Effect of a Complex Lutein Formula in an Animal Model for Light-Induced Retinal Degeneration

Yin-Pin Cheng\textsuperscript{1}, Chia-Ying Ke\textsuperscript{2}, Chih-Chieh Kuo\textsuperscript{1}, and Yih-Jing Lee\textsuperscript{2}

\textsuperscript{1}Center of Research and Development, Uni-President Biotech, Tainan 70955 and
\textsuperscript{2}School of Medicine, Fu-Jen Catholic University, Hsinchuang, New Taipei City 24205, Taiwan, Republic of China

Abstract

Several retinal degenerative diseases cause vision loss and retinal cell death. Currently, people face prolonged exposure to digital screens, rendering vision protection from light exposure a critical topic. In this study, we designed a complex lutein formula (CLF) by combining several natural compounds: \textit{Calendula officinalis}, \textit{Lycium barbarum}, \textit{Vaccinium myrtillus}, \textit{Cassia obtusifolia}, and \textit{Rhodiola rosea}. In addition, we evaluated the protective effects of the formula on retinal functions in an animal model for light-induced retinal degeneration. We employed electroretinography to analyse retinal function, and conducted a histological examination of the morphological changes in the retina treated under various conditions. We revealed that the retinal function in animals exposed to light for 7 days decreased significantly; however, the retinal function of animals that had received the CLF exhibited superior performance, despite light exposure. In addition, a greater portion of the outer nuclear layer (ONL) (i.e. the nuclei of photoreceptors) in these animals was preserved compared with the animals that had not received the formula after 7 days of light exposure. These results revealed that our dietary CLF supplement attenuated retinal function loss resulting from long-term light exposure.

Key Words: electroretinogram, light expose, lutein, photoreceptor, retinal degeneration

Introduction

Retinal diseases such as glaucoma, optic nerve atrophy, diabetic retinopathy, retinitis pigmentosa and age-related macular degeneration (AMD) cause vision loss in human (47). These degenerative retinal diseases often result in irreversible damage to retinal cells, such as ganglion cell loss caused by intraocular hypertension (31), and may lead to blindness. Currently, people face prolonged exposure to digital screens such as mobile phones and tablet computers, rendering vision protection from light exposure a critical topic. We therefore used a long-term light expose animal model to mimic the retinal degeneration cause by this condition.

Numerous natural products are considered to be beneficial for vision protection. \textit{Calendula officinalis}, commonly known as marigold, belongs to the Asteraceae family. The flowers of this plant are widely acknowledged as a folk medicine in Europe, China, and India. Numerous studies have reported that marigold has anti-inflammatory (11), antioxidant (1, 9, 13) and wound healing (40, 44) properties. Marigold flowers contain carotenoids and flavonoids (24, 45), most of which are found in their petals. Carotene and xanthophyll are two major groups of carotenoids. The total carotenoid concentration (mg/g of dry weight) in marigold was 7.71% in petals and 1.61% in pollens, and the main carotenoid was lutein, which was found in 15% to 25% (2). Furthermore, the 2 major carotenoids in the human macula and retina are lutein and zeaxanthin (17). Many reports have revealed that the
dietary supplementation of lutein and zeaxanthin with carotenoid-rich foods could increase macular pigment density and reduce the risk of progression towards advanced AMD (7, 27, 36).

Fructus Lycii, also known as *Lycium barbarum* (*Gouqizi* in Mandarin Chinese) belongs to the Solanaeace family. It has a long history of usage as a dietary supplement for improving vision (41). Recent studies have indicated that its active components, including *Lycium barbarum* polysaccharides (49, 54), zeaxanthin, and lutein (55), protect the retina against oxidative damage in instances of light-induced retinopathy and cataracts (20).

*Vaccinium myrtillus* fruit, commonly known as bilberry, is a member of the Ericaceae family. During World War II, bilberry gained popularity among pilots in the Royal Air Force, who claimed that eating bilberry jam improved their night vision (37). In the past few years, bilberry has been reported to induce visual enhancements by improving night vision (8), eliminating retinal inflammation (35), and protecting retinal cells (34, 38). Anthocyanins are the most vital and richest of the active components in bilberry that induce these biological activities influencing vision (28), and they have been found to have potential antioxidative effects (12).

