Role of Extracellular Vesicles in retinitis pigmentosa

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ABSTRACT:

Senses are our contact to the environment and the eyes are considered the most important sensory system. It is known that up to eighty percent of all of our perceptions are mediated by the sight\(^1\). The human eye is a complex and organized system with a high level of specialization\(^2\). Also, it is considered an immunological privileged organ then, in order to preserve the sight, developed molecular and cellular mechanisms to limit the immune response\(^3,4\).

The retina is a complex anatomic and functionally structure, were the light information process starts\(^14\). The retina extends back of the eyeball, from the inner surface to the ciliary body. It is in contact with the vitreous body internally and with the choroid externally\(^15\). Eleven layers composed the retina. Starting from the back of the eye, we find the RPE, the photoreceptor outer segments (POS), the photoreceptor inner segments (IS), the external limiting membrane (ELM), the photoreceptor outer nuclear layer (ONL), the outer plexiform layer (OPL), where photoreceptor cells synapse with interneurons, the inner nuclear layer (INL), containing bipolar, amacrine and horizontal cells, the inner plexiform layer (IPL), where interneurons synapse with the ganglion cell layer (GCL), the nerve fiber layer (NFL), and the inner limiting membrane (ILM)\(^16\). Due, their structural complexity, the retina can be separate in two functional parts: the internal part or neural retina and the external part or non-neural retina. The neural retina transforms the light into electrical impulses and send it to the brain through the optic nerve. The non-neural retina, that that includes RPE and Bruch’s membrane, maintains the integrity of the barrier between the choroid and the retina, known as external blood retinal barrier (BRB)\(^17\).

Retinitis Pigmentosa (RP) is a group of inherited neurodegenerative diseases in which rod photoreceptors die due to a genetic mutation, whereas cone photoreceptors
disappear secondarily, once rods are gone. While the initial disease symptoms (i.e. night blindness) are comparatively mild, the secondary loss of cones ultimately leads to complete blindness. The disease affects approximately 1 in 3,000 to 7,000 people among the working age population in the developed world\textsuperscript{51} and is characterized by strong genetic heterogeneity with causative mutations in more than 100 genes\textsuperscript{52}. RP was described for the first time in 1857 by the ophthalmologist Franciscus Cornelius Donders\textsuperscript{53}.

Loss of night vision is the first sign of the disease and normally starts in childhood. Afterwards, in the peripheral vision, blind spots appear and tunnel vision is produced when these spots merge. The disease progresses affecting the central vision and hindering daily tasks, such as reading, driving, and recognizing faces, eventually causing blindness in adulthood\textsuperscript{54}. Around 50 to 60\% of RP cases show autosomal recessive inheritance while 30 to 40\% is produced by autosomal dominant heredity. Moreover, 5 to 15\% of RP is produced by X-linked trait\textsuperscript{55}.

In 4 – 8\% of human RP cases, the disease is caused by mutations in genes encoding for cGMP specific PDE\textsubscript{6}\textsuperscript{57,58}. The PDE\textsubscript{6} family, commonly known as photoreceptor phosphodiesterase, entails three genes PDE\textsubscript{6}A, PDE\textsubscript{6}B, and PDE\textsubscript{6}C\textsuperscript{59}, encoding a key protein in phototransduction cascade and the intracellular cGMP level maintenance\textsuperscript{60,61}. In rods, the PDE\textsubscript{6} catalytic core is a heterodimer of PDE\textsubscript{6}A and B subunits, whereas in cones the enzyme consist in two PDE\textsubscript{6}C subunits giving rise to a catalytic homodimer\textsuperscript{59,61}.

A mutation in the B subunit of PDE\textsubscript{6} produces a defective protein. The non-functional enzyme fails to hydrolyze cGMP, causing its accumulation\textsuperscript{57,62}. Notably, elevated cGMP levels in dying photoreceptors were found to correlate with increased activity of Poly ADP ribose polymerase (PARP)\textsuperscript{50,63}.

**Poly-ADP-ribose (PAR)** metabolism is a post-translational modification involved in many cellular pathways such as transcription, DNA repair, and cell death\textsuperscript{64}. Although the enzyme activity presents beneficial role in cell physiology, PARP is also implicated in human diseases such cancer and neurodegenerative disorders. In neurodegenerative diseases, including hereditary retinal degeneration, PARP over activation may consume cellular substrates, producing a subsequent cell death\textsuperscript{63,65,66}. The precise mechanisms leading to cell death remain unknown and no adequate treatment is available. Poly ADP
ribose polymerase (PARP) over activity is involved in photoreceptor degeneration and, in mice models, its pharmacological inhibition protects the retina.

