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The efficacy of Saffron Repron® in counteracting the progression of retinitis pigmentosa: Neuroprotection and resilience

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ABSTRACT

The mutations observed in the various forms of retinitis pigmentosa (RP) affect genes coding for rod-specific proteins and direct the progression of different forms of dystrophy toward total blindness. The present investigation aims to explore the protective effects of Saffron Repron®, in a mouse model of RP. Saffron was administered orally to pregnant females and weaned pups for 120 days. At different time points (P30, P60, P90, and P120), visual function, retinal function, cone lifespan, morphology, gene expression, and protein level were analyzed. The results indicate that chronic saffron treatment effectively slows the progression of long-term damage caused by genetic mutations in both the morphology and function of retinal neurons. Cellular mechanisms responsible for this action appear complex and, probably, due to coordinated and synergistic activities by its chemical components. Here we provide evidence that saffron is able to modulate the epigenetic pathway involved in neuroinflammation. Biochemical and molecular measures suggest that early saffron treatment may induce a form of adaptation known as acquired resilience.

1. Introduction

Retinitis pigmentosa (RP) groups a series of inherited retinal dystrophies characterized by progressive degeneration of photoreceptors and abnormalities of the retinal pigment epithelium. It has an incidence of 1–5/10000.[1] Hundreds of mutations involving more than 200 genes have been identified. These mutations have both common and rare variants.[2] RP is fully included among the orphan diseases for which there is currently no definitive cure. The high genetic heterogeneity involves every aspect of photoreceptor biology, including development, phototransduction, protein transport, and recycling. All these factors, if not regulated lead to cell death.[3] Typically, vision loss appears early in young adults and the disease progresses inexorably to blindness. The first sign of the disease is night blindness and a progressive restriction of the visual field (tunnel vision) due to the loss of rods in the peripheral retina where they predominate. Later, the patients experience a progressive decline in visual acuity and impaired color discrimination caused by the gradual disappearance of cones in the central retina. [4] Although cones account for less than 5 percent of all retinal photoreceptors in most mammals, their role in human vision is critical and their degeneration leads to a condition of legal blindness. The most severe clinical sign of RP, caused by cone-mediated vision loss, is not a consequence of the primary genetic defect but derives from the secondary degeneration of cones which is, in turn, caused by decreased survival factors, altered glucose metabolism, oxidative stress, and increased inflammatory mediators.[5,6] It is therefore clear that saving even a fraction of cones can be crucial to ensure patients with residual serviceable visual function.[7]

One of RP's studied and known animal models is rd10. [8–10] This model represents one of the autosomal recessive forms of RP (incidence in patients up to 20 %) caused by a spontaneous point mutation at the level of the Pde6b gene, which turns out to be one of the most represented even in patients. [11]

Numerous treatments and therapeutic strategies have been proposed

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to slow RP progression and preserve cone function and survival. [7,12,13] Among the several treatments recently proposed, many are based on botanicals or nutraceutical products, which are known to counteract processes common to all forms of RP such as stress-oxidation and neuroinflammation. [5,14–17]

Recently, data have been published showing that saffron supplementation was able to slow down the progression of retinal diseases in AMD, Stargardt, glaucoma, and diabetic maculopathy patients.[18]

Oxidative stress plays a relevant role in visual pathologies [19] and the use of saffron was primarily suggested by its antioxidant molecular components. Preclinical data in animal models and cell cultures provided evidence of two relevant characteristics of saffron neuroprotective capacity, the protective efficacy of saffron is strictly related to its chemical composition, (ratio among chemical components, saffron Repron), and multiple ways of action are activated suggesting an integrated activity of all the components.[20] Apparently, the neuroprotective efficacy of saffron seems to include control of neuroinflammation and increased capacity to cope with dis-metabolic responses by increasing tissue resilience.

Growing evidence from pharmacological studies has also shown that saffron may exert a variety of beneficial effects in addition to neuroprotection, like anticonvulsant, antidepressant, anxiolytic, antioxidant, anti-inflammatory, hypolipidemic, antiatherogenic, antihypertensive, and even anticancer. [22–24] Based on phytochemical studies, the pharmacologically active constituents of saffron include bitter principles (e.g., picrocrocin), volatile agents (e.g., safranal, which can be obtained by hydrolysis of picrocrocin), and coloring substances (e.g., crocetin and its glycosides, crocins, which give saffron its characteristic color). [21,23]

All these data allowed us to hypothesize that preventive treatment with saffron Repron might be effective in slowing retinal degeneration in RP as well. In this study, a group of pregnant rd10 females were treated with an oral dose of 10 mg/kg/day saffron during their gestational period until the pups were weaned. Saffron treatment was then continued, again per os, in pups up to P120, and visual function and retinal morphology were analyzed at various time points. Interestingly, both parameters resulted improved in treated animals compared to control, for a long period of time, never reached before using different neuroprotective strategies. Altogether, we provide evidence that retinal tissue, even in animal models genetically predisposed to neurodegenerative diseases, can adapt and counteract dysmetabolic events thus delaying the onset and progression of the disease. The results obtained, if confirmed in humans, could represent a successful strategy to increase the capability of the system to counteract damage, including the genetic one, a process called acquired resilience.[25]

2. Methods

2.1. Animals

The rd10 mouse is affected by a phosphodiesterase 6b mutation (Pde6brd10/rd10) rooted in a C57Bl/6 J genetic background. In these mice, the degeneration of rod photoreceptors starts around the 18th day after birth, followed by cone photoreceptor degeneration, which winds up in 60 days.[8] These mice were maintained under a constant room temperature and exposed to a 12-hour light/dark cycle, ensuring that light intensity did not exceed 60 lux. All procedures on these animals complied with adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and both Italian and European standards and were sanctioned by the Italian Ministry of Health and the Ethical Committees of the University of Pisa (n° 719/2022-PR).

2.2. Saffron testing chemical composition

According to international patent W02015/145316, owned by Hortus Novus srl, each batch of saffron undergoes a series of analytic tests to determine if the chemical composition of the spice meets the established requirements for possessing neuroprotective activity (checked out by the Repron trademark). According to the ISO 3632 standard referenced,[26] an aqueous extract of saffron is analyzed using a UV-Vis spectrophotometer. The spectrum is acquired in the wavelength range between 200 and 600 nm, as the main molecules (crocins, picrocrocin, and safranal), responsible for the organoleptic properties of the spice, absorb at 440 nm, 330 nm, and 257 nm, respectively. The determined quantities are three: coloring power E440, indicative of the crocin content; aromatic power E330, indicative of the safranal content; and bitterness power E257, indicative of the picrocrocin content. These three quantities are then compared with the tabulated values in the standard, and the saffron's class is determined, with class 1 saffron of top quality and with the highest market value. All quantities must be referred to the dry weight of saffron. The ISO standard also requires an evaluation of the moisture, which in the case of saffron in stigmas must be less than 12 %. In addition to spectrophotometric analysis, saffron is analyzed using High-Performance Liquid chromatography (HPLC-DAD). Specifically:10 mg of saffron is extracted using 10 ml of methanol/water 50/50 by volume. The extraction is carried out at room temperature, in darkness, under magnetic stirring for 1 hour. The extract is then analyzed as follows: stationary phase, C18 5μm column, Evo (Phenomenex); gradient elution phase, initial conditions 95 % water, 5 % acetonitrile; after 30 minutes, linearly reaching 100 % acetonitrile. The neuroprotective activity of saffron depends on its chemical composition, particularly its crocins content. Specifically, we have focused on the content of the two most abundant: trans-crocetin bis (β-d-gentiobiosil) ester (T1) and trans-crocetin (β-d-gentiobiosil) (β-d-glucosyl) ester (T2), which are present in all saffron varieties regardless of geographical origin.

The two crocins have been quantified using the following formula: [27]% i-th crocin= (E440 * A_i*W_i)/(10 *ξ)

The concentration of the i-th crocin is referred to the dry weight of saffron and is determined taking into account the coloring power E440, the molecular weight Wi of the crocin, the relative area of the i-th crocin (the ratio of the area of the specific crocin to the total area of the chromatogram at 440 nm), and ξ, the molar extinction coefficient which for trans crocins is 89000[28]. The results of these measures indicate that for saffron to meet the Repron standards and to possess neuroprotective activity, the percentage concentrations of the two crocins must be equal to or greater than the given values of 17 % for T1 and 8 % for T2. Saffron was tested at the beginning of the procedure and then re-tested at the end to verify the maintenance in time of the chemical composition.

