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REVIEW ARTICLE

Citicoline and Retinal Ganglion Cells: Effects on Morphology and Function

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Abstract: ***Background*:** Retinal ganglion cells (RGCs) are the nervous retinal elements which connect the visual receptors to the brain forming the nervous visual system. Functional and/or morphological involvement of RGCs occurs in several ocular and neurological disorders and therefore these cells are targeted in neuroprotective strategies.

Cytidine 5-diphosphocholine or Citicoline is an endogenous compound that acts in the biosynthesis of phospholipids of cell membranes and increases neurotransmitters' levels in the Central Nervous System. Experimental studies suggested the neuromodulator effect and the protective role of Citicoline on RGCs. This review aims to present evidence of the effects of Citicoline in experimental models of RGCs degeneration and in human neurodegenerative disorders involving RGCs.

***Methods*:** All published papers containing experimental or clinical studies about the effects of Citicoline on RGCs morphology and function were reviewed.

***Results*:** In rodent retinal cultures and animal models, Citicoline induces antiapoptotic effects, increases the dopamine retinal level, and counteracts retinal nerve fibers layer thinning. Human studies in neurodegenerative visual pathologies such as glaucoma or non-arteritic ischemic neuropathy showed a reduction of the RGCs impairment after Citicoline administration. By reducing the RGCs' dysfunction, a better neural conduction along the post-retinal visual pathways with an improvement of the visual field defects was observed.

***Conclusion*:** Citicoline, with a solid history of experimental and clinical studies, could be considered a very promising molecule for neuroprotective strategies in those pathologies (*i.e*. Glaucoma) in which morpho-functional changes of RGCc occurs.

Keywords: Retinal ganglion cells, citicoline, neuroprotection, neurodegeneration, glaucoma, ischemic optic neuropathy, pattern electroretinogram.

1. INTRODUCTION

Functional and/or morphological alterations of retinal ganglion cells (RGCs) occur in several diseases of the visual system (*i.e*., diabetic retinopathy, glaucoma, demyelinating optic neuritis, ischemic optic neuritis). Cytidine 5-diphosphocholine or Citicoline is an endogenous molecule that has a role in the biosynthesis of phospholipids of cell membranes and increases neurotransmitters' levels in the Central Nervous System (CNS). Several *in vitro* and *in vivo* studies have shown that Citicoline has neuromodulatory and neuroprotective properties in RGCs and it is able to reduce their morpho-functional impairment. For this reason, Citicoline has been studied for its neuroenhancement potential on impaired RGCs in several human diseases. Studies have been performed with the administration of Citicoline in patients suffering from Open Angle Glaucoma (OAG) or Non-Arteritic Ischemic Optic Neuritis (NAION) and showed remarkable findings in rescuing dysfunctional RGCs. In this review, we present evidences about the effects of Citicoline in experimental models of RGCs degeneration and in human neurodegenerative disorders involving RGCs.

2. RETINAL GANGLION CELLS PROPERTIES AND MECHANISMS OF DEGENERATION

Retinal ganglion cells are part of the inner retina and with their axons constitute the optic nerve, bringing visual inputs to the cerebral structure [1]. In this way light runs to become an image. The RGCs bodies reside in the inner retina, while the long axons are located into the retinal nerve fiber layer

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(RNFL), forming the optic nerve head. Once these fibers go through the lamina cribrosa, they are surrounded by the myelin that speeds up the velocity of the neuronal transmission of the optic nerve. For the lack of myelin into the ocular globe, the long unmyelinated axons require high levels of energy for the genesis of the action potentials that posteriorly to the ocular globe are substituted by the high efficacious saltatory action potentials [2, 3].

2.1. Mechanisms of RGCs Degeneration in Pathologies of the Visual System (Mithocondrial Optic Neuropathies, Glaucoma, Ischemic Optic Neuropathies, Diabetes, Multiple Sclerosis, Central Nervous System Degenerative Diseases)

Since RGCs are the connecting ring between the sensory retina and the neuro-retina, they may be affected in several pathologies of the visual system, with both visual and neurological pathogenesis. Indeed, RGCs loss is currently the hallmark of several optic neuropathies, where damage to RGCs axons occurs at the level of the optic nerve head (ONH). Optic neuropathies refer to pathologies of the optic nerve characterized by lack of function and/or degeneration of neuronal axons with consequent cell loss for RGCs apoptosis inducing optic nerve atrophy.

Glaucoma and optic neuritis are the two major degenerative causes of optic nerve damage. Optic neuropathies may have different causes: inheritance, inflammation, ischemia, trauma, nutritional depletion, toxic damage [3].

RGCs loss, optic nerve degeneration, and consequent visual loss are very common clinical manifestation of mitochondrial diseases [4]. For instance, the optic nerve mitochondria are very copious in the ocular globe where myelin is absent, and a smaller amount can be found behind the lamina cribrosa.

This distribution is the potential cause of the remarkable susceptibility of these cellular organelles to mechanisms of cellular dysfunction as failure of the respiratory chain, production of oxidative reagents and finally apoptosis [2, 5, 6].

Leber's hereditary optic neuropathy (LHON) [7, 8] and dominant optic atrophy (DOA) [9-11] are the two most frequent mitochondrial non-syndromic hereditary diseases involving the optic nerve [12]. Both diseases affect precociously and preferentially the small axons of the papillo-macular bundle, which are responsible for the fine central vision and color detection [2, 13-15]. Larger axons are less involved and are surrounded by the glial tissue replacing the lost fibers [2, 16, 17]. This early prevalent involvement of the small axons has been recently documented also by functional testing [18, 19]. Remarkably, in LHON, electron microscopy reveals the persistence of axonal degeneration, even after the clinical onset of the disease [2, 16]. Hence, the neuronal degeneration proceeds long after.

DOA is another mitochondrial optic neuropathy often diagnosed at early ages due to the advancing central visual loss in both eyes progressing in adult life [2, 13, 20, 21]. Patients display centro-cecal scotomas and color vision loss (tritanopia), associated to temporal optic disc pallor at the beginning, and optic atrophy by time [22]. Overall, despite the different evolution, LHON and DOA have both a preferential involvement of the papillo-macular bundle [2].