To date no effective treatments for RP are available\textsuperscript{53,87}. Nutritional supplements were used to limit the diseases progression. Among them, Vitamin A, B-carotene, Docosahexaenoic Acid (DHA), and lutein, showing a limited effect\textsuperscript{53}. Gene and cell therapies are under development\textsuperscript{53,87,88}. In 2018 the food and drug administration (FDA) approved the first retina gene therapy for RP65 mutations, that causes LCA and RP. Moreover, gene therapy trials for RPGR, PDE6B, MERTK, and RLBP1 mutations are currently ongoing\textsuperscript{53}. However, it is expected that gene therapy only halts or slows the progression of the disease\textsuperscript{53} since the absence of long-term benefits were reported, probably due to the low transduction efficiency of recombinant gene vectors\textsuperscript{88}. Moreover, the use of high dose recombinant gene vectors may produce toxicity\textsuperscript{88}. Cell therapy in RP aims to differentiate photoreceptors from stem cells in vitro to replace lost cells and restore the vision. The implanted cells should have the capacity to integrate, survive, and signal correctly to bipolar cells\textsuperscript{53}. In RP patients, a bone marrow–derived stem cells treatment reported the improvement of quality live 3 months after treatment. Unfortunately the treatment efficiency was deteriorated and lost at 12 months\textsuperscript{87,88}. The problem seems to be the low transduction efficiency of recombinant gene vectors. However, a higher dose produced toxic effects\textsuperscript{88}.

In advanced stages of RP, retinal prostheses or subretinal implants can be an option. The Argus II Retinal Prosthesis System is available in the USA. In Europe, we have the Alpha-IMS (developed in Tübingen University). These implants stimulate the visual pathway downstream of the photoreceptors. The visual system restoration is modest and rudimental, nevertheless it allows the perception of movement and shape\textsuperscript{53,88}.

In RP several PARP inhibitors developed for cancer treatments and approved by FDA were tested, in vitro and in vivo, in the last years in order to verify its neuroprotective capacity in photoreceptors in the rd1 mouse model. PARP inhibitor PJ-34, R503, ABT-888 (Veliparib) and Olaparib (LynparzaTM) were evaluate in rd1 retinas. Despite PARP inhibitors display mechanistic similarities their structural differences affect their pre-clinical potency and the drug tolerability in patients\textsuperscript{89}. R503 and ABT-888 exhibited adverse effects whereas PJ-34 and Olaparib inhibitors shown neuroprotective effects. Among this two drugs Olaparib presented stronger photoreceptor protection\textsuperscript{66}. 
Olaparib is a PARP inhibitor that was initially approved by the FDA in December 2014 as a monotherapy for ovarian cancer. In addition, it is used in patients with metastatic breast cancer and is also under clinical trial III in prostate cancer. Unlike other PARP inhibitors, Olaparib seems to have multiple ways of action. As seen in other molecules, Olaparib competes with NAD+, blocking the PAR chains formation by PARP. Additionally, Olaparib inhibits PARP1, PARP2, and PARP3s leading to the inability to recruit the appropriate DNA repairing factors. This fact produces the accumulation of single strand breaks, followed by the double strand breaks, as well as the collapse of replication forks.

The rd1 and rd10 mice are two animal models that hold a mutation in the gene encoding the beta subunit of the PDE6, mapped on chromosome 5, which protein catalyzes cGMP into guanosine monophosphate (GMP). Due to the mutation, cGMP accumulates causing, as a consequence, photoreceptor cell death. Mice homozygous for the rd1 and rd10 mutations occur naturally and are characterized by a rapid degeneration rod-like photoreceptor cells, remaining only the cones, which eventually die as well.

Rd1 mice was reported by Kepler for the first time in 1924. The animal carries a murine leukaemia provirus insertion in intron 1 and a second nonsense mutation (stop codon) in exon 7. In 2002 Chang et al. described the rd10 mouse that contains a missense mutation (R560C) in exon 13 of the PDE6B gene. Despite both mutations occur at PDE6B there are differences between them. In rd1 mice the protein expression and activity remains undetectable whereas in rd10 the PDE6 activity decrease significantly, but is detectable at postnatal day (P) 10. Moreover, in rd1 mice the peak of degeneration occurs before the complete retinal structures development at P13. In contrast, in rd10 mice, the peak of rod photoreceptor cell death take place when the retina is matured at P18. The residual enzyme activity in rd10 retinas may reduce the toxic cGMP accumulation at early stage of the disease and explain why degeneration is slower in rd10 compared with rd1. Thus, the rd10 is considered a better mouse model than rd1 for developing new treatments for RP.

