SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of Therapeutic Interventions for Age-Related Visual Impairment Using Functional and Molecular Biological Approaches

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"Aging is the progressive accumulation of changes with time that are associated with or responsible for the ever-increasing susceptibility to disease and death which accompanies advancing age."

Harman (1981, p. 7124)

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1. Introduction

Life expectancy has increased dramatically over the past decades. Recent estimations suggest that by 2050, the global population aged over 60 could nearly triple. With advancing age, the risk of degenerative eye diseases also rises, with the prevalence of vision impairment exceeding 20% in individuals over 80 years of age. A significant proportion of age-related eye diseases lack effective treatment in clinical practice, making it crucial to explore therapeutic methods capable of preserving retinal function and preventing age-related vision loss.

Ayahuasca is a traditional brew used for centuries, primarily in South America during religious ceremonies, for its hallucinogenic effects. The traditional ayahuasca beverage is composed of β-carboline-rich plants (e.g., Banisteriopsis caapi, Peganum harmala) combined with plants containing N,N-dimethyltryptamine (DMT) (e.g., Psychotria viridis, Diplopterys cabrerana), achieving the most intense and long-lasting psychoactive effects.

In recent decades, DMT has emerged as a promising candidate for treating various diseases. Due to its Sigma-1 (Sig-1R) receptor agonist activity, it has been suggested to have neuroprotective effects. Harmaline, a β-carboline, is a reversible inhibitor of the monoamine oxidase enzyme type A (MAO-A), thereby potentiating the effects of tryptamines (e.g., DMT) by inhibiting their breakdown. Harmaline has received significant attention in the past decade, with potential applications in cancer, inflammatory, and neurodegenerative disorders, as well as in treating cognitive decline. Recent studies have demonstrated that MAO enzymes play a key role in inducing oxidative stress, as the breakdown of neurotransmitters generates hydrogen peroxide and reactive oxygen species. Thus, inhibiting this enzyme may mediate antioxidant effects.

Regular physical activity plays a significant role in enhancing defense mechanisms against oxidative stress, i.e., activating the antioxidant system. Numerous studies suggest that exercise has a dose-dependent effect, indicating that the benefits of exercise increase with its intensity. However, recent research has highlighted the importance of age, suggesting that in older individuals, high-intensity exercise does not confer noticeable advantages over low-intensity exercise and may even accelerate the process of aging due to its oxidative stress-enhancing effects.

Our main research objective was to test the efficacy of potential therapeutic interventions in preventing age-related ocular diseases. Furthermore, we aimed to precisely elucidate the functional and molecular effects of these preventive methods. In our first experiment, we

examined the biological effects of the two primary components of ayahuasca, DMT and harmaline, on ischemia-reperfusion (I/R) injury in the eye, which is crucial in the etiology of several age-related eye diseases. The primary goal of our second experiment was to determine whether high-intensity exercise or recreational physical activity is more beneficial for the eyes of elderly animals, and to identify molecular and functional changes in the eye among groups with different activity levels. In both studies, we assessed the functional performance of the retinal neural layer using electroretinographic measurements, evaluated retinal morphology through histological analysis, and examined protein expression changes via western banalysis,with a focus on proteins involved in cell death, oxidative stress and antioxidant effects.

2. Materials and Methods

2.1. Animals

For our first experiment, we used 12-weHC-old male Sprague Dawley (SD, 420–460 g) rats. The animals arrived at 10 weHCs of age and underwent a 2-weHC acclimatization period. During this time, the rats were housed in cages in a windowless animal facility room with a 12-hour light/dark cycle and a constant temperature of 24°C. The animals had ad libitum access to water and standard rodent chow. Humane care was provided according to ARVO (Statement for the Use of Animals in Ophthalmic and Vision Research) and NIH (National Institute of Health) guidelines. All methods used in the study were approved by the The Institutional Animal Care Committee of the University of Debrecen (permit number: 12/2019/DEMÁB).

For our second experiment, we included 3-month-old and 18-month-old male Wistar rats. The older rats arrived at the animal facility at 10 months of age and remained untreated until they reached 18 months. The younger rats arrived at 10 weHCs of age and underwent a 2-weHC acclimatization period. Housing conditions and feeding protocols were identical to those described previously. However, since the planned active cycles on the running wheel were scheduled for the morning hours, we artificially reversed the 12-hour dark/light cycle to optimize experimental conditions. As Wistar rats are nocturnal, the active running period was aligned with the onset of their “nighttime,” which corresponds to their active phase. Humane care for the Wistar rats followed ARVO and NIH guidelines, and all experimental methods were approved by the Workplace Animal Experimentation Committee of the University of Debrecen (permit number: 3/2022/DEMÁB).

2.2 Experimental Protocols and Groups

2.2.1 Investigation of the Anti-Ischemic Effects of DMT and Harmaline in Sprague Dawley Rats

The aim of our first experiment was to examine the biological effects of the two primary components of the ayahuasca brew, N,N-dimethyltryptamine (DMT) and harmaline, on ischemia-reperfusion (I/R) injury in the eye.

After a two-weHC acclimatization period (at 12 weHCs of age), the animals were randomly divided into groups (n = 10 per group): vehicle-treated (the ligated eye served as the diseased

control – DC, while the non-ischemic eye was used as the healthy control – HC), DMT- and harmaline-treated (DHT), and harmaline-treated (HT) groups.

The DMT solution was administered via an osmotic minipump capable of subcutaneous delivery at a constant rate for one weHC. This approach was intended to maintain a consistent DMT concentration and avoid its strong hallucinogenic and serotonergic side effects. The daily dose of DMT was 10 mg/kg/day. Harmaline and vehicle were administered twice daily by oral gavage. Due to the limited capacity of the minipumps (2 ml), harmaline could not be delivered via this method. Given harmaline's relatively short half-life and reversible inhibitory effect on the MAO-A enzyme, it was administered orally to both treatment groups at 10 mg/kg/day, twice daily. Harmaline was dispersed in mucilago hydroxethylcellulose, so mucilago was also used in the vehicle-treated group.

Considering extremely short half-life of DMT without MAO-A inhibition, a DMT-only group was not established to minimize the number of animals used (in line with the 3R ethical principles). For the healthy control (HC) group, the right (non-ligated) eyes of untreated Sprague-Dawley rats were used, further reducing the number of animals sacrificed.

