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Original Research Article (Experimental)

Allium cepa exerts neuroprotective effect on retinal ganglion cells of pterygopalatine artery (PPA) ligated mice

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ABSTRACT

Background: Repeated failure to rescue the damaged retinal ganglion cells (RGCs) by various drugs has warranted the need to screen common herbal compounds available in the form of various eye formulations for their efficacy.
Objective: We aimed to investigate the neuroprotective effect of pretreatment with aqueous extract of *A. cepa* in Ischemia/Reperfusion (I/R) induced retinal injury.
Methods: Ischemia was induced for 2 h by pterygopalatine artery (PPA) ligation in C57BL/6J mice, followed by reperfusion. The neuroprotective role of oral pretreatment with aqueous extract of *A. cepa* (300 mg/kg) was analyzed with respect to control and injury only group at 7, 14, and 28 day after the surgery for expression of different genes in the retina by Real-Time PCR.
Results: Molecular analysis at different time points showed increased expression of BCl-2, GDNF, GFAP, and Brn3b in the retina at 14 and 28 day after *A. cepa* treatment in comparison to the injury alone group. However, at shorter time point (7th day), the expression of these genes was pronounced in the injury only group in comparison to the injury and pretreated group.
Conclusion: Pretreatment with aqueous extract of *A. cepa* may protect from the neuronal damage in I/R-induced retinal injury in mice by altering the expression of neurotrophic factor.

1. Introduction

Retinal ischemia, one of the leading causes of sight impairment or blindness, is associated with grave retinal ailments like ischemic optic neuropathy (ION), rubeotic glaucoma, and diabetic retinopathy [1–4]. In general, ischemia occurs because of the blockage in the arteries, which could lead to serious complications like stroke or heart attack. Stenosis or occlusion of carotid artery can lead to ocular ischemic syndrome (OIS) [5,6]. One of the most common type of OIS is Amaurosis fugax, which is retinal ischemia resulting into transient blindness [7]. In case of ION, there is inadequate blood supply to the optic nerve, causing loss of retinal ganglion cells (RGCs) [8].

Previous studies suggest that retinal ischemia/reperfusion (I/R) models imitate various medical conditions like retinal vascular occlusion and acute glaucoma [9]. Glaucoma is one of the devastating neurodegenerative disorder [10] affecting millions of people worldwide. According to Liang et al. the total number of people affected in 2013 was 64.3 million and is expected to be 76.0 million by 2020 [11]. The prevalence of glaucoma in India is about 11.9 million [12] and by 2020, India will be the second most affected nation with glaucoma. Thus, it is imperative for researchers to revisit the prognostic and therapeutic strategies to rescue the damaged retinal ganglion cells affected by the disease. The approved drugs like Timolol and Letanoprost have been shown to cause toxic effects and have failed to repair the damaged RGCs [13,14]. Timolol has been used for glaucoma treatment for more than 30 years, however, even its topical administration may have adverse effects [15]. In view of this, the conventional treatment approach is inadequate [16], and awaits alternative interventions.

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Various herbal extracts have been used as therapeutics since several years. It may not be out of context to state that 70% of the Indian population and almost 90% of the African population is dependent on traditional medicine, whether or not preclinically tested. 90% of Chinese general hospitals reportedly have units for traditional medicine, where *Ginkgo biloba, Allium sativum,* and *Panax ginseng* are the most common and top-prescribed herbal products, yet the evidence-based approach is still lacking. *Allium cepa (A. cepa)* is one of the commonly used botanical product in our routine diet, because of its broad mechanism of action (as antioxidant, antidiabetic, anticancer, antiatherogenic), and has even been tested for its therapeutic efficacy on damage induced by nicotine [17]. It is also a rich source of organosulphur elements like thiosulphimates and cepaenes, and flavanols like quercetin, kaempferol, and myricetin [18]. The antioxidant role of quercetin and its derivatives present in *A. cepa* are well known [19]. It has been shown that quercetin also helps in prevention of other eye disorders like age-related macular degeneration (AMD) and cataract [20]. While the methanolic extracts of *A. cepa* are believed to exert neuroprotective effects on brain I/R injury [21].