Additionally, retinal cell survival depends of adequate reception and processing of the information and appropriate cellular communication. Initially, the extracellular vesicles (EVs) were recognized as a mechanism for discharging useless cellular components.
EVs include a heterogeneous group of particles release from cells. Currently, EVs are classified in three categories: exosomes, microvesicles or ectosomes and apoptotic bodies, based in their biogenesis, mechanism of release, and size\textsuperscript{96,109}. Apoptotic bodies are released from the plasma membrane of dying cells, and they have a diameter range from 200 nm to 5 mm\textsuperscript{96}. Microvesicles (MVs), or ectosomes, are shed from the plasma membrane and their size are between 100-800 nm\textsuperscript{96}. The ectosome nomenclature derives from the term ectocytosis used to describe the shedding of vesicles from the plasma membrane in stimulated neutrophils\textsuperscript{102}. Exosomes, which are 30-150 nm in diameter, are the EVs best characterized and as explained above their release depends of the MBV formation\textsuperscript{98,109}.

The EVs cargo includes nucleic acids, proteins, lipids, and metabolites and can be modified depending on the cell type, stimulus, environment, and cell damage. Nowadays, the EVs cargo of more than 40 species are studied by more than 1,000 studies according to the Vesiclepedia\textsuperscript{127}, a community compendium for EVs cargo.

Although the EVs activity in neurodegeneratives diseases is identified, our knowledge is the field is still limited\textsuperscript{150} and there are controversial studies about their positive or negative role\textsuperscript{130,151}. Then, EVs were define as a double-edged sword since they can promote the disease progression or they can favour the homeostasis maintenance, sequestering neuro-toxic components and therefore protecting the cells from degenerate\textsuperscript{130,151}.

In the CNS, EVs release was reported in neurons, astrocytes, microglial cells, and oligodendrocytes\textsuperscript{130,150}. Many properties were attributed to EVs in the nervous system including their role in neural networks development and remodelling\textsuperscript{130}, neuron-neuron and neuron-glia communication\textsuperscript{130,147,152,153}, regeneration\textsuperscript{147,152}, neuroprotection, immunomodulation synaptic plasticity regulation\textsuperscript{130,147} and vascular integrity\textsuperscript{147}. EVs activity was reported in different neurodegeneratives diseases such as Alzheimer’s and Parkinson’s Diseases\textsuperscript{130,150,151,152}, Frontotemporal Dementia\textsuperscript{151} Huntington’s disease and Amyotrophic lateral sclerosis\textsuperscript{130,150}, multiple sclerosis\textsuperscript{150}. Moreover, EVs relevance was reported in some retinal diseases like dry eye, corneal rejection after transplantation, uveitis, AMD, glaucoma\textsuperscript{154} rhegmatogenous retinal detachment (RRD)\textsuperscript{149}, corneal inflammation and RD\textsuperscript{148}. 
Growing evidence has elucidated their roles in cell–cell communication by carrying nucleic acids, proteins, and lipids that can, in turn, regulate behavior of target cells. Nevertheless, the role of EVs in blinding diseases, such as RP, is far from being understood.

As described in other pathologies, the EVs release and cargo are modified in retinal diseases, according to the state of the cell and the environment\textsuperscript{155}. Furthermore, EVs can impact the fate of their target or recipient cells. Taking all the information exposed into consideration, it is possible that EVs in damaged retinas, such as in RP patients, are different from those released from healthy retinas. Thus, the hypothesis proposed herein is that retinal EVs released from damaged cells are different in terms of number and cargo and they influence their recipient cells. Also, there is a connection between PARP and EVs activity in retinitis pigmentosa.

Section from rd1 and rd10 mice and organotypic retinal explants from rd10 were used to investigate cellular communication by EVs. CD9 and CD81 tetraspanins were studied to investigate EVs activity at tissue level by immunostaining. Inhibition of PARP activity was performed using Olaparib. Immunohistochemistry was carried out to evaluate PARylated proteins and immunostaining was performed to determinate rhodopsin (rho) expression, Müller glia cell activity, and cyclic guanosine monophosphate (cGMP) levels after olaparib treatment. Also, immunofluorescence was used to study EVs and their colocalization with cilia in rd10 retinae after PARP inhibition. EVs were isolated using ultrafiltration and size exclusion chromatography or a commercial isolation kit, depending on downstream applications. Nanosight analysis, electron microscope, Fluorescence-Activated Cell Sorting (FACs), dot blot, and proteomics were used to characterize the EVs. Moreover, rd10 retinaes were treated with EV from wt and vice-versa. Immunostaining assays against CD9, CD81, rho, and IBA-1 (microglia marker) were carried out after EVs treatments. TUNEL assay was used to evaluate cell viability, thickness, and row photoreceptor number in the outer nuclear layer (ONL) after Olaparib and EVs treatments.

EVs release changes with the age in wt mice and also under retinal degeneration in rd1 and rd10 in different retinal layers. PARP inhibition by Olaparib rescues photoreceptors and also modify the EVs release and cargo in rd10 mice. The EVs release was increased in rd10 retinae and the protein cargo was modified under retinal degeneration. Moreover, EVs from rd10 retinae had the ability to damage wt retinas and
something similar was produced after treated rd10 retinae with EVs from wt. This data strongly suggests the implication of EVs in retina development and degeneration.


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