2.2.1.1 Ischemia Model

Deep anesthesia was induced using an intramuscular injection of ketamine/xylazine combination (100/10 mg/kg). Alongside general anesthesia, topical anesthesia was applied to the left eye using oxybuprocaine-containing eye drops. Once the animals no longer responded to painful stimuli, ischemia was experimentally induced in the left eye of SD rats using a ligation technique based on a previously established protocol from our research group.

During ligation, the eyeballs were slightly elevated from their sockets, and a thick, atraumatic surgical suture was placed behind the eyeballs. Using a polyethylene cannula, a slipknot was created, encircling the optic nerve, retinal vessels, and retrobulbar connective tissue. Ischemia was initiated by tightening and securing the knot to apply pressure on the retinal vessels. The cessation of blood flow was confirmed macroscopically using an ophthalmoscope; whitening of the fundus indicated the onset of global ischemia. The eyes were protected from desiccation during anesthesia with a carbomer-based eye gel. The ischemic period lasted 60 minutes. At the end of this period, the knot was released, and the ligature was removed to restore blood flow.

2.2.1.2 Ultrasonographic Examination of the Eye

Retinal blood flow was assessed using the Vevo 3100 ultrasound system. During the examination, the animals were under deep anesthesia and positioned prone on a heated warming pad set to 39°C to maintain body temperature. Water-based inert ultrasound gel was applied to the corneal surface, and the eye was visualized in the longitudinal macula (LMAC) view. Standard Color Doppler signals were recorded at a depth of 9 mm, and pulsed-wave (PW) Doppler images were captured to evaluate blood flow in the posterior short ciliary artery (55, gate size: 0.27 mm). Blood flow was assessed before (baseline) and after (during reperfusion) the 1-hour I/R injury. The images were analyzed semi-quantitatively to evaluate the presence of flow under baseline and reperfusion conditions.

2.2.1.3 Osmotic Minipump Implantation

Osmotic minipumps (2 ml capacity) were implanted in the interscapular region of the animals immediately following the ischemic period, utilizing the existing deep anesthesia. The skin over the implantation site was shaved and disinfected with 10% povidone-iodine solution. An incision (1 cm) was made along the midline of the interscapular region, and a hemostat was used to create a subcutaneous pocket by repeatedly opening and closing its jaws. Once the pocket was appropriately sized, the minipump was inserted, and the incision was closed with non-absorbable sutures, followed by a second disinfection. This procedure was also performed on the harmaline- and vehicle-treated groups without actual minipump implantation (sham surgery), ensuring equal stress exposure across treatment groups.

2.2.2 Investigation of the Dose-Dependent Effects of Regular Exercise in an Aging Rat Model

The main aim of our second experiment was to examine the effects of different intensities of exercise initiated during aging on visual function.

The following groups were established:
Group I: Young, 3-month-old rats = young control (YC) group (no exercise) (n=10).
Group II: Aging (18-month-old), inactive (physically inactive) control group (AIC): housed in a cage without a running wheel, representing the physically inactive population (n=12).
Group III: Aging voluntary/recreational runners (18-month-old) (ARR): housed in cages equipped with a running wheel, allowing voluntary use (2 animals per cage, n=12).
Group IV: Aging forced runners (18-month-old) (AFR): subjected to high-intensity exercise in a closed running wheel under continuous supervision, 6 days/weHC (n=12).

For forced runners, exercise intensity was gradually increased to prevent injuries. Initially, rats were placed in the stationary running wheel for 5 minutes to familiarize themselves with

the device, after which it was set to a speed of 5 m/min. The speed was increased daily by 0.5 m/min until reaching the target 13 m/min. Once this speed was achieved, exercise duration was increased daily by 1 minute, starting from 1 minute. The goal was to reach a maximum of 20 minutes of running per session. Training sessions were conducted once daily in the morning, corresponding to the onset of the animals' active phase (nighttime).

2.3. Western blot

After completing both experiments, we conducted western blot analysis to examine protein expression changes in the eyes of the animals. Humane euthanasia was performed using an overdose of general anesthetic. The eyeballs were then carefully removed from the orbits and frozen in liquid nitrogen (n=5 per group). To extract protein fractions, the eyeballs were ground into powder and mixed with homogenization buffer. The solution was further homogenized using a bladed disperser. The mixture was centrifuged at 2000 rpm for 10 minutes at 4°C, and the supernatant, containing cytosolic and mitochondrial fractions, was collected. The remaining pellet, containing the nuclear fraction, was resuspended in a homogenization solution containing Triton X-100 detergent and incubated on ice for 1 hour. The nuclear fraction solution was then centrifuged again at 14,000 rpm for 10 minutes at 4°C, and the supernatant containing nuclear proteins was collected. The cytosolic and mitochondrial supernatants were centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant obtained contained the cytoplasmic protein fraction.

The total protein concentration in each fraction was determined using the BCA method with 10 µl of each supernatant measured by spectrophotometry. The remaining supernatant was prepared for SDS-polyacrylamide gel electrophoresis by adding Laemmli buffer in a 1:1 ratio and boiling for 5 minutes. Proteins from the eye homogenates were separated by molecular weight on 12% polyacrylamide gels. Proteins were transferred from the gel onto nitrocellulose membranes. Non-specific binding sites on the membranes were blocked using a 3% BSA solution, after which they were incubated overnight with primary antibodies in TBST buffer.

Primary antibodies used in the first experiment: anti-histone H3 recombinant rabbit monoclonal antibody; anti-beta-actin mouse monoclonal antibody; anti-MMP9 rabbit polyclonal antibody; anti-PARP1 polyclonal antibody; GFAP rabbit polyclonal antibody; anti-NFκB p65 rabbit polyclonal antibody; anti-HSP70 mouse monoclonal antibody.

Primary antibodies used in the first experiment: anti-histone H3 recombinant rabbit monoclonal antibody; anti-beta-actin mouse monoclonal antibody; anti-BDNF rabbit monoclonal antibody; anti-MAOB monoclonal antibody; GFAP rabbit polyclonal antibody;

anti-SIRT6 rabbit monoclonal antibody; anti-PARP1 polyclonal antibody; anti-NFκB p65 rabbit polyclonal antibody; anti-SOD1 rabbit polyclonal antibody; anti-HSP70 mouse monoclonal antibody.

