Research Article

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Efficacy of a New Antioxidants Blend in Protecting RPE Cells in vitro and in Improving the Visual Performance of Sport Pilots

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ABSTRACT

*The **rationale** of this study stems from the consideration that vision is a dynamic process, during which photopigments of photoreceptor cells (rods and cones) are continuously consumed and regenerated by retinal pigmented epithelial (RPE) cells. The whole process of vision triggered by light radiation generates free radicals which are potentially toxic to the cells in the central retina (macula), which is the main player in vision. Therefore, the central retina contains carotenoid pigments (mainly lutein and zeaxanthin) with the double function of shielding the cells from light radiation and providing free radical-scavenging action due to their antioxidant power. Nonetheless, visual performance (including visual acuity, contrast sensitivity and stereoscopic perception) can be hampered by bright light and by sudden changes in illumination conditions, such as during the transit from the dark to intense light. Therefore, **objective** of this study has been to investigate whether the treatment with a food supplement regimen containing different antioxidant and protective elements shown to protect from photo-oxidative damage retinal pigment epithelial (RPE) cells, might also improve retina functions during challenging light conditions. We present here our **findings**, showing the protective effects on human RPE cells in vitro of lutein and cyanidin-3-glucoside (C3G) against photo-oxidative stress. Accordingly, the results of a retrospective analysis of a case series show that sport motorcycle test pilots (who need for their profession an optimal visual performance) taking a commercially available food supplement containing a blend of antioxidant and protective molecules (lutein, C3G, verbascoside and zinc), improved their visual abilities and reduced their sensibility to glaring lights in a dose/time-dependent fashion. In **conclusion**, these results suggest that a food supplement may increase the antioxidant defense of the retina, thus improving the visual performance also during challenging illumination conditions, hence increasing the safety of individuals finding themselves in such situations.*

Keywords

Cyanidin-3-glucoside, Food supplement, Lutein, Visual performance.

Introduction

An excessive generation of reactive oxygen species (ROS) overwhelming the endogenous antioxidant capacity of cells and tissues results in oxidative stress. This phenomenon is nowadays recognized to be a major contributor to the detrimental effects of aging, and to the pathogenesis of many neurodegenerative diseases, including photoreceptor-based retinopathies [1]. ROS generation is triggered by external factors, such as environmental agents (e.g. pollution, cigarette smoke and sunlight), and by internal physiologic reactions linked to the energetic metabolism. The intracellular increase of ROS may damage cellular components like lipids, proteins, carbohydrates, and DNA, thus activating specific pathways linked to cell senescence [2], eventually inducing cell death by apoptosis. Normally, the retina has a high oxygen consumption rate and is exposed to pro-oxidizing agents (such as sunlight or artificial illumination) impinging on its high content

of polyunsaturated fatty acids concentrated in photoreceptor membranes. Therefore, the retina is highly susceptible to oxidative stress [3-5]. In fact, both photoreceptors and RPE cells produce relevant amounts of ROS because of their intense metabolism due to different reasons. First, photoreceptors are continuously exposed to light which activates their many photosensible molecules; second, the polyunsaturated fatty acids of which the photoreceptor membranous disks are rich, are highly susceptible to oxidation damage; third, RPE cells continuously recycle by phagocytosis the photoreceptor disks exhausted or damaged by oxidative stress, finally resulting in accumulation of ROS also in the RPE. These events in turn cause a buildup of the oxidation byproduct (lipofuscin) of lipids and lipoproteins containing photo-oxidable fluorophores [6,7]. As a consequence, the oxidative stress induced by high levels of light radiation eventually results in the apoptosis of photoreceptors and inner retina neuronal cells [8,9] beside affecting also the phagocytic function of the RPE [10], thus exacerbating the whole process. For instance, the excessive and continuous free radical production in rod photoreceptor cells has been involved as an important early event in the course of at least two major sight-threatening retinal diseases: age-related macular degeneration (ARMD) and diabetic retinopathy (DR). Moreover, visual acuity, contrast sensitivity and stereoscopic vision all depend on the efficiency of light perception by photoreceptor cells and the downstream signal transmission through the optic nerve to the visual cortex. In turn, such efficiency is hampered by oxidative stress of the visual system, mostly affecting photoreceptor cells, their visual pigments and the RPE deputed to their recycling. Therefore, the retina contains an elevated concentration of antioxidants aimed at protecting as much as possible the retina from oxidative damage. Lutein, together with its stereoisomers zeaxanthin and meso-zeaxanthin, are the only carotenoids present in the human retina [11] with the highest amount concentrated in the macula, where lutein dominates in the periphery, while zeaxanthin and meso-zeaxanthin become predominant in the center [12,13]. These molecules are not naturally synthesized by cells, but must be acquired through the diet. Lutein is found in several fruits and vegetables [14,15]. Besides having anti-oxidative and anti-inflammatory properties [16,17], lutein may also filter blue light thus protecting photoreceptors from light-induced damage [12].

Cyanidin-3-glucosyde (C3G) is a common anthocyanin that is present in edible parts of plants and has been reported to help vision because of its potent antioxidant and anti-inflammatory activity [18,19]. Moreover, C3G can participate in the process of rhodopsin regeneration in the outer retina [20]. In the presence of oxidative stress, the antioxidant and anti-inflammatory properties of lutein and/or C3G may result in improved retinal function by blunting photoreceptor cell death [21,22].

Complementing the protective efficacy of lutein and C3G, zinc and verbascoside are also compounds potentially useful against retinal diseases. Zinc is an essential microelement and an antioxidant involved in keeping normal ocular functions [23], and its presence in food supplements has been shown to reduce the progression of age-related macular degeneration [24]. Verbascoside is a glycoside endowed with anti-oxidative, anti-inflammatory and neuroprotective properties, which is found in several medicinal herbs [25]. It has a poor bioavailability, nonetheless there is evidence that its presence in food supplements may reduce oxidative stress in the eye, and inhibit apoptotic death of retinal cells *in vitro* [26,27].

Given the proven efficacy of each of these antioxidant compounds in the protection from photooxidative damage, it is reasonable that their association in a multicomponent mixture might benefit its efficacy. Accordingly, the choice of combining different antioxidants in complex mixtures resulted to be a promising approach for the treatment of retinal neurodegenerative diseases [28]. The use of such mixtures may take advantage of their ability to affect multiple targets, thus exerting a synergistic effect on the diseased tissues [29]. For instance, using individual antioxidants had no significant beneficial effect on the treatment of photoreceptor degeneration in the rd1 model of retinitis pigmentosa, whereas treatment with a complex mixture dramatically blunted rod degeneration [30].

We decided to verify this hypothesis by showing the protective efficacy of lutein and C3G either alone or in association on human RPE cells *in vitro* exposed to high intensity white light illumination. In parallel, we evaluated in a retrospective analysis the visual performance of sport motorcycle test pilots who were taking a new commercial food supplement containing lutein, C3G, verbascoside and zinc. The profession of this category of riders requires optimal stereoscopic vision to perceive and evaluate distances and unpredicted obstacles on the road, and a quick adaptation to different light conditions, as during the run at high speed, they may cross tunnels, thus passing from bright outside illumination to the poor inside illumination. The results obtained from this small, limited pool of subjects corroborate the hypothesis of a good efficacy of these compounds in protecting and even improving the visual abilities of these individuals.