2.3. Saffron Repron treatment

The experimental protocol was set off during the pregnancy of the female mice. The animal was treated with an oral solution of Saffron Repron 10 mg/kg/day [29] from day of gestation 10 up to the delivery and then throughout the lactation of the pups, up to P18 when the pups were able to drink on their own. Separated at P18, the animals were treated with the same dose of saffron as the mother had been treated with, up to P30, P60, or P120. Although data on embryonic and neonatal development are limited, available toxicological evidence indicates that low doses of saffron extract are generally well-tolerated in rodent models without inducing teratogenic or toxic effects.[30,31] Additionally, throughout the study, no signs of maternal toxicity, adverse pregnancy outcomes, or developmental abnormalities were observed, supporting the safety of this dose in pregnant mice and pups. A control age group was also treated with no saffron delivered. The weight of the animals was checked every two days, and the dose was adjusted according to their weight. Also, water intake was measured every two days, and the treatment was always delivered at the same time of the day.

We decided to maintain the same dosage across developmental stages based on several considerations. Firstly, the selected dose of 10 mg/kg/day has been shown in previous studies to be well-tolerated across different life stages in rodent models without inducing

toxicity.[29] Secondly, maintaining a consistent dose allowed for a more straightforward comparison of effects across developmental periods, reducing variability that could arise from dose adjustments. Additionally, given that the dosage was weight-adjusted every two days, the actual amount of saffron each animal received was proportionate to its growth, ensuring consistent exposure relative to body weight. To prepare the solution, saffron was simply dissolved in water and left stirring for approximately one hour before being administered to the animals.

2.4. Behavioral test: Prusky water maze

To assess visual acuity under photopic conditions, the Prusky water maze was used with the same group of 12 mice at various ages (30, 60, 90, and 120 days).[32] The visual stimuli consisted of computer-generated square-wave black and white gratings with a fixed luminance of 39.95 cd/m^2 and spatial frequencies ranging from 0.087 to 0.550 cycles/degree. These stimuli were displayed on gamma-linearized monitors and generated using MATLAB 2022 and PsychToolbox. [33]

The mice underwent initial training at 20 days old, and following the method outlined in, [34] the spatial frequency was progressively adjusted to determine the threshold of each animal in locating the platform. During the test, if the animal made the right decision, one cycle was added to the screen to enhance the spatial frequency of the stimulus. The time spent away from the threshold was kept to a minimum by carrying out this process for the low spatial frequencies until a mistake was made. After an error, more trials were conducted until either four consecutively accurate responses were produced or seven correct choices were made in a block of 10 trials.

The highest spatial frequency at which the mice consistently located the platform at least 75 % of the time was recorded as the visual acuity marker. Using the spatial frequency with the 75 % performance that was last achieved is a reliable and effective method of quantifying the visual acuity of our animals. Even though, it may slightly underestimate their understanding compared to the frequency expected by chance (50 %) because the performance declined rapidly at the threshold. [35]

2.5. Electroretinogram (ERG)

Dark-adapted animals (a total of 43 mice) were anesthetized with an intraperitoneal injection of urethane (20 % physiological saline solution) at a dose of 0.1 ml/10 g body weight. The reference electrode (earth) was implanted on the scalp. ERGs were recorded in total darkness using coiled gold electrodes in contact with corneas moistened by a thin layer of gel (Lacrinom, Farmigea, Pisa, Italy). Responses were digitalized at 12.8 kHz using a computer interface (LabVIEW 6.1; National Instruments, Austin, TX, US), amplified differentially, and band-pass filtered at 0.1–500 Hz before being recorded on a disk for further processing. Animals were housed and light stimulated inside a 30 cm diameter Ganzfeld sphere coated inside with highly reflecting white paint. Six calibrated neutral density filters were utilized to control the intensity of the light stimulation, which was provided by a white light electric flash (SUNPACK B3600 DX, Tecad Company, Tokyo, Japan). A single flash of increasing intensity (1.71 ×10–5–377.2 cd*s/m2, 0.6 log unit increments) was presented to mice six times for scotopic ERG recordings, with an inter-stimulus interval spanning from 20 s for dim flashes to 45 s for the strongest flashes. Fifteen minutes after the onset of a stable background of saturating intensity for rods (30 cd/m2), isolated cone (photopic) components were registered by superimposing the test flashes (0.016–377.23 cd* s/m2) over the constant background. The amplitude of the scotopic and photopic b-waves were measured from the baseline to the b-wave peak. The data were analyzed with the LabVIEW 2019 program (National Instruments, Austin, TX, US).

2.6. Immunohistochemistry

Whole eyes were collected from mice treated with saffron and untreated mice at 30, 60, and 120 days of age. A total of 30 mice (5 for each group) were used for immunohistochemistry. The animals were deeply anesthetized with an i.p. injection of Urethane and sacrificed by cervical dislocation. Immunostaining on vertical sections was performed as reported in. [36] Briefly, retinal sections were incubated overnight at 4°C, with the primary antibody (Table) in 1 % BSA solution and the next day were incubated with the appropriate secondary antibody for two hours at room temperature. Following that, sections were incubated with ethidium homodimer or DAPI solution in PBS (1:5000, Sigma-Aldrich, Merck Group, Burlington, Massachusetts, United States). Finally, they were covered with a mounting medium (Vectashild, Vector Laboratories, Inc., Newark,

United States). A Nikon confocal microscope was used to obtain the images, with a 20 × (air) and 40 × (water) objective. Z-stack capture was done for the PSD95/PKCα staining. After that, the images were processed using analytic software (ImageJ). Cone counting was performed on retinal whole mounts. The immunostaining on the whole mount was performed as reported by.[18] Briefly, the primary antibodies (Table 1) were incubated with the retinas at 4°C for 3 days in a 1 % BSA and 0.1 % Triton solution. The retinas were then treated with the secondary antibody for 2 days. Finally, the retinas were opened in whole mount, placed on microscope slides with the photoreceptor side up, and coated with Vectashild. Nikon mod. NiE fluorescent microscope with Nikon mod. DS-Ri2 digital camera was used to capture the images. The 10x objective was used for reconstruction of the whole retina and the various images acquired were merged by using Nis software to rebuild the entire retinal surface. Cone counting was performed by acquiring images at 20x on the focal plane of the outer segments of the cones. For each sample, 8 different regions of the retina, spaced along the dorso-ventral and naso-temporal meridians, were acquired. images and counting were performed by the Nis software. The sample area used covers approximately 15 % of the total retinal area.

2.7. Western Blot

Protein lysates were obtained with 25 μL of RIPA-modified lysis buffer. [37] Samples were quantified with Bradford assay (Bio-rad, Hercules, California, United States) according to the manufacturer's instructions. The procedure of electrophoretic run of protein samples, antibodies incubation, and analysis has been described previously in. [38] Briefly, 25 μg of each cell protein extract was mixed with the Laemmli 2X (Bio-rad, Hercules, California, United States) solution and resolved by electrophoresis SDS-PAGE, supported by the use of precast stain-free gel (Bio-rad, Hercules, California, United States). After activation, the separated proteins were transferred to PVDF membranes (Bio-rad, Hercules, California, United States). The membranes were incubated with the antibody in Table 1. All the proteins of interest were normalized to the total protein content. [39] The densitometry analysis was undertaken using Bio-Rad ImageLab software 6.0 (Bio-rad, Hercules, California, United States).

Table 1
Antibodies used for Immunohistochemistry and Western Blot.

Antibody	Host	Company	Work dilution	Application
Anti-Opsin B	Rabbit	Millipore	1:500	IHC/WB
Anti-Opsin R/G	Rabbit	Millipore	1:500	IHC/WB
Anti-Rhodopsin	Mouse	Millipore	1:200/1:1000	IHC/WB
Anti-PSD95	Mouse	Millipore	1:200	IHC
Anti-PKCa	Rabbit	Millipore	1:200	IHC
Anti-SIRT1	Mouse	Abcam	1:1000	WB
Anti-NF-κB	Mouse	Millipore	1:1000	WB
Anti-Rabbit Alexa 568	Goat	Biorad	1:1000	IHC
Anti-Rabbit Alexa 488	Goat	Biorad	1:500	IHC
Anti-Mouse Alexa 568	Goat	Biorad	1:500	IHC
Anti-Mouse HRP	Goat	Millipore	1:5000	WB
Anti-Rabbit HRP	Goat	Millipore	1:5000	

IHC, immunohistochemistry; WB, western blot.