The root of *Rhodiola rosea*, also called golden root or rose root, has been used for centuries in Asian and European traditional medicine. *Rhodiola rosea* contains more than 20 chemical compounds, including essential oils, phenylpropanoids, phenylpropanoid glycosides, phenolic acids, flavonoids, triterpenoids, proanthocyanidins, and cyanogenic glycosides (14, 43). Among these compounds, proanthocyanidins have been reported to considerably engage in bioactivities by inducing antioxidative (48) and anti-inflammatory effects (3). However, the effect of proanthocyanidins on vision has yet to be reported.

*A Cassia obtusifolia* seed from the Fabaceae family, *Juemingzi*, is a traditional Chinese medicine for vision improvement, and has recently been used to treat hyperlipemia in diabetic patients (10). Its active compounds have also been reported as antioxidants with neuroprotective properties (22, 23). Several compounds such as lactones, triterpenoids, benzoic acid, naphthopyrones, anthrones, and anthraquinones have been reported from *Cassia obtusifolia*, among which anthraquinones appeared to be most effective (25, 26, 51). A recent study reported that Cassia seed extracts exhibited clear antioxidative and vasodilatative effects (30), which may evidence the visually protective potential of this plant.

Because these natural products exhibited potentially protective effects on sight, we designed a complex lutein formula (CLF) by combining the following natural compounds: *Calendula officinalis*, *Lycium barbarum*, *Vaccinium myrtillus*, *Cassia obtusifolia* and *Rhodiola rosea*. Afterward, we evaluated the potential effect of vision protection in CLF by conducting a retinal function test and morphological examinations in an animal model for light-induced retinal degeneration.

**Materials and Methods**

**Formula Preparation**

Five traditional herbal preparations, namely *Calendula officinalis*, *Lycium barbarum*, *Vaccinium myrtillus*, *Cassia obtusifolia* and *Rhodiola rosea*, were purchased from Chuang Song Zong Pharmaceutical Co. Ltd. (Kaohsiung, Taiwan) and were mixed at the ratio 9:6:2:2:1. The selection and proportion of these traditional herbal medicines were determined by screen tests of their capacity of anti-oxidation and anti-free radicals, such as Prussian blue Oyaizu method (39) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay (50). The mixture was then submerged in an ethanol solution for 3 h for extraction. After refluxation, the extract was filtered, concentrated, sterilised and stored in a refrigerator until it was needed for use. The active component, lutein, was quantified as the indicator of the CLF by conducting high-performance liquid chromatography (HPLC), for which a Shimadzu Prominence system (CBM-20A), pump (LC-20AT), UV/Vis detector (SPD-M20A), and autoinjector (SIL-20) were used. A reverse-phase Inertsil 5 ODS-2 (Nacalai, 4.6 mm id × 250 mm) column was used. All standard and sample solutions were filtered through a 0.45-μm membrane filter before HPLC processing.

**Animal Model**

Sprague-Dawley rats (200 to 300 g) were obtained from BioLASCO Technology (Taipei, Taiwan) and were preserved in a normal light environment of 100–120 lux. The animal protocols were approved by the Animal Care and Use Committee of Fu-Jen Catholic University. Light damage was induced through 7 days of exposure to a cyclic, bright, and luminous light (5000 lux; 12-h in dark/12-h in light), as adopted from Joly *et al.* and modified for this study (21). After the bright light period, the rats were returned to the normal light environment in the animal care facility. The animals were categorised into 2 groups: (1) Control group: Gavage-feeding with water, instead of any supplements, once per day after light exposure; and (2) CLF-treated group: gavage-feeding with daily CLF supplementation after light exposure. Fig. 1 shows a simplified version of the experimental design. The CLF dosage used in the experiment was
set at 104 mg/kg/day of active lutein for the rats, which is equivalent to 1000 mg/day for humans.

**Electroretinogram (ERG)**

To examine the physiological function of the retina, an ERG test was applied to both eyes of the animals before exposure to light as well as once a week afterward, until the experiment was complete. After 2 h under the dark condition, the rats were anaesthetised using Zoletil 50 (intraperitoneal injection of 50 mg/kg; Virbac, France). Under a dim red light, the rats were laid in a suitable position for ERG recording by using an RETIport ERG system (AcriVet, Germany). A gold foil electrode positioned and fixed to the corneal surface with super gel served as the active electrode. In addition, a genuine grass platinum subdermal needle electrode inserted subcutaneously into the epicranium served as the reference electrode, and an electrode attached to the ear served as the ground electrode. For ERG waveform analysis, the a-wave amplitude was measured from the prestimulus baseline to the most negative trough, whereas the b-wave amplitude was measured from the a-wave trough to the most positive peak of the evoked response. The a- and b-wave amplitudes before the light expose of each rats were used as the denominator (100%), and those in the control and experimental groups following light exposure, and every week after the light expose were divided by their own denominator and presented in percentage to obtain the recovery rate. ERGs were recorded before light exposure and once weekly thereafter for 4 weeks.