The following morning, unbound primary antibodies were removed with three 10-minute washes in TBST. Membranes were then incubated for 45 minutes with secondary antibodies conjugated to horseradish peroxidase (anti-rabbit or anti-mouse antibodies). After the incubation, additional washing steps were performed. Proteins of interest were visualized using ECL™ Prime™ western blotting reagent and the LI-COR C-DiGit® blot scanner, which detects chemiluminescent light intensity to reveal differences between groups. Blots were analyzed using ImageJ software, with normalization to background and standardization to a housHCeeping protein (histone H3 or beta-actin). For each treatment group, three western blot results per protein were analyzed from five animals.

2.4 Histology

After humane euthanasia of the rats, the eyeballs (n=5 per group) were carefully excised from the orbits. To ensure proper orientation during analysis, a ligature was placed on the superior corneal section of the eyeballs. Paraformaldehyde was injected into the eyeballs with a fine needle to fix the internal structures, followed by immersion in paraformaldehyde solution for 24 hours. The next day, corneas were incised, and the eyes were rinsed under running water for 1 hour to remove residual paraformaldehyde. Thereafter, samples were stored in 70% alcohol until further histological processing. The next step was dehydration (70%, 90%,100% ethanol), followed by clearing (xylene) and wax infiltration/embedding, and finally the paraffin-embedded eye tissue blocks were sectioned frontally with a microtome into 5 μm-thick sections.

Histological analysis was performed on sections near the optic nerve in the posterior-inferior segment of the retina.

The sections were deparaffinized, rehydrated through a descending alcohol series, and stained with hematoxylin and eosin (H&E). Hematoxylin staining was applied for 10 minutes, followed by a 10-minute wash under running water, during which the sections turned blue. Eosin staining was performed for 5 minutes. Images were captured from the inferior optic nerve region of the retina using a Nikon Eclipse 80i microscope at 40× magnification. Retinal thickness measurements were performed using Nikon NIS-Elements BR software (Ver5.41.00).

2.5 Statistics

Statistical analysis was performed using GraphPad Prism software (version 9.1.2). Gaussian distribution was assessed with the Shapiro-Wilk normality test. Data with normal distribution were analyzed using one-way analysis of variance (ANOVA), while non-normally distributed data were analyzed using the non-parametric Kruskal-Wallis test. For comparisons across multiple time points, two-way ANOVA was used. Results were considered significant if p-values were less than 0.05 ($p < 0.05$). Significance levels were indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$. All data are presented as mean ± standard error of the mean (SEM).

3. Results

3.1 Investigation of the Effects of DMT and Harmaline on Ocular Ischemia-Reperfusion Injury

3.1.1 Ocular Ultrasonography

Arterial blood flow in the retina, assessed using Color and PW (pulse wave) Doppler echography, was restored after the ischemic period. The peak blood flow velocity in the ciliary arteries ranged between 100 and 300 mm/s during both the baseline and post-reperfusion phases, indicating complete reperfusion following the injury.

3.1.2 Electroretinography

To assess the extent of retinal damage, the results from the treatment groups were compared to those of the healthy control (HC) group (vehicle-treated, non-ischemic) and expressed as percentages. The highest percentages were observed in the harmaline-treated group, where the a-waves were recorded at 42.92 ± 1.35% and the b-waves at 35.38 ± 0.89% relative to the 100% values of the healthy control. These were significantly higher ($p < 0.0001$) than the untreated ischemic control (DC) group values for a- and b-waves (29.49 ± 0.79% and 20.08 ± 0.45%) and the DMT and harmaline-treated group (DHT) values (22.76 ± 0.52% and 13.82 ± 0.24%).

In the DHT group, the percentages for a- and b-waves were lower than those in the DC group, with significant differences observed (22.76 ± 0.52% and 13.82 ± 0.24% vs. 29.49 ± 0.79% and 20.08 ± 0.45%; $p < 0.0001$).

3.1.3 Western Blot

Based on the Western blot results, the GFAP expression profile significantly differed between the HC and DHT groups (70.59 ± 8.879 vs. 123.0 ± 17.56; $p < 0.05$). In contrast, no statistically significant differences were observed between the HC and HT (70.59 ± 8.879 vs. 97.20 ± 10.16; $p > 0.05$) or the HC and DC groups (70.59 ± 8.879 vs. 97.93 ± 6.342; $p > 0.05$).

The expression of the HSP70 protein also varied between treatment groups. Significant differences were found between the HC and DHT groups, as well as between the HT and DHT groups (122.6 ± 8.638 vs. 87.72 ± 7.993, $p < 0.05$, and 120.7 ± 10.92 vs. 87.72 ± 7.993, $p < 0.05$, for HC vs. DHT and HT vs. DHT, respectively). Although the DC group showed a seemingly lower value (100.9 ± 7.191) than the healthy untreated group, this difference did not

reach statistical significance. No statistically significant difference was detected between the HC and HT groups ($p > 0.05$).

For MMP9, protein levels changed in a pattern opposite to that of HSP70. Significant differences were observed when comparing the HC and DHT groups, as well as the HT and DHT groups (16.11 ± 8.909 vs. 78.08 ± 11.85, $p < 0.05$, and 19.79 ± 9.925 vs. 78.08 ± 11.85, $p < 0.05$, for HC vs. DHT and HT vs. DHT, respectively). Protein levels were lower in the HC and HT groups but increased in the DC and DHT groups. The MMP9 expression levels in the HC and HT groups were similarly low, with no significant differences between them. Additionally, the DC group, which exhibited apparently higher MMP9 expression, did not show a statistically significant difference (16.11 ± 8.909, 19.79 ± 9.925, and 46.30 ± 15.06 for the HC, HT, and DC groups, respectively; $p > 0.05$ in all comparisons.