2.8. RT-qPCR

Total RNA from retina samples was extracted using miRNeasy Micro Kit (Qiagen, Hilden, Germany). The extracted RNA was quantified by NanoDrop Lite spectrophotometer (ThermoFisher Scientific, Nanodrop Technologies, Wilmington, DE, US) and then retro-transcribed using RT2 First Strand Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The obtained cDNA (n = 3; 5 ng/uL) was used for the analysis of multiple genes by using the Custum RT^2 Profiler PCR Array (Qiagen, Hilden, Germany) (Figure S2). The expression analysis was carried out by the Gene Globe Data Analysis Center (Qiagen, Hilden, Germany).

Then further gene expression analyses were performed in cDNA samples (n = 5; 5 ng/uL). Real-time PCR was performed using RT^2 SYBR Green Mastermix (Qiagen, Hilden, Germany) and specific primers RT^2 qPCR Primer Assay (Catalog No. 330001, Qiagen, Hilden, Germany) (Table 2). GAPDH was used as the housekeeping gene. The mRNA expression was calculated using the ΔΔCt method.

2.9. Statistical analysis

Using the Origin Lab 8.0 application, one-way ANOVA was used for the statistical comparisons of ERG parameters, followed by *t*-tests with Bonferroni correlation (MicroCal, Northampton, MA, United States). GraphPad Prism Software was used to analyze the data, for the remaining studies. A corresponding parametric or non-parametric test was carried out depending on the results of the normality test. The legend of each graphic contains pertinent information concerning statistical testing. Results were considered statistically significant for p-values under 0.05.

3. Results

3.1. Treatment with Saffron Repron improves visual and retinal function

A behavioral test was used to evaluate, in cooperating animals, visual function at different time points during the progression of the neurodegenerative disease. Animals in the Saffron Repron treatment or untreated group were trained to perform the Prusky Water Maze (PWM) test and by means of this test it was possible to follow the decay of visual function longitudinally in the animals themselves. Specifically, the results obtained by PWM behavioral tests showed that rd10 mice treated with Repron saffron retained better visual acuity than untreated controls at all the analyzed time points. At P30, the average visual acuity in the Repron-treated group was 0.308 ± 0.055 cycles/degree, compared to 0.205 ± 0.062 cycles/degree in the control group ($P = 0{,}0004$). At P60, treated mice maintained a visual acuity of 0.297 ± 0.012, while controls significantly declined to 0.121 ± 0.054 ($P < 0.0001$). By P90, the treated group had a visual acuity of 0.21 ± 0.022, still higher than the control group, which declined further to 0.126 ± 0.052 ($P = 0{,}0043$). On day 120 (P120), visual acuity in the treated group was 0.166 ± 0.024, compared to 0.107 ± 0.015 in controls ($P = 0{,}0465$). The difference in visual decline between the Repron saffron-treated group and the control group is evident in Fig. 1. While visual loss in control mice begins at P30 and reaches a plateau between P60 and P90, in treated mice visual acuity is not significantly different between P30 and P60 (Table in Fig. 1) demonstrating that visual function remains constant in this time window; from P60 onwards, there is a decline in visual acuity which, although evident at P90 and P120, is still greater compared to the animals in the untreated control group. Therefore, in saffron Repron-treated animals, the beginning of visual impairment is delayed by 30 days compared with untreated animals. The curves in Fig. 1C show the percentages of correct responses by the animals to the different spatial frequencies presented. Treated animals consistently maintained higher correct response rates, with 90 % at P60 compared to 50–60 % in controls, indicating better preservation of visual function over time. The slope of the response curves in saffron-treated animals was also more gradual, indicating a slower rate of visual decline compared to the untreated group.

In other groups of animals, ERG recordings were performed for each animal under scotopic and photopic conditions to assess retinal neuron activity, in particular, the ability of photoreceptors to respond to light stimuli and to pass the signal to ON bipolar cells. The ERG sensibility curves in functions to light stimuli are shown in Fig. 2. Fig. 2A illustrates the response curves of the b-wave amplitude in early degenerating rd10 mice (aged P30) for both scotopic and photopic ERG in the Repron saffron-treated group (in orange) compared to its control group (in black). The retinal function in the late degenerating rd10 mice is illustrated in Fig. 2B showing the b-wave light intensity response curves of the scotopic and photopic ERG in Repron saffron-treated mice (orange line) aged P60 and P120 and their corresponding controls (black line). Fig. 2C shows the decay of scotopic and photopic ERG responses at the high light intensity stimulus (377 cd*s/m2), at different time points. For the scotopic ERG, it was evident that the treatment was effective in preserving the b-wave amplitude of the scotopic ERG at P60 (Repron-treated group 101.97 ± 15.10 vs control group 0) and P120 (Repron-treated group 65.38 ± 30.84 vs control group 0). Saffron Repron treatment was also effective in preserving the function of the cone pathway as shown by the photopic ERG where the amplitude of the b-wave was significantly larger in treated animals at all-time points analyzed (P30 Repron-treated group 215.98 ± 35.16 vs control group 109.22 ± 12.93; P60 Repron-treated group 103.15 ± 11.46 vs control group 75.28 ± 9.21; P120 Repron-treated group 94.47 ± 4.03 vs control group 28.30 ± 28.30).

Table 2
Primers used for the RT^2 qPCR.

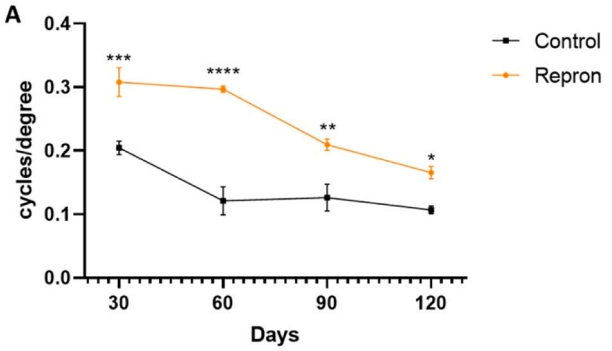
Name	GeneGlobe ID	Detected Transcript
Guanine nucleotide-binding protein alpha transducing 1 (GNAT1)	PPM03513A−200	NM_008140
Arrestin 3, retinal (ARR3)	PPM04815A−200	NM_133205
Guanine nucleotide-binding protein, alpha transducing 2 (GNAT2)	PPM06964A−200	NM_008141
cyclic nucleotide-gated channel beta 1 (CNGB1)	PPM06962A−200	NM_001195413

3.2. Treatment with saffron Repron preserves the lifespan of cone photoreceptors

Cone-opsin B+R/G labeling was carried out to examine the preservation of cone lifespan following saffron Repron treatment. This marker is specific for the cone visual pigment (Fig. 3). The whole retinas of saffron-treated animals and their corresponding controls at all-time points are shown in Fig. 3A; the panels to the right end of the figure showed the magnified area of the corresponding whole retina of Fig. 3A where outer segments of cones were visible. When compared to the treatment group, it can be noticed that a large majority of the control group cones lose their outside segments while those of treated animals are substantially preserved. A quantitative determination of the residual cone number is reported in the bar graph of Fig. 3B which showed that at P60 and P120 about 50 % of the outer segments are preserved in treated animals, while in untreated animals the loss is nearly complete.

