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Optic Nerve Changes in Ascorbic Acid-deficient Rats

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Purpose: To examine changes in the optic nerves of mutant Wistar rats (OD rats) with a hereditary defect in ascorbic acid-synthesizing ability, since there is a lack of information on the role of ascorbic acid in the optic nerve.

Methods: OD rats fed a ascorbic acid-deficient diet and deionized water starting 20 days after birth were killed 3 weeks after the start of the experiment. Control rats were fed a usual diet and deionized water containing 2 mg/ml of ascorbic acid. In the recovery experiment, rats fed on a usual diet and deionized water for 3 weeks after weaning were then fed the same deficient diet for 2 more weeks and deionized water containing 2 mg/ml of ascorbic acid. Pair-fed control OD rats received the same amount of usual diet as the experimental OD rats ate on the previous day with free access to deionized distilled water containing 2 mg/ml of ascorbic acid. The optic nerve was examined by transmission electron microscopy.

Results: The ultrastructural study showed that in the ascorbic acid-deficient rats the number of myelinated nerve fibers in the optic nerve was significantly decreased and the lamellae of the myelinated axons were thin. In the recovery rats the abnormal morphology persisted. The optic nerve of the pair-fed control rats showed no abnormal findings.

Conclusion: These findings suggest that the optic nerve needs ascorbic acid for the maintenance of its cell structure probably due to the antioxidative action of ascorbic acid on the lipid membrane of myelin lamellae.

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Key Words: OD rats, ascorbic acid-deficiency, ascorbic acid, optic nerve, electron microscopy

Introduction

It is well known that a deficiency of ascorbic acid induces scurvy and hemorrhages in ocular tissues and mucous membranes[1]. Ascorbic acid is also essential for the formation of the mucopolysaccharide components of the ground substance of connective tissue, particularly hydroxyprolin. This may be related to corneal wound healing. Ascorbic acid is reported to be a coenzyme in collagen metabolism[2]. The antioxidant ascorbic acid is known to prevent cerebral tissue degeneration[3]. In spite of these important roles of ascorbic acid, many physiological aspects of ascorbic acid remain obscure. Most animals except humans, other primates and guinea pigs are capable of synthesizing ascorbic acid from carbohydrate precursors. Since most species of animals can produce ascorbic acid in the body, it is difficult to study the effect of ascorbic acid deficiency in the eyes of experimental animals. A colony of mutant Wistar rats (OD rats) with a hereditary defect in ascorbic acid-synthesizing ability has been established[4,5]. To study the role of ascorbic acid in the optic nerve, we examined by electron microscopy the morphological changes in the optic nerve of OD rats.

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Materials and Methods

Animals and Diets

OD rats with a hereditary defect in ascorbic acid-synthesizing ability (Nippon Clea Incorporated, Tokyo) were fed a usual diet containing no ascorbic acid and deionized distilled water starting 20 days after birth. These rats become ascorbic acid deficient since they cannot produce ascorbic acid. Ascorbic acid deficient rats were killed 3 weeks after the start of the experiment because they rarely survive longer. Control rats were fed a usual diet and deionized water containing 2 mg/ml of ascorbic acid. The rats were killed under Nembutal anesthesia. Blood was drawn from the heart or aorta for measurement of the serum levels of ascorbic acid, Cu, Zn and Mg by a standard atomic absorption spectrophotometer. The eyes were enucleated and the optic nerves were examined by electron microscopy. In the recovery experiment rats were fed the deficient diet for 3 weeks after weaning and then given

deionized water containing 2 mg/ml of ascorbic acid for 2 weeks. The animals were treated according to the ARVO resolution on Use of Animals in Research. Rats were housed in cages with stainless steel wire bottoms and maintained at a constant room temperature of 21° C with 12 h light and dark cycles at the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine. In the pair-fed control group, infant rats were suckled by their normal mother rats for 3 weeks, then given the same amount of usual diet that ascorbic acid-deficient rats took on the previous day with free access to water containing 2 mg/ml of ascorbic acid. They were killed under Nembutal anesthesia at 6 weeks of age.