The histopathology of DOA [23] shows loss of the RGCs of the macular area, with an overall normal appearance of the rest of the retina.

In glaucoma, one of the most common ophthalmic neurodegenerative diseases and cause of irreversible blindness globally [24], a gradual degeneration of RGCs and optic nerve damage produces the progressive visual field loss and ultimately severe vision loss. Classically, this manifests as an initial permanent loss of peripheral vision, leading to central vision defects as the disease progresses [25, 26]. At the present, there is no curative treatment for glaucoma, and only exist therapies targeting elevated intraocular pressure (IOP) level, the major risk factor [27, 28]. Because glaucoma is a complex and multifactorial disease, with variation in its progression, and vision loss continues to occur in some patients, despite well-controlled IOP [29], it is thought that secondary RGCs degeneration plays an important role in the pathology [30].

Various mechanisms are thought to contribute to RGCs loss**.** For instance, RGCs apoptosis is recognized as the earliest manifestation of cell death in glaucoma, and we know that up to 40 % of RGCs are lost before visual field defects can be observed by standard clinical tests [31]. Other mechanisms identified in order to explain neurodegeneration in glaucoma are: a) axonal transport failure through the lamina cribrosa that is the primary place of transport dysregulation–[32]; b) the mechanical theory for that elevated IOP conduces the compression of the nerve fibers, causing a dysregulation of anterograde and retrograde axoplasmic transport [33]; c) ischemic theory, characterized by blood perfusion deficit with hemodynamic abnormalities at the site of the optic nerve head, associated or not with elevated levels of IOP [34]; d) autophagy dysregulation [35]: autophagy is the orderly self- degradation and recycling of dysfunctional cells and is useful for the turnover of cytoskeletal and organelle [36]; these mechanisms can be upregulated in response to cellular stress [37] and downregulated in many neurodegenerative disorders, including Alzheimer's and Parkinson's diseases [38]; e) increased levels of amyloid beta (Aβ) in RGCs as in Alzheimer disease, therefore anti-Aβ therapies have been identified as a therapeutic target to prevent RGCs degeneration [39]; f) another possible reason for the variability in response to IOP elevation and extent of glaucomatous RGCs damage is that IOP levels do not really represent the pressure levels at the ONH [40]. The change in IOP relative to orbital cerebral spinal fluid pressure (CSFP), the so-called trans-lamina cribrosa pressure difference (TLPD) or a change in the time-dependent relationship between the pulse-synchronous changes in IOP and orbital CSFP, might better represent the pressure situation at the ONH [40-42].

The intraorbital, intracanalicular and intracranial portions of the optic nerve receive their main blood supply from the pial vessels from the ophthalmic artery. Branches of the retinal arteries supply the ONH: the peripapillary choroidal vessels and the short posterior ciliary arteries.

Anterior ischemic optic neuropathy (AION) is the most frequent type of acute optic neuropathy in patients above 50 years of age, resulting from blockage of the blood supply of the optic nerve head from the posterior ciliary artery circulation [43]. The pathology of AION is similar to other stroke events, however the blood supply of the RGCs cell body in the retina is distinct from that supplying RGCs axons in the optic nerve [44]. The optic nerve stroke, an ophthalmological emergency, presents with sudden and severe visual loss, peripapillary hemorrhages, disruption of normal nerve architecture, RGCs apoptosis and permanent vision impairment. AION can be non-arteritic (NAION) and arteritic (AAION) [3]. In most cases NAION pathology is self-limiting with a partial resolution of the visual loss. AAION instead leads more frequently to bilateral blindness [3]. Corticosteroids are commonly administered as soon as the diagnosis is made; however emerging neuroprotective treatment for RGCs in ischemic optic neuropathies is proved to be useful [45].

RGCs are targeted also in the pathogenesis of diabetic retinopathy (DR) [46, 47]. Recent reports on macular RGCs in diabetic patients (with and without diabetic retinopathy) by using optical coherence tomography (OCT) to calculate changes of thickness of the inner neuronal layers did not exhaustively answer this question. Thus, morphological studies could not agree with the possibility of RGCs loss preceding the onset of initial microangiopathy in DR, as described by studies using other *in vivo* techniques**,** and histological sections from postmortem human retinae and animal models with diabetes [47-56].

Functionally, it has been described that in frank insulin-dependent diabetic (IDDM) patients, the early electrofunctional impairment consists into an abnormal neuronal conduction along the visual pathways, that involves later the macular innermost retinal layers proceeding to the middle and outer retinal layers [57]. In type 1 diabetes patients without retinopathy and with a disease duration shorter than 6 months, the early abnormal Pattern Electroretinogram (PERG) responses display an impairment of the innermost retinal layers [58-60] suggesting preferential and early RGCs abnormalities in diabetes.

RGCs' damage is also present in demyelinating diseases such as multiple sclerosis (MS). Apart from optic neuritis which consists into the acute inflammation of the optic nerve, and it is the most frequent first event of this disease, all other components of the visual pathway from the photoreceptors to the visual cortex may be affected. Recently, however, loss of RGCs has also been documented in non-optic neuritis (N-ON) eyes with significant thinning of RGCs axons observed by retinal tomography [61]. The significant RNFL and RGCs layer thinning revealed by OCT in N-ON-eyes of MS patients as compared to controls described in many reports is, however, considerably less than the loss typically reported in optic neuritis eyes.

Animal and human studies of multiple sclerosis show evidence of retrograde trans-neuronal retinal ganglion cell degeneration in the visual system [62, 63] by magnetic resonance imaging (MRI) [64] and diffusion tensor imaging [65]. This damage is preferential for the temporal RNFL fibers supplying the central part of the visual field [66] and can be evaluated also functionally by recording the PERG and the visual evoked potentials (VEP) [67]. We firstly described that there exists a correlation between functional (PERG) and morphological (RNFL thickness) changes in MS patients previously affected by optic neuritis. In addition, a reduction in RNFL thickness was also observed in MS patients without optic neuritis [68]. Beyond visual pathologies, RGCs are involved also in neuro-degenerative diseases of the brain. Hinton *et al*. [69] first described histologically that there exist retinal abnormalities in Alzheimer Disease (AD), observing disrupted ganglion cells, RNFL thinning and optic nerve degeneration as compared to normal subjects. These evidences were confirmed by further studies [70] and provide an anatomical input for the use of *in vivo* retinal imaging as a potential marker of AD [71-78] . These retinal morphological findings in AD are highly correlated with the impairment of the electrophysiological responses originated from the innermost retinal layers (abnormal PERG responses with delayed implicit times and reduced amplitudes) [77, 79].