Materials and Methods

Photooxidative stress *in vitro*

Cell culture: ARPE-19 cells purchased from ATCC. Cells were grown in DMEM-F12 (ATCC, cat. no. 30-2006) supplemented with 1% penicillin/streptomycin, 10% FBS, at 37°C in a humidified atmosphere containing 5% CO_2.

White light irradiation and measurement of viability and free radicals: ARPE-19 cells were seeded at a density of 12 x 10^3 cells per well into 96-well plates and left to adhere overnight at 37°C in a humidified incubator at 5% CO_2. After 24 hours the culture medium was replaced with serum-free medium (SFM) with or without 100 μM lutein (L), 100 μM cyanidin-3-glucoside (C3G) or with a mix of the two molecules each at 100 μM for 1 hour at 37°C in the incubator. At the end of the incubation time, cells were exposed at 2 x 10^5 lux of a common white light LED lamp (5000 K) for 15 minutes. Afterwards, the treatment medium was replaced with normal culture growth medium and the cells incubated for 24

hours. At the end of this recovery period, the intracellular content of reactive oxygen species (ROS) was analyzed using the reaction with 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, Life Technologies, Invitrogen™, USA; cat. no. D-399). Briefly, adherent ARPE-19 cells were washed once with SFM and incubated with 10 µM H2DCFDA (loading buffer) at 37°C for 30 minutes. The loading buffer was then removed, and the cells returned to SFM conditions. The fluorescence intensity generated by ROS (λex = 492 nm, λem = 517 nm) was measured with the VarioskanTM (Thermo Fisher Scientific, USA). The same plate was then used to estimate cell viability with the soluble CKK-8 assay (Sigma-Aldrich, cat. no. 96992). To this purpose, 10 µl of CKK-8 solution in 100 µl of SFM were added to each well for 1.5 hours at 37°C in a humidified atmosphere containing 5% CO2. At the end of the incubation time, the absorbance was measured at 450 nm with the plate reader Synergy 2 (BioTek, Thermo Fisher Scientific, USA). O.D. values were used to evaluate cell survival and to normalize ROS levels.

Clinical observations

The retrospective observational study included 17 healthy sport motorcycle test pilots working at a test facility located near the town of Giarre, in Sicily (Italy). The subjects were all males, aged between 24 and 55 years (Figure 2A), with a non-corrected binocular far-sight visual acuity (VA: Snellen optotype at a distance of 4.5 meters) between 2/10 and 15/10, and a non-corrected binocular near-sight VA (Jaeger optotype) between 8/10 and 12/10 (Figure 2B). All parameters were evaluated according to the protocol of the Italian Health Ministry (https://www.patente.it/normativa/allegato-a-alla-circolare-del-25-07-2011-n-0017798?idc=1650). Shortly, glare sensibility and glare recovery were evaluated measuring the binocular corrected far-sight photopic VA directly in the presence of a glaring light of 500 lux (sensibility), or after 30 seconds of rest in the dark (recovery), to give time to the recycling mechanisms in the retina to restore VA. Binocular corrected far-sight twilight VA was measured by dimming the ambient light to 2 lux (as opposed to the 500 lux to measure daylight visual acuity). Binocular corrected far sight contrast sensitivity (CS) was measured under photopic conditions by adjusting the contrast on the electronic optotype. Results are reported as the percentage of contrast attenuation that still allowed the correct reading of the smallest characters read at the 100% of contrast. Stereoscopic vision was measured by the TNO stereotest with red/green glasses and seven plates (carrying figures that can be seen only when both eyes cooperate to give stereoscopic vision) presented at 40 cm of distance, to establish and quantitate the stereoscopic perception expressed in seconds of arc (measuring retinal disparity or binocular parallax): the higher the value, the lower the stereopsis.

A subjective evaluation was also conducted, asking each subject how he would rate on a scale 0-5 (where 0 is optimal, and 5 is the highest perception of the defect) eye fatigue at the end of the day (asthenopia), glare sensibility, visual perception either in daylight or at twilight.

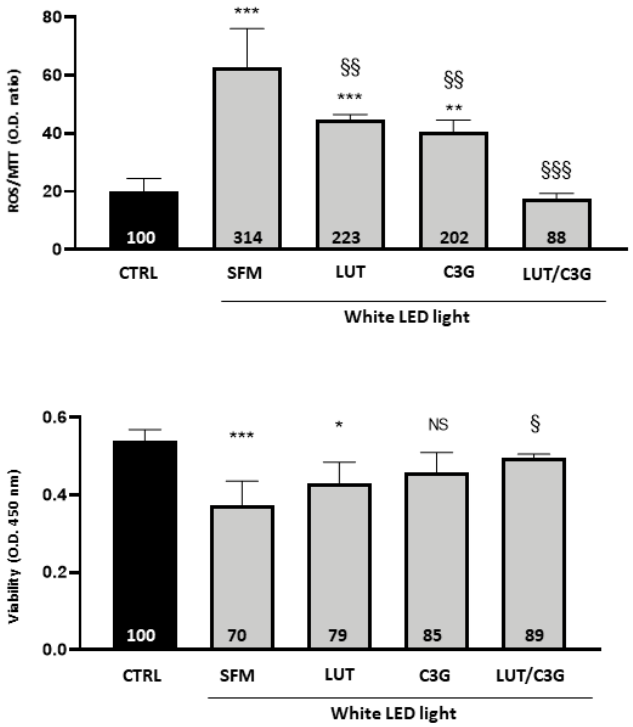

Figure 1: Phototoxicity in ARPE-19 cells. Intracellular ROS content and cell viability in ARPE-19 cells under dark condition (CTRL) or irradiated at 2 x 105 lux with a white LED light in presence of SFM either alone or added with 100 µM lutein (LUT), 100 µM C3G or their mix; A) Intracellular ROS content measured by the H2DCFDA assay. O.D. values were normalized to the respective viability O.D. values. Percent values are indicated at the bottom of each column. (B) Cell viability was evaluated by the CKK-8 assay and reported as mean ± SD of O.D. values. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ vs. CTRL; § $p \leq 0.05$; §§ $p \leq 0.01$; §§§ $p \leq 0.001$ vs. SFM. One-way ANOVA followed by Tukey's test.

The treatment with the food supplement, spontaneously chosen by the pilots on advice from their consultant sport physician, consisted of four pills per day (two in the morning and two in the evening) for the first 5 days, and then two pills per day (one in the morning, one in the evening) in the prosecution of the treatment, of a commercially available food supplement (Meramirt XG®, Sooft Italia SpA, Italy) containing an association of FloraGlo lutein (6 mg from an extract of marigold flowers titrated at 20% in lutein), cyanidin-3-glucoside (20 mg, from a black rice extract titrated at 20% in C3G), verbascoside (2 mg from an extract of *Verbascum thapsus* L. leaves titrated at 10% in verbascoside) and Zinc (5 mg). A group of 5 pilots took the treatment for 10 days, while the remaining 12 continued for 70 days (Figure 2). Evaluation of the parameters under analysis was done before the start of treatment and at the end of the respective treatment time as part of the routinary examinations that this category of pilots usually do.