A similar trend was observed in the NFκB protein expression profile based on Western blot analysis: for NFκB p65, the DC group significantly differed from both the HC and HT groups (53.21 ± 6.409 vs. 3.350 ± 1.850, $p < 0.0001$, and 53.21 ± 6.409 vs. 19.19 ± 7.140, $p < 0.01$,

Similarly, the levels of poly-ADP-ribose polymerase (PARP1) protein were higher in the DC and DHT groups (52.47 ± 9.597 and 73.26 ± 19.42), while lower levels were observed in the

HC and HT groups (11.22 ± 6.145 and 17.41 ± 4.640). No statistically significant differences were detected between the aforementioned pairs of groups ($p > 0.05$ for both DC vs. DHT and HC vs. HT comparisons). However, significant differences were found in comparisons between the HC and DC ($p < 0.05$), DC vs. HT ($p < 0.05$), HT vs. DHT ($p < 0.01$), and DHT vs. HC ($p < 0.01$) groups.

3.1.4 Histology

Based on the histological results, significant differences were observed in total retinal thickness among the various groups. In the control group (DC), the thickness of the retina subjected to ischemia-reperfusion (I/R) injury was significantly reduced compared to the corresponding non-ischemic (HC) value (106.2 ± 2.608 μm vs. 147.0 ± 5.394 μm; $p < 0.0001$). The same significant difference was noted in both treated groups: the thickness of the I/R-injured retina was reduced compared to the non-ischemic values (108.5 ± 1.716 μm vs. 138.2 ± 4.382 μm and 122.5 ± 3.267 μm vs. 150.6 ± 6.283 μm in the DHT and HT groups, respectively; $p < 0.0001$ for both comparisons).

When comparing the non-ischemic values across the different treatment groups, no significant differences were found among them (138.2 ± 4.382 μm vs. 150.6 ± 6.283 μm vs. 147.0 ± 5.394 μm for the DHT, HT, and HC groups, respectively). Significant differences were observed among the ischemic groups: the retinal thickness of the HT group was significantly greater than the averages measured in the DHT and DC groups (122.5 ± 3.267 μm vs. 106.2 ± 2.608 μm and 122.5 ± 3.267 μm vs. 108.5 ± 1.716 μm for HT vs. DC and HT vs. DHT, respectively; $p < 0.05$ for both comparisons).

No statistically significant differences were detected between the values of the DHT and DC groups (106.2 ± 2.608 μm vs. 108.5 ± 1.716 μm for DHT and DC groups, respectively; $p > 0.05$).

3.2 Dose-Dependent Effects of Regular Exercise on Aging in Rats

3.2.1 Electroretinography

The young (non-running) control group (YC) showed significantly higher a-wave amplitudes compared to the older groups under scotopic measurements. Among the three older groups with varying activity levels, there were no significant differences in a-wave amplitudes, except at a high light intensity of 25,000 mcd·s/m², where the older, forced running (AFR) group exhibited significantly lower a-wave amplitudes.

Regarding a-wave implicit times, pronounced differences were observed between the groups at low (10 mcd·s/m²) and very high (10,000, 30,000 mcd·s/m²) light intensities. The a-wave implicit time was significantly prolonged in the AFR group compared to the YC group and the other older groups. At high light intensities, implicit time prolongation was observed not only in the forced running group but also in the older inactive control (AIC) and older recreational running (ARR) groups compared to the young control group.

Under low-intensity scotopic conditions, the YC group exhibited significantly lower b-wave amplitudes than the older groups. This difference diminished and eventually reversed at higher light intensities and during photopic measurements. Among the older groups, the ARR group consistently showed higher b-wave amplitudes than the AIC and AFR groups during scotopic measurements. This difference was not observed after light adaptation during photopic measurements.

The b-wave implicit time in the young control group was significantly shorter than that of the older groups.

3.2.2 Western blot

We detected a significantly elevated MAO-B level in the AIC and AFR groups compared to the YC and ARR groups (60.08 ± 7.314 and 69.17 ± 5.032 vs. 23.39 ± 4.938 and 35.98 ± 6.74, $p < 0.05$). There was no significant difference between the YC and ARR groups (23.39 ± 4.938 vs. 35.98 ± 6.74; $p > 0.05$).

The expression of the GFAP protein was significantly higher in the AIC and AFR groups compared to the YC group (107.9 ± 7.929 and 119.8 ± 8.034 vs. 62.7 ± 6.644; $p < 0.001$ and $p < 0.0001$), while the GFAP level in the ARR group was significantly lower than that of the AFR group (89.05 ± 6.185 vs. 119.8 ± 8.034; $p < 0.05$).

Significantly lower SIRT6 expression was observed in the AIC and AFR groups compared to the YC group (103.8 ± 6.455 and 89.51 ± 5.9 vs. 134.6 ± 10.88; $p < 0.01$ and $p < 0.0001$).

The SIRT6 level in the ARR group was closer to that of the YC group (122.2 ± 8.278 vs. 134.6 ± 10.88; $p > 0.05$).

The expression of BDNF was significantly reduced in the AFR group compared to the YC group (27.01 ± 4.476 vs. 49.73 ± 6.539; $p < 0.05$). However, the ARR group showed no significant differences compared to either the YC or AIC groups (38.54 ± 4.392 vs. 49.73 ± 6.539 and 38.36 ± 5.65; $p > 0.05$).

The expression of PARP1 was significantly higher in the elderly physically active groups (ARR and AFR) than in the YC group (91.34 ± 16.52, 101.9 ± 15.01 vs. 42.57 ± 5.45, $p < 0.05$), while the AIC group's results (64.61 ± 10.92) did not differ from the YC group.

In our second experiment, NFκB expression followed a pattern similar to PARP1: there was no significant difference among the elderly groups, but its expression was significantly higher in elderly animals compared to the YC group (114.9 ± 12.92, 124.1 ± 13.93, 91.47 ± 10.58 vs. 12.59 ± 4.06, comparison of AIC, ARR, AFR vs. YC groups, $p < 0.0001$).

We also examined the expression of SOD1 and HSP70 proteins in the eye. Based on the results of the experiment, neither age nor exercise influenced the expression of these proteins in the eye: no significant differences were detected between the groups.

4. Discussion

Aging is an inevitable and irreversible process that triggers characteristic changes in the biological functions and physical appearance of an organism. With advancing age, the incidence of eye diseases increases. The primary goal of our research group was to identify a preventive therapeutic option to avoid or delay age-associated eye diseases. The efficacy of these interventions was supported by functional, morphological, and molecular studies. Additionally, we aimed to uncover molecular pathways that could be pharmacologically targeted to provide therapeutic options in the treatment of age-related pathologies.