**Histological Study**

The animals were sacrificed 4 weeks after light exposure. After perfusion with 4% paraformaldehyde (Showa, Japan), the eyes were enucleated, embedded in a tissue freezing medium, cryostate-sectioned, and immersed in 4% paraformaldehyde in a phosphate buffered saline solution (pH 7.4). The preparations were stained by conducting hematoxylin and eosin staining to assess the retinal histology and to measure the thickness of the outer nuclear layer (ONL). The ONL thickness was evaluated by modifying a method adopted from LaVail (29) and Bok (5). The first measurement was conducted at a distance of 250 µm from the optic nerve head, with subsequent 250-µm areas defined as the periphery. Five defined points were measured within each 250-µm area, and the mean thickness was calculated. An upright microscope (Leica DM2500, Germany) and a digital camera system (Leica DFC420, Germany) were used to conduct morphological assessments and measurements.

**Statistical Analyses**

SPSS software (version 19, IBM, NY, USA) was used for statistical analyses. One-way Analysis of variance (ANOVA) and the Bonferroni multiple comparisons test were conducted for statistical analyses, and $P < 0.05$ was considered a statistically significant difference.

**Results**

**Histological Study of the Retina**

Histological analysis of the unexposed (normal) retina revealed 3 distinct layers of nerve cell bodies: ONL, which contains the nuclei of photoreceptors; the inner nuclear layer (INL), which contains the nuclei of bipolar, amacrine, and horizontal cells; and ganglion cells, which are the nuclei of optic nerves (Fig. 2A). For the control group, which did not receive the CLF, the thickness of the ONL decreased significantly 4 weeks after light exposure (Fig. 2B). This result is consistent with those of previous studies, which have reported that light-induced retinopathy mainly affects the ONL, not other layers of the retina (52, 57). Histological imaging also revealed that the loss of ONL.
A Complex Lutein Formula Keeps Retinal Cells and Function

was comparatively less for animals that received CLF supplementation (Fig. 2C). The ONL thickness of these groups was approximately 20% of that in the control group and 48% of that in the CLF-treated group. These results implied that the CLF may protect retinal cell loss caused by long-term light exposure.

Because ONL thickness may vary between the central and the peripheral retina, the analysis of ONL thickness was also conducted on different areas in the retina. Fig. 3 illustrates the results of the quantitative analysis on ONL thickness in normal animals and those in the control group (water) and the CLF-treated group 4 weeks after light exposure. The detail data of the ONL thickness was also shown in Table 1. Animals in the control group exhibited a statistically significant reduction in mean ONL thickness in both the nasal and temporal aspects compared to normal animals. After CLF treatment, the ONL thickness was similar to normal animals in both nasal and temporal directions.

Fig. 2. Histological studies on retinal cell layers with identical preparations. (A) Normal retina; (B) retina of the control group, which exhibited fewer nuclei in the ONL; and (C) retina of rats that received CLF after light exposure, which exhibited more cells preserved in the ONL compared with the control group. Scale bar = 50 µm.

Fig. 3. ONL thickness with various preparations of the retina. ONL thickness in different areas of the retina, from nasal to temporal, were measured every 250 µm. The normal retina retained an average thickness of 62-78 µm. However, in the light-exposed control group, the ONL thickness decreased to 10-20 µm. After CLF treatment, the average ONL thickness was 28-39 µm in different areas. One-way ANOVA and the Bonferroni multiple comparisons test were conducted for analysis. *indicates $P < 0.05$ compared with the normal group; #indicates $P < 0.05$ compared with the control group.
and temporal retinal hemispheres, from 63 to 80 µm (normal eyes) to 10 to 20 µm (control group) (Fig. 3). However, in the CLF-treated group, the retinal morphology was preserved, and the mean ONL thickness improved significantly compared with the control group (28 to 37 µm in the CLF-treated group; Fig. 3). These results suggested that the CLF may preserve ONL thickness for up to 4 weeks after light exposure. By contrast, light exposure did not influence INL thickness compared with nonexposed (normal) rats (data not shown).