2.2. Assessment of RGCs Damage: Morpho-functional Tests for Evaluating RGCs Impairment in Human Eye

In current clinical ophthalmology, measurements of RGCs status are generally limited to non-invasive techniques: structural measurements of the RNFL and ONH by OCT, or functional measurements with perimetry (visual field) or electrophysiology (Pattern Electroretinogram) provide estimation of RGCs integrity. These surrogate measurements, while clinically useful, are several levels far from estimating actual RGCs loss.

2.2.1. Perimetry

Testing the visual field has been for many years the gold standard for qualification of visual deficits in many ocular and brain diseases. The standard automated perimetry measures achromatic differential light sensitivity with the purpose of quantifying visual function and RGCs loss. A correlation between perimetric sensitivity and histological RGCs counts has been demonstrated in experimental glaucoma in nonhuman primates [80, 81] and in human glaucoma patients [82, 83]. However, the sensitivity values depend on subjective visual detection of the stimuli, and therefore are influenced by several factors besides RGCs such as pre-retinal media irregularities, integrity of the visual pathway, higher visual processing, reaction time and attention.

2.2.2. Electrophysiology

The function of RGCs can be assessed non-invasively in experimental models of optic neuropathies by means of the electroretinographic techniques that study the bioelectrical activity of inner retina neurons. This knowledge comes from the crucial experiment done by Maffei and Fiorentini [84] in the cat, that showed that the Electroretinogram (ERG) in response to contrast-reversing patterns at constant mean luminance (PERG) was extinguished after optic nerve cut, for a retrograd loss of RGCs, with no alteration of the photoreceptors as the standard Flash-ERG was in fact intact**.** From this evidence, we know that the PERG selectively measures the functional activity of RGCs. In glaucoma models, the PERG amplitude may be reduced before histological loss of RGCs; PERG abnormalities are reversible whether IOP is

lowered or increases in presence of IOP elevation [85-88]. Under particular electrofunctional conditions, the Flash-ERG shows a slow component that reflects the bioelectrical activity of the elements of the proximal retina and is altered in experimental models of RGCs impairment (positive Scotopic Threshold response, pSTR; negative Scotopic Threshold Response, nSTR; Photopic Negative Response, PhNR; Oscillatory Potentials, OPs; multifocal ERG, mfERG) [89].

2.2.3. Morphology

The retinal and ONH morphology can be also explored *in vivo* by optical coherence tomography [5]. OCT provides objective measurements of anatomical structures related to RGCs. The most commonly used parameter is the peripapillary RNFL thickness. The RNFL is made up mostly of RGCs axons and its thickness as measured with OCT has a strong correlation with optic nerve axon count in experimental glaucoma in non-human primates [90, 91]. This objective test has been recently applied also to describe the fate of RGCs in LHON. During the first six months of disease presentation, there exist an acute failure of the papillo-macular bundle temporal fibers and swelling of the adjacent RNFL in the superior/inferior quadrants [92]. After this time, a remarkable loss of fibers occurs in all sectors, the nasal being mostly spared [92]. OCT RNFL thickness is also a useful measurement to distinguish patients that have visual recovery, with a relative sparing of RNFL, from those patients with no recovery of vision [92]. Swelling of the papillo-macular bundle in the temporal/inferior quadrants can also be found in unaffected carriers by OCT [93]. However, the usage of OCT in following-up optic neuropathies that involve RGCs is limited by some other cellular contributions beside RGCs. The RNFL contains a significant and variable contribution of glial and vascular component. Because of these non-axonal contributions, the correspondence between RNFL thickness and the number of axons varies between retinal locations, stage of disease, and among individuals, reducing the accuracy of RNFL thickness as a parameter of RGCS damage in glaucoma. Another parameter commonly used as a surrogate of remaining RGCs in optic nerve diseases is the neuroretinal rim (NRR), which is the continuation of the RNFL at the optic nerve head, containing all RGCs axons before they leave the eye. NRR measurements are influenced by remodeling and biomechanical changes in the connective tissues of the optic nerve head. Additionally, the ganglion cell layer also contains a significant number of displaced amacrine cells, varying from ~ 3% in central retina to 80% in the periphery [94]. For all these limitations, advances in imaging, labelling techniques, and transgenic medicine are making enormous strides in experimental ocular neurodegenerative diseases, providing knowledge on the ocular disease pathophysiology, its progression and testing new therapeutic avenues. Many efforts are on the way for the objective quantification of the number of remaining or lost RGCs.