Over the time different animal models have been used to validate therapies and to develop understanding of the disease pathogenesis. The ischemic models include both chemical induced (cobalt chloride), as well as surgical models (middle cerebral artery occlusion (MCAO), common carotid artery (CCA) occlusion, or pterygopalatine artery (PPA) ligation) [22]. PPA ligation model has been used to screen drugs for retinal ischemia. The novel model was first used to induce retinal ischemia by occluding External carotid artery (ECA) and pterygopalatine artery by a group of researchers from Japan and France [3,23]. Studies suggest that ligation of both PPA and ECA induces histological damage and deficits in the retina, without affecting the mice brain [3]. Our previous reports have shown that PPA ligation can be used to study transient ischemia in mice [1,22]. Hideaki Hara and Takashi Ishibashi, patented astaxanthin, showing retinal protective action in the above said PPA ligation model.

Therefore, in this study, we analyzed the mRNA expression of Glial Fibrillary Acidic Protein (GFAP), B-cell lymphoma 2 (BCl-2), Glial Cell Derived Neurotrophic Factor (GDNF), and Brn3b in *A. cepa* pretreated mice subjected to PPA ligation-induced I/R injury and indicate a neuroprotective role for *A. cepa* in preservation of RGCs.

2. Methodology

2.1. Preparation of A. cepa extract

A. cepa aqueous extract was prepared at Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, India. Initially, the red onion bulbs and outer scales (500 g) were chopped in boiling water (1100 ml) followed by sonication for 30 min, placed in shaking incubator at 100 rpm (37 °C) for 24 h, filtered, and concentrated under reduced pressure. The yield was calculated with respect to dry weight (9.78% w/w). The dried extract was tested for toxicity according to the OECD guidelines.

2.2. Animals

All the experiments were performed on C57BL/6J male mice (8–10 weeks, 25 g–30 g) as per Institutional Animal Ethical Committee (IAEC) approval **(67/IAEC/390R)**. The animals were given free access to feed and water while exercising control over humidity and temperature in the animal house. The animals were housed under 12 h light/dark cycle. The animals were divided into three groups: Normal Control, Injury only, and Injury and 300 mg/kg *A. cepa* pretreatment. Details of groups are provided in Fig. 1. The mice were allowed to reperfuse for 7-days, 14-days, and 28-days, after which the animals were sacrificed and end point analysis were done.

2.3. Administration of A. cepa

Fresh aqueous extract of *A. cepa* (300 mg/kg) was administered by oral gavage 24 h prior to the surgery.

2.4. Surgery

The animals were anaesthetized intraperitoneally with Xylazine (50 mg/ml)/Ketamine (50 mg/ml). The I/R retinal injury was induced as described earlier [1,2,22]. Briefly, mice were fixed in a supine position and approximately 1.5 cm midline incision was made under a stereozoom microscope (Leica S6D microscope + EC3 Camera). The muscles were retracted and the right CCA was exposed. The bifurcation was traced and the ECA was ligated with a suture (Ethicon; 7.0). Upon ligation of ECA, bifurcation of internal carotid artery (ICA) was traced and PPA was ligated with the suture (Ethicon; 7.0). Ischemic condition was maintained for 2 h. After 2 h, the ligated sutures were removed and the blood circulation was allowed to reperfuse. After 7, 14, and 28 days of reperfusion, the animals were sacrificed, and the ipsilateral eyes were enucleated and stored for retina isolation.