Retinal Function Assay Through ERG

To examine the potential effects of the CLF on retinal function, we conducted ERG. Fig. 4 shows the representative ERG recordings obtained from animals in the control, and CLF-treated groups. Fig. 1 illustrates the treatment details for these groups. The a- and b-wave amplitudes are summarised as the mean recovery rate. Seven days of light exposure caused a significant reduction in a- and b-waves (approximately 20% and 30%, respectively) of the normal range (Fig. 4, A and B). At 1 and 2 weeks after light exposure, the recovery rates for the a- and b-waves did not differ statistically in these groups, whereas that for the b-waves was significantly higher in the CLF-treated group 3 and 4 weeks after light exposure (Fig. 4B). According to these results, we conclude that the effect of recovery in the b-waves starts at week 3 in the CLF-treated group.

Discussion

In our animal model for light-induced retinopathy, bright light exposure resulted in photoreceptor

### Table 1. Thickness of ONL in different preparations

<table>
<thead>
<tr>
<th>Location (mm)</th>
<th>Normal</th>
<th>Control</th>
<th>CLF</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2.25</td>
<td>63.8 ± 8.8</td>
<td>10.8 ± 2.7*</td>
<td>36.8 ± 2.4*,#</td>
</tr>
<tr>
<td>-2</td>
<td>63.5 ± 11.2</td>
<td>10.9 ± 1.8*</td>
<td>37.5 ± 3.9*,#</td>
</tr>
<tr>
<td>-1.75</td>
<td>65.2 ± 8.1</td>
<td>12.4 ± 1.7*</td>
<td>34.2 ± 4.1*,#</td>
</tr>
<tr>
<td>-1.5</td>
<td>68.0 ± 8.6</td>
<td>13.1 ± 1.7*</td>
<td>34.2 ± 4.9*,#</td>
</tr>
<tr>
<td>-1.25</td>
<td>69.8 ± 8.5</td>
<td>14.1 ± 1.8*</td>
<td>34.6 ± 3.3*,#</td>
</tr>
<tr>
<td>-1</td>
<td>70.6 ± 5.5</td>
<td>14.3 ± 2.6*</td>
<td>35.9 ± 3.5*,#</td>
</tr>
<tr>
<td>-0.75</td>
<td>71.7 ± 6.5</td>
<td>15.1 ± 2.4*</td>
<td>35.2 ± 6.1*,#</td>
</tr>
<tr>
<td>-0.5</td>
<td>69.6 ± 6.4</td>
<td>16.1 ± 2.4*</td>
<td>33.0 ± 3.7*,#</td>
</tr>
<tr>
<td>-0.25</td>
<td>69.3 ± 6.8</td>
<td>20.3 ± 4.5*</td>
<td>28.8 ± 2.2*,#</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.25</td>
<td>67.5 ± 16.7</td>
<td>16.5 ± 2.6*</td>
<td>32.9 ± 3.2*,#</td>
</tr>
<tr>
<td>0.5</td>
<td>69.0 ± 14.3</td>
<td>15.4 ± 1.7*</td>
<td>33.2 ± 3.5*,#</td>
</tr>
<tr>
<td>0.75</td>
<td>75.3 ± 9.5</td>
<td>16.1 ± 1.1*</td>
<td>33.2 ± 2.5*,#</td>
</tr>
<tr>
<td>1</td>
<td>72.6 ± 13.1</td>
<td>14.6 ± 2.0*</td>
<td>32.0 ± 2.2*,#</td>
</tr>
<tr>
<td>1.25</td>
<td>78.3 ± 8.7</td>
<td>15.3 ± 2.6*</td>
<td>30.8 ± 3.6*,#</td>
</tr>
<tr>
<td>1.5</td>
<td>80.9 ± 13.3</td>
<td>14.2 ± 1.2*</td>
<td>33.1 ± 2.8*,#</td>
</tr>
<tr>
<td>1.75</td>
<td>68.8 ± 5.7</td>
<td>13.5 ± 2.5*</td>
<td>34.7 ± 4.8*,#</td>
</tr>
<tr>
<td>2</td>
<td>72.3 ± 11.1</td>
<td>13.2 ± 1.8*</td>
<td>31.0 ± 3.7*,#</td>
</tr>
<tr>
<td>2.25</td>
<td>64.7 ± 5.6</td>
<td>13.3 ± 1.6*</td>
<td>28.1 ± 3.7*,#</td>
</tr>
</tbody>
</table>