3. RGCS: A TARGET FOR NEUROPROTECTIVE STRATEGIES

Although the treatment of optic neuropathies is mainly dependent on etiological control, such as anti- inflammation/autoimmune regulation for optic nerve and IOP control for glaucoma, evidences have shown that etiology-targeted therapy is not sufficient to hinder the process of RGCs apoptosis [95]. Since the RGCs are the specialized cells between the receptive retina and the visual pathways and they are involved in several neuro-degenerative with progressive loss of retinal neurons, RGCs are targeted for sparing neurodegeneration and proceeding to neuroprotection for neuronal survival. Neuroprotection is defined as any intervention that can prevent RGCs death. Several natural and synthetic compounds, with neuroprotective properties, by direct rescue of RGCs or neutralization of the deleterious effects of toxic factors, have been studied [96]. Among neuroprotective agents, neurotrophic factors are a group of growth factors that acts in neuronal development and rescue. The high neuroprotective effects of neurotrophic factors, have suggested these molecules as optimal candidates for neurodegenerative disorders [97]: brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell-line derived neurotrophic factor (GDNF) and nerve growth factor (NGF). Neuroprotective compounds enclose also 1) glutamate antagonists, since glutamate-induced excitotoxicity has been implicated as a common pathogenic mechanism in a broad group of neurological diseases, including Alzheimer's disease and glaucoma [98]; 2) gingko-biloba extracts that is an anti-oxidant capable of penetrating into the mitochondria and therefore can be of benefit as neuroprotective agents. Ginkgo biloba contains certain substances, including poly-phenolic flavonoids which may theoretically prevent oxidative stress in the mitochondria and thereby protect RGCs [99]; 3) the neurotoxic effect of N-methyl-D-aspartate (NMDA) is mediated by calcium influx into neural cells, followed by apoptosis and cell death [100], therefore, calcium-channel blockers (CCBs), seem to be a rational alternative for neuroprotection in glaucoma by improving local blood flow in ischemic tissues by inducing vasodilation [101]; 4) against the oxidative stress that causes RGCs death, a number of antioxidants such as Coenzyme Q10 (CoQ10), and natural substances such as polyphenolic flavoids in green tea, coffee, wine and dark chocolate; anthocyanosides in bilberry; vitamins including thiamin (vitamin B1) has been proposed for RGCs rescue [102]; 5) it has also been suggested that brimonidine (an alpha 2 adrenergic agonist) may prevent RGCs death by direct interaction with alpha-2 adrenergic receptors, leading to reduced accumulation of extracellular glutamate and blockade of NMDA receptors [103]; 6) evidence in the literature points to a possible role for nitritic oxide in RGCs degeneration, however preclinical evidence regarding the effect of nitritic oxide synthase (NOS) neurodegeneration is inconclusive [104] and NOS inhibitors have not yet been tested in any clinical study; 7) stem cell transplantation has gained significant interest for the possible application in the treatment of neuro-degenerative diseases. The most important therapeutic potential of stem cells is in the ability to produce novel cell types and to induce RGCs regeneration [105]; nevertheless, RGCs replacement would require that the cells become integrated into the complex circuitry and be capable of synapsing at precise brain locations. Among all these neuroprotective agents for RGCs sparing and visual loss prevention, with very little proof of efficacy, Citicoline, instead, an endogenous compound that can be administered exogenously with a neuroprotective role, has been widely studied in mod-

els of neurodegenerative diseases and has a solid history of experimental and clinical findings showing positive effects on the RGCs morpho-functional impairment [106-112]. We here report the experimental studies *in vivo* and *in vitro* that supported the administration of Citicoline in human pathologies of the visual system and of the brain (see below).

4. CITICOLINE: MECHANISMS OF ACTION AND ITS EFFECTS ON RGCS

4.1. Mechanisms of Action of Citicoline Cytidine Diphoshocholine

(CDP-choline or Citicoline) is a mononucleotide consisting of ribose, cytosine, pyrophosphate, and choline whose chemical structure corresponds to 2-oxy-4-aminopyramidine [113]. It derives from endogenous choline and contributes to the synthesis of cell membrane phospholipids.

The formation of this compound from phosphorylcholine is the rate-limiting step of this biosynthetic pathway [113]. When administered exogenously, Citicoline has high bioavailability in injectable route or in oral solution (more than 90%) and is quickly metabolized to cytidine and choline in the liver and in the gut wall.

Cytidine is then metabolized to uridine. Uridine crosses the blood-brain barrier, and then it is converted to cytidine triphosphate (CTP). The free choline, instead, is phosphorylized into phosphocoline, and then it combines with CTP to form Citicoline. Citicoline elimination occurs in two phases, mainly *via* respiratory CO2 and urinary excretion [114]. Citicoline is not toxic in animal models: the LD50 of a single intravenous dose of Citicoline is 4600 mg/kg and 4150 mg/kg in mice and rats respectively, while the orally lethal dose has not been determined because no animals ever died at the maximum oral dose [115,116]. The Citicoline dosage used for therapeutic purposes in humans is 500-2000mg which corresponds to 7-28 mg/kg in a person of approximately 70 Kg.

Results from many studies have confirmed a high safety profile also in humans. Only few adverse reactions related to gastrointestinal discomfort, uneasiness and irritability have been reported, while there are no data about liver or kidney insufficiency caused by Citicoline [117].

Citicoline may act as a phosphatidylcholine (PtdCho) precursor [118]. The CDP-choline pathway of PtdCho byosynthesis was first described more than 50 years ago. In 1957 Eugene P. Kennedy revealed that in the phospholipid metabolism the synthesis of PtdCho is mediated by a nucleoside which was the CDP-choline. So, the pathway that leads to PtdCho is known as CDP-choline or Kennedy pathway [119].

Moreover, Citicoline contributes to the settle of cardiolipin levels, which is part of the inner mitochondrial membrane and is essential for mitochondrial electron transport, maybe through the inhibition of the enzymatic hydrolysis of cardiolipin by phospholipase A2. Cardiolipin serves also as a component in the synthesis of sphingomyelin which is another part of membrane phospholipid [120, 121]. Phosphoplids are fundamental constituents of cell membranes and are important in homeostasis conservation. Phospholipids also contribute to the cell organization, activities of different enzymes and interaction between receptors and intracellular signals. Additional specific functions of neuronal membranes include nerve impulse conduction and neurotransmission [122]. Furthermore, the stimulation of their synthesis may increase the function of the visual pathway elements like retinal photoreceptors, retinal ganglion cells and/or axons.

Catecholaminergic neurotransmission is affected by Citicoline. In fact, Citicoline is a choline donor in the acetylcholine biosynthesis improving its levels in the hippocampus and neocortex and increases levels of dopamine in the corpus striatum *via* enhancing tyrosine hydroxylase activity and inhibiting dopamine reuptake at nerve terminals. Additionally, Citicoline can increase levels of noradrenaline in the cerebral cortex and hypothalamus and levels of serotonin in the cerebral cortex, striatum, and hypothalamus [113, 123-125].

Dopamine is one of the most important neurotransmitter in mammalian retina, while noradrenaline and adrenaline have been found also in bovine and rat retinas [126, 127]. Hence, visual improvement after treatment with Citicoline may also be related to the enhancement of catecholaminergic neurotransmission. Among other important roles, Citicoline may reduce brain glutamate activity by increasing expression of excitatory amino acid transporter-2 (EEAT2) [128], counteracts the deposition of beta-amyloid [129] and increases the number of oligodendrocyte precursor cell as shown *in vivo* and *in vitro* in a murine model of experimental autoimmune encephalomyelitis and in the cuprizone model of toxic induced demyelination [130].