In our first experiment, we investigated the effects of two components of the traditional ayahuasca brew - DMT and harmaline - on ischemia-reperfusion (I/R) injury in the eye. The results showed that the harmaline-treated (HT) group exhibited the largest a- and b-wave amplitudes and the most preserved retinal thickness among the I/R groups. In contrast, the combined administration of DMT and harmaline (DHT group) had adverse effects on the visual organ, as the a- and b-wave amplitudes did not reach the average levels measured in untreated ischemic animals. To explain this surprising result, we examined the expression of several proteins in the eye that play critical roles in cell death (PARP1), inflammation (NFκB, GFAP), tissue destruction and remodeling (MMP9), and cellular homeostasis (HSP70).

Poly-ADP-ribose polymerase 1 (PARP1) is a key regulator of various cellular functions, such as cell proliferation, differentiation, cell death, inflammatory responses, and mitochondrial function. Although increased PARP1 expression may act as a protective factor during aging due to its role in DNA repair, its overactivation can lead to a specific type of programmed cell death, which several studies suggest plays a significant role in retinal damage, hereditary retinal diseases, age-related macular degeneration, and ischemia-reperfusion injury of the eye. In our study, harmaline reduced PARP1 expression in the eye compared to the DC and DHT groups, potentially contributing to its retinoprotective effect. Similarly, previous publications reported that PARP1 inhibition reduces microglial activation and consequent inflammatory responses, thereby mitigating neuronal damage.

The activation of the NFκB pathway is critical for cell survival as it regulates apoptosis, proliferation, and survival. Due to these effects, the NFκB pathway plays an essential role in the pathomechanism of several neurodegenerative diseases. Consistent with the literature, I/R injury increased the level of the NFκB p65 subunit in the eye in our experiments. Harmaline prevented the elevated expression of NFκB p65, suggesting that harmaline attenuated the inflammatory cascade triggered by I/R injury. This anti-inflammatory effect could be

retinoprotective, as evidenced by ERG results in the HT group. Surprisingly, when DMT was combined with harmaline (DHT), no reduction in NFκB expression was observed compared to the diseased control group, indicating that DMT counteracted harmaline's protective potential. This could be attributed to the higher PARP1 levels observed in the DHT group compared to the HT and DC groups, though the difference between the latter groups was not statistically significant.

Matrix metalloproteinases (MMPs) are responsible for extracellular matrix degradation and tissue remodeling. Under extreme intraocular pressure (e.g., in glaucoma), which leads to ischemia in the eye, MMPs have been shown to exhibit significant overexpression in various ocular tissues. In our experiment, harmaline treatment mitigated I/R injury-induced MMP9 overexpression, resulting in MMP9 levels in the HT group similar to those in the healthy controls. Conversely, MMP9 expression was significantly higher in the DHT group compared to the HT and HC groups, aligning with the expression patterns of NFκB and PARP1 proteins previously described.

Glial fibrillary acidic protein (GFAP) is a widely used marker of astrocyte activation, and its expression is known to increase in pathological conditions of the central nervous system. A study reported that GFAP inhibition protects photoreceptors during retinal detachment. In our first experiment, GFAP expression did not change significantly in the HT group compared to the DC and HC groups, while the combined administration of DMT and harmaline likely enhanced astrocyte activity, leading to significantly elevated GFAP expression in the animals' eyes.

The 70 kDa heat shock protein (Hsp70) is responsible for maintaining the physiological structure of proteins and protein complexes. Hsp70 induction offers several therapeutic advantages, including neuroprotective effects by inhibiting the release of pro-inflammatory cytokines and astrogliosis. Hsp70's anti-inflammatory properties are multifaceted, affecting multiple molecular pathways, including the inhibition of NFκB, MMPs, and reactive oxygen species production. According to our western blot results, DMT adversely influenced Hsp70 expression, as its levels were significantly lower in the DHT group than in the HC animals. In contrast, harmaline-treated animals exhibited Hsp70 levels comparable to healthy controls, with no statistically significant differences detected.

The electroretinographic results of our first experiment are consistent with the changes observed in the western blot analysis. The HT group exhibited the highest a- and b-wave amplitudes among the ischemic groups, indicating the protective effect of harmaline, an MAO-A inhibitor. The molecular basis of this retinoprotective effect may lie in the increased

expression of the chaperone protein Hsp70 and the reduced expression of PARP1, NFκB, and MMP9—key mediators of apoptosis, inflammatory response, and tissue destruction. However, DMT counteracted this protective effect, demonstrating a retinal-damaging effect when combined with harmaline.

Histological findings further supported the protective effect of harmaline on the eye. Among the ischemic groups, the harmaline-treated animals exhibited the most preserved retinal layer thickness, significantly exceeding the averages of the DC and DHT groups.

The role of serotonin (5-HT) in retinal degenerative diseases is debated. Systemic inhibition of the MAO-A enzyme leads to reduced breakdown of 5-HT and norepinephrine. The molecular structure of DMT closely resembles serotonin, enabling it to activate serotonin receptors, particularly the 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} subtypes. 5-HT_{1A} receptor agonists have demonstrated neuroprotective effects in animal models of central nervous system ischemia, traumatic brain injury, excitotoxicity, and Parkinson's disease. Their protective effect has also been confirmed in light-induced retinal damage. The mechanisms underlying the protective effects of 5-HT_{1A} receptor stimulation in the central nervous system and retina are not fully understood, but they may involve increased synthesis of antioxidant enzymes and anti-apoptotic proteins.

The combined use of the MAO-A inhibitor harmaline and DMT may lead to excessive stimulation of 5-HT receptors. Excessive production of 5-HT has been implicated in conditions such as valvular and coronary artery diseases, peripheral vascular disorders, and diabetic nephropathy. A 2021 study reported increased serotonin, 5-HT_{2A} receptor, and MAO-A expression in renal I/R injury, suggesting that 5-HT and its metabolism play a significant role in the I/R damage process. Furthermore, inhibition of 5-HT_{2A} receptors reduced MAO-A expression and mitigated oxidative damage. Elevated reactive oxygen species production could result from the oxidative degradation of 5-HT by MAO-A, a process preventable by reducing serotonin synthesis, blocking 5-HT_{2A} receptors, or inhibiting the MAO-A enzyme. In a model of light-induced retinal damage in BALB/c mice, intraperitoneal administration of a 5-HT_{2A} receptor antagonist preserved retinal thickness and integrity. Activation of the 5-HT_{2C} receptor may also contribute to I/R injury; in isolated retinal ganglion cells, 5-HT_{2C} receptor blockade protected against glutamate overstimulation-induced cell death.