3.2.1. Treatment with saffron Repron modifies protein and gene expression of photoreceptors

Western blotting of rhodopsin and cone-opsin B+R/G protein expression obtained at all time points in treated and corresponding controls are shown in Figs. 4A and 4B. A substantial increase in the expression of rhodopsin is observed in the treated group at P30, reaching approximately 200 % of control levels, while in the control group, cone-opsin remains unaffected at around 100 %. A significant increase in the



B

Sample group	Summary	P Value
Ctrl p60 vs. Ctrl p30	**	0,0072
Ctrl p90 vs. Ctrl p30	*	0,0138
Ctrl p120 vs. Ctrl p30	***	0,0010
Repron p60 vs. Repron p30	ns	0,9995
Repron p90 vs. Repron p30	***	0,0009
Repron p120 vs. Repron p30	****	<0,0001
Repron p90 vs. Repron p60	**	0,0042
Repron p120 vs. Repron p60	****	<0,0001

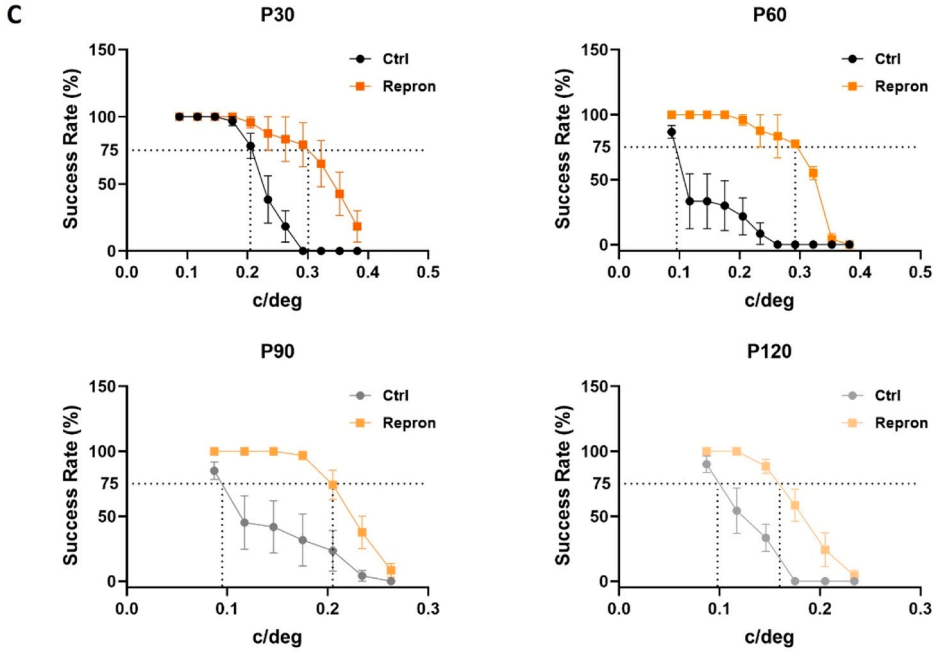


Fig. 1. Visual acuity assessed by Prusky water maze test. A. Visual acuity expressed as cycles/degree in mice treated with Repron and in the control mice, at different time points (P30, P60, P90, P120). The mice were divided into 2 groups: control (n = 6) and Repron (n = 6); the same animal performed the test at different time points. One-way ANOVA followed by Tukey's multiple comparisons test. *$P \leq 0.5$ (Repron P120 vs ctrl P120); * *$P \leq 0.05$ (Repron P90 vs ctrl P90); * **$P \leq 0.01$ (Repron P30 vs ctrl P30); * ** *$P \leq 0.001$ (Repron P60 vs ctrl P60). B. Table of the significant values between the control mice at different time points and the saffron-treated mice at different time points. Two-way ANOVA followed by Tukey's multiple comparisons tests. C. Success rates (percentage of correct trials) for each group in response to each spatial frequency.

treated group, in rhodopsin levels are also observed at P60, with levels around 220 % of controls. Cone-opsin values showed a modest increase, maintaining levels close to 100–120 %. At P120, rhodopsin levels in treated animals were about 130 % compared to controls, while cone-opsin levels showed an increase to approximately 120 % of control levels. The increase in rhodopsin levels at P60 demonstrates a delay in the degeneration of the rods (carriers of the mutation responsible for the disease), which also affects the subsequent degeneration of the cones, which in fact, showed a trend in the increase of cone-opsin levels at P120 compare with control group.

The results of a study on specific genes for the correct functionality of the photoreceptors CNBG1 (photosensitive channel), GNAT1 (transducin 1), found in rod photoreceptors, and ARR3 (cone-arrestin), GNAT2 (transducin 2), found in cone photoreceptors, are shown in Fig. 4D. At P30, gene expression in the treated group was notably higher, with GNAT1 reaching about 300 % and ARR3 around 180 % of control levels. At P60, the increase became more pronounced, with CNGB1 at approximately 250 %, GNAT1 at 400–500 %, and ARR3 at 250 % of control levels. GNAT2 levels remained consistent at around 100 % across all time points. By P120, the trend in gene expression increase was lost, with all genes, except for ARR3, showing values comparable to controls. All these data are in agreement with each other and confirm the results obtained with the analysis to assess retinal function (ERG in Fig. 2), in particular confirming the preserved state of the photoreceptors and bipolar cells.

3.2.2. Morphology of Outer and Inner Retinal Cells in control and saffron Repron treatment

The visual pigment rhodopsin the specific protein responsible for light absorption, is mainly localized in the disk membranes of the outer segments of rods. In panel A of Fig. 5, at P30 the retinas of saffron-treated animals show increased rhodopsin labeling compared to their control. As degeneration progresses (P60 and P120) the labeling for rhodopsin is completely absent in controls but in the group treated with Repron at P60, a residual staining was again present. At P120 also the Repron treated group had lost the marking for rhodopsin. In the same sections, however, and at all-time points, the animals treated with Repron show a greater number of lines of photoreceptors (ONL, cell nuclei stained in red) compared to the relative controls. Furthermore, it is well known that second-order retinal neurons undergo regressive modifications at times of phase I remodeling after photoreceptor death. Typically, photoreceptor terminals and their synaptic machinery in the