Statistical evaluation, considering the small number of subjects under analysis, was done by the non-parametric Wilcoxon matched-pairs signed rank test, considering the paired values of each subject before and after treatment.

A p value < 0.05 was considered statistically significant.

Results

In vitro effect of lutein and C3G

The efficacy of lutein and C3G in the protection of the human RPE cell line ARPE-19 was evaluated in the presence of a white light LED lamp irradiation at $2x10^5$ lux and 5000 K. Under these conditions, intracellular ROS dramatically increased to 314% of control values (Figure 1A), and cell viability decreased to 70% (Figure 1B). The presence of lutein or C3G at 100 µM blunted the toxic effects of light irradiation, so that ROS were now significantly lower than SFM values, although still significantly higher than control values. Cell viability also appeared to be significantly improved in presence of C3G. The association of lutein and C3G, each at 100 µM fully prevented the increase of ROS and restored a cell viability not significantly different from control.

Effects of the food supplement on visual abilities of test pilots

From a retrospective analysis of the medical records of the group of 17 test sport motorcycle pilots, two groups were extrapolated, one of 5 individuals (group A) who followed the advised regimen with the food supplement for 10 days, and another of 12 subjects (group B) who followed the indicated regimen for 70 days. The two groups were similar for age (35 ± 13 group A; 42 ± 10 group B) (Figure 2A), binocular uncorrected far-sight VA (9.6/10 ± 4.3/10 group A; 8.8/10 ± 5.2/10 group B), and binocular uncorrected near-sight VA (10.8/10 ± 1.8/10 group A; 9.2/10 ± 3.5/10 group B) (Figure 2B).

The short treatment time (10 days) with the food supplement resulted in a general trend of improvement for all parameters, which however did not reach statistical significance likely because of the small size of the group, and the variability of results. Treatment for a longer time (70 days) resulted in a significant improvement of all measured parameters.

Far-sight binocular uncorrected VA measured on a decimal scale improved from 9.6 ± 4.3 to 13.8 ± 5.9 for group A (Figure 3A; p=0.06) and all individuals experienced such improvement (Figure 3C). A significant improvement (p=0.01) was observed for individuals of group B, with a shift from 8.8 ± 5.2 to 11.3 ± 5.9 (Figure 3B). Among these subjects, only one out of twelve did not show any improvement (Figure 3C).

Similar results were obtained for binocular uncorrected near-sight VA. Pilots in group A showed a non-significant (p=0.25) improvement from 10.8 ± 1.8 to 13.2 ± 3.9, involving only three out five subjects (Figures 3A and 3D). The values for pilots of group B significantly (p=0.03) improved from 9.2 ± 3.5 to 10.2 ± 2.6. In this case, only three subjects over twelve showed no signs of improvement (Figures 3B and 3D).

Critical for pilots running at high speed is the possibility to conserve a good vision also under glaring conditions. The measure of corrected binocular far-sight VA in the presence of a glaring light (Figure 4) shows some improvement (11.0 ± 1.4 to 13.4 ± 2.3; p=0.12) in group A (Figure 4A), and a more robust improvement (12 ± 1.8 to 15.6 ± 2.2; p=0.01) in group B (Figure 4B). Only one pilot out of five in group A and one pilot out of twelve in group B showed no improvement in this test (Figure 4C). Accordingly, a quick recovery of VA after glaring is necessary to maintain a steady control of the vehicle at high speed. Figure 5 shows the values of corrected binocular far-sight VA measured 30 seconds after glaring before and at the end of the treatment period with the food supplement. A consistent improvement for all subjects (Figure 5A and C) was seen in group A (from 10.2 ± 2.1 to 14.0 ± 2.2; p=0.06), which became significant for eleven out of twelve pilots in group B (from 12.1 ± 1.7 to 15.6 ± 2.2; p=0.001) (Figure 5B and C).

Corrected binocular far-sight VA recorded under twilight conditions also improved in all pilots of group A (Figure 6A and C), increasing from 11.4 ± 1.3 to 14 ± 2.2 (p=0.06), and in eleven out of twelve pilots of group B, from 11.9 ± 1.8 to 15.3 ± 2.5 (p=0.001) (Figure 6B and C).

Contrast sensitivity values for the two treatment groups are given in Figure 7. Improvement in this case consists in the ability to discriminate letters on the optotype with a lesser contrast, which translates in a decreased value measured as percent of the original contrast value set at 100%. Four out of five pilots of group A treated for a short time (Figures 7A and C) showed an average 5% improvement, from 21 ± 5.5 to 16 ± 2.2 (p=0.125). Seven out of 12 pilots treated for 70 days showed an average 6.2% improvement (Figures 7B and C), from 23.7 ± 9.6 to 17.5 ± 6.6 (p=0.015).

Stereoscopic vision (Figure 8) is a consequence of binocular frontal vision, and allows the exact perception of the shape, depth, and distance of an object. It is measured in seconds of arc, and also in this case lower values indicate a higher resolution of depth perception. The short treatment time of the 5 pilots in group A resulted in an average highly variable improvement observed in three out of five subjects (Figures 8A and C) from 210” ± 180” to 138” ± 161” (p=0.25). A significant improvement was observed in seven out of twelve pilots (Figures 8B and C) treated for 70 days, with average values shifting from 380” ± 170” to 280” ± 185” (p=0.015).

Finally, figures 9 and 10 consider the perception of the variation after food supplement treatment of four different parameters measured on a subjective scale from 0 (no defect perceived) to 5 (highest perception of the defect). Pilots of group A (Figure 9A) registered non-significant average improvements of eye fatigue at the end of the day (from 2.6 ± 1.7 to 1.2 ± 1.1; p=0.125); glare sensitivity (from 1.4 ± 1.9 to 1.0 ± 1.4; p=0.50); photopic perception (from 2.6 ± 1.7 to 1.2 ± 1.3; p=0.125); scotopic perception (from 2.8 ± 1.9 to 1.6 ± 1.1; p=0.125). Figure 10A-D shows that four out five improved for eye strain, photopic and scotopic perception, while only two out of five improved for glare sensitivity. Pilots of group B (Figure 9B) showed significant improvements of all parameters: eye strain at the end of the day (from 2.9 ± 1.0 to 2.3 ± 1.1; p=0.03); glare sensitivity (from 3.1 ± 1.1 to 2.1 ± 1.0; p=0.002); photopic perception (from 2.5 ± 1.4 to