Note: Data are shown as mean ± SD. Location indicates distance from optic nerve head, negative value means to the nasal side, and positive value means to the temporal side. One-way ANOVA and the Bonferroni multiple comparisons test were conducted for analysis. *indicates $P < 0.05$ compared to the normal group; #indicates $P < 0.05$ compared to the control group.
cell loss, which is due to apoptosis, and presented as a decrease in ONL thickness. This result is consistent with those reported in light-induced retinal degeneration models (15, 18, 42), which have confirmed that the death of photoreceptor cells characterised by apoptosis was a common final pathway. In our experiments, the animals that received CLF exhibited a greater preservation of photoreceptor cells after light exposure, suggesting that CLF may exert a protective effect from long-term light exposure for photoreceptors. Two major types of carotenoids are present in the macular area of the retina (i.e. lutein and zeaxanthin). Zeaxanthin exists in the central region, whereas lutein is found mainly in the peripheral region of the retina (6). These carotenoids are concentrated mainly in the axons and outer segments of the photoreceptors (46). Moreover, these carotenoids have 2 functions in the retina: the first is essential for photoprotection against oxidative stress, and the second entails forming a filter for blue light (53). Because oxidative stress is considered a major cause of AMD and light-induced retinal degeneration (19), the antioxidative effects of xanthophylls and lutein should be beneficial for patients diagnosed with these diseases.

A previous study reported that light-induced retinal damage was triggered by the excess absorption of photons from rhodopsin (16), a visual pigment ubiquitously found in photoreceptors. In this study, we also evaluated the retinal function of animals through ERG recordings. Our data (Fig. 4) revealed that both a- and b-waves decreased at the end of 7-day light exposure (at Week 0). The b-waves in the ERG recordings of rats that received CLF supplementation increased significantly after 3 weeks and 4 weeks of light exposure, but a-waves did not improve in the same rats. Although our finding was based on a limited use of animals, it confirmed that the proposed CLF might exert a protective effect on the retina after long-term light exposure. Numerous clinical cases have indicated that lutein and zeaxanthin supplementation strengthened macular pigments and preserved visual function in cases of early-stage AMD (32, 33). Possible mechanisms for this retinal protective effect in lutein and zeaxanthin may involve reducing phototoxic oxidative damage and altering the expression of inflammatory genes, such as monocyte chemotactic protein 1, interleukin-8, and complement factor H (4). *Lycium barbarum* is a commonly used herbal medicine and reported may provide protective effects in diabetic retinopathy (20). Our CLF preparation included five natural plant extracts containing lutein and zeaxanthin (such as *Calendula officinalis* and *Lycium barbarum*) in addition to anthocyanins and proanthocyanidins. Proanthocyanidins and anthocyanins have been shown to be excellent antioxidative substances (12, 48, 56). Faria et al. reported that the anthocyanin derivate from extracts of *Vaccinium myrtillus* performed high protective effect of the liposome membranes toward oxidation (12). Extract of *Rodiola rosea* was also found to protect cultured cells against ultraviolet light and oxidative stress (48). The multiple components in our CLF would provide multiple effects on protecting photoreceptors from apoptosis cause by long-term light exposure. Our investigation results regarding the proposed CLF revealed that daily CLF supplementation led to the preservation of more photoreceptors and improved retinal function 4 weeks after long-term light exposure, and ERG was conducted both before and after light exposure. The proposed CLF may provide protective diet support for patients diagnosed with light-induced retinal degeneration or AMD.

**Acknowledgments**

This project was granted by the Uni-President Biotech Co. Ltd., Tainan, Taiwan. YPC and CCK were employees in the Center of Research and Development of the Uni-President Biotech Co. Ltd. when the work processed, but not while submitting the manuscript. There is no conflict of interest for authors.

**References**

25. Kitanaka, S., Nakayama, T., Shibano, T., Ohkoshi, E. and Takido, M.
26. Kitanaka, S. and Takido, M. Studies on the constituents in the
11. Della Loggia, R., Tubaro, A., Sosa, S., Becker, H., Saar, S. and
14. Ganzera, M., Yayla, Y. and Khan, I.A. Analysis of the marker
12. Faria, A., Oliveira, J., Neves, P., Gameiro, P., Santos-Buelga, C.,
27. Kvansakul, J., Rodriguez-Carmona, M., Edgar, D.F., Barker, F.M.,
208 Cheng, Ke, Kuo and Lee
19. Della Loggia, R., Tubaro, A., Sosa, S., Becker, H., Saar, S. and
18. Della Loggia, R., Tubaro, A., Sosa, S., Becker, H., Saar, S. and
11. Della Loggia, R., Tubaro, A., Sosa, S., Becker, H., Saar, S. and