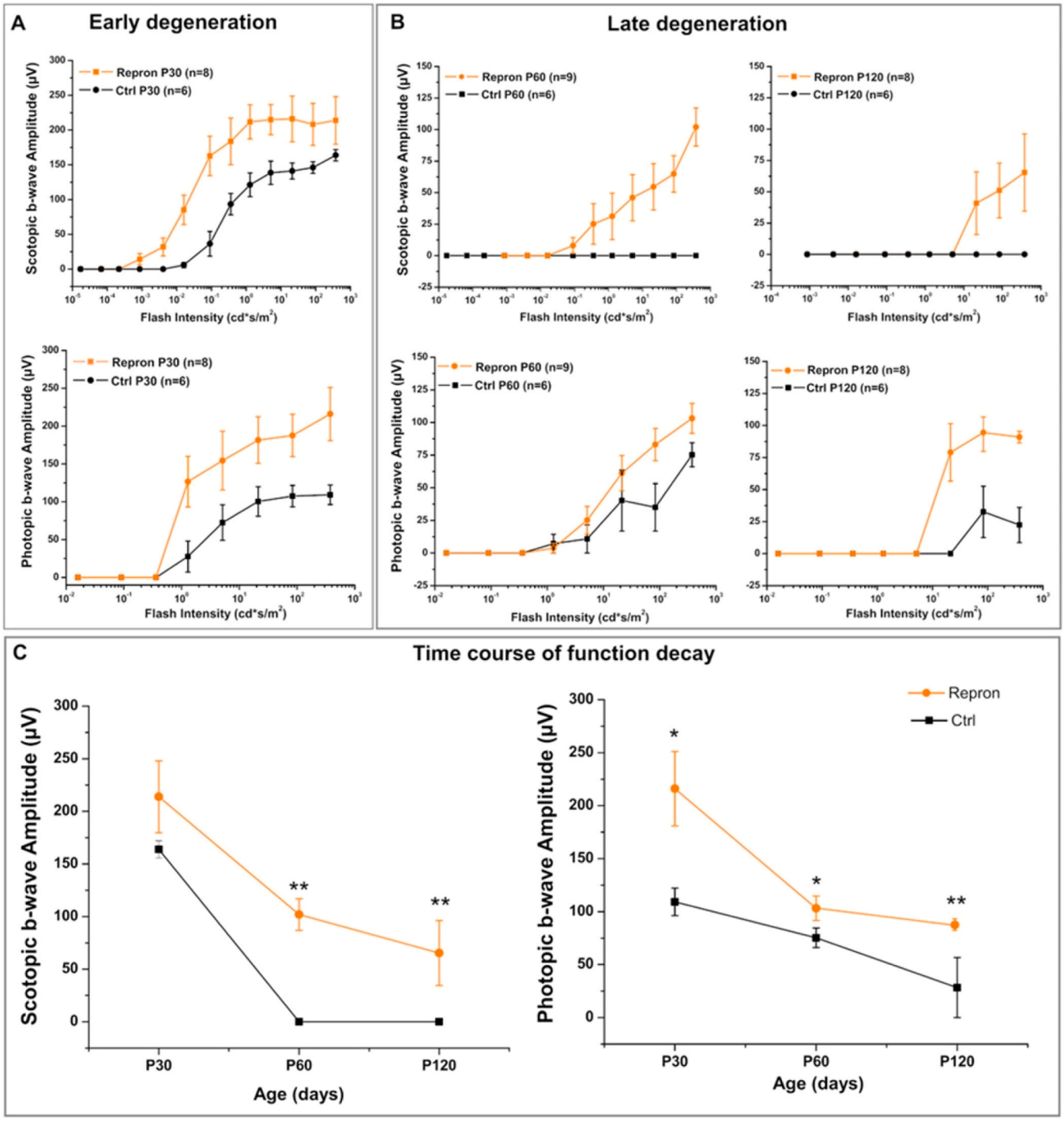


Fig. 2. Retinal function assessed by ERG recording. A. Scotopic and photopic b-wave amplitude as a function of light stimulus intensity at the early stage of degeneration, in treated (orange line) and control mice (black line) at P30. B. Scotopic and photopic b-wave amplitude at the late stage of degeneration, P60 and P120, with the same color legend as A. Note that for easier appreciation of the late response decay in treated mice, the amplitude scale of the graphs in B is doubled. C. Time course of the decay of the amplitude of scotopic and photopic b-waves at the highest flash intensity (377 cd*s/m2). The mice were divided into 6 different groups: control P30 (n = 6), Repron P30 (n = 8), control P60 (n = 6), Repron P60 (n = 9), control P120 (n = 6) and Repron P120 (n = 8). Control indicates rd10 untreated mice while Repron rd10 mice treated with saffron Repron 10 mg/kg/day. One-way ANOVA test was performed: *$P \leq 0.5$ (photopic b-wave: Repron P30 vs ctrl P30, Repron P60 vs ctrl P60); * *$P \leq 0.05$ (Scotopic b-wave: Repron P60 vs ctrl P60, Repron P120 vs ctrl P120; Photopic b-wave: Repron P120 vs ctrl P120).

outer plexiform layers gradually disappear while arborizations from postsynaptic cells to rods (rod bipolar and horizontal cells) retract significantly.[40] In panel B of Fig. 5, the dendritic terminals of rod bipolar cells were examined using the protein kinase C alpha (PKCα). When compared to saffron Repron animals, the control mouse retina displayed considerably weaker PKC immunoreactivity in the inner plexiform layer (IPL) and outer plexiform layer (OPL). In the retinas of control animals at P60 and P120 the bipolar cells underwent a retraction of their dendrites (indicated by the arrows), which was more severe than that of treated groups. The contours of the photoreceptor synaptic ribbons in the outer plexiform layer (OPL) were studied using the detection of postsynaptic density protein-95 (PSD-95). The results showed that the

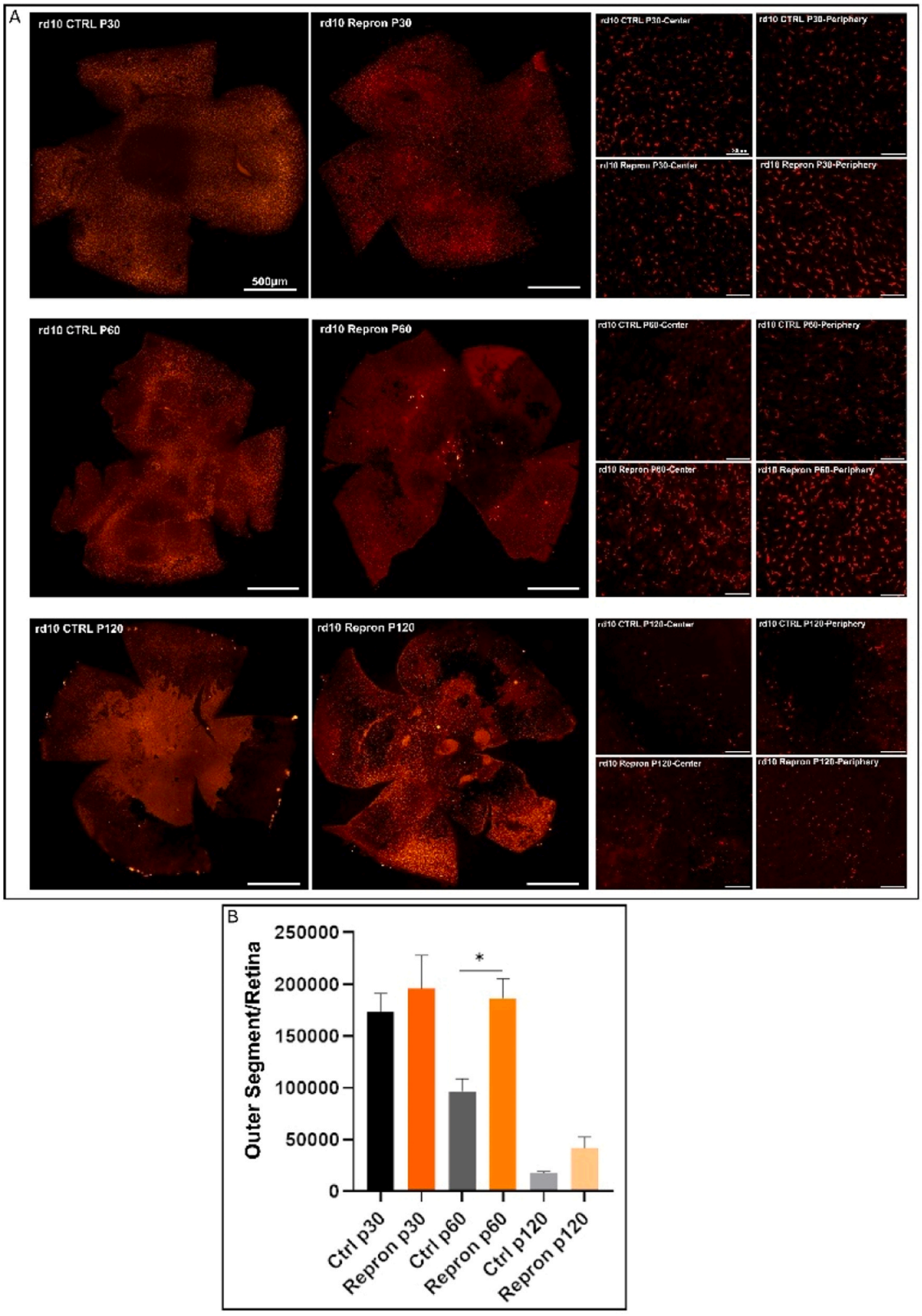


Fig. 3. Cones lifespan preservation. A. On the left cone-opsin B+R/G stained whole mount retinas of Repron P30, P60, and P120 treatment groups and the respective untreated mice control. In the right-hand panels: high-magnification microscope images of central and peripheral retinal areas stained for cone opsins from all the experimental groups. Note that residual cones in the control groups have lost their outer segment while in the respective treated group, a substantial number of cones appear still unharmed. B. Counts of the outer segment in the retina from the Repron treated group at P30 (dark orange bar), P60 (orange, bar), and P120 (light orange bar) and from the respective pathological control (untreated mice) (black bar, dark gray bar, and gray bar respectively) (n = 5 for each group). One-way ANOVA followed by Tukey's multiple comparisons tests. $*P \leq 0.5$ (Repron p60 vs ctrl p60).