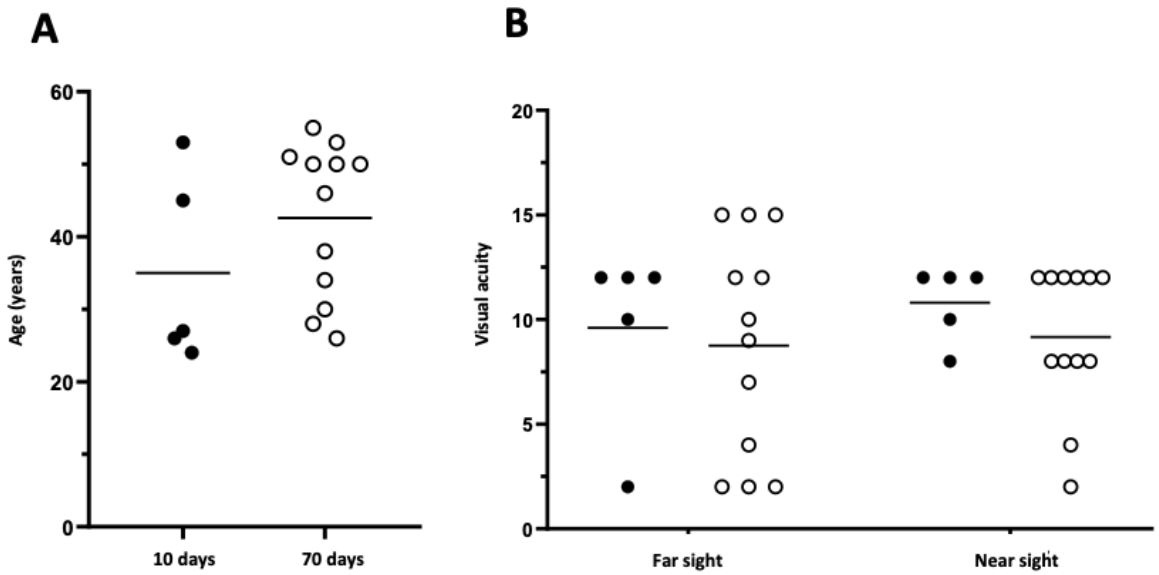

Figure 2: *Enrolment values of test pilots*. The enrolment values of pilots included in group A (black circles) or in group B (empty circles) is shown for age in years (A) and for visual acuity in decimals (B).

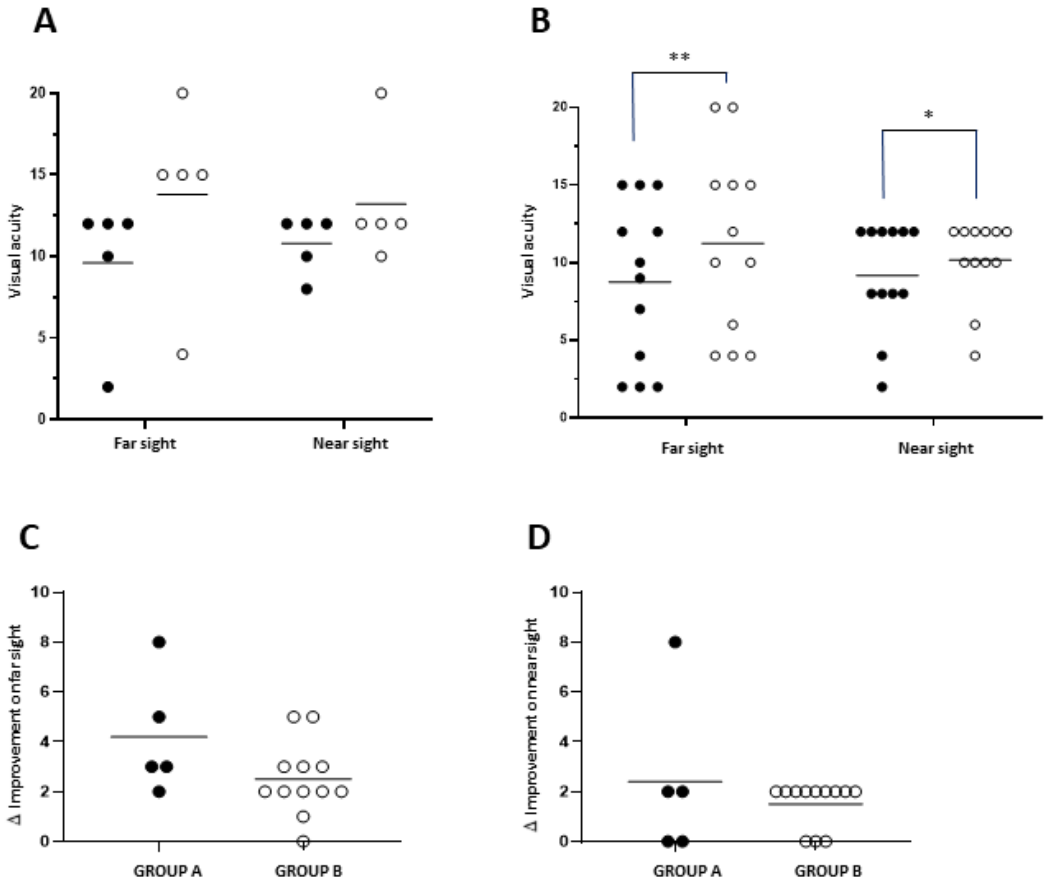

Figure 3: *Changes in visual acuity after treatment with the food supplement*. Binocular uncorrected far-sight and near-sight visual acuity values in decimals are shown for group A pilots (A) and group B pilots (B). Black circles before treatment, empty circles after treatment. The delta difference calculated subtracting the value before treatment from the value after treatment showing the extent of the improvement is shown for the far-sight visual acuity (C) and for the near-sight visual acuity (D). Black circles Group A, empty circles Group B. A zero value corresponds to a subject with no improvement; positive values give the measure of the improvement. * $p \leq 0.05$; ** $p \leq 0.01$ by the Wilcoxon matched-pairs signed rank test.

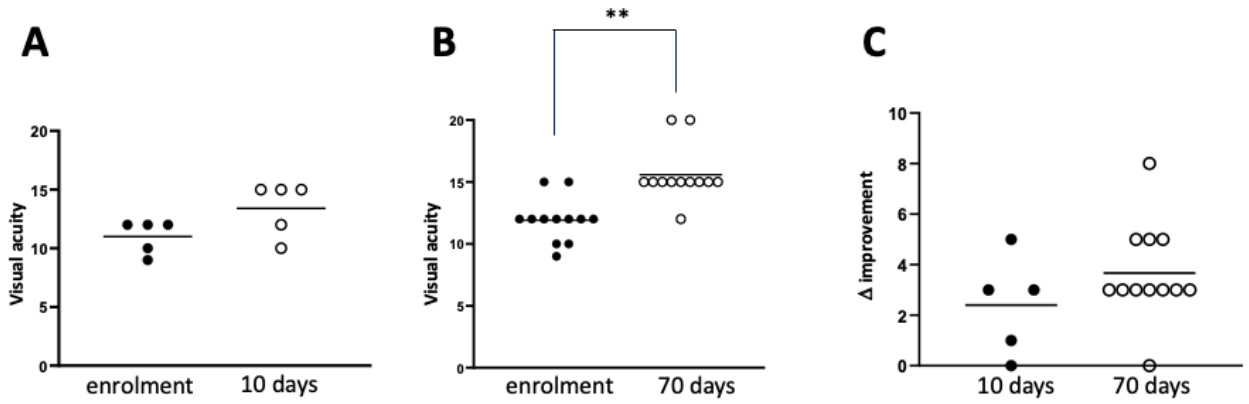

Figure 4: ***Glare sensitivity*.** Binocular corrected far-sight visual acuity was measured in the presence of a glaring light shined on the subject eyes. (A) subjects of Group A. (B) subjects of Group B. Black circles before treatment, empty circles after treatment. (C) Delta difference calculated subtracting the value before treatment from the value after treatment showing the extent of the improvement. Black circles Group A, empty circles Group B. A zero value corresponds to a subject with no improvement; positive values give the measure of the improvement. ** $p \leq 0.01$ by the Wilcoxon matched-pairs signed rank test.