I/R injury triggers an inflammatory response that can lead to cell death and tissue destruction in the eye, particularly in the retina. Our findings indicate that the MAO-A enzyme inhibitor harmaline counteracts the pathological processes associated with I/R injury. Adhering to the serotonin-centered hypothesis, we propose that MAO-A inhibition may reduce post-

ischemic oxidative stress by inhibiting the oxidative deamination of 5-HT. Considering the conflicting data in the literature regarding the role of 5-HT in pathological conditions, we hypothesize that there is a delicate balance between physiological and pathological concentrations of 5-HT in the eye, which was disrupted by the combined administration of DMT and harmaline. In the HT group, the elevated 5-HT concentration likely did not reach the threshold to cause harm, instead activating protective effects in the eye through reduced serotonin metabolism (lower oxidative stress). In contrast, in the DHT group, the 5-HT agonist DMT and the inhibited breakdown of endogenous serotonin may have jointly led to serotonin overstimulation.

Emerging evidence suggests that selective serotonin reuptake inhibitors (SSRIs), which increase serotonin levels in the central nervous system, impair visual quality and lead to retinal pigment epithelium (RPE) atrophy by reducing the thickness of the foveal and perifoveal macular ganglion cell complex. DMT has high affinity for 5-HT_{2A} receptors, which are abundantly located in the RPE and densely distributed in the claustrum, a region associated with the visual cortex. The adverse effects of DMT on the visual organ may be mediated by the (over)activation of 5-HT_{2A} receptors. Additionally, DMT may inhibit serotonin transport (SERT), further increasing 5-HT concentrations in the retina.

Another possible explanation for DMT's adverse effects on the visual organ is that it acts on other receptors. The effects of ayahuasca components have not yet been studied on the visual organ or retinal cells; therefore, a receptor localized in the eye may exist to which DMT binds with high affinity, mediating harmful effects. One such candidate is the Sigma-2 (Sig-2R) receptor, for which DMT is a proven ligand. Activation of Sig-2R can induce cell death and may play a key role in neurodegenerative diseases. Blocking Sig-2R has protective effects against ischemic and light-induced retinal damage, reinforcing the receptor's role in the pathomechanism of degenerative processes.

DMT is a promising drug candidate for the treatment of several diseases, including PTSD (post-traumatic stress disorder), depression, and brain and kidney I/R injury. However, the results of our first experiment highlight the importance of thoroughly investigating the effects of any promising drug candidate across all organ systems to mitigate potential side effects. In this light, further research is required to elucidate the effects of DMT on the visual organ. An unexpected benefit of the experiment was recognizing the therapeutic potential of MAO-A inhibition, which may play a significant role in preventing and treating eye diseases associated with I/R injury in the future.

In our second experiment, we examined the dose-dependent effects of physical exercise on the visual organ in an aging animal model, focusing on structural and functional aspects and molecular markers related to oxidative stress, inflammation, and neuroprotection. Age-related retinal functional decline can be effectively monitored using electroretinography. The results of our second experiment align with the literature, indicating that unfavorable changes in ERG parameters predict retinal degeneration during the aging process. Our findings show that recreational and forced physical exercise have different effects on the visual health of aging Wistar rats, highlighting the complex interplay between physical activity and ocular aging. We confirmed that voluntary physical activity exerts more beneficial effects on the aging retina by mitigating its functional decline.

Our ERG analysis revealed significant differences in a-wave amplitudes between young and aged rats, suggesting reduced numbers electrical activity of photoreceptors or in the aged groups. This was particularly evident at lower light intensities, where rod-type photoreceptors—responsible for scotopic (low-light) vision—are predominantly active. At higher light intensities (mixed rod-cone response) under scotopic conditions, the difference in a-wave amplitudes was no longer significant. Based on the literature, this may indicate that cones are less affected by the aging process. In contrast, forced physical exercise may have harmful effects on cones, as photopic measurements - dominated by cone activity - showed significantly lower a-wave amplitudes in the AFR group.

The prolonged implicit time suggests reduced responsiveness of photoreceptors. In the case of the a-wave, an extension of the implicit time was observed in the AFR group, particularly at very low and high light intensities. This may be explained by the fact that under extremely low light intensity (10 mcd·s/m²), rods in the AFR group are more difficult to excite. The prolonged implicit time at high light intensities may indicate that strenuous physical activity adversely affects cone function. No significant differences were observed between the aged groups for mixed rod-cone responses.

The analysis of the b-wave provided further insight into the effects of aging and exercise on retinal cells. Surprisingly, in young animals under scotopic conditions, particularly at low light intensity (when rods are active), lower b-wave amplitudes were measured. This could be due to slower rod maturation compared to cones. This observation is supported by the fact that at increasing light intensities, when cones are also activated, higher wave amplitudes were detected in young animals compared to the aged groups. Among the aged groups, the animals engaging in recreational exercise performed the best, particularly under scotopic conditions.

This suggests that voluntary exercise most effectively preserves the condition of retinal cells, especially the postsynaptic cells involved in rod signal transmission.

Consistent with the trends observed for a-waves, the implicit times of b-waves were also prolonged in the aged groups compared to the young control animals. Unlike the differences observed in a-waves, this prolongation of implicit time persisted even at higher light intensities, indicating that both the cone and rod postsynaptic pathways are affected by the aging process, reducing their responsiveness. When comparing b-wave implicit times among the aged groups, a unique pattern emerged. Interestingly, the pattern of b-wave amplitudes matched the trend observed in implicit times. Under scotopic conditions, the recreational exercise group exhibited significantly longer implicit times (with higher b-wave amplitudes); however, this difference diminished with increasing light intensity and disappeared under photopic conditions. In contrast, the forced exercise group exhibited shorter implicit times with lower wave amplitudes under scotopic conditions at low light intensities. This might suggest that in the AFR group, the signal transmission pathway became simplified, potentially involving a reduced number of synaptic cells, resulting in shorter implicit times. This explanation aligns with the observed reduction in b-wave amplitudes.