Electron Microscopy

Immediately after Nembutal anesthesia, the eyes were enucleated rapidly, and the optic nerves were removed during fixation with 4% glutaraldehyde in 0.05 M cacodylate buffer for 1h and postfixed in 1% osmium tetroxide in veronal acetate buffer for 1h after an overnight washing with 0.05 M cacodylate buffer containing 0.44 M sucrose. The fixed materials were dehydrated through a series of ethanols. Optic nerves were embedded in Luveak 812. Ultrathin sections were made with a Porter-Blum MT 2 microtome (Sorvall, Norwalk, CT) and examined with Hitachi H300 (Tokyo, Japan) and JEOL JEM-1210 (Tokyo, Japan) electron microscopes. Ultrathin sections for electron microscopy were cut from the optic nerves about 3 mm behind the globe. The myelinated axons per 600 μm^2 field at the central part of the optic nerve were counted on electron micrographs at a magnification of 2,000. The diameter of a myelinated axon was measured by the method of Trapp and Bernsohn[6]. The thickness and number of myelin lamellae were measured on enlarged electron micrographs at a magnification of 30,000 with a slide caliper.

Statistical Analysis

Data were analyzed by unpaired Student's t-test and Fisher's test with least significant difference. Differences were considered significant at $P<0.05$. All values represent mean ± SD.

Results

Overt Characteristics

Growth retardation was noted in the ascorbic acid-deficient rats by the end of the first week of the experiment. After 3 weeks on the diet, the ascorbic acid-deficient rats were much smaller than the controls (Fig. 1). Most of the ascorbic acid-deficient rats showed hair loss and hemorrhages around the eyes and gait disturbance, and some had conjunctival secretions. By the end of the 5th week of the experiment the ascorbic acid-deficient rats died. The pair-fed control rats were small, but looked healthy.

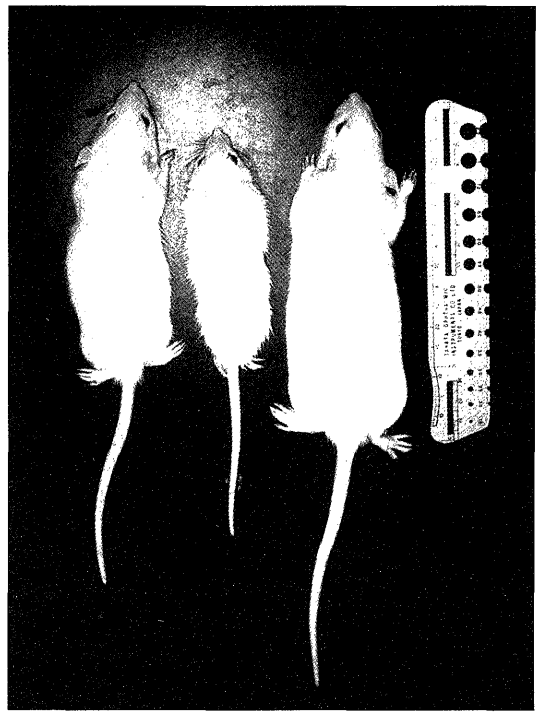

Figure 1. Gross appearance of the animals.
There are obvious differences in a rat which has recovered from ascorbic acid deficiency (left) (5 weeks after weaning), an ascorbic acid-deficient rat (center) (3 weeks after weaning) and a control rat (right) (3weeks after weaning).

Body Weight

The body weight of the ascorbic acid-deficient rats was significantly lower than that of the controls ($p<0.0001$) (Table 1). The pair-fed control rats also

Table 1. Body weight

Groups (n=12)	Weeks after start of experiment 1	3	5
Control	34.2±5.3	96.7±8.8* ** ***	161.1±13.2* **
Ascorbic acid-deficient	30.9±4.9	31.4±6.4*	
Recovery	31.4±3.2	31.7±9.9 **	65.5±6.3*
Pair-fed control	30.3±4.6	34.9±4.4 ***	68.3±7.7 **

*, **, *** P<0.0001

weighed significantly less than the controls ($p<0.0001$) (Table 1).