4.2. Citicoline: Experimental *in vitro* and *in vivo* Studies

4.2.1. Experimental "in vitro" Studies

Oshitari *et al*. [109] explored the neuroprotective effect of Citicoline on damaged RGCs in cultured retina by the quantitative analysis of TdT-dUTP terminal nick-end labeling (TUNEL) staining and the evaluation of the number of regenerating neurites (Table **1**).

Various concentrations of Citicoline (0.01 μmol/l, 0.1 μmol/l, 1 μmol/l, 10 μmol/l) were incubated in culture media. Control retinas did not get any agent. The TUNEL-positive ratio on cultured retina treated with 0.1-10 μmol/l Citicoline was very low, and the number of regenerating neurites was higher than in control retinas. Authors suggested that Citicoline has an anti-apoptotic effect in cell death mechanism related to mitochondria and can assert neurite regeneration.

Few years later, after exposing cultured rat retinas (Sprague–Dawley rats) to high glucose, which is toxic to RGCs, the same author explored the consequence of BDNF, neurotrophin-4 (NT-4) and Citicoline administration on apoptosis and neurite regeneration [110]. The effect of these neurotrophic factors and of Citicoline was evaluated by the numbers of TUNEL positive and caspase-3 and -9-immunopositive cells in the RGCs layer.

Retinas incubated in high glucose and integrated with BDNF, NT-4, or Citicoline had significantly lower numbers of TUNEL-positive and caspase-3 and -9-immunopositive cells in the RGCs layer. Furthermore, the numbers of regenerating neurites were significantly higher.

Also Matteucci *et al*. [111] investigated the neuroprotective role of Citicoline in retinal cells against high glucose induced apoptosis and synaptotoxicity. Primary retinal cell cultures were treated with high glucose for 96 hours, showing an increase in apoptosis which was reduced when 100 μM Citicoline were given. Furthermore, thanks to Citicoline, synaptophysin levels were comparable to those of control cultures. The same authors also explored another *in vitro* condition relevant to neuroretinal degeneration and believed to be significant in glaucoma pathophysiology: glutamate-induced excitotoxicity.

Primary retinal cultures were supplemented with 100 μM Citicoline and 24 h later were exposed to excitotoxic damage by 100 μM glutamate for 25 min. Control retinal cultures were treated only with iso-osmolar mannitol.

TUNEL-positive nuclei were counted and apoptosis was expressed as percentage of apoptotic cells over total cells. Results showed that Citicoline significantly reduced the apoptotic rate in glutamate-treated cells compared to controls ($p<0.05$) [111].

4.2.2. Experimental "in vivo" Studies

Some experimental "*in vivo*" studies have confirmed the neuromodulator effect of Citicoline and its protective role on neuronal cells like retinal ganglion cells (Table **2**). In 2002, Rejadak *et al*. [106] examined the *in vivo* effects of Citicoline on catecholamine levels in the rabbit retina. Adult male Albino rabbits at the age of 3 months (2- 3 Kg) were divided in two groups. Six rabbits got intraperitoneal injections of 50 mg/Kg Citicoline twice daily for 7 days, while 4 rabbits got vehicle injections twice daily for 7 days. Then all animals were sacrificed and isolated retinas were obtained and homogenized in 0.5 ml of saline. Catecholamines were then evaluated by HPLC and their levels was normalized to tissue weight. Animals treated with Citicoline revealed a statistically significant increase in dopamine concentration (t test p=0.027; Mann-Whitney U test p=0.017) and an increase in adrenaline concentration (t test p=0.06; Mann-Whitney U test p=0.051) which was not statistically significant, while the noradrenaline concentration did not change (t test p= 0.08; Mann- Whitney U test p= 0.19) [106].

To quantify protection offered by both Citicoline and lithium to RGCs survival, Schuettauf *et al*. [107] used a model of optic nerve crush (ONC). Authors also determined whether their effects are mediated by increased expression of the antiapoptotic protein Bcl-2. Two groups of adult rats were subjected to ONC and contralateral sham operations. Animals were previously treated with intraperitoneal injections of vehicle, Citicoline sodium (1g /kg daily for 7 days and then 300 mg/kg daily), lithium chloride (30 mg/kg daily) or both drugs together. Fluorogold was injected bilaterally into superior colliculi 1, 5 or 19 days after ONC and labeled cells were counted under fluorescence microscope while Bcl-2 expression was evaluated by immunohistochemistry [107]. Results showed that there was a reduction of RGCs density after crush in those rats treated with vehicle, while this reduction was lower in rats treated with both Citicoline and lithium.

Furthermore, Bcl-2 immunoreactivity was higher in the lithium and Citicoline group.

The neuroprotective effect of Citicoline was also investigated in a model of retinal damage induced by kainic acid (KA) by measuring the thickness of the various retinal layers [108]. KA was injected into the vitreous of rat eyes and Citicoline (500 mgkg-1) was injected intraperitoneally twice daily for 1, 3 and 7 days.

Additionally, the expression of choline acetyltransferase (ChAT) and tyrosine hydroxylase (TH) was explored by immunohistochemistry. In the inner nuclear layer and inner plexiform layer, it was observed an important cell loss after Ka- injection and ChAT and TH immunoreactivities had almost vanished. When Citicoline was prolonged for 7 days the reduction of retinal thickness was weakened.

KA is an analogue of glutamate, so authors concluded that Citicoline might exert its neuroprotective action in glutamate- mediated cell death [108]. In 2015, Zerbini *et al*. [112] explored the protective effect of Citicoline eye drops (2%) against the toxic effect of prolonged hyperglycemia in a mouse model of type 1 diabetes. After eight months of diabetes, *in vivo* analysis of the retina was performed using the Spectralis HRA (Heidelberg Retinal Angiography) + OCT. A reduction of retinal layers' thickness, like nerve fiber

Table 1. Summary of experimental *"in vitro"* studies evaluating the effect of citicoline on RGCs.

