Effect of Long-Term Treatment of L-Ornithine on Visual Function and Retinal Histology in the Rats

Kenji Sakamoto,*a Asami Mori,a Tsutomu Nakahara,a Masahiko Morita,b and Kunio Ishiia

a Department of Molecular Pharmacology, Kitasato University, School of Pharmaceutical Sciences; 5–9–1 Shirokanedai, Minato-ku, Tokyo 108–8641, Japan; and b Healthcare Products Development Center, Kyowa Hakko Bio Co., Ltd.; 2 Miyukigaoka, Tsukuba, Ibaraki 305–0841, Japan.

Received July 4, 2014; accepted October 5, 2014

L-Ornithine (L-Orn) is one of non-proteinogenic amino acids, abundant in freshwater clams and commercially available as an oral nutritional supplement. L-Ornithine is metabolized by ornithine-δ-aminotransferase. Deficiency of this enzyme causes gyrate atrophy of the choroid and retina, an autosomal recessive hereditary disease characterized by the triad of progressive chorioretinal degeneration, early cataract formation, and type II muscle fiber atrophy, with hyperornithinemia. However, it is unknown whether long-term L-ornithine supplementation affects visual function and retinal histology. The aim of the present study is to determine the effect of long-term supplementation of excess amounts of L-ornithine on visual function and retinal histology in rats. Male Brown Norway rats at six weeks of age were allowed free access to chow containing 4% (w/w) L-ornithine (the high ornithine diet) or that containing 4% (w/w) casein (the control diet) for 49 weeks. The dose of L-ornithine calculated from the food intake was approximately 0.8 g/d/animal, which was 100 times higher than the recommended dose for healthy humans. The amplitude of the a-wave of the scotopic rod-cone electroretinogram and the number of cells in the ganglion cell layer in the L-ornithine-treated group were larger than those in the control group 49 weeks after initiating the test diet. No functional or histological damage to the retina was seen up to 49 weeks after the start of the high-ornithine diet. The present study demonstrated that long-term supplementation of very high doses of L-ornithine for at least 49 weeks did not induce retinal damage.

Key words L-ornithine; gyrate atrophy of the choroid and retina; retinal ganglion cell

* To whom correspondence should be addressed. e-mail: sakamotok@pharm.kitasato-u.ac.jp

© 2015 The Pharmaceutical Society of Japan
Regulations for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kitasato University. Male Brown Norway rats at six weeks of age were purchased from Charles River Japan (Yokohama, Japan). The environment of the animal room was kept at 25°C with a 12h:12h light–dark cycle. All animals were fed and watered ad libitum.

**Study Design** Before starting to feed the test diet, ERG was taken from all of the rats as described below. The rats were allowed free access to the rat chow containing 4% (w/w) 1-Orn (the high Orn diet) or that containing 4% (w/w) casein (the control diet) for 49 weeks. The chow containing 4% amino acid was previously used in the amino acid safety study for 12 weeks, and the dose of amino acid is almost maximum dose for the rats in the safety test for one year.22–24) ERG and blood samples were taken from all of the rats 16, 30, and 49 weeks after starting to feed the test diet. After measuring ERG and collection of blood samples at 49 weeks, the rats were sacrificed with an overdose of sodium pentobarbital, and both eyes were enucleated for histological analysis.

**Measurement of Plasma 1-Orn Concentration** In order to determine the plasma concentration of 1-Orn, blood samples were collected into heparinized tubes, and the plasma was separated by centrifugation. The plasma was deproteinized with sulfosalicylic acid (50 mg/mL plasma). Amino acid concentrations were quantified using an amino acid analyzer (JLC-500/V: JEOL, Tokyo, Japan) as described previously.25)

**Electroretinography** Scotopic ERG was recorded from animals that had been dark-adapted overnight. A contact lens electroencephalogram electrode with a white light-emitting diode (Mayo, Inazawa, Japan) was placed in contact with the electroencephalogram electrode with a white light-emitting diode (Mayo, Inazawa, Japan) was placed in contact with the retina. A contact lens electroencephalogram electrode, a reference electrode, and a grounding electrode were placed, as described previously.26–30) Background light intensity was 25.1 cd/m² for the test diet, and 25.8 cd/m² for the control diet. ERG and blood samples were taken from all of the rats 16, 30, and 49 weeks after starting to feed the test diet. After measuring ERG and collection of blood samples at 49 weeks, the rats were sacrificed with an overdose of sodium pentobarbital, and both eyes were enucleated for histological analysis.

