 46:564-569, 1982
(8) Innes JR, Strachan IM, Triger DR: Unilateral Kayser-Fleischer ring. *Br J Ophthalmol* 70:469-470, 1986
(9) Owen CA Jr, Hazelrig JB: Metabolism of Cu64 labelled copper by the isolated rat liver. *Am J Physiol* 210:1059-1064, 1964
(10) Owen CA Jr: Absorption and excretion of Cu64 labelled copper by the rat. *Am J Physiol* 210:1203-1206, 1964
(11) Sayato Y, Nakamuro K, Hasegawa T: Lipid peroxidation induced by copper in rat erythrocytes. *Eisei Kagaku* 37:137-142, 1991
(12) Sansinanea A, Cerone S, Najle R: Lipid peroxidation in erythrocyte membranes of sheep with chronic copper poisoning. *J Clin Biochem Nutr* 17:65-72, 1994
(13) Saenko EL, Skorobogat'ko OV, Yaropolov AI: Interrelation between structure and protective action of normal and pathological ceruloplasmins during copper-induced lysis of red blood cells. *Biochem Int* 22:749-757, 1990
(14) Forman SJ, Kumar KS, Redeker AG, Hochstein P: Hemolytic anemia in Wilson disease: clinical findings and biochemical mechanisms. *Am J Hematol* 9:269-275, 1980
(15) Toyokuni S, Okada S, Hamazaki S, Fujioka M, Li JL, Midorikawa O: Cirrhosis of the liver induced by cupric nitrilotriacetate in Wistar rats. An experimental model of copper toxicosis. *Am J Pathol* 134:1263-1274, 1989
(16) Frieden E, Hsieh HS: The biological role of ceruloplasmin and its oxidase activity. *Adv Exp Med Biol* 74:505-529, 1976
(17) Harris ZL, Klomp LW, Gitlin JD: Aceruloplasminemia: an inherited neurodegenerative disease with impairment of iron homeostasis. *Am J Clin Nutr* 67:972-977, 1998
(18) Miyajima H: Aceruloplasminemia. *Rinsho Shinkeigaku* 40:1290-1292, 2000
(19) Miyajima H, Fujimoto M, Kohno S, Kaneko E, Gitlin JD: CSF abnormalities in patients with aceruloplasminemia. *Neurology* 51:1188-1190, 1998
(20) Gitlin JD: Aceruloplasminemia. *Pediatr Res* 44:271-276, 1998
(21) Okamoto N, Wada S, Oga T, Kawabata Y, et al: Hereditary ceruloplasmin deficiency with hemosiderosis. *Hum Genet* 97:755-758, 1996
(22) Virata SR, Kylstra JA, Peiffer RL: The ocular effects of intralenticular iron foreign bodies in rabbits. *Ophthalmic Surg* 26:142-144, 1995
(23) Kishimoto N, Ohkuma H, Uyama M: Detection of destruction of anionic sites in the outer blood-retinal barrier and damage caused by iron. *Nippon Ganka Gakkai Zasshi* 95:130-139, 1991
(24) Welch RB: Two remarkable events in the field of intraocular foreign body: (1)The reversal of siderosis bulbi. (2)The spontaneous extrusion of an intraocular copper foreign body. Trans Am *Ophthalmol Soc* 73:187-203, 1976
(25) Lu ZY, Gong H, Amemiya T: Aluminium chloride induces retinal change in the rat. *Toxicol Sci* 66:253-260, 2002