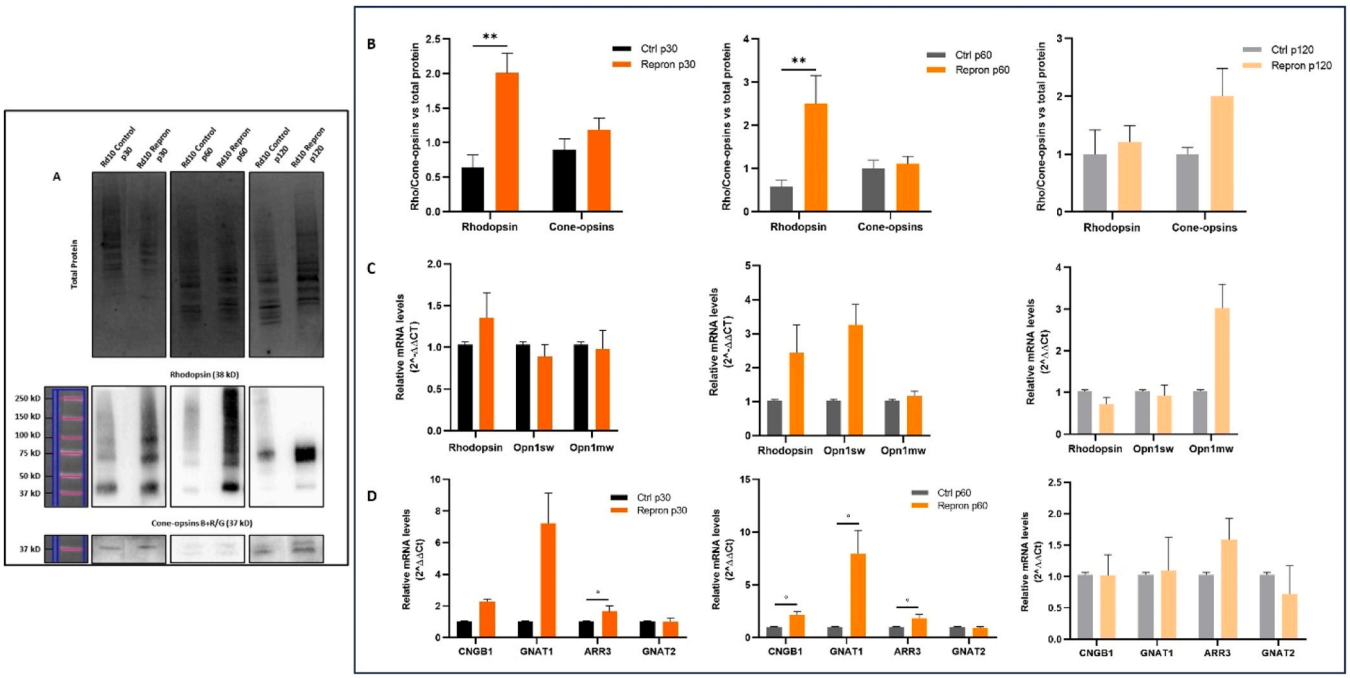


Fig. 4. Photoreceptors protein and gene expression. A. Bands of Total Protein, Rhodopsin, and Cone-opsins. B. Histogram of the quantification, by optical densitometry, of Rhodopsin and Cone-opsin at all-time point treatment and at the corresponding controls. n = 7 for each group. The bar represents the value obtained after the normalization of each value with Total Protein and on the mean of the control group. Student's *t*-test followed the Mann-Whitney *U* test ± SEM * $*P \leq 0.005$. C. Gene expression levels for Rhodopsin Opn1sw and Opn1mw extrapolated from the heat map gene analysis but vs the corresponding control, in all time point treatment and the corresponding controls. n = 3 for each group. D. gene expression of CNGB1 coding light-sensitive channel, GNAT1 coding trasducin1, ARR3 coding cone-arrestin, and GNAT2 coding trasducin 2 in all-time point treatment and the corresponding controls. n = 5 for each group. GAPDH was used as the housekeeping gene and data was expressed as fold-change relative to the control group (made = 1) Student's *t*-test followed Mann-Whitney *U* test ± SEM $^{\circ}P = 0.057$. Bar color legend: Control P30 (black bar), Repron P30 (dark orange bar), Control P60 (dark gray bar), Repron P60 (orange, bar), Control P120 (gray bar), and Repron P120 (light orange bar).

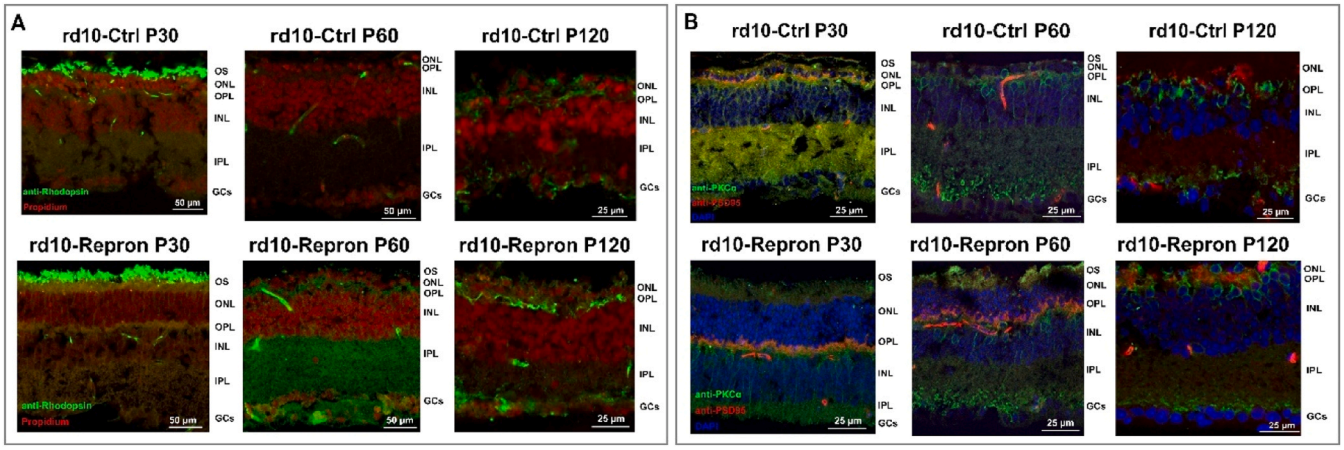


Fig. 5. Retinal cells survival and morphology after Repron treatment. Retinal sections from all-time points of treated and control rd10 mice. A. Rhodopsin staining in green and nuclei in red. Note the extensive rod degeneration and low number of visual cell rows in control mice compared to treated ones. B. In green are Rod bipolar cells stained with PKCα, in red the synaptic terminals of the visual cells are labeled with PSD95. GCs, ganglion cell layer; IPL, inner plexiform layer, INL, inner nuclear layer, OPL outer plexiform layer, ONL, outer nuclear layer. Scale bar 50 µm and 25 µm.

ribbon contours of treated mice retain a better structure than that of control mice. This may suggest that the presynaptic contacts between photoreceptors and bipolar cells of treated mice are better preserved.

3.2.3. Efficacy of saffron Repron in modulating oxidative stress and inflammation in retinal tissue

The treatment's impact on inflammatory and oxidative stress pathways was also investigated. A possible cause of secondary death of the cones in RP could be the onset of inflammatory and/or oxidative stress processes as triggered by the death of rods. The effects of saffron Repron delivery on inflammatory and oxidative stress pathways are summarized in Fig. 6. It is seen that the expression of 36 genes implicated in these pathways is affected by the Saffron treatment. Specifically, the heat map shows that saffron Repron treatment succeeds in some yet-to-be-defined way in modulating the expression of genes related to oxidative stress and inflammation. It can be assumed that the combination of the effects reported above is at the origin of the protective efficacy of Saffron.