**Histological Evaluation** Histological evaluation methods have been described previously.28–30) Briefly, animals were euthanized with an overdose of sodium pentobarbital. Both eyes were enucleated and fixed with a Davidson solution (37.5% ethanol, 9.3% paraformaldehyde, 12.5% acetic acid) for 24 h at room temperature. Fixed eyes were dissected through the optic nerve head in the vertical meridian with a microtome blade (PATH BLADE+PRO by Kai, Matsunami Glass, Kishiwada, Japan) and embedded in paraffin after the lens had been removed. We used a microtome (HM325, Microm International, Walldorf, Germany) and a microtome blade (PATH BLADE+PRO by Kai, Matsunami Glass) to make 4-μm thickness, horizontal sections through the optic nerve head. Sections were stained with hematoxylin and eosin and examined for morphometry. Using a light microscope (Optiphot-2, Nicon, Tokyo, Japan), the total number of the cells in the retinal ganglion cell layer (GCL) was manually counted in a region beginning 1 mm from the center of the optic nerve head and ending 1.25 mm from the center of the optic nerve head (for a retinal length of 0.25 mm). Digital photographs [digital camera (Senamal, Micronet, Kawaguchi, Japan) connected to a light microscope] were taken so that ca. 0.25 mm of retina appeared in each photograph, with sections ca. 1 mm from the center of the optic nerve head chosen. The thickness of the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL), and the outer nuclear layer (ONL) was then measured.

**RESULTS**

**Changes of Body Weight, Food Intake and Plasma 1-Orn Concentration** There was no significant difference in the body weight at the end the experimental period (Fig. 1A) and daily food intake throughout the experimental period (Fig. 1B) between the control diet group and the high Orn diet group. The mean food intake during the experimental period in the control diet group was 18.8±1.1 g/d/animal, and that in the high Orn diet group was 19.0±1.2 g/d/animal. The dose of 1-Orn calculated from the food intake was approximately 0.8 g/d/animal. The plasma 1-Orn concentration in the high Orn diet group was higher than that in the control diet group 16, 30 and 49 weeks after starting to feed the test diet (Fig. 1C).

**Electroretinography** There was no significant difference between the control diet group and high Orn diet group in the amplitudes of scotopic rod-cone a-wave and b-wave, and photopic cone b-wave, before starting to feed the test diet (Pre in Fig. 2). The amplitude of scotopic rod-cone a-wave in the high Orn diet group was significantly higher than that in the control diet group, 16 and 49 weeks after staring to feed the test diet (Fig. 2A). The similar tendency was observed 30 weeks after staring to feed the test diet. The amplitude of scotopic rod-cone b-wave in the high Orn diet group tended to be higher than that in the control diet group, 16, 30 and 49 weeks after starting to feed the test diet (Fig. 2B). In contrast, there is no significant difference in the amplitude of photopic cone b-wave between the two groups, 16, 30 and 49 weeks after starting to feed the test diet (Fig. 2C).

**Histological Analysis** The number of the cells in GCL 49 weeks after starting the test diet was significantly smaller than that before starting to feed the test diet (Pre) (Figs. 2D,
E). High Orn diet significantly reduced the decrease of the number of the cells in GCL 49 weeks after starting to feed the test diet. The thickness of IPL, INL, OPL and ONL 49 weeks after starting to feed the test diet was smaller than that before starting to feed the test diet (Pre) (Figs. 2D, F). There is no significant difference in the thickness of IPL, INL, OPL and ONL between the control diet group and the high Orn diet group 49 weeks after starting to feed the test diet.
DISCUSSION

In the present study, we determined the effect of long-term supplementation of excess amount of L-Orn on visual function and retinal histology in the rats. The result in the present study indicated that long-term supplementation of L-Orn for at least 49 weeks did not cause accumulation of L-Orn in the plasma, nor induce the retinal damages at the dose used in the present study (approximately 0.8 g/d/animal) in the rat. Instead, long-term supplementation of excess amount of L-Orn augmented the amplitude of the a-wave of the scotopic rod-cone electroretinogram, and reduced the decreases of the number of the cells in the ganglion cell layer by aging. The present study suggested that supplementation of L-Orn would not affect the retinal function and histology in healthy subjects.