2.5. RT-PCR

The expression of different cell markers (GDNF, BCl-2, Brn3b, and GFAP) was analyzed through real-time PCR (Applied Biosystems). RNA was isolated from retina using Qiagen RNeasy Mini kit and quantified using a UV spectrophotometer (Beckman–Coulter DU730 UV/Vis Spectrophotometer). Using cDNA synthesis kit (Verso Thermo Fisher Scientific), total RNA was converted into cDNA (as per the manufacturer's protocol). The cDNA was stored at −20 °C for further analysis and 20 ng was later used as a template against specific primers (Sigma and Eurofins, Genomics) (Table 1) for amplification using the Sybr Green master mix (Applied Biosystems) as per the following protocol: initial denaturation at 95 °C for 10 min; 35 cycles of denaturation (95 °C for 1 min), annealing (63 °C for 1 min) and extension (72 °C for 1 min); and final extension at 72 °C for 1 min (35 cycles). The beta actin was used as the house-keeping gene.

2.6. Statistical analysis

Normality of the data was analyzed by Kolmogorov–Smirnov test (K–S test). One sample t-test was applied for comparison of means (M±SD) with normative values. For comparison of two different groups, independent t-test was applied. All the statistical tests were two-sided and performed at a significance level of $p < .05$. The analysis was conducted using IBM SPSS Statistics Software (Version 22.0).

3. Results

3.1. A. cepa increases Brn3b expression

Brn3b mRNA expression analyzed by real time PCR was found to be downregulated with time after the I/R injury (Group II) ($p = .076$, .015, .087 for 7, 14, and 28 day, respectively). However, *A. cepa* pretreatment group (Group III) showed an increased expression with time ($p < .001$, $p = .074$, .003 for 7, 14, and 28 day, respectively) (Fig. 2).

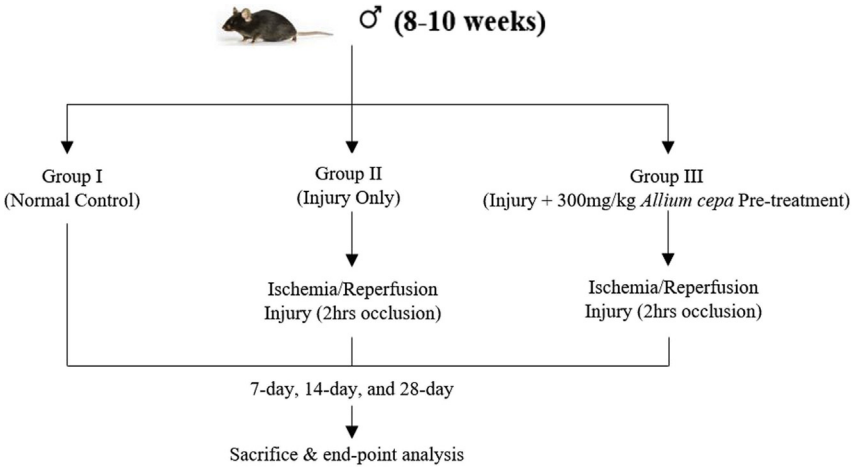

Fig. 1. Schematic representation of the experimental plan.

Table 1
Primer sequence for different marker genes used in real-time PCR.

Target Gene	Primer Sequence (5′→3′)
Brn3b	F: GCAGTCTCCACTTGGTGCTTACTC
(Sigma)	R: TTCCCCCTACAAACAAACCTCC
BCl-2	F: GCCCTTCGGAGTTTAATCAG
(Eurofins Genomics)	R: TACACTTGCACACACACGCT
GFAP	F: ACAGACTTTCTCCAACCTCCAG
(Sigma)	R: CCTTCTGACACGGATTTGGT
GDNF	F: TGGGCTATGAAACCAAGGAG
(Sigma)	R: CAACATGCCTGGCCTACTTT
B-actin	F: AGCCATGTACGTAGCCATCC
(Sigma)	R: CTCTCAGCTGTGGTGGTGAA

3.2. *A. cepa induces BCl-2 expression*

Molecular analysis (RT-PCR) of *A. cepa* pretreated mice retina revealed alteration in mRNA expression of BCl-2 at all the time points analyzed, with an increasing trend in expression with days after reperfusion.

We found statistically significant expression in both injury only ($p < .001$, $p = .010$, and .039) and *A. cepa* pretreated group ($p < .001$, $p = .030$, .002) at 7, 14, and 28 day as compared to the normal control (Fig. 3).