Next, we studied the protein expression of SIRT1 (epigenetic

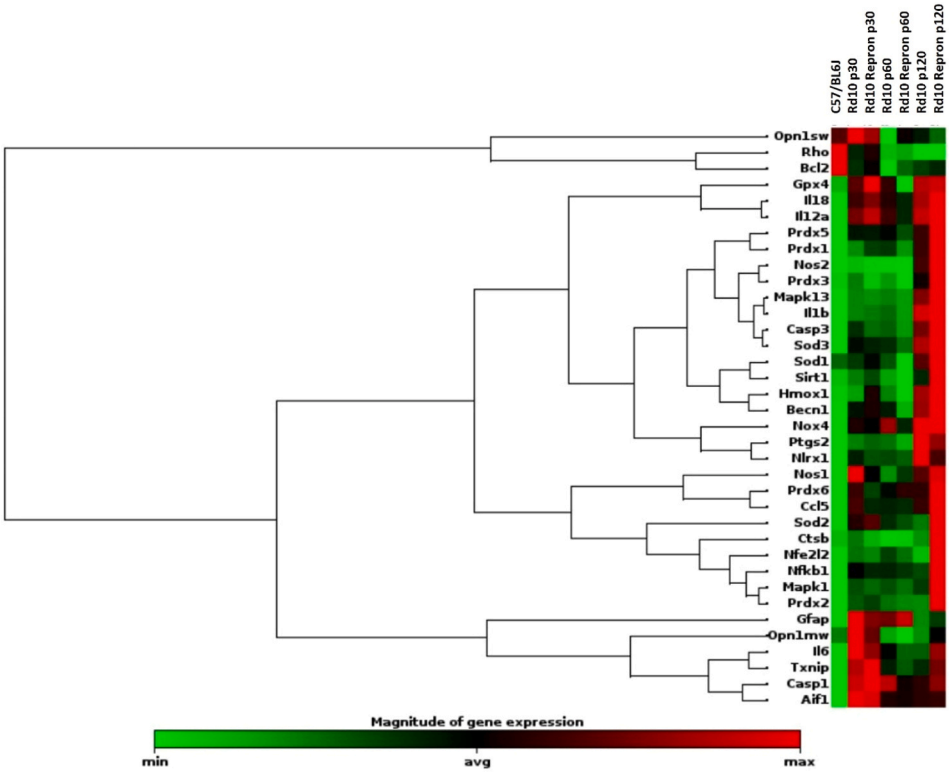


Fig. 6. Heat map of specific photoreceptor genes and those involved in inflammation, apoptosis, and oxidative stress. Columns report the transcriptional expression levels of 36 genes in groups treated with saffron Reprona all-time points and control at all time points, compared to the expression levels of control C57/BL6 mice (n = 3). Each square represents the mean of the values normalized to the mean expression of GAPDH. The red color indicates the highest upregulated expression levels, while the green color indicates the lowest downregulated expression levels.

modulator) and NF-κB, knowing that SIRT1 inhibits NF-κB signaling directly through deacetylation of the p65 subunit of the NF-κB complex. From Fig. 7B, significantly at P60, there was an increase in the levels of SIRT1 in Repron-treated animals by about 70 % compared to controls and a subsequent decrease in NF-κB around 60 %. It was also possible to observe how gene expression of IL1b, a pro-inflammatory marker, decreases significantly by over 80 % in all groups of Repron-treated animals compared with their controls; also, Bcl2, an anti-apoptosis marker, increases significantly in Repron-treated animals by about 70 % at P60 and 120 % at P120 compared with their controls, indicating a decrease

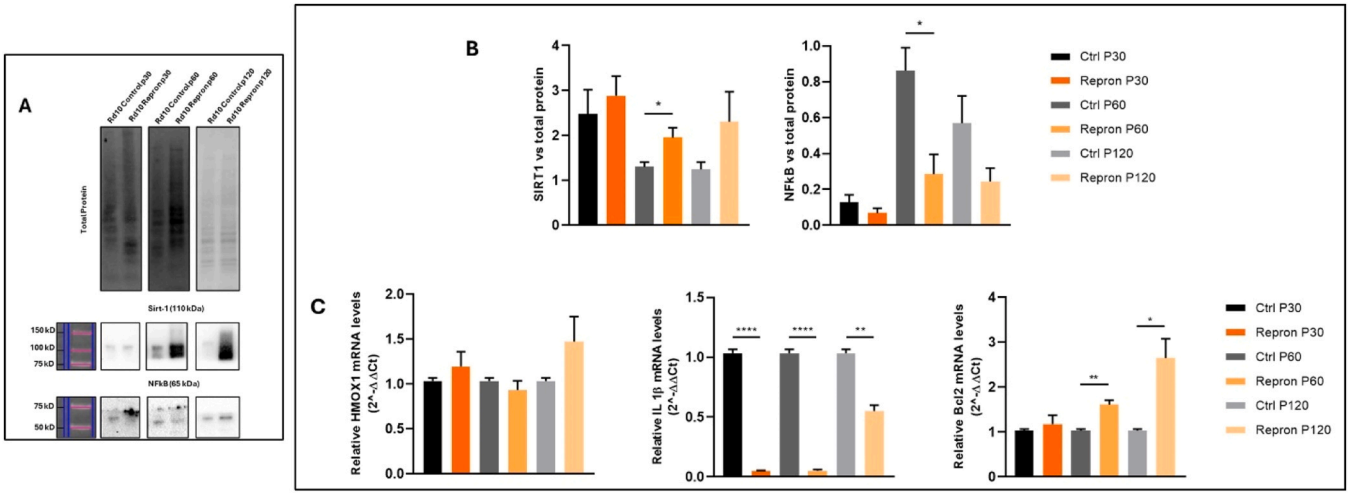


Fig. 7. Real-time PCR and WB analysis of inflammation markers. A. Bands of Total Protein, Sirt-1, and NF-κB p65 active subunit. B. Histogram of the quantification, by optical densitometry, of Sirt-1 and NF-κB p65 active subunit at all-time point treatment and the corresponding controls. n = 6 for each group. The bar represents the value obtained after the normalization of each value with Total Protein and the control group means. Student's *t*-test followed the Mann-Whitney *U* test ± SEM *$P \leq 0.05$ (Sirt-1 and NF-κB, Repron P60 vs ctrl P60). C. Gene expression levels for HMOX1, IL1β, and Bcl2 extrapolated from the heat map gene analysis but vs the corresponding control, in all time point treatments and the corresponding controls. n = 3 for each group. GAPDH was used as the housekeeping gene and data was expressed as fold-change relative to the control group (made=1) Student's *t*-test followed Mann-Whitney *U* test ± SEM *$P \leq 0.5$ (Bcl2 Repron P120 vs ctrl P120), * *$P \leq 0.05$ (IL1β Repron P120 vs ctrl P120), * ** *$P \leq 0.001$ (IL1β Repron P30 vs ctrl P30 and Repron P60 vs ctrl P60). Bar color legend: Control P30 (black bar), Repron P30 (dark orange bar), Control P60 (dark gray bar), Repron P60 (orange, bar), Control P120 (gray bar), and Repron P120 (light orange bar).

in cell death pathway.

The results described here demonstrate a multi-target action of saffron treatment that probably lies in the great therapeutic potential of the spice; furthermore, the experimental plan conducted in this study that involves the administration from the pregnant mice activates the epigenetic modifications that make the retina resilient to the genetic damage to which it is subjected. This notion, in addition to being demonstrated with the data obtained from the SIRT1-NfkB pathway, is also demonstrated by behavioral results using PWM on animals treated starting from P18. In fact, Fig. S1 shows how the treatment in rd10 mice starting from P18 has a significant protective efficacy at P30 (0,38 cycles/degree vs 0,21 cycles degree) but already at P60, this activity is lost with visual acuity in the Repron P18 group decreasing to approximately 0.22 cycles/degree, similar to the control group. This result encourages us to hypothesize that, given the genetic nature of the disease and the high tolerability of the treatment, early treatment with saffron Repron may represent a high-profile tool for slowing down the neurodegeneration typical of RP.

4. Discussion

The main result reported in this work is that oral treatment with saffron Repron slows the progression of neurodegenerative remodeling of the retina and maintains visual function up to times previously unmatched by previous nutraceutical strategies. [41,42] RP is a genetic disease of the retina, and it is ruthless in causing blindness. Cone photoreceptors degenerate and die after rod visual cells and the cause for this secondary death is still under something of the cloud. One of the main objectives for improving RP patients' quality of life in recent years has been the use of natural strategies for slowing down retinal disorders.

Previous research carried out in our and other groups shows that, regardless of the involved genetic mutation, administration of natural compounds such as the flavonoids Naringenin and Quercetin, could reduce cone degeneration caused by the genetic death of rods in different mutations of in-vivo models of RP.[18,19,43,44] These observations prompted us to test the efficacy of retina degenerations of saffron, one of the oldest and most expensive spices that have been used for millennia and one of the foods with the highest nutraceutical potential. There is evidence to suggest that saffron is an effective treatment for neurodegenerative conditions including retinal degeneration.[36,45–50] Numerous studies have demonstrated the strong connection between saffron chemical properties and its medicinal benefits.[20,36,51]

A chemical investigation of numerous saffron samples revealed that the ratio among components is critical and the quantity of the two most prevalent crocins—trans-crocetin di-(-D-gentiobiosyl) ester and trans-crocetin (-D-gentiobiosyl) (-D-glucosyl) ester—determines the neuroprotective action of this spice. However, not all batches of saffron offer the same grade of neuroprotection[51] and the chemical composition is critical. This composition has been defined and patented (Patent: US-10561697-B2) and named saffron Repron. To be called Repron, saffron must have a weight concentration of the two main crocins (%mg crocin/100 mg dry saffron), trans-crocetin bis (β-d-gentiobiosil) ester and trans-crocetin (β-d-gentiobiosil) (β-d-glucosyl) ester, which must be greater than and/or equal to certain values, defined in the patent. Recent data have shown that to get the best degree of protection all the components appear relevant (polar and apolar in addition to crocins) altogether they seem to act in a synergist manner. [20]

In the present study, for the first time, we find that the treatment with saffron Repron from the rd10 pregnant female improved the morphology and function of the retina in progeny. It is important to highlight that most research focuses on the direct administration of nutraceuticals to pups affected by RP rather than on administration to mothers during pregnancy. Therefore, further studies are needed to fully understand the effect of nutraceutical treatments given to pregnant mice with RP and their impact on the retinal health of their offspring. However, since it is a genetic disease, the possibility of intervention from the prenatal stages could represent an advantage for retinal health even in adulthood.