In the present study, the dose of L-Orn calculated from the food intake was approximately 0.8 g/d/animal. In human, the recommended dose of L-Orn is 1.5 g/d. Calculated from the body weight, the dose of L-Orn in the present study was 100 times higher than that recommended in human. The plasma L-Orn concentration in the high Orn diet group was ranging from 200 to 300 µM, and that in GA patients reported in the previous studies was ranging from 500 to 1000 µM, much higher than that in the high Orn diet group. The present study demonstrated that long-term supplementation of very high dose of L-Orn did not induce accumulation of L-Orn that was found in GA patients, suggesting that supplementation of L-Orn at the recommended dose is safe in healthy subjects.

Very high concentration of L-Orn has been reported to induce lesions of RPE. Kuwabara et al.12 reported that intravitreal injection of L-Orn (1 M, 10 µL) led to edema and degeneration of RPE in the rats. Because the volume of the vitreous body of the rats is approximately 50 µL, and that in GA patients is ranging from 200 to 300 µM, much higher than that in the high Orn diet group. The present study demonstrated that long-term supplementation of very high dose of L-Orn did not induce accumulation of L-Orn that was found in GA patients, suggesting that supplementation of L-Orn at the recommended dose is safe in healthy subjects.

In the present study, although the amplitudes of scotopic and photopic ERG were not much affected by aging, the number of the cells in the ganglion cell layer and thickness of photoreceptors at the end of the experiment (55 weeks old) were smaller than those before starting to feed the test diet (6 weeks old). These results are consistent with the results of the previous reports using Wistar and Wistar/SR rats. It is widely known that the amplitude of the a-wave in rod cone electroretinogram reflects the function of the rod and cone cells. The function of the retinal ganglion cells very little affect the amplitude of electroretinogram. The amplitude of a-wave is not always consistent with the thickness of the outer nuclear layer, i.e., the number of the nuclei of the rod and cone cells. For example, in a mouse model of retinitis pigmentosa, although there were two or three rows in the outer nuclear layer, the a-wave was almost flat.29 We speculate that L-Orn may conserve the function of the rod and cone cells in the aged retina.

El-Sayyad et al.34 demonstrated that depletion of the activities of anti-oxidant enzymes, such as superoxide dismutase, catalase and glutathione-S-transferase, occurred in the aged retina. Therefore, it is possible that the increases of reactive oxygen species cause the decrease of the number of the cells in the ganglion cell layer. As far as we know, there is no report showing that L-Orn itself has an anti-oxidant activity. However, L-Orn is a central part of the urea cycle, which allows for the disposal of excess nitrogen by conversion of ammonia into urea in the liver. It is well-known that the availability of cycle intermediates is one of the factors to control the urea cycle. When the level of L-Orn in the liver is high, the urea cycle proceeds more rapidly. Therefore, a high level of L-Orn will accelerate the detoxication of ammonia, one of neurological toxins. L-Orn is known to be converted to l-arginine and l-citrulline in the urea cycle. However, the plasma level of l-arginine and l-citrulline was not changed in the present study (data not shown), suggesting that l-arginine and l-citrulline is not likely involved in the effects of L-Orn found in the present study. L-Orn is known to be metabolized to polyamines, such as spermine and spermidine.37 Spermine is reported to scavenge free radicals,38 and to modulate the activity of N-methyl-D-aspartic acid receptor, that is involved in the mechanism of neuronal cell death in various pathophysiologic situations. In the present study, we cannot clarify the change in the plasma levels of the polyamines, and whether the polyamines have the similar effects to L-Orn. Further analyses are clearly needed to clarify the underlying mechanisms.

In conclusion, the present study first demonstrated that long-term supplementation of very high dose of L-Orn, which is 100 times higher than the recommended dose for human, for at least 49 weeks did not induce the retinal damages in the rats. The present study suggests that supplementation of L-Orn at the recommended dose is safe in healthy subjects.

Acknowledgments Kunio Ishii received a research Grant from Kyowa Hakko Bio Co., Ltd. Masahiko Morita is an employee of Kyowa Hakko Bio Co., Ltd.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES