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Scientific Research

Suppression of *N*-Methyl-*N*-Nitrosourea-Induced Retinal Damage in Mice by Oligonol, an Oligomerized Polyphenol Formulation

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Abstract

Oligonol is a lychee fruit-derived functional food that contains oligomerized polyphenol compounds. Oligonol exhibits a number of beneficial biological effects, primarily due to its antioxidant activity. Retinitis pigmentosa (RP) is an inherited chronic degenerative disease affecting retinal photoreceptor cells. There is currently no effective therapy capable of stopping or reversing the progression of the disease. In RP, apoptosis of photoreceptor cells resulting from oxidative damage is considered to be the final common pathway. In this report, we present an evaluation of the suppressive activity of Oligonol against *N*-methyl-*N*-nitrosourea (MNU)-induced retinal damage in mice, which is a commonly used animal model of RP. Both intraperitoneal and oral administration of Oligonol reduced the loss of photoreceptor cells 7 days after MNU injection, as evaluated by histological staining. Photoreceptor cells derived from MNU-treated mice exhibited increased TUNEL-positive staining, suggesting increased DNA fragmentation, a hallmark of apoptosis. Oligonol treatment reduced the number of TUNEL-positive cells. Additionally, Oligonol suppressed MNU-induced retinal production of 8-hydroxydeoxyguanosine (8-OHdG), a marker of oxidative stress. Moreover, Oligonol attenuated the MNU-induced decrease in the visual activity of mice, as evaluated by the visual cliff test. Oligonol, therefore, effectively suppresses NMU-induced retinal degeneration.

Keywords

Oligonol; Oligomerized Polyphenols; Retinitis Pigmentosa; *N*-Methyl-*N*-Nitrosourea; Retinal Degeneration; Antioxidant

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1. Introduction

Retinitis pigmentosa (RP) is an inherited eye disease characterized by a slowly progressive retinal degeneration. The main symptoms are decreased vision at night and loss of peripheral vision in the early stages of the disease, occasionally leading to blindness in the later stages, as reviewed in [1]. It is estimated that approximately 1 out of 4000 people is afflicted by RP [1]. Pathologically, degeneration of photoreceptor cells is commonly observed, usually starting with the death of rod cells, followed by degeneration of cone cells [1]. Mutations in over 100 genes considered causative or related to RP have been reported [2], accounting for the differences in disease progression and outcome between the patients and making treatment difficult. There are no effective treatments currently recognized that are capable of arresting the progression of the disease or reversing the condition. Although gene therapy and stem cell implants are expected to start a new era in RP treatment in the future [3]-[5], effective therapy aimed at the suppression of photoreceptor degeneration is urgently required.

To date, therapeutic approaches using vitamins A and E, or vitamin A in combination with nutritional supplements docosahexaenoic acid or lutein have been suggested to suppress retinal degeneration [6] [7]. Death of the photoreceptor cells was proposed to be caused by oxidative damage [8] [9]. Oral administration of the antioxidant *N*-acetylcysteine was reported to effectively slow the progression photoreceptor cell death in animal models of RP [10]. Since it was also suggested that all types of RP converge on the common pathway of apoptotic photoreceptor cell death, anti-apoptotic compounds were postulated to be potentially useful for the treatment of RP [11]. However, considering the extremely long duration of this disease, pharmacological or nutritional interventions need to be not only effective, but also safe for long term use to be applicable in slowing the progression of the disease.

Flavonoids are plant polyphenols that exhibit a variety of biological activities, including antioxidative, anti-inflammatory, anti-carcinogenic, anti-allergic, anti-bacterial, and anti-thrombotic properties [12]-[16]. Among flavonoid compounds, proanthocyanidins exhibit stronger protective activity against free radical-induced lipid peroxidation and DNA damage, as compared to vitamins C, E, and β-carotene [17]. However, proanthocyanidins are found as polymers in crude plant materials [18], which may lower their absorption and bioavailability following ingestion.

Oligonol is a commercially available, manufactured dietary ingredient containing oligomerized polyphenols, namely monomeric and oligomeric proanthocyanidins, derived from lychee and other fruits [19] by a fragmentation of proanthocyanidine polymers from crude fruit extracts [20]. Such oligomerized polyphenol compounds are considered to be easily absorbed and have higher bioavailability than polyphenol polymers abundant in raw fruits and plants [21]. Oligonol has been reported to be safe enough for use as a dietary supplement [19] [22], while exhibiting strong antioxidant capacity [21]. The safety of Oligonol was established in studies evaluating acute and subchronic administration, as well as its genotoxicity [19]. Growing *in vitro* and *in vivo* evidence suggests that Oligonol has a number of beneficial biological effects, including amelioration of potassium bromate-induced renal toxicity in rats [21], attenuation of dysregulated expression of adipokines in adipocytes [23] and diabetic liver damage [24], inhibition of dextran sulfate-induced colitis [25], and inhibition of influenza virus proliferation [26]. These effects are believed to be related to the antioxidative activity of Oligonol. In humans, Oligonol intake was shown to reduce fatigue during exercise [27], attenuate exercise-induced increases in serum inflammatory markers such as proinflammatory cytokines and cortisol level [28], and ameliorate abdominal obesity [29].

N-methyl-*N*-nitrosourea (MNU), an alkylating agent, was reported to induce retinal degeneration in mice, rats, and other animals following systemic administration, providing a simple model of retinal degenerative diseases, including RP [30]. Although the exact mechanism underlying MNU-induced retinal cell death is not fully understood, oxidative stress was recently proposed to be a contributing factor [31]. The strong antioxidative activity and excellent safety profile of Oligonol provided a compelling rationale to evaluate its potential suppressive activity against MNU-induced retinal degeneration. The results show that both systemic injection and oral administration of Oligonol effectively suppress retinal degeneration in MNU-administrated mice.

2. Materials and Methods

2.1. Oligonol

Oligonol was produced by oligomerization of polyphenol polymers in the extract of lychee fruit pericarps, and is

commercially available at present (Amino Up Chemical Co., Ltd., Sapporo). The procedure used for the production, as well as an analysis of the polyphenol composition of Oligonol, was described elsewhere [32]. Briefly, Oligonol contains 30% - 50% oligomeric polyphenols (component monomers of catechin and epicatechin, dimers of polycyanidins, and epicatechin trimers). Oligonol powder was dissolved in saline at a time of use.

2.2. Mice

Male C57BL/6 mice were purchased from Sankyo Lab Service Co., Ltd. (Tokyo, Japan). All animal experiments were performed in accordance with the Ethical Committee Guidelines for Animal Experimentation, Teikyo University.

2.3. Reagents

MNU (Chem Service, West Chester, PA, USA) was dissolved in 0.9% saline solution containing 0.05% acetic acid. 3-Aminobenzamide was purchased from Sigma (St. Louis, MO, USA) and dissolved in 0.9% saline. Oligonol was supplied by Amino Up Chemical Company (Sapporo, Japan) and was dissolved in 0.9% saline. Solutions of MNU, 3-aminobenzamide and Oligonol were prepared at the time of administration.

2.4. Histological Analysis of MNU-Induced Retinal Degeneration

Mice (7 weeks old) received single intraperitoneal (i.p.) injection of MNU (60 mg/kg) on day 0. Immediately after MNU injection and daily over the next 6 days (days 1 - 6), 10, 100, and 1000 mg/kg of Oligonol was administered once daily by oral (p.o.) gavage or at a dose of 100 mg/kg by i.p injection. As a positive control, four mice were administered 3-aninobenzamide (50 mg/kg) by subcutaneous (s.c.) injection concurrent with the MNU treatment. In the control (untreated) group, respective vehicle solutions were administered i.p. or p.o. On day 7, mice were killed by cervical dislocation, and the eyes were quickly removed and fixed for 30 min in methacarn (60% methanol, 30% chloroform and 10% acetyl acid). The fixed eyes (one eye per mouse) were embedded in paraffin and 7-μm-thick sections were cut parallel with the maximum circumference of the eyeball through the optic disc. The sections were stained with hematoxylin and eosin (HE) and observed at ×100 magnification using an Olympus BX51 light microscopy.

2.5. Quantification of Photoreceptor Cell Loss

To evaluate the damage of the photoreceptor cells, the photoreceptor cell ratio was calculated as [(outer retinal thickness)/(total retinal thickness)] × 100%, as previously described [31]. Outer retinal thickness denotes the distance from the outer nuclear layer to the pigment epithelium layer. Total retinal thickness denotes the distance from the internal lining membrane to the pigment epithelium.

2.6. TUNEL Staining

Histochemical staining using the terminal deoxynucleotidyl-transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) method was performed to detect DNA fragmentation as a marker of apoptosis [33] using a commercially available kit (*In situ* cell death detection kit, Roche, Mannheim, Germany). Retinal sections collected on days 1 or 2 following MNU injection from Oligonol- or vehicle-treated mice were prepared as described above. Following deparaffinization, TUNEL staining of the sections was performed according to the manufacturer's protocol.

2.7. Detection of 8-OHdG

To examine the formation of 8-hydroxydeoxyguanosine (8-OHdG), an indicator of DNA modification by reactive oxygen species, paraffin-fixed retinal sections were deparaffinized and blocked with normal serum. Following blocking, sections were incubated with anti-8-OHdG antibody (Japan Institute for the Control of Aging, Shizuoka, Japan) overnight at 4˚C. The antibody binding was visualized using the standardavidin-biotin-alkaline phosphatase complex (ABC-AP) method (ABC-AP kit from Vector Laboratories, UK) with the Vector Red substrate (Vector Laboratories, UK).

2.8. Visual Cliff Test

Gross visual activity of the mice was evaluated using a visual cliff test performed using a technique described by Krishnamoorthy *et al.* [34], with minor modifications. A clear glass plate (60 × 60 cm) was set 1 m above the ground. A black-and-white checkerboard (30 × 30 cm total dimensions, with 1×1 cm squares) was placed at the center of the lower surface of the glass plate. The edge of the checkerboard represented the virtual "cliff". To test the visual activity of mice, each mouse was placed in the center of the checkerboard, and the time spent on the checkerboard without leaving its area was recorded. Behavior was measured only for the first 60 sec, since general behavioral factors become a dominant influence affecting mouse location more than visual activity beyond that time point. Measurements were repeated on the next day by another operator, with similar results obtained.

2.9. Statistical Analysis

Data are expressed as means ± standard deviations. Statistical analysis was conducted using Dunnett's test with $P < 0.05$ considered statistically significant.

3. Results

3.1. Suppression of MNU-Induced Photoreceptor Cell Death by Oligonol

A single systemic injection of MNU was previously shown to induce apoptosis and a loss of photoreceptor cells within 7 days [29]. In current study, we evaluated the effect of Oligonol on the MNU-induced photoreceptor cell loss using histological analysis. Based on the results of a subchronic study that has detected no adverse effects, weight loss, or alterations in food consumption with Oligonol administration at doses up to 1000 mg/kg in rats [19], we administered Oligonol at doses of 100 mg/kg i.p. and 10 - 1000 mg/kg p.o. As shown in **Figure 1**, the outer nuclear layer (ONL) was markedly diminished, *i.e.* the number of nuclei and the thickness of the photoreceptor layer (PRL) were reduced on the 7th day following a single MNU injection. These observations confirm past reports of MNU-induced loss of photoreceptor cells [29]. Daily administrations of Oligonol, both by the i.p. and p.o. route, for 7 consecutive days significantly suppressed the loss of ONL and PRL.

MNU modifies DNA in the photoreceptor cell nuclei to yield a 7-methylguanosine DNA adduct, resulting in an activation of poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme [29]. Since a single s.c. injection of PARP inhibitor 3-aminobenzamide was reported to inhibit MNU-induced photoreceptor cell apoptosis in rats [30], PARP activation is thought to be a key event in the apoptotic cascade in the photoreceptor cells. We examined the effect of 3-aminobenzamide as a positive control, showing that 3-aminobenzamide administration

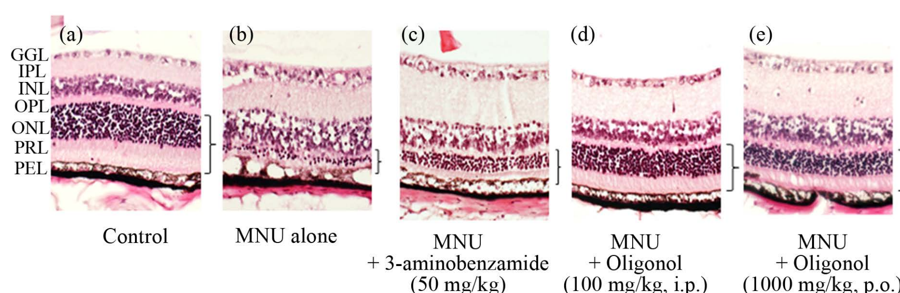

Figure 1. Histology of retina of MNU-treated mice stained by hematoxylin and eosin. C57BL/6 mice were treated with vehicle (A) or MNU (60 mg/kg) by i.p. at start of experiment (day 0; B-E). MNU-treated mice received a single s.c. injection of 3-aminobenzamide (50 mg/kg) soon after the MNU injection on day 0 (C). Oligonol (100 mg/kg, D; 1000 mg/kg, E) was administered daily by i.p. injection for 7 days, starting from day 0. On day 7, mice were sacrificed and the eyes were fixed. The sections were stained with hematoxylin and eosin. Images were obtained at ×200 magnification. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRL, photoreceptor layer; PEL, pigment epithelial layer. The right parentheses indicate the outer retinal thickness, measured as the distance between the outer nuclear layer and the pigment epithelium layer.

moderately suppressed the photoreceptor cell loss in MNU-treated mice.

To quantify the suppressive effect of Oligonol against MNU-induced photoreceptor cell loss, we calculated the ratios of the photoreceptor cell layer thickness to the total retinal thickness (**Figure 2**). MNU significantly reduced the photoreceptor cell layer ratio, whereas i.p. administration of 100 mg/kg Oligonol significantly atenuated MNU-induced damage to the photoreceptor cells. Since Oligonol is intended to be used as a food supplement, we examined the effectiveness of its oral administration at doses between 10 and 1000 mg/kg. Suppression of the effect of MNU was observed even at the 10 mg/kg dose, with the magnitude of the effect comparable to the effect observed following 100 and 1000 mg/kg Oligonol p.o. treatments. Oligonol did not completely abolish the effects of MNU on the photoreceptor cells even at the 1000 mg/kg dose. Interestingly, 3-aminobenzamide administration marginally protected against the MNU-induced photoreceptor cell loss in this experiment, in contrast to the findings of past studies which have reported a complete suppression of the deleterious effects of MNU after a single administration of 50 mg/kg 3-aminobenzamide. This discrepancy may be caused by the difference in the experimental system used, particularly the difference in the animal species; conceivably, higher doses of the agent are required to obtain a similar effect in mice as compared to rats.

To examine whether Oligonol inhibits the MNU-induced apoptosis of photoreceptor cells, retinal sections were stained by TUNEL to detect DNA fragmentation, a hallmark of apoptosis. Administration of a high dose of MNU (100 mg/kg) resulted in an increase in the number of TUNEL-positive cells in the outer nuclear layer, which includes the nuclei of photoreceptor cells, on day 2 and, more extensively, on day 3 (**Figure 3**, left). Oligonol treatment attenuated the MNU-induced increase in intensity of TUNEL staining on days 2 and 3, suggesting that it suppressed the induction of photoreceptor cell apoptosis by MNU.

MNU-induced oxidative stress leads to photoreceptor cell apoptosis [35]. 8-OHdG, a product of DNA oxidation, is one of the most commonly used markers of oxidative stress. MNU reportedly increases 8-OHdG levels in the outer nuclear layer of the retina 12 h after the i.p. injection of MNU. We detected 8-OHdG in both the outer and inner nuclear layers of MNU-treated mice, whereas no 8-OHdG was found in the retinal tissue of control (untreated) mice. Importantly, Oligonol reduced 8-OHdG levels in both layers following MNU administration, suggesting that Oligonol suppresses MNU-induced oxidative stress (**Figure 3**, right).

3.2. Oligonol Rescues Loss of Visual Activity Caused by MNU

Since Oligonol suppressed MNU-induced retinal degeneration in the histological analysis, we used the “visual

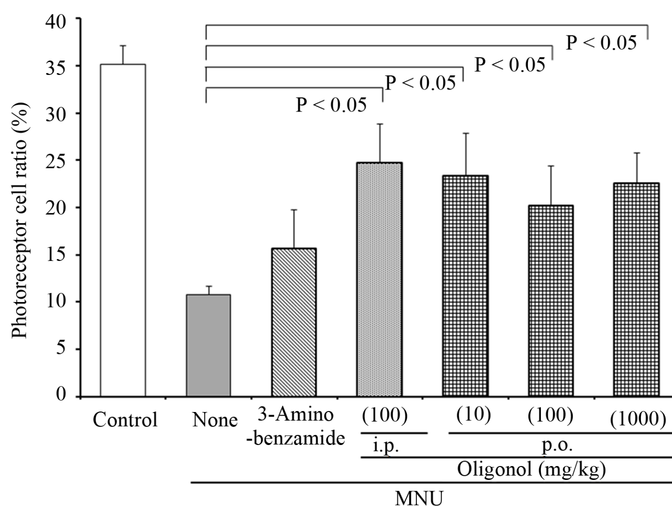

Figure 2. Suppression of MNU-induced photoreceptor cell damage by Oligonol i.p. or p.o. administration. MNU (60 mg/kg) was injected i.p. into C57BL/6 mice on day 0. Oligonol (100 mg/kg, i.p. or 10, 100, and 1000 mg/kg, p.o.) was administrated on 7 consecutive days from day 0, and 3-aminobenzamido (50 mg/kg) was injected s.c. only on day 0 (n = 4 for each treatment group). On day 7, mice (n = 4) were sacrificed and the eyes were fixed. The loss of the photoreceptor cells on day 7 was quantified as described in Materials and Methods.

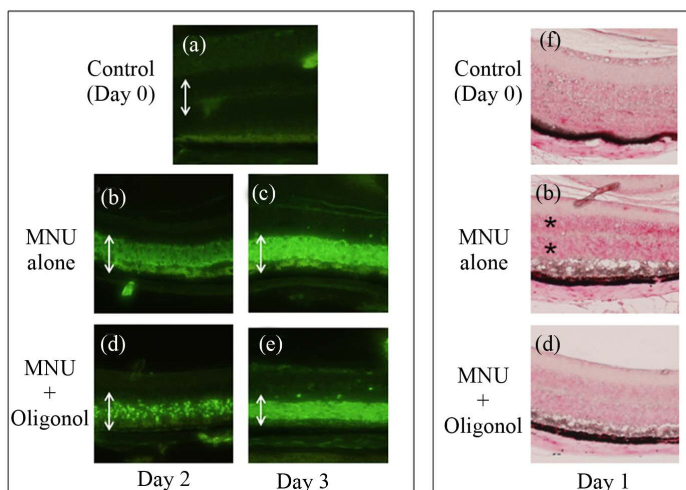

Figure 3. TUNEL stain and detection of 8-OHdG in the retinal sections of the MNU-injected mice with or without Oligonol administration. MNU (100 mg/kg) was injected i.p. on day 0. For the preparation of day 1 section, Oligonol (100 mg/kg) was injected i.p. on day 0. Oligonol injection was repeated on day 1, and on day 2 in case of the sections prepared from tissue collected on day 2 or day 3. Left; TUNEL stain. Bidirectional arrows indicate the thickness of the outer nuclear layer (ONL). Right; detection of 8-OHdG. The asterisks (*) represent 8-OHdG-positive lesions: the layers marked by the lower and upper asterisk correspond to ONL, and the inner nuclear layer (INL).

cliff test" to study whether Oligonol can reverse the MNU-induced impairment in visual activity. Animals that underwent the cliff test were administered 100 mg/kg dose of MNU. Higher dose of MNU (in comparison to the dose used in the experiments studying histological changes) was used to achieve a clear deficiency in visual activity in mice.

Mice hesitate to pass out from the central checkerboard area, because the edges are perceived as a "virtual cliff" (see **Figure 4(a)**). As shown in **Figure 4(b)**, a majority of the control mice spent the entire 60 sec of the assay on the checkerboard, while all of the MNU-treated mice exited the checkerboard during the first 15 sec. This finding clearly demonstrates the impairment of visual activity by MNU. Daily administration of Oligonol (100 mg/kg, i.p., as well as 1000 mg/kg, p.o.) significantly extended the length of time that the mice spent on the checkerboard. In this experiment, the effect of 3-aminobenzamide was not significant, although the mean value of the length of time that mice treated with both MNU and 3-aminobenzamide spent on the checkerboard was higher than that of mice treated with MNU alone. This result suggests that administration of Oligonol by both i.p. and p.o. routes rescues the MNU-induced loss of visual activity.

4. Discussion

Photoreceptor cell death is the common pathway in RP [4]. Since MNU injection specifically induces apoptosis of retinal cells within several days in animals [30], MNU-induced retinal damage is a good model for RP. Using this model of retinal degeneration, previous studies have demonstrated retinoprotective effects of the apoptosis inhibitor 3-aminobenzamide [30], calcium antagonists nimodipine [36] and docosahexaenoic acid [37], anthocyanins extracted from black soy beans [38], nicotinamide [39], and curcumin [40]. In search of the remedy for RP, very low toxicity is a key requirement for compounds aimed at preventing retinopathy, since retinal damage in RP patients develops through a gradual process that progresses over decades.