Authors	Years	Cultures	Citicoline concentration	Outcomes
Oshitari T *et al*. [109]	2002	Cultured mouse retina	0.01 μmol/l, 0.1 μmol/l, 1 μmol/l, 10 μmol/l	TUNEL analysis and assessment of the number of regenerating neurites on damaged RGCs.
Oshitari T *et al*. [110]	2010	Cultured rat retinas	NA	The number of apoptotic cells and the number of regenerating neurites after high glucose exposition.
Matteucci A *et al*. [111]	2014	Cultured rat retinas	100 μM	TUNEL-positive nuclei and synaptophysin immunostaining in glutamate-induced and high-glucose induced RGCs damage.

Table 2. Summary of experimental *"in vivo"* studies evaluating the effect of Citicoline on RGCs.

Authors	Years	Animals	Citicoline Concentration	Outcomes
Rejadak R *et al*. [106]	2002	Albino rabbit	50 mg/Kg	Retinal catecholamine concentration.
Schuettauf F *et al*. [107]	2006	Adult rat	1g/kg	RGCs density and Bcl-2 immunoreactivity after ONC.
Park CH *et al*. [108]	2005	Adult rat	500 mgkg-1	Thickness of retinal layers and immunoreactivities of ChAT and TH after KA induced retinal damage.
Zerbini G *et al*. [112]	2015	Mouse	2% (39,19mM)	Retinal layers and choroidal thickness

layer, ganglion cells/inner plexiform layer, ganglion cells complex and a reduction of choroidal thickness were found in diabetic mice compared to sham-treated control mice. Instead, Citicoline- treated control mice didn't show a significant reduction of the same retinal layers and the inverse correlation between mean blood glucose levels measured during the study and RNFL thickness demonstrated in sham-treated diabetic mice was lost after treatment with Citicoline.

5. CITICOLINE: CLINICAL STUDIES IN HUMAN PATHOLOGIES INVOLVING RGCS

There is an extensive literature about the positive effect on memory and behavior obtained by pharmacological treatment with Citicoline in several pathologies involving the Central Nervous System (see Fioravanti and Yanagi for a review [131]).

In different pathologies involving the optic nerve, such as glaucoma or NAION, there is a similar involvement of the RGCs that occurs in AD (see all that previously reported). This condition suggested us since 1996, the possibility of reach a reduction of visual dysfunction that may occurs in glaucoma or in NAION by using a treatment with Citicoline administrated in different ways: intramuscular, oral or eye drops.

The clinical studies in human pathologies involving RGCs are summarized in Table **3**.

5.1. RGCs Function after Intramuscular Administration of Citicoline in Glaucoma

In 1999, we published our first work about the treatment with Citicoline in patients with open angle glaucoma (OAG) [132].

In this randomized clinical trial we evaluated forty glaucomatous patients with medium visual field defects [Humphrey 24-2 perimetry (HFA) with mean deviation (MD) between - 2 and - 6 dB]. The enrolled patients were randomly divided into two groups for similar characteristics of visual field deficits: Citicoline group (GC, n= 25 eyes) and placebo group (GP, n= 15 eyes). The study was designed as follow. Both GC and GP patients, during the time of the entire study were treated with ocular hypotensive therapy and IOP was still lower than 18 mmHg. In an initial period of 60 days, in GC patients was performed a treatment with Citicoline, at the dosage of 1000 mg/ day intramuscularly (Neuroton, Nuovo Consorzio Sanitario, Rome, Italy); GP patients received placebo (physiologic solution with additives). This first period of treatment was followed by 120 days of wash-out (day 180). At the end of washout, the GC patients were separated into two groups: in one Group (10 patients, GC1 group) the washout was prolonged for further 120 days; in a second group (15 patients, GC2 group) was administrated a second 60-day period of Citicoline treatment followed by a second 120-day period of washout. In GP patients, at day 180, the washout was extended for another 180 days. In all patients enrolled in study, the function of the RGCs was evaluated by recording PERG responses. We considered as "Main Outcome Measures" the PERG P50 implicit time and PERG P50-N95 amplitude. During the all period of follow-up (360 days), GP patients displayed PERG values similar (analysis of variance: ANOVA $p>0.05$) to those observed in baseline condition.

In GC patients, after treatment with Citicoline a significant improvement ($p<0.01$) of the values of PERG parameters, with respect to those of GP patients was found. After the first washout, the improvement in PERG values was still maintained. After a second period of washout, GC1 patients showed values of PERG parameters non-significant different ($p<0.05$) with respect to those detected at baseline and with respect to GP patients ones. In GC2 patients, the second period of Citicoline treatment induced a further significant increase in PERG P50-N95 amplitude and significant shortening of the PERG P50 implicit time was detected. In this study, the administration of Citicoline induced an increase of the RGCs function with consequent enhancement of the neural conduction along the visual pathways as suggested by the shortening in the VEP P100 implicit times and by the increase in VEP N75-P100 amplitudes.

Of these 40 patients enrolled in this first study, 24 glaucomatous patients were enrolled in a second study in which the period of follow-up was extended to 8 years [133]. Twelve patients were treated, in addition to the ocular hypotensive therapy, with Citicoline, at the dosage of 1000 mg/day intramuscularly (Neuroton, Nuovo Consorzio Sanitario, Rome, Italy) every 2 months followed by 4 months of wash-out (16 cycles of treatment); and in 12 patients the ocular hypotensive therapy was performed exclusively.

The additional periods (16 cycles of 2-months period each during a total period of 8 years) of Citicoline treatment induced a greater (ANOVA $p<0.01$) improvement of the values of PERG P50 implicit time and PERG P50- N95 amplitude with respect to pre-treatment conditions and when compared to glaucomatous patients treated exclusively. The PERG improvement induced an enhancement of VEP re-

Table 3. Summary of clinical studies evaluating the effects of Citicoline in human pathologies involving RGCs.