Serum Ascorbic Acid and Trace Elements

At the end of the 3rd week the serum ascorbic acid level of the ascorbic acid-deficient rats was undetectable; in the control group it was 3.7 μg/L. At the end of the 2 weeks of recovery, the serum ascorbic acid level was 2.3 μg/L. The serum ascorbic acid level of the pair-fed control rats was 2.3 μg/L (Table 2). There was no significant difference in the serum ascorbic acid level between the recovery and control groups. There was no significant difference in the serum levels of Zn, Cu and Mg in ascorbic acid-deficient, control and pair-fed control rats (Table 3).

Table 2. Serum ascorbic acid level

Groups	n	serum ascorbic acid (μg/ml)
Control	12	3.7±2.4
Ascorbic acid-deficient	12	undetectable
Recovery	12	2.3±0.6
Pair-fed control	12	2.3±1.0

Serum ascorbic acid level is not significantly different in control, recovery and pair-fed control groups ($p>0.1$).

Table 3. Serum Zn, Cu and Mg levels

Groups	n	serum Zn level (μg/ml)	serum Cu level (μg/ml)	serum Mg level (μg/ml)
Control	12	121±37	114±19	4.0±1.3
Ascorbic acid-deficient	7*	119±17	108±9	4.1±1.2
Pair-fed control	12	131±9	119±16	3.2±0.6

* Enough blood was not taken in 5 rats.
Serum Zn , Cu and Mg levels are not significantly different in control, ascorbic acid-deficient and pair-fed control groups ($p>0.1$).

Electron Microscopic Findings

TEM revealed significantly fewer myelinated axons in the optic nerves of ascorbic acid-deficient [Fig. 2(B) and 3(B)] and ascorbic acid-recovery rats [Fig. 2(C) and 3(C)] than in those of control rats [Figure 2(A) and 3(A)] (Table 4). In the controls [Fig. 3(A)] and pair-fed control rats [Fig. 3(D)], the axons of the myelinated optic nerve fibers contained mitochondria and neurotubules, but ascorbic acid-deficient [Fig. 3(B)] and recovery rats [Fig. 3(C)] had vacuoles and destroyed mitochondria and tubules. The fiber density in the myelinated axons of ascorbic acid-deficient [Fig. 3(B)] and ascorbic acid-recovery rats [Fig. 3(C)] was obviously decreased. Unmyelinated axons in the optic nerve were more numerous in the ascorbic acid-deficient [Fig. 2(B) and 3(B)] and ascorbic acid-recovery rats [Fig. 2(C) and 3(C)] than in the control rats [Fig. 2(A) and 3(A)]. In transverse sections of ascorbic acid-deficient [Fig. 3(B)] and ascorbic acid-recovery rats [Fig. (3C)], the myelin sheaths of optic nerves were thin, and undeveloped axons were numerous. The diameter of myelinated fibers was not significantly different between the ascorbic acid-deficient and recovery groups and the control and pair-fed control groups ($p>0.1$) (Table 5). Myelin sheath thickness and number of lamellae in ascorbic acid-deficient and ascorbic acid-recovery rats were significantly different from those of the control and pair-fed control rats ($p<0.01$) (Table 6 and 7). The myelin index (ratio of the axon diameter to the myelinated nerve fiber diameter) in ascorbic acid-deficient and ascorbic acid-recovery rats was significantly larger than that in the control and pair-fed control rats ($p<0.01$) (Table 8). The numbers of myelinated axons and of the lamellar

Table 4. Number of myelinated axons in optic nerve (600 μ m^2)

Groups	Number of fields examined	Number of myelinated axons
Control	121	147±20 *
Ascorbic acid-deficient	133	110±18 * ** ***
Recocery	96	114±18 ***
Pair-fed control	78	148±35 **

* $P<0.0001$, ** $P<0.0001$, *** $P=0.06$.