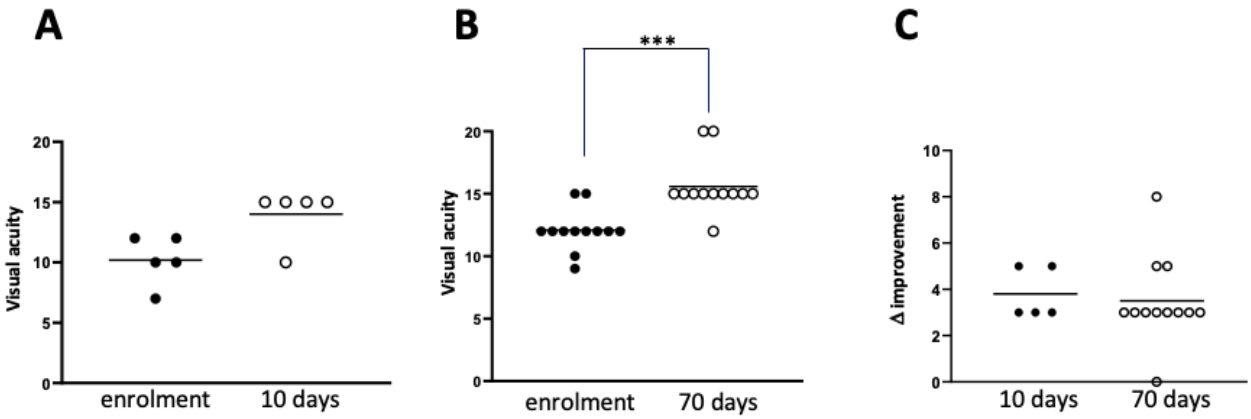

Figure 5: ***Recovery from glare.*** Binocular corrected far-sight visual acuity was measured thirty seconds after the shining of a glaring light on the subject eyes. (A) subjects of Group A. (B) subjects of Group B. Black circles before treatment, empty circles after treatment. (C) Delta difference calculated subtracting the value before treatment from the value after treatment showing the extent of the improvement. Black circles Group A, empty circles Group B. A zero value corresponds to a subject with no improvement; positive values give the measure of the improvement. *** $p \leq 0.001$ by the Wilcoxon matched-pairs signed rank test.

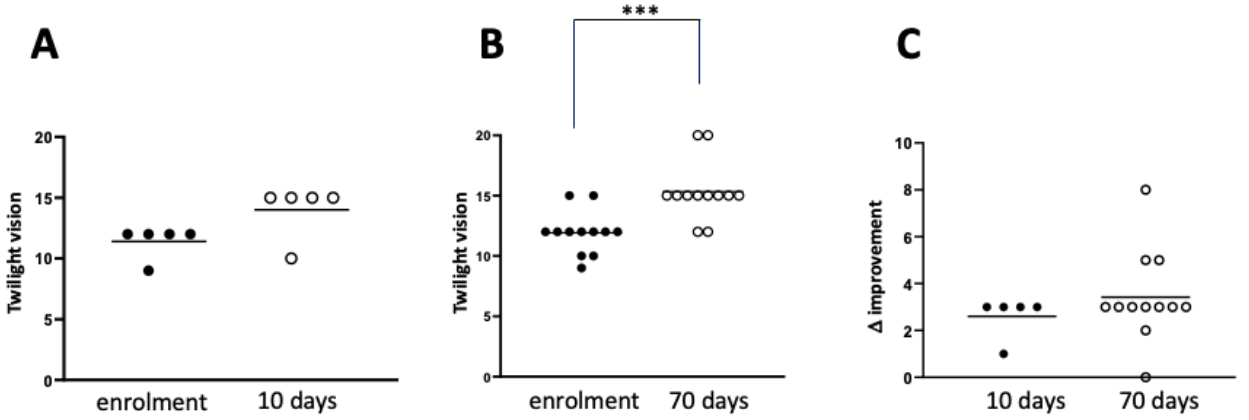

Figure 6: ***Visual acuity under twilight conditions*.** Binocular corrected far-sight visual acuity was measured after dimming the ambient light to 20 lux to simulate a twilight. (A) subjects of Group A. (B) subjects of Group B. Black circles before treatment, empty circles after treatment. (C) Delta difference calculated subtracting the value before treatment from the value after treatment showing the extent of the improvement. Black circles Group A, empty circles Group B. A zero value corresponds to a subject with no improvement; positive values give the measure of the improvement. *** $p \leq 0.001$ by the Wilcoxon matched-pairs signed rank test.

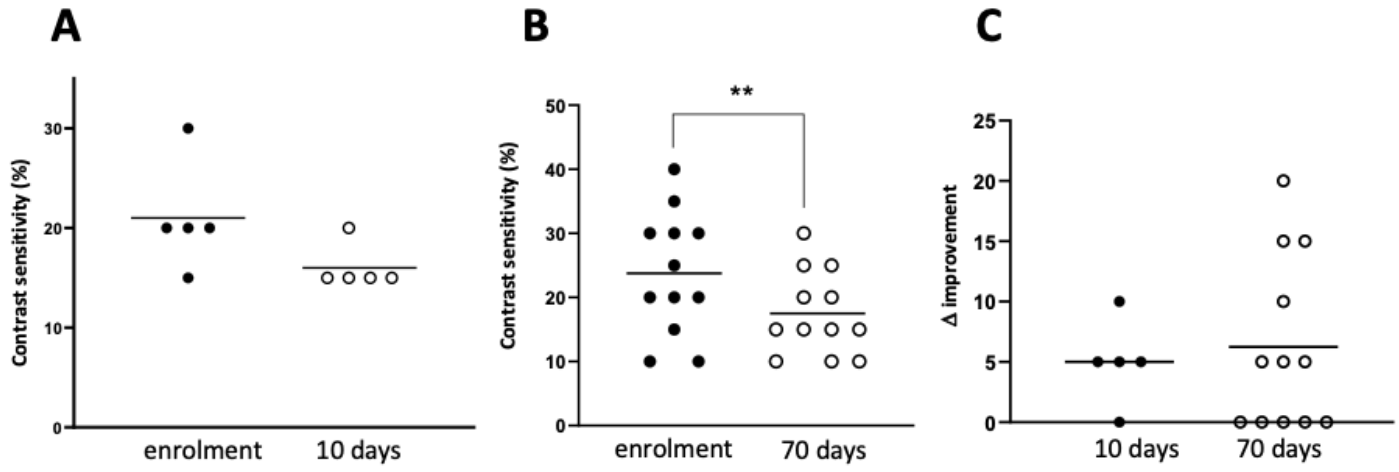

Figure 7: ***Contrast sensitivity*****.** Binocular corrected far-sight visual acuity was measured after progressive decrease of the contrast on the electronic optotype, reporting results as the lowest percentage of contrast attenuation that still allowed the correct reading of the smallest characters read at the 100% of contrast. (A) subjects of Group A. (B) subjects of Group B. Black circles before treatment, empty circles after treatment. (C) Delta difference calculated subtracting the value after treatment from the value before treatment showing the extent of the improvement (in this case the improvement is measured by a decrease of the value). Black circles Group A, empty circles Group B. A zero value corresponds to a subject with no improvement; positive values give the measure of the improvement. *** $p \leq 0.001$ by the Wilcoxon matched-pairs signed rank test.