Authors	Year	Study Population	Adm	Dosage	Schedule of Treatment	Follow-up	Results
Parisi V. *et al* [132]	1999	OAG with MD -3/-6 dB	IM	1000mg/day	2 cycles of 60 days of treatment each followed by 120 days of was-out	360 days	Increase in PERG P50/N95 Amplitude and shortening in PERG P50 Implicit Time and relative VEP improvement
Parisi V. *et al* [133]	2005	OAG with MD -3/-6 dB	IM	1000mg/day	14 cycles of 60 days of treatment each followed by 120 days of was-out	8 years	Increase in PERG P50/N95 Amplitude and shortening in PERG P50 Implicit Time and relative VEP and Visual Field improvement
Parisi V. *et al* [135]	2008	OAG with MD -2/-14 dB	IM Oral	1000mg/day 1600mg/day	2 cycles of 60 days of treatment each followed by 120 days of was-out	360 days	Increase in PERG P50/N95 Amplitude and shortening in PERG P50 Implicit Time and relative VEP improvement. Non-significant differences between IM and Oral treatment.
Parisi V. *et al* [45]	2008	NAION	Oral	1600mg/day	2 cycles of 60 days of treatment each followed by 120 days of was-out	360 days	Increase in PERG P50/N95 Amplitude and shortening in PERG P50 Implicit Time and relative VEP, Visual Field and Visual Acuity improvement.
Parisi V. *et al* [141]	2015	OAG with MD -2/-14 dB	Eye drops	3 drops/day	120 days of treatment followed by 60 days of was-out	180 days	Increase in PERG P50/N95 Amplitude and shortening in PERG P50 Implicit Time and relative VEP and Visual Field Improvement.

Abbreviations: Adm = administration; OAG = Open Angle Glaucoma; NAION = Non-Arteritic Ischemic Optic Neuropathy; IM= intramuscular; PERG = Pattern Electroretinogram; VEP = Visual Evoked Potentials.

sponses suggesting that the increase in RGCs function lead to an enhancement of the neural conduction along the whole visual pathways.

The reduction of the glaucomatous visual pathways dysfunction due to the treatment with Citicoline induced also a reduction of the visual field defects. In fact, at the end of follow-up (96 months), the MD values of HFA 24–2 perimetry were reduced with respect to those observed at baseline. The changes in MD were significantly related (Pearson's Test) to the increase PERG P50- N95 amplitudes ($r = 0.81944$, $p < 0.001$) and to the shortening in PERG P50 implicit times ($r = -0.7526$, $p < 0.001$). All that reported about the effects induced by intramuscular treatment with Citicoline on the RGCs function may provide an explanation about the evidence of Virno *et al*. [134], who observed during a period of follow-up of 10 years that glaucomatous patients treated with Citicoline showed a stable improvement of the "not perceived area" at the perimetric examination.

5.2. RGCs Function after Oral Administration of Citicoline in Glaucoma and in Non-arteritic Ischemic Optic Neuropathy

Since 2005 Citicoline is available for oral administration, and in 2008 we published a work in which were presented the results about the comparison between the oral and the intramuscular treatment with Citicoline in patients with glaucoma [135]. In this study, 60 OAG eyes with moderate visual field defects (HFA with MD between -2 and -14 dB) were studied. All OAG patients, treated with ocular hypotensive therapy whit IOP lower than 18 mmHg, were randomly divided on the basis of age and visual field defects into three group:

1) Group NT-OAG: 20 OAG patients in which no additional pharmacological treatment was performed;
2) Group TI-OAG: 20 OAG patients treated with a daily intramuscular dose of 1000 mg Citicoline (Cebroton 1000s, Tubilux Pharma, Pomezia, Rome, Italy);
3) Group TO-OAG 20 OAG (patients treated with a daily oral dose of 1600 mg Citicoline (Cebrolux, Tubilux Pharma). The treatment with Citicoline (in oral or intramuscular administration) was prescribed according to the following protocol: 0–60 days: first period of pharmacological treatment with Citicoline;61–180 days: first period of washout and follow-up at the sixth month; 181–240 days: second period of pharmacological treatment with Citicoline; 241–360 days: second period of washout and follow-up at the twelfth month.

After the two periods of treatment (oral or intramuscular administration) of Citicoline, a significant (ANOVA: $p<0.01$, vs baseline) improvement of RGCs function (objectively evaluated by the increase of the PERG P50-N95 amplitude) was detected. The increase in retinal function induced an enhancement of the neural conduction along visual pathways (objectively evaluated by the shortening in the VEP P100 implicit time). After the two periods of washout a partial regression of this improvement was found, but the values of PERG parameters were still improved when com-

pared to baseline ones. It is worth noting that non-significant (ANOVA: $p< 0.05$ TI-OAG vs TO-OAG) differences between the PERG values detected in OAG patients treated with oral or intramuscular administration of Citicoline were found. More later, Citicoline was available in oral solution and this is an important step, since it is well known that when the Citicoline is administrated in oral solution, the bioavailability is of about 98% [136, 137]. By using Citicoline in oral solution (Neukron Ofta, Omikron Italia, Rome, Italy), in OAG patients in which was documented a history of the progression of the disease despite controlled IOP (at least a loss of 1 dB/year of the MD in the three years before the enrollment in the study), it was observed that the mean rate of MD progression was significantly reduced to −0.15 (±0.3) dB/year after 2 years of treatment with Citicoline (4 cycles of 4-months period each followed by 2-months period of washout) [138]. Also in this case, the changes in visual field have been ascribed to the effects of Citicoline to an enhancement of the RGCs function and of the neural conduction along the visual pathways.

It is also worth noting that the treatment with Citicoline in oral solution was also able to reduce the loss of retinal fibers in OAG patients with a documented history of progressive loss of retinal fibers [139].

A treatment with oral Citicoline was also suggested in patients with non-arteritic ischemic optic neuropathy. In this study, twenty-six patients with NAION were enrolled. They were randomly divided into two age-similar groups: 14 patients (T-NAION group) had oral treatment with Citicoline (Cebrolux-Tubilux, Italy, 1600 mg/ day) for 60 days and 12 patients (NT-NAION group) had no treatment during the same period. This first period of treatment was followed by a period of wash out (days 60–180) At day 180, T- NAION patients has been submitted to a second period of Citicoline treatment (days 181–240) followed by a second period of wash-out (days 241–360).

At the end of the two periods of Citicoline treatment (days 60 and 240), in T- NAION patients a significant enhancement (ANOVA: $p < 0.01$ vs baseline and vs NT-NAION) of the RGCs function as suggested by greater PERG P50-N95 amplitude and by the reduction in PERG P50 implicit time.

This improvement was observed together with an enhancement of the whole visual pathways function as suggested by the amelioration of the values VEPs parameters and the increase of the visual acuity. After washout, the functional improvement of RGCs (PERG changes) persisted when compared to the baseline PERG values [45].