The complex ERG results of our second experiment highlight the intricate and multifaceted effects of physical exercise on the eye. Our data suggest that voluntary, recreational exercise has a more positive impact on the functional state of the eye than forced exercise in older age.

According to the literature, the aging process leads to increased cell death in the retina, resulting in a reduction in retinal layer thickness. The results of our second experiment were consistent with these findings. Significant retinal thinning was detected in the aged inactive control (AIC) group. Our experiment highlights the importance of physical activity, as both voluntary and forced exercise prevented retinal atrophy, maintaining retinal morphology similar to that of the young control (YC) group. The preservation of retinal thickness and the favorable changes observed in electroretinogram (ERG) measurements, particularly in the group engaging in voluntary exercise, support our hypothesis that physical activity may mitigate various aspects of age-related retinal degeneration. These findings align with the existing literature, which emphasizes the beneficial effects of regular exercise on retinal health through mechanisms such as increased production of trophic factors, reduced oxidative damage and inflammation, improved metabolic functions, and enhanced blood flow in retinal tissues.

In our second experiment, we also aimed to investigate the molecular changes induced by exercise in the eye. To this end, and to provide explanations for the ERG and histological findings, we examined the expression of several proteins in the eye that play key roles in

oxidative stress (MAO-B), tissue remodeling and inflammation (GFAP, NFκB), regulation of cellular functions (PARP1, NFκB), and neuroprotective processes (BDNF, SIRT6, SOD-1, HSP70).

Monoamine oxidases (MAO-A and MAO-B) are located on the outer mitochondrial membrane and metabolize several key neurotransmitters in the central nervous system. Increased MAO-B expression is a hallmark of aging, neuroinflammatory, and neurodegenerative conditions. During neurotransmitter breakdown (oxidative deamination), aldehydes and H_2O_2 are produced, which can lead to oxidative damage and neuronal loss. In our experiment, MAO-B expression was significantly elevated in the AIC and AFR groups compared to the YC and ARR groups, with no differences observed between the YC and ARR groups. This indicates that recreational running was able to prevent the increase in MAO-B expression. Conversely, this finding suggests that intense physical exertion may cause greater oxidative damage in aged rats.

The increased MAO-B expression was accompanied by a simultaneous rise in the levels of glial fibrillary acidic protein (GFAP). GFAP synthesis was significantly elevated in the AIC and AFR groups compared to the young animals, indicating increased astrocyte activation. Recreational physical activity prevented the excessive expression of GFAP.

Age-related regulatory changes, oxidative stress, inflammation, and genomic instability can lead to cellular senescence and apoptosis. We examined the levels of SIRT6, a key regulator of genomic stability, in the eye. This cytoprotective protein activates DNA-protective and repair functions under oxidative stress. SIRT6 deficiency has been shown to result in reduced lifespan and aging-like phenotypes in rodent models. In our experiment, SIRT6 expression was significantly lower in the AIC and AFR groups compared to the YC and ARR groups, indicating the detrimental effects of strenuous physical activity in aging.

Similar to SIRT6 expression, BDNF levels were also reduced in the AFR group compared to the young animals, potentially contributing to the detrimental effects of strenuous exercise. BDNF is a neurotrophin that plays a key role in the development of the central nervous system, promoting neuronal survival and differentiation. In aging, the neuroprotective role of BDNF has been well established. It has been shown to exert protective effects on ganglion cells, preserving the dendritic network and mitigating vision loss caused by elevated intraocular pressure, as demonstrated in experimental glaucoma models. Additionally, BDNF has proven retinoprotective against damage induced by hypoxia and glucose deprivation.

In our second experiment, the expression patterns of PARP1 and NFκB yielded surprising results that seemingly contradicted conclusions drawn from the first experiment.

It is known that elevated PARP1 levels and excessive activity can lead to a specific type of cell death, known as PARthanatos. PARthanatos may play a critical role in the pathomechanism of retinal damage, particularly in hereditary retinal degeneration, age-related macular degeneration, and ischemia-reperfusion injury in the eye. On the other hand, increased PARP1 activity has been associated with longer life expectancy. Under physiological levels of oxidative stress, PARP1 has been shown to have cytoprotective effects. These findings suggest that the balance of PARP1 activity in the body is extremely delicate: while mild oxidative stress-induced PARP1 expression during physiological aging is likely protective, processes associated with high oxidative stress and substantial PARP1 activation (e.g., I/R injury) may promote cell death. In our second experiment, both moderate and forced physical activity increased PARP1 expression in the eye compared to the young control group. Considering the histological results, we hypothesize that, in this case, elevated PARP1 synthesis mediates a protective effect.

NFκB influences the expression of both pro-apoptotic and anti-apoptotic genes, which play critical roles in various pathological conditions. Like PARP1, NFκB is a "double-edged sword": it can activate cytoprotective mechanisms to support cell survival or promote cell death by stimulating intracellular pathways involved in DNA damage and inflammatory responses. Its regulation is closely tied to PARP1 and SIRT6. According to our western blot results, significantly higher NFκB levels were detected in all aged groups compared to the YC group. Physical activity did not significantly reduce NFκB expression in the eye, suggesting that the beneficial effects of exercise are not mediated through the suppression of the NFκB pathway.

Enhanced oxidative stress may play a pivotal role in initiating and maintaining cellular aging, leading to cell damage and death. To clarify the impact of physical exercise on the antioxidant enzyme system, we examined the levels of SOD1 and HSP70 proteins in the eye. Surprisingly, no significant differences were detected between the groups for either protein, suggesting that their expression does not significantly decline during aging and that the protective effects of regular physical activity are not mediated by the induction of these proteins.