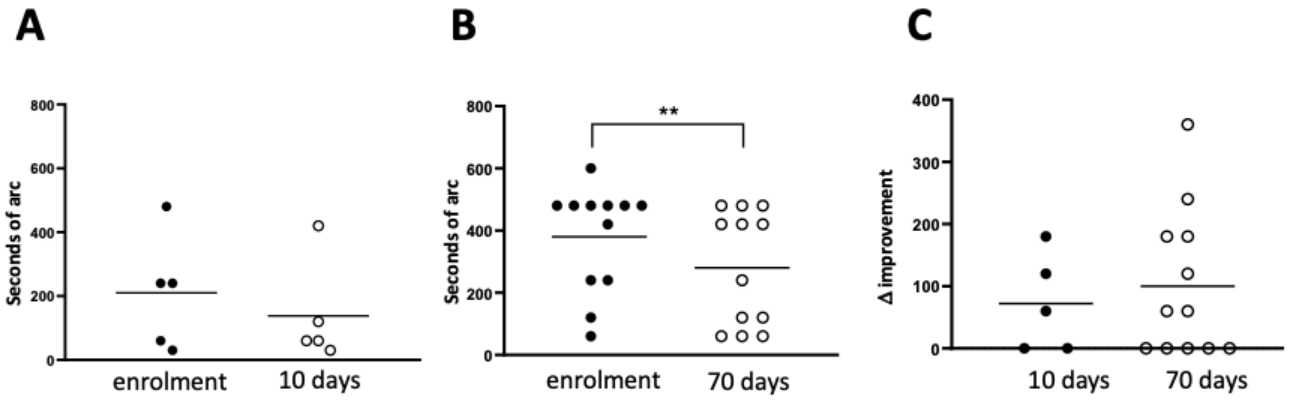

Figure 8: ***Stereoscopic vision*****.** Stereoscopic vision was measured with the aid of TNO plates, registering the seconds of arc at which stereoscopic vision is first obtained: the lower the value, the better the stereoscopic ability. (A) subjects of Group A. (B) subjects of Group B. Black circles before treatment, empty circles after treatment. (C) Delta difference calculated subtracting the value after treatment from the value before treatment showing the extent of the improvement (in this case the improvement is measured by a decrease of the value). Black circles Group A, empty circles Group B. A zero value corresponds to a subject with no improvement; positive values give the measure of the improvement. *** $p \leq 0.001$ by the Wilcoxon matched-pairs signed rank test.

1.5 ± 1.0; p=0.007); scotopic perception (from 3.0 ± 1.3 to 2.0 ± 1.1; p=0.008). Figure 10A-D shows that within the group of twelve pilots the improvement for eye strain occurred in six subjects; for glare sensitivity in nine subjects; for photopic perception in eight subjects and for scotopic perception in eleven subjects.

Discussion

We have presented here data showing the cooperative effect of lutein and C3G in protecting RPE cells from intense white light irradiation (Figure 1), mostly due to their antioxidant, free radical scavenging activity, and the shielding effect of lutein towards blue light. These results are highly consistent with what recently shown in a rat model of oxidative photostress, in which the association of lutein, C3G, verbascoside and zinc given by intragastric administration prevented oxidative stress, inflammation, gliotic and apoptotic responses in the retina, preserving the ERG response and protecting photoreceptor cells from death, with higher efficacy than each component alone [31].

Most relevant are the clinical observational data here reported, obtained from the retrospective analysis of the visual performance of sport motorcycle test pilots taking the food supplement for different lengths of time, and showing improvements of all the parameters under evaluation (Figures 2-10). The action of driving,