Oligonol is a widely available nutritional supplement with an established safety profile. The safety of Oligonol was examined in the animal acute and chronic toxicity studies [19]. In addition, supplementation of Oligonol for healthy human volunteers was carried out at doses of 100 mg/day and 400 mg/day for 92 days and all the biochemical parameters were within the normal range [29]. In this paper, both oral and intraperitoneal administra-

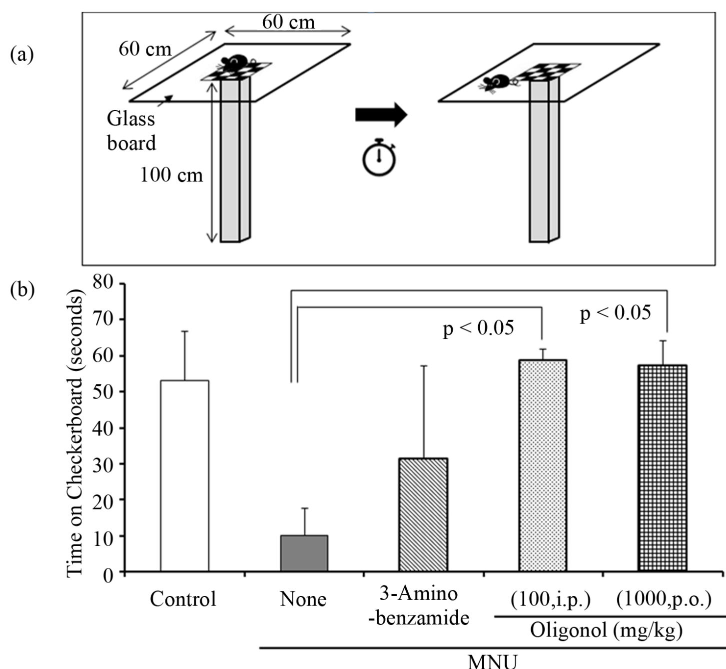

Figure 4. Estimation of the gross visual activity of mice by visual cliff test. (a) The schematic view of the apparatus used for the cliff test. (b) MNU (100 mg/kg) was administered to mice by i.p. injection on day 0. Oligonol was administered i.p. (100 mg/kg) or p.o. (1000 mg/kg) to MNU-injected mice on 7 consecutive days, starting at day 0. On day 10, the visual cliff test was performed as described (n = 6). The length of time each mouse spent on the central checkerboard was measured for 60 sec.

tion of Oligonol suppressed MNU-induced photoreceptor cell loss as evidenced by the outcomes of the histological analysis. It is noteworthy that even the 10 mg/kg of daily oral administration exert the significant effect, since it is comparable with the expected intake in humans who use oligonol as a food supplement.

To explore the mechanism of Oligonol to suppress the MNU-induced retinal damage, we studied the effect on emergences of retinal cell apoptosis and the marker of oxidative stress. We obtained the results that Oligonol attenuated the apoptosis and the intensity of 8-OHdG, which were augmented by MNU. Although the suppression of apoptosis was not complete, it corresponds well with the observation of incomplete inhibition of MNU-induced reduction in the photoreceptor cell layer thickness by Oligonol. Although 8-OHdG was detected in both the outer and inner nuclear layer, apoptosis was only observed in the outer nuclear layer. This finding suggests that the photoreceptor cells are highly sensitive to oxidative stress. In spite of the reduction in 8-OHdG, MNU-induced apoptosis was not completely abolished by the Oligonol administration, suggesting that a mechanism other than oxidative stress contributes to the induction of photoreceptor cells apoptosis. Further experiments are warranted to identify the specific mechanism.

Using the visual cliff test, we found that Oligonol suppressed not only the loss of photoreceptor cells but also the visual activity deficit in mice treated by MNU. In the next study, a functional evaluation in MNU-treated mice with and without Oligonol administration using electroretinographic recordings would be highly informative.

Based on the findings of our current study, we propose Oligonol, a dietary supplement comprising oligomerized polyphenols as a new candidate for the treatment of RP and other retinal degenerative disease. However, comparison between Oligonol and other substances which are reportedly effective for the MNU model is required to evaluate the efficacy and the safety. In addition, a number of factors remain to be investigated before Oligonol can be recommended for application in human patients. The active ingredients of Oligonol and the exact mechanism underlying the observed protection against the MNU-induced photoreceptor cell apoptosis need to be elucidated. Furthermore the effective administration schedules should be tested. Finally, the efficacy of Oligonol in a genetically inherited mouse model of RP also needs to be clarified.

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