A limitation of our experiments is that the histological analysis only focused on retinal layer thickness, without performing immunofluorescence analysis, which could have provided precise insights into specific cell layers and cell types. Consequently, based on ERG results, it is unclear whether differences in amplitude size are due to changes in cell numbers or activity. Another limitation of our study is that during the western blot analysis, we used the entire eye bulb rather than isolating the retina, meaning that the observed protein expression changes reflect the whole eye. The vast majority of recent research focuses exclusively on retinal

function and protein expression profiles, with only a few studies considering the eyeball as a functional unit. Oxidative stress, a key factor in the pathomechanism of many eye diseases, damages not only the retina but also other parts of the eye, contributing to vision loss. Therefore, we chose to examine the entire eye to provide a more comprehensive understanding of its condition.

Physical inactivity is the fourth leading cause of death worldwide, with nearly one-third of the global population not meeting the minimum recommendations for health benefits. Between 6-10% of all deaths from non-communicable diseases worldwide are attributed to physical inactivity, a figure that reaches up to 30% for ischemic heart disease. Recognizing the proven role of exercise in maintaining health and the rising trend of inactivity worldwide, there has been growing scientific and commercial interest over the past decade in identifying bioactive, orally administrable compounds that mimic or amplify the effects of exercise. These compounds have been dubbed "exercise pills" or "exercise mimetics."

The potential of "exercise mimetics" is supported by research demonstrating that factors circulating in the blood of trained aged mice, when administered to sedentary aged mice, improved their physical and cognitive states and enhanced neurogenesis in the central nervous system. These pharmacological agents, if designed with precise molecular targets and effective formulations, could offer substantial benefits, particularly for individuals unable to engage in physical activity due to underlying medical conditions.

Based on our experimental results, it is conceivable that inhibitors of the monoamine oxidase (MAO) enzyme system could serve as excellent active compounds for "exercise pills." MAO inhibitors have been used for decades in the treatment of mood disorders and Parkinson's disease, and their potential application in other neurodegenerative conditions has also been suggested. Exercise increases serotonin (5-HT) levels in the body, which promotes neurogenesis in the central nervous system. MAO-A inhibition not only reduces oxidative stress in the eye but also elevates serotonin concentration (similar to the effects of exercise), which may mediate neuroprotective effects.

MAO-B inhibitors, on the other hand, prevent apoptosis by stabilizing mitochondrial membranes, inducing anti-apoptotic processes, preventing increased mitochondrial membrane permeability and swelling, reducing mitochondrial membrane potential, and inhibiting the release of cytochrome C. Through these mechanisms, they effectively block various apoptotic processes.

5. Summary

The results of these two experiments conducted by our research team have highlighted the indisputable role of the monoamine oxidase enzyme system in ophthalmic conditions associated with ischemia-reperfusion injury and aging, offering a potential therapeutic target for the prevention and treatment of these diseases. Based on the findings of our first experiment, the MAO-A inhibitor harmaline reduced I/R damage of the eye by decreasing the expression of NFκB, PARP1, and MMP9, while increasing Hsp70 levels. However, the combined administration of DMT and harmaline had the opposite, detrimental effect on the eyes of Sprague Dawley rats. In our second experiment, we demonstrated that in elderly Wistar rats, low-intensity physical exercise was more beneficial compared to high-intensity regimen. Increased exertion caused elevated oxidative stress in the body due to the high expression of MAO-B, which could trigger a harmful cascade. Along with MAO-B, there was also an increase in GFAP levels, which may indicate accelerated inflammation. The expression of the protective factors, SIRT6 and BDNF, significantly decreased in the group subjected to forced exercise. Conversely, recreational running had a protective effect on the visual system, and the group averages were comparable to the results of the young control animals, suggesting that low-intensity physical activity is more suitable for maintaining health in old age.

6. Novel Findings of the Dissertation

- Based on ERG and histological analysis, harmaline treatment exerts a protective effect against ischemia-reperfusion injury in the eyes of young male Sprague Dawley rats by reducing the expression of NFκB, MMP9, and PARP1 proteins.
- The combination of DMT and harmaline had detrimental effects on the eyes of male Sprague Dawley rats affected by ischemia-reperfusion injury, as demonstrated by ERG and histological analysis. This adverse effect may be attributed to elevated expression of GFAP, NFκB, MMP9, and PARP1 proteins, along with decreased expression of the HSP70 chaperone protein.
- Voluntary exercise initiated in advanced age (18 months) has a more favorable impact on the functional state of the eye compared to forced exercise in a Wistar rat model.
- Forced exercise reduced the expression of the protective SIRT6 and BDNF while increasing the expression of MAO-B and GFAP proteins in the eyes of aged (24-month-old) male Wistar rats. In contrast, recreational exercise mediated opposite, more beneficial effects.
- Regular physical activity over six months, regardless of intensity, mitigates the reduction in retinal layer thickness in the eyes of aged (24-month-old) Wistar rats.

7. Publications

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List of publications related to the dissertation

1. **Szilágyi, A.**, Takács, B., Szekeres, R., Tarjányi, V., Nagy, D., Priksz, D., Bombicz, M., Kiss, R., Szabó, A. M., Lehoczki, A., Gesztelyi, R., Juhász, B., Szilvássy, Z., Varga, B.: Effects of voluntary and forced physical exercise on the retinal health of aging Wistar rats.
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IF: 5.3 (2023)

2. **Szilágyi, A.**, Takács, B., Szekeres, R., Tarjányi, V., Bombicz, M., Priksz, D., Kovács, A. I., Juhász, B., Frecska, E., Szilvássy, Z., Varga, B.: Therapeutic Properties of Ayahuasca Components in Ischemia/Reperfusion Injury of the Eye.
Biomedicines. 10 (5), 1-19, 2022.
DOI: http://dx.doi.org/10.3390/biomedicines10050997
IF: 4.7

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3. Takács, B., **Szilágyi, A.**, Priksz, D., Bombicz, M., Szabó, A. M., Pelles-Taskó, B., Rusznyák, Á., Haimhoffer, Á., Gesztelyi, R., Szilvássy, Z., Juhász, B., Varga, B.: Electroretinographical Analysis of the Effect of BGP-15 in Eyedrops for Compensating Global Ischemia-Reperfusion in the Eyes of Sprague Dawley Rats.
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8. Key words

Retina, aging, ischemia-reperfusion, Sprague Dawley rat, Wistar rat, ayahuasca, DMT, harmaline, recreational physical activity, forced physical activity, electroretinography (ERG), Western blot, histology

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