Table 5. Diameter of myelinated nerve fibers in optic nerve (μm)

Groups	Number of myelinated fibers counted	Diameter
Control	154	0.81±0.25
Ascorbic acid-deficient	164	0.76±0.31
Recocery	146	0.78±0.28
Pair-fed control	123	0.84±0.32

The diameter of myelinated fibers in the 4 groups is not significantly different ($p>0.1$).

Table 6. Thickness of myelin sheath in optic nerve (nm)

Groups	Number of myelin sheath counted	Thickness
Control	123	142.1±33.9 *
Ascorbic acid-deficient	137	81.1±33.8 * ** ***
Recocery	124	88.5±38.5 ***
Pair-fed control	115	143.7±39.5 **

* $P<0.0001$, ** $P<0.0001$, *** $P=0.103$

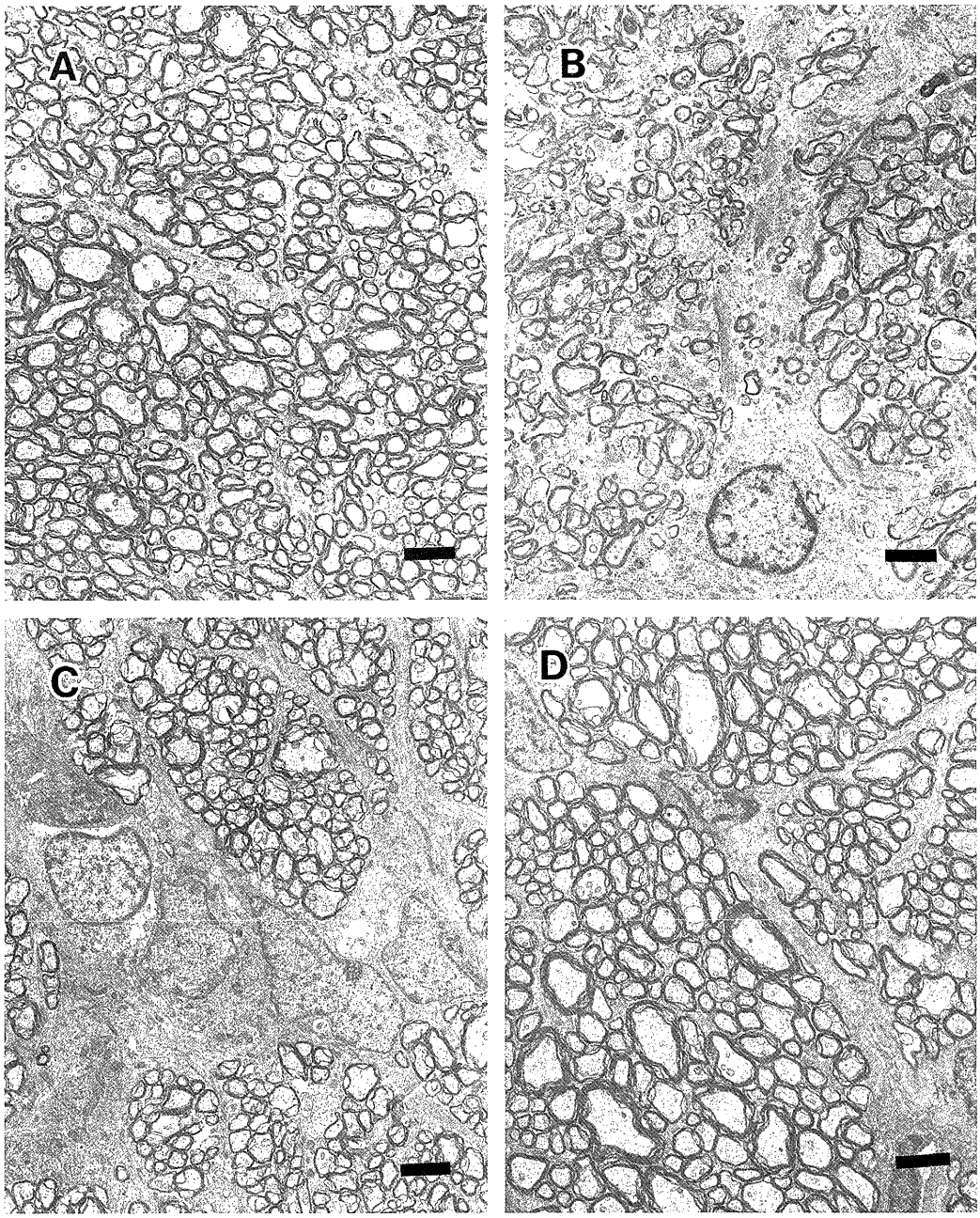

Figure 2A,B,C,D. Electron micrographs of transverse sections of optic nerves.
In a control rat (A) and a pair-fed control rat (D) myelin lamellae are compactly arranged. In an ascorbic acid-deficient rat (B), fewer axons and undeveloped myelin are seen. In an ascorbic acid-recovery rat (C), fewer axons and undeveloped myelin are seen. Bar=4 μ m

Table 7. Thickness of lamellar layers of myelinated fibers in optic nerve (nm)

Groups	Number of myelin sheath counted	Number of layers			
Control	148	10.9±2.8	*		
Ascorbic acid-deficient	154	5.6±2.7	*	**	***
Recocery	136	5.4±2.3			***
Pair-fed control	115	11.8±3.2		**	

* P<0.0001, ** P<0.0001, *** P=0.197

Table 8. Myelin index (ratio of axon diameter to fiber diameter)

Groups	Number of myelinated nerve fibers counted	Myelin index			
Control	154	0.71±0.12	*		
Ascorbic acid-deficient	164	0.81±0.09	*	**	***
Recovery	146	0.80±0.13			***
Pair-fed control	123	0.72±0.15		**	

* P<0.0001, ** P<0.0001, *** P=0.527

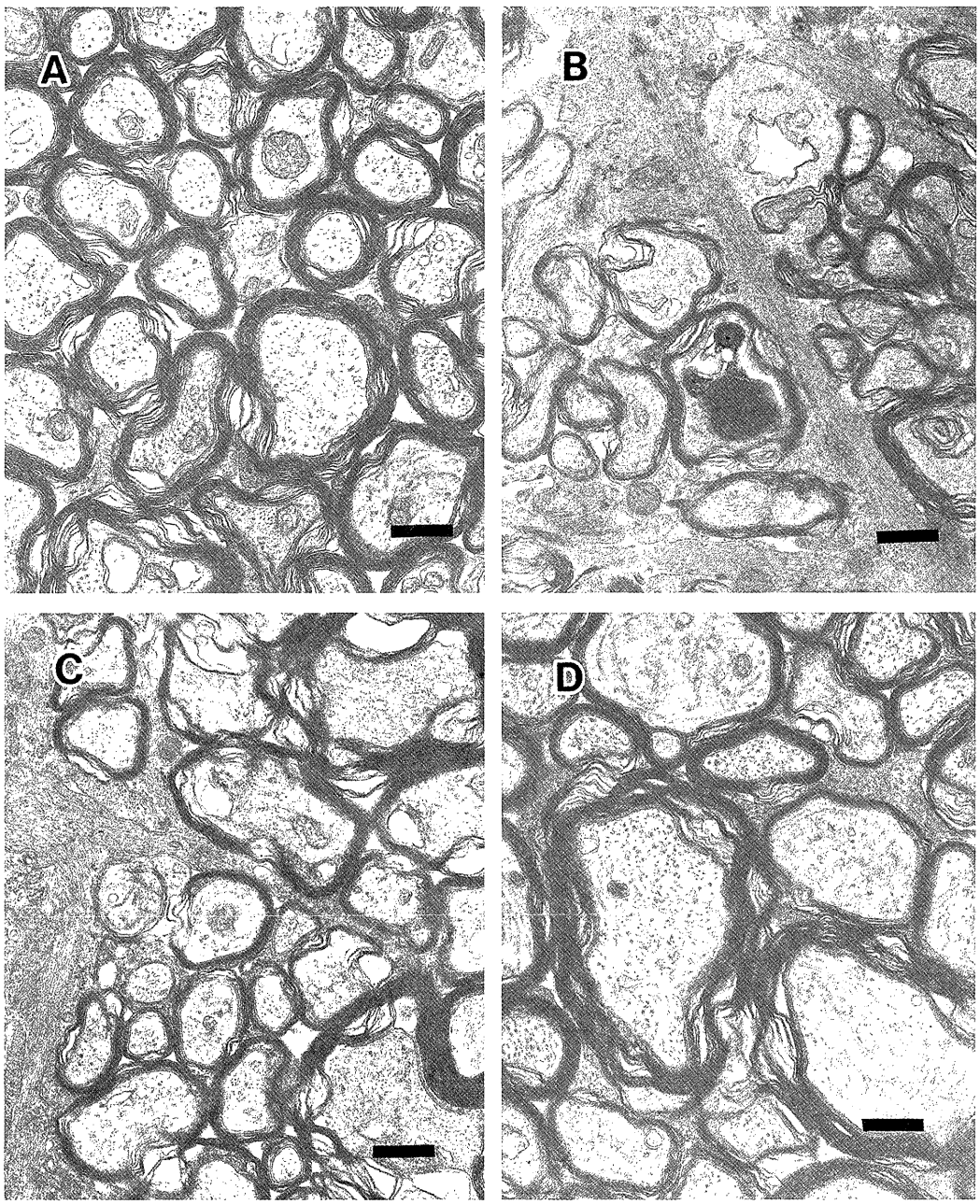

Figure 3A,B,C,D. High magnification of electron micrographs of transverse sections of optic nerves. In a control rat (A) and a pair-fed control rat (D) the myelin sheaths are thick, and the arrangement of myelin lamellae is regular. The axon contains mitochondria and microtubules, but no vacuoles. In an ascorbic acid-deficient rat (B) and an ascorbic acid-recovery rat (C) the myelin sheaths are thin and the number of lamellae is obviously decreased. The axon contains destroyed mitochondria and microtubules and vacuoles. Bar=500nm

layers of myelinated fibers and the thickness of the myelin sheath in the optic nerves were significantly reduced in the recovery and ascorbic acid-deficient rats, compared with those of the control and pair-fed control rats (Table 4, 5, 6 and 7). The optic nerves of control and paired-fed control rats showed no abnormalities.

Discussion

In this study, the serum ascorbic acid level was significantly lower in the ascorbic acid-deficient rats than in the control group ($P<0.005$), and the weight gain in ascorbic acid-deficient rats was lower than in the controls. OD rats fed a usual diet with deionized water developed ascorbic acid deficiency, a low serum level

of ascorbic acid, poor weight gain and physical abnormalities, which have been noted in other reports[2,5], and rarely survived longer than 3 weeks after ascorbic acid-deficient feeding was started.

Electron microscopy of the optic nerve confirmed that the rats fed a usual diet and deionized water in the present study were certainly deficient in ascorbic acid. In the pair-fed control group the optic nerve showed none of the histopathologic changes seen in the ascorbic acid-deficient rats. Thus, changes in the ascorbic acid-deficient rats were not due to maldevelopment.

In this study, the numbers of fibers and of myelin lamellae in the optic nerves were significantly lower in the ascorbic acid-deficient rats than in the control and pair-fed control rats. Even when the ascorbic acid-deficient rats were supplemented with ascorbic acid, their optic nerves did not recover: the numbers of myelinated axons and of lamellar layers of myelinated nerve fibers, and the thickness of myelin sheaths in optic nerves was not significantly different between the ascorbic acid-deficient and the recovery rats. It appears that axons continue to degenerate after the resumption of a normal diet and water supplemented with sufficient ascorbic acid. This means that when the CNS is once destroyed it never recovers.

Our series of studies on the effect of deficiencies of several trace elements showed similar results in the present study[7-10]. One of the assumed causes is a decrease of superoxide dismutases which contain Zn, Cu and Mg. In the present study, however, Zn, Cu and Mg were not reduced in ascorbic acid-deficient condition. Thus the effect of Zn, Cu and Mg on the optic nerve may be neglected.

Antioxidant systems of the CNS act in concert to protect cerebral tissue from oxidative damage. Ascorbic acid and glutathione act as a first defense line in this process, serving as redox buffers and free radical scavengers to limit the reactivity of radical intermediates generated by aerobic metabolism[11]. Both are effective scavengers of peroxyl and hydroxyl radicals, superoxide anion, and singlet oxygen[12,13]. Through their action against radicals generated in the aqueous phase, ascorbic acid and glutathione peroxidase protect and recycle α-tocopherol and prevent membrane lipid peroxidation[14,15]. Since myelin is a lipid membrane, the disturbance of these preventive mechanisms by ascorbic acid-deficiency may lead to decreased numbers of fibers and myelin lamellae in the optic nerve. Such actions of ascorbic acid on the myelinated nerve have not been reported yet. The optic nerve needs ascorbic acid for the maintenance of its cell structure.

There is a report on the possible involvement of ascorbic acid in the regulation of myelin gene activity in the CNS[16]. But, to best of our knowledge, it has not been reported that ascorbic acid is need for the development of myelinated nerve fibers. We assume that the same disturbance due to ascorbic acid deficiency could occur in nerves other than the optic nerve.

References

1. Hoyt CS 3d. Vitamin metabolism and therapy in ophthalmology. *Surv Ophthalmol* 24: 177-190, 1979
2. Kurata T. Ascorbic acid. Japan Vitamin Soc ed., Handbook of Vitamin, Water soluble vitamins, Kagaku Dojin, Kyoto. 171-191, 1989
3. Rice ME, Lee EJ, Choy Y. High levels of the ascorbic acid, not glutathione, in the CNS of anoxia-tolerant reptiles contrasted with levels in anoxia-intolerant species. *J Neurochem* 64:1790-1799, 1995
4. Mizushima Y, Harauchi T, Yoshizaki T, Makino S. A rat mutant unable to synthesize ascorbic acid. *Experientia* 40; 359-361, 1984
5. Horio F, Ozaki K, Yoshida A, Makino S, Hayashi Y. Requirement for ascorbic acid in a rat mutant unable to synthesize ascorbic acid. *J Nutr* 115:1630-1640, 1985
6. Trapp BD, Bersohn J. Essential fatty acid deficiency and CNS myelin. Biochemical and morphological observations. *J Neurol Sci.* 37: 249-266, 1978
7. Dake Y, Amemiya, T. Electron microscopic study of the optic nerve in copper deficient rats. *Exp Eye Res* 58: 277-81, 1991
8. Mishima K, Dake Y, Amemiya T, Nishimura M. Electron microscopic study of optic nerves of macular mice. *Exp Eye Res* 63: 85-90, 1996
9. Gong H, Amemiya T. Optic nerve changes in manganese-deficient rats. *Exp Eye Res* 68: 313-320, 1999
10. Gong H, Amemiya T. Optic nerve changes in zinc-deficient rat. *Exp Eye Res* 72: 363-369, 2001
11. Meister A. On the antioxidant effect of ascorbic acid and glutathione. *Biochem Pharmacol* 44: 1905-1915, 1992
12. Nishikimi M. Oxidation of ascorbic acid with superoxide anion generated by the xanthine-xanthine oxidase system. *Biochem Biophys Res Comm* 63: 463-468, 1975
13. Basaga HS. Biochemical aspects of free radicals. *Biochem Cell Biol* 68: 989-998, 1990
14. Seregi A, Schafer A, Komlos M. Protective role of ascorbic acid content against lipid peroxidation. *Experientia* 34: 1056-1057, 1978
15. Vatassery GT, Smith WE, Quach HT. Ascorbic acid, glutathione and synthetic antioxidants prevent the oxidation of vitamin E in platelets. *Lipids* 1043-1047, 1989
16. Laszkeiwicz I, Wiggins RC, Konat G. Ascorbic acid upregulates myelin gene expression in C6 glioma cells. *Metab Brain Dis* 7: 157-164, 1992