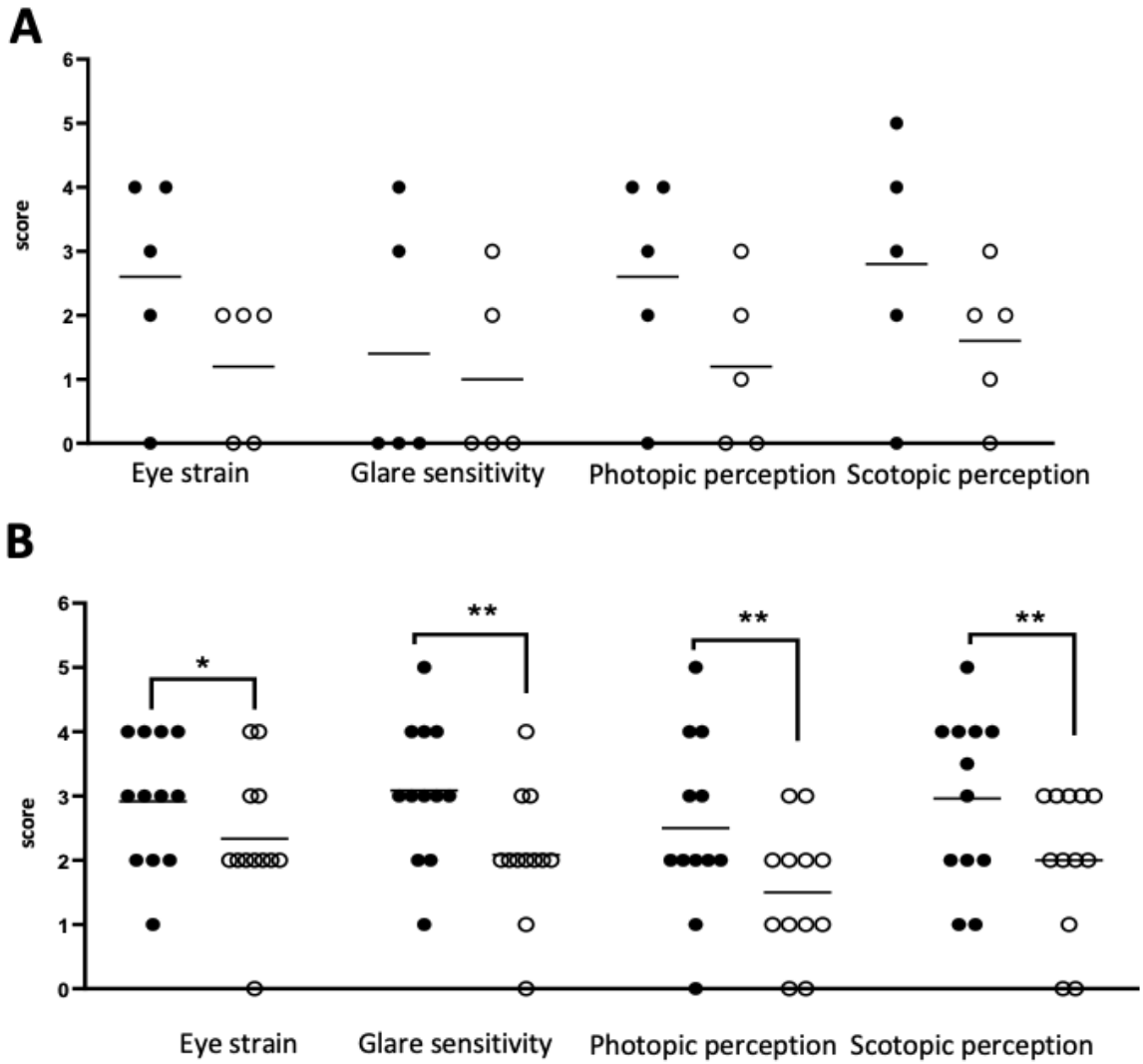

Figure 9: ***Subjective evaluation*****.** The subjective perception of vision parameters like eye strain at the end of the day, glare sensitivity and the ability of seeing during the day or at night has been rated by each pilot on a scale from 0 to 5, where 0 corresponds to an optimal performance, and 5 to the worst. (A) subjects of Group A. (B) subjects of Group B. Black circles before treatment, empty circles after treatment. ** $p \leq 0.01$ by the Wilcoxon matched-pairs signed rank test.

and even more when driving at high speed, requires optimal visual abilities, in order to have a full perception of the surrounding environment and all the possible obstacles that might present along the path, under variable light situations. Therefore, VA under photopic, scotopic and glaring conditions, contrast sensitivity, stereoscopic vision for correct depth perception, all contribute to the safety and the driving performance of pilots, either sport pilots, or even normal drivers in every day's traffic.

The maintenance of the efficiency of our visual system depends on the protection mechanisms that allow the continuous functioning of the photoreceptors (rods and cones) under stressing light conditions. Antioxidant defense in the retina is provided by intracellular enzymes mostly present in RPE cells: superoxide dismutase, catalase, glutathione peroxidase and reductase, peroxiredoxin; Zn is a critical co-factor for the functioning of catalase. Non enzymatic antioxidants like vitamins A, C and E are widely distributed among cells and the extracellular space; the macular pigment and rod outer segment disc membranes contain lutein and zeaxanthin working both as filters for high energetic blue light, and as free-radical scavengers. The melanin present in RPE cells works in preventing light scattering, as an antioxidant and as a filter for UV and blue light [32,33]. However, all this weaponry is not enough to protect the retina over a long lifetime; in fact, a common pathology like age-related macular degeneration (AMD) is the consequence of continuous oxidative stress, and the inadequacy of the antioxidant system for a full prevention of the related damages [32]. Therefore, since vitamins and carotenes must be taken through the diet, the use of food supplements to replenish and improve the antioxidant defenses of the eye is a strategy now widely used to prevent retinal diseases [28].

In our study, visual performance (which includes VA under different lighting conditions, adaptation to glaring conditions, contrast

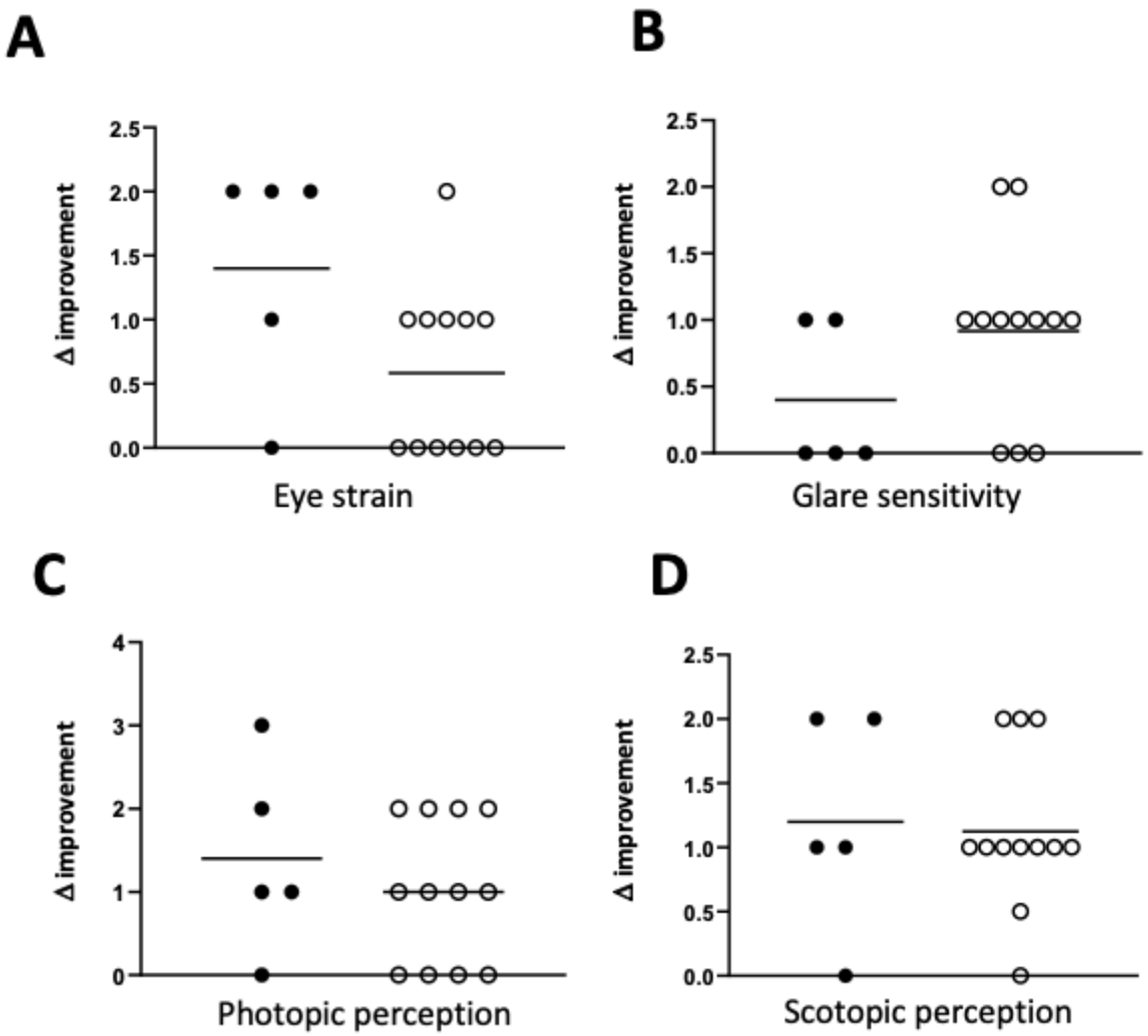

Figure 10: ***Delta improvement on subjective evaluation*****.** The delta difference per each parameter considered (A: eye strain; B: glare sensitivity; C photopic perception; D: scotopic perception) was calculated subtracting the value after treatment from the value before treatment showing the extent of the improvement (in this case the improvement is measured by a decrease of the value). Black circles Group A, empty circles Group B. A zero value corresponds to a subject with no improvement; positive values give the measure of the improvement.

sensitivity and stereoscopic vision) appears to improve in the vast majority of subjects treated with the food supplement, reaching statistical significance in the larger group of 12 individuals treated for 70 days. All the ingredients of the food supplement likely contributed to this effect.