Visual function tested with behavioral technique showed that saffron-treated mice maintained reasonably good visual performance longer than control mice. Accordingly, at the all-time points tested the visual resolution of treated animals was always significantly higher than in control.

Electrophysiological recordings provide evidence that amplitudes of the scotopic and photopic responses observed in saffron-treated mice compared to controls are significantly higher. In treated groups a small scotopic response to high luminance stimuli can be detected as late as at P120. This indicates that although small a visual contribution from rods is still present at a very late stage of degeneration. Behavioral and physiological data match well with morphological analyses. Two immunohistochemical studies provide evidence that both rod and cone visual cells are preserved for long term in treated mice.

In agreement with functional data connections between photoreceptors and their postsynaptic neurons were better preserved in Repron treated groups. The integrity of PKCα and the presence of PSD95 are still observed in the treated group up to P120. These findings suggest that saffron coordinately interacts with complex molecular and cellular processes to slow down the progression of downstream events probably activated mainly by neuroinflammation. It has been suggested that Saffron treatment activates tissue resilience [26] which is the ability to respond to stress by regulating the activation of self-protective mechanisms, like microglia activation, production of cytokines, and proinflammatory molecules leading to neuroinflammation and eventually acceleration of disease progression. Interestingly saffron regulates calcium influx through the purinergic P2X7 receptor, [20,51,52] which is involved in glia neuro connection and, as recently discussed, plays a critical role in glaucoma and diabetic retinopathy.

Once established that there was an improvement in the function and the morphology of the retina, we tried to study a possible mechanism activated by saffron Repron treatment.

Several evidence in the literature shows that the bioactive constituents of saffron are able to modulate different cellular pathways through the interaction with micro-RNAs and epigenetic modifiers. In a microarray experiment conducted in a model of light-induced retinal degeneration. [53] It was clear that saffron was able to modulate the activity of many genes, and "unclassified genes" that we now know to be micro-RNAs. In that experiment it became clear that saffron was able to regulate protein synthesis. In another recent review the results obtained in independent studies on the interaction of the main constituents of saffron such as crocins, picrocins and saffranal, with epigenetic modulators such as SIRT1, HDAC1 and HDAC3 were reported.[53] The results of these works agree with those obtained from the analyses we conducted on the gene expression of different pathways including inflammation, oxidative stress and apoptosis. It should be emphasized that saffron is a phytocomplex that includes a variety of compounds that act on different targets, but the neuroprotective efficacy shown by saffron Repron has been mainly related to the high percentage of crocins and crocetins present (Patent: US-10561697-B2), so the modulatory activity of SIRT1 and consequently the downstream modulation of the Nf-Kb pathway [53] with consequent decrease in inflammatory markers (IL-1β) and increase in anti-apoptotic ones (Bcl2) could be due precisely to the allosteric modulation of crocetins towards SIRT1 as also demonstrated by Krishnaswamy et coworkers.[54] Moreover, several independent studies show that the inhibition or silencing of SIRT1 can have various negative effects on retinal neuroprotection, including: increased oxidative stress, greater apoptosis of retinal cells through the FOXO1-p53 pathway, mitochondrial dysfunction reducing the energy efficiency of retinal cells and compromising their function, and finally, increased inflammation leading to greater activation of NF-κB and excessive production of pro-inflammatory cytokines, accelerating retinal degeneration. It has also been demonstrated that SIRT1 activators, such as resveratrol, are effective in promoting neuroprotection.[55]

Furthermore, also in vitro studies on rat brain microglial cells demonstrate the ability of crocins and crocetins to reduce the release of pro-inflammatory markers such as IL-1β and to reduce cell death;[55] also in vivo studies of neurotoxicity show the neuroprotective effects of crocetins through the modulation of Nd-Kb activation and the reduction of markers pro-inflammatory, increased antioxidant markers (HO-1) and reduced cell death.[56,57]

The results obtained in this study where saffron Repron is administered to pregnant mothers, continued during lactation and up to adulthood, significantly improve all retinal aspects examined in animals treated up to P120, even in comparison with a conventional treatment started in puppies at P18 (Fig. S1). This prolonged neuroprotective activity is attributable to the early treatment which, in addition to epigenetically modulating the response to a genetically imposed stress, also interacts in the developmental phases of neuronal circuits making them more resistant to stress and probably with a delay in the activation of death program. This ability of the system to respond and "self-defense" from adverse events to date is known as resilience. This attractive hypothesis requires further research to clarify the processes through which saffron acts on the retina, on photoreceptors, bipolar cells, and the outgoing circuitry (ganglion cells and visual pathways).

Currently there is no viable treatment to slow the progression of RP or to replace the eye health that has already been lost. The use of saffron as an adjunct in the treatment of RP represents a therapeutic opportunity of great clinical relevance, especially considering the lack of effective treatments to significantly slow the progression of the disease or restore lost vision. RP is a degenerative retinal disease of genetic origin, leading to the progressive loss of photoreceptors and impairment of visual function. In this context, saffron, with its powerful neuroprotective and antioxidant properties, could become not only an innovative treatment but also a crucial one in the management of this condition. Saffron is known for its high safety profile and availability in the form of supplements, representing a non-invasive adjunct therapy that can be easily integrated into existing therapeutic protocols. Given the lack of effective therapeutic options to slow RP progression, the introduction of saffron could fill a significant therapeutic gap, improving the quality of life of patients. The treatment acts on common cellular mechanisms involved in retinal degeneration in RP, such as chronic inflammation, oxidation, and apoptosis of retinal cells. Since these mechanisms are present in many genetic forms of RP, the use of saffron could prove mutation-independent, opening the door to a broad and versatile therapy, useful for a larger number of patients, regardless of the genetic mutation underlying the disease.

Additionally, the administration of Repron has already been tested in patients with AMD and Stargardt's disease [27,58,59] and validated for its safety and ease of use. Being a non-invasive treatment, it does not require surgical or complex procedures, making it an easily integrable therapy in patients' daily treatments. Its use as a dietary supplement, without significant side effects, makes it practical for patients already on other medications or treatments.

Therefore, the clinical translation of saffron as an adjunct treatment for RP could represent one of the next frontiers in managing this condition. Clinical studies exploring the efficacy of the treatment in real patient groups, even in the more advanced stages of the disease, could provide crucial data for official approval by regulatory agencies, making the treatment available to a wider patient population. Furthermore, adopting a saffron-based treatment could be combined with other emerging therapies for RP, such as gene therapy, retinal implants, or assistive devices. Saffron, being non-invasive and free of significant side effects, fits perfectly into an integrated and multifactorial therapeutic approach, potentially improving overall responses in RP patients.

5. Conclusions

In summary, the use of saffron as an adjunct in the treatment of Retinitis Pigmentosa has substantial clinical relevance, as it offers a safe, accessible, and non-invasive solution in a field where slowing disease progression is currently difficult. Although this study used only an animal model of RP, the results provide a promising foundation for the exploration of similar treatments in humans. Animal models, particularly murine models, are essential tools for testing the efficacy of potential therapies before clinical application. While there may be differences between animal and human species, the basic biological mechanisms of RP, such as inflammation, oxidative stress, and cell death, are similar. Therefore, the data collected could have significant translational relevance, contributing to the development of more targeted treatments for human patients with RP. However, further clinical studies in humans will be necessary to confirm the treatment's efficacy and determine the safety and optimal therapeutic approach for patients.

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