5.3. RGCs Function after Eye Drops Solution Administration of Citicoline in Glaucoma

Citicoline is now available in eye drop solution. About the effects of Citicoline in eye drop solution, our first study aimed to assess the presence in the vitreous when the Citicoline is administered in eye drops solution [140]. Therefore, in five eyes of CD1 mice Citicoline 1% and 2% was administrated at the dosage of two drops per day for three days. After this treatment, the liquid chromatography and spectrometry mass (LC-MS/MS) was used to analyze the vitreous and the Citicoline was discovered. Thus, Citicoline reaches the vitreous and this constitute a rationale to believe that this molecule may induce an effect on the glaucomatous damaged ganglion cells and their fibers.

Therefore, we performed a second study in which it was evaluated in OAG patients the effects of the treatment with Citicoline administrated in eye drops on the RGCs function and on the neural conduction along the visual pathways [141]. Forty-seven OAG patients, with moderate visual field defects (HFA with mean MD between -2 and -10 dB) and with IOP less than 18 mmHg with ocular hypotensive therapy completed the study. Of these, in 24 OAG eyes Citicoline was administrated by eye drops (Citicoline sodium salt: 0. 2g, Hyaluronic acid: 0. 02g, Benzalkonium chloride 0.001 g, Water for injection up to 10 ml, OMK1®, Omikron Italia, Italy, 3 drops /day) (GC eyes) during a period of 4-months that was followed by 2-months of Citicoline washout; other 23 OAG eyes were only treated exclusively with ocular hypotensive therapy (GP eyes).

The results of this study confirmed all that previously observed by intramuscular or oral Citicoline treatment. In fact, after treatment with Citicoline eye drops a significant (ANOVA $p<0.01$ vs baseline) enhancement of the RGCs function as suggested by the increase in PERG P50-N95 amplitude and the shortening in PERG P50 implicit time was found. This functional enhancement leads to a better neural conduction along the visual pathways and this is documented by the significant correlation (Pearson's Test: $r= 0.76396$, $p<0.001$) between the improvement in PERG P50-N95 amplitude and the shortening in VEP P100 implicit times. In addition, a significant correlation (Pearson's Test: $r= 0.6345$, $p<0. 001$) was observed between the values of the PERG P50-N95 amplitude at baseline and the relative differences after treatment and this suggests that a better functional enhancement of the RGCs may be reached in those eyes that presented a greater dysfunction at baseline condition. After washout, the values of PERG parameters were similar to baseline ones. In patients treated exclusively with beta-blocker non –significant (ANOVA $p>0.05$ vs baseline) changes of the PERG parameters values during the period of follow-up were found. It was very interesting to detect that after Citicoline eye drops treatment, an improvement of the visual field (increase in MD greater than 1 dB) was observed on 17/24 (71%) OAG eyes. Consequently, the individual changes in MD induced a positive mean progressive rate (0.56 dB). In all GP eyes (23 out of 23) no changes lower than 1.0 dB of the MD values were found. The visual field changes can be ascribed to a direct effect of Citicoline administrated in eye drop solution on the RGCs with consequent functional enhancement. This is supported by the significant correlation (Pearson's Test: $r=- 0.7437$, $p<0.001$) between the increase in PERG P50-N95 amplitude and the changes in MD detected in OAG eyes treated for four months with eye drops containing Citicoline. An important point is that none of the patients enrolled in the above-mentioned studies, in which Citicoline was administrated intramuscularly, orally or by eye drops reported any general or ocular side effects.

CONCLUSION

Morphological and functional impairment of RGCs occurs in several human neurodegenerative pathologies. In particular, patients with OAG or NAION present a great visual dysfunction primarily related to an involvement of RGCs. In OAG and in NAION patients, the treatment with Citicoline induces a functional improvement of RGCs with consequent better neural conduction along the visual pathways resulting in positive changes in the psychophysical responses (stabilization or improvement of the visual field defects). In OAG patients, the functional enhancement of RGCs was similarly detected when Citicoline was administrated intramuscularly [132-135], orally [45, 135], or by eye drops [141].

Citicoline is an endogenous substance that acts as an intermediary in the synthesis of phosphatidylcholine (a major phospholipid in the neuronal membrane) through the activation of the biosynthesis of structural phospholipids in neuronal membranes, increases the metabolism of cerebral structures, inhibits phospholipid degradation and induces an increase in the levels of different neurotransmitters and neuromodulators, including noradrenaline in the Central Nervous System. In addition, a mechanism of dopamine-like action has also been supposed (see Fioravanti and Yanagi for a review [131]).

Regarding the direct effect of Citicoline on the RGCs, literature reports several interesting works where in experimental models of cultured retina enriched by Citicoline it was possible to reduce the RGCs degeneration [109- 111] and in animal model of diabetes, in which the administration of eye drops containing Citicoline may rescue the loss of RGCs due to the iperglycemic effects [112].

Thus, the observed effect of Citicoline on the RGCs function in OAG patients can be ascribed to different/concomitant factors: a direct effect on the RGCs structure [106-112] and/ or to a neuromodulator effect on the retinal neurotransmitters (see Secades for a review) [113]. Actually, the possibility of influencing the progression of visual dysfunction in glaucomatous patients (as in other pathologies such as NAION) has constituted a constant effort for years. Toward this end, an improvement of RGCs morphology and function was hypothesized by the administration of several molecules (see paragraph "RGCs a target for neuroprotective strategies"), however, among these ones, Citicoline presents a solid history of experimental and clinical studies in which the beneficially effects of this molecule on the RGCs morphology and function are extensively reported. All this let us to believe that Citicoline is, at this moment, a very promising molecule for neuroprotective strategies.

AUTHOR'S CONTRIBUTION

Vincenzo Parisi: design research, performed research, collected data, wrote paper.

Francesco Oddone: supervised the manuscript and obtained permission from all coauthors for the submission of any version of the manuscript and for any changes in the authorship.

Lucia Ziccardi: wrote the introduction and collected data

Gloria Roberti: wrote the second part of the review and collected data

Gianluca Coppola: collected data

Gianluca Manni: revised and approved the manuscript

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

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