Anthocyanins, and C3G among these, despite a low bioavailability after ingestion, easily cross the blood-retinal barrier, and can be found in ocular tissues and the retina [34]. C3G exerts its functions mainly through its phenolic metabolites produced in the gastrointestinal tract, such as protocatechuic acid (PCA), phloroglucinol-aldehyde (PGA), vanillic acid (VA) and ferulic acid (FA) [35]. PCA and PGA are considered the main bioactive phenolic metabolites produced by phase I metabolism. PCA can increase the antioxidant capacity of cells by increasing the activity of endogenous antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), thus attenuating lipid peroxidation in photoreceptors' membranes [36]. An *in vivo* study on rabbits exposed to photo-oxidative stress showed that C3G and its metabolites, especially protocatechuic acid (PCA) and ferulic acid (FA), protected the structure and function of cellular photoreceptors from visible light-induced retinal degeneration. Such protection appears to depend on their ability to induce the expression of the transcription factor Nrf2 which, binding to the antioxidant responsive element (ARE), induces the expression of antioxidant enzymes such as HO1, thus conferring protection from oxidative damage to retinal cells [37-39]. Moreover, the activation of the Nrf2/ARE pathway activates the glutamate-cysteine ligase [40], hence increasing the endogenous expression of the antioxidant glutathione and protecting neuronal cells from glutamate excito-toxicity [41].

The oral intake of anthocyanosides leads to a reduction of the adaptation threshold to vision in the dark, thus improving

nocturnal visual acuity and allowing the recovery of the transient refractive defect induced by "work at a screen terminal", as well as the subjective symptoms of eyestrain in healthy subjects [42]. This effect is likely a consequence of the stimulation by C3G of rhodopsin regeneration in rods [43], that are the photoreceptors diffused throughout the retina (with the exception of the foveola, populated by cones only), deputed to light perception under low illumination or in the dark. The general improvement on visual function by C3G could be related to the above effect on rhodopsin, but also to the effects on the metabolism of RGC facilitating their survival and functioning under stress conditions [42,44,45]. Moreover, the protective antinflammatory effects of C3G are also extended to RPE cells, as shown by a study in which the stress on RPE cells was given by inflammatory events triggered by 4-hydroxyhexenal-(HHE-), and the presence of C3G resulted in a decrease of inflammatory markers and apoptotic death, ultimately improving RPE cell survival [46].

Finally, anthocyanins (including C3G) have been shown both *in vitro* and *in vivo* to be able to activate endothelial NO synthase, thus improving endothelial function [47], and to protect endothelial cells from high glucose induced oxidative damage like in diabetic retinopathy [48,49]. An improvement in capillary retinal blood circulation and perfusion might also improve visual functions [50]. In fact, a treatment for 30 days with anthocyanins resulted in a significant improvement of the VA and CS of adult myopic subjects, thus implicating also an effect on the ciliary muscle, lens pliability and accommodation [51].

Verbascoside is also known for its antioxidant, antinflammatory and neuroprotective effects, linked to its ability to decrease the level of pro-inflammatory cytokines, like IL-6, IL-13 and TNFα [52]. The antinflammatory effects of verbascoside also depend on its capacity to inhibit the release of arachidonic acid (AA) and histamine from mastocytes, and the release of NO from macrophages [53,54]. Its antioxidant activity derives from its stimulatory effects of the Nrf2/ARE pathway [53,55], leading to the expression of antioxidant enzymes like HO1 and the concomitant inhibition of the ARE repressor BACH1 (*BTB Domain and CNC Homolog 1*) [56], thus in good cooperation with the effects of C3G. A peculiar activity of verbascoside is the inhibition of metalloproteases MMP2 and MMP9 [57], the latter being implicated in extracellular matrix degradation correlated with the detrimental effects on the retina of short wavelength light irradiation [58].

Lutein effects on eye and retinal health and defense are largely known since many years. Lutein is highly present in the human retina, especially at the macular level, where together with its stereoisomer zeaxanthin forms the macular pigment. Lutein and zeaxanthin are characterized by their blue light filtering and anti-oxidant properties. Thanks to these properties, the macular pigment can protect the underlying retinal structures such as RPE and photoreceptors from light induced damage [59]. Moreover, a clinical study on Chinese drivers found that a supplementation with lutein may improve driving at night together with other spatial discrimination tasks carried out under twilight conditions [60].

Last, but not least, zinc in humans plays relevant roles as an antioxidant and anti-inflammatory agent. Despite being a redox-inert metal, zinc works as an antioxidant through the catalytic action of copper/zinc-superoxide dismutase, the stabilization of cellular membranes, the protection of sulfhydryl groups in proteins, and the increase of metallothionein (MT) activity [61]. In addition to the well-known enzymatic antioxidants, MTs are arousing much interest in cell antioxidant protection. It is a family of low mw and cysteine rich proteins able to capture and neutralize free radicals through a redox-dependent mechanism involving zinc binding and release. Such Zn-MT redox cycle is an antioxidant defense system also found in the ocular surface, lens, retina and RPE [33]. Zinc supplementation clinical studies in adults reported a lower incidence of infections, decreased oxidative stress, and decreased production of inflammatory cytokines [62]. In fact, zinc may prevent the dissociation of the pro-inflammatory transcription factor NF-κB from its inhibitory protein, and in so doing it avoids the nuclear translocation of NF-κB thus inhibiting the subsequent inflammatory process [63].

This is the first report to our knowledge concerning a clinical observation on humans of this original formula comprising an association of lutein, C3G, verbascoside and Zn given as a food supplement. A similar study was published some years ago with a food supplement containing a mix of anthocyanosides, procyanidolic oligomers, lutein and vitamins A and E on the visual performance of aircrew members when wearing night vision goggles (NVG) [64]. The endpoint of this study was the analysis of visual evoked potentials (VEP) triggered by an alternating checkerboard pattern on a video screen. The VEP response was impaired by the use of NVG, and efficiently restored by the oral treatment with the food supplement with the ability to enhance foveal selectivity, central photoreceptors sensitivity and magnocellular fibers efficiency [64]. Lutein with omega-3 in food supplements has mainly been linked to protection of the retina from degenerative events due to age and oxidative stress, such as in the case of age-related macular degeneration [65,66].

In conclusion, our data support the use of the food supplement based on lutein, C3G, verbascoside and zinc both for the defense of the retina from age and oxidative insults, and for the improvement of visual performance when given to healthy subjects challenged by specific tasks requiring a good visual perception.

The limit of this study is that it is a retrospective observational study on a limited number of individuals. However, the results obtained grant for a longer, better organized, prospective clinical study, with either a cross-over design, or a run-in observational period.

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