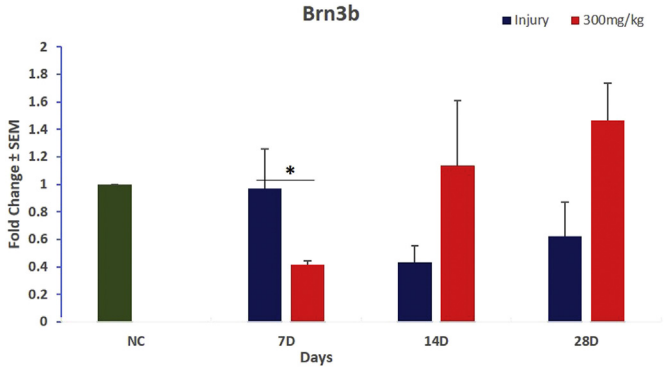

Fig. 2. Fold change in the gene expression of Brn3b for injury and 300 mg/kg A. *cepa* group estimated through Real Time PCR analysis for 7-day, 14-day, and 28-day after PPA surgery in ipsilateral eye. The values were normalized against the endogenous control (β-Actin) (n = 3). The data was found to be significant at $P \leq .05$ (* = **.022**).

3.3. *Upregulated GFAP expression in both injury and A. cepa pretreated group*

In comparison to the normal control, GFAP was found to be upregulated when analyzed by real time PCR at 14 and 28 day ($p = .002$, .013 and .004, <.001 for injury only and injury + pretreatment group, respectively) (see Fig. 4).

3.4. *Increased expression of GDNF in A. cepa pretreatment*

mRNA expression of GDNF was analyzed in the *A. Cepa* treated mice and was found to be upregulated at various time points. The expression was highly significant in both injury only ($p = .004$, .026 at 7 and 14 day, respectively) and injury + pretreatment group ($p < .001$, $p = .021$, .003 at 7, 14, and 28 day, respectively) with respect to the normal control group (Table 2) (see Fig. 5).

4. Discussion

Retinal ischemia, a clinical condition associated with many vision impairing diseases, can lead to RGC damage, and hence, necessitates the need to investigate treatment approaches. PPA ligation-induced retinal ischemia model has been used to test different therapeutic strategies. The model is characterized by increased GFAP expression, thinning of retinal vasculature, and decreased retinal thickness [1,2]. We have previously reported a decrease in the inner plexiform layer (IPL) thickness as well as an increase in GFAP levels after the I/R surgery in mice [2]. In the current study, we analyzed the differential mRNA expression of genes upon oral administration of *A. cepa* using Real-Time PCR. The blood retinal barrier (BRB) in the eye acts as a physical deterrent for drug delivery as well as harmful substances. Retinal capillaries contain tight junctions, which makes their permeability poor for even smaller substances like sodium [24]. However, fenestrated capillaries present in the choroid are highly permeable to molecules with low molecular weight. *A. cepa* contains quercetin as an active component. Different *in vitro* and *in vivo* studies suggest that quercetin is able to cross the BRB or Blood brain barrier [25], thereby, reducing the cell apoptosis [26,27]. In our study, comparison between injury and pretreated group revealed that most of the genes like BCl-2, GDNF, and Brn3b show an upregulation at 7 day in the injury group, and *A. cepa* treatment failed to exert any significant effect at 7 day. It may be hypothesized that at 7-day time point, the elevated expression could be due to an immediate protective

Table 2
Real-Time PCR data mean, S.D + S.E, P-Value for BCl-2, Brn3b, GFAP, and GDNF genes at different time points (7-day, 14-day, and 28-day). The values were normalized against absolute controls. The P-values are indicated with respect to the normal control. The data was found to be statistically significant at P ≤ .05

Genes	Time Points	Injury Only (Group II)			Injury + 300 mg/kg pretreatment (Group III)		
		Mean	SD+S.E	P-Value	Mean	SD+S.E.	P-value
BCl-2	7D	.418115	.0911550 + .0372139	<.001	.198191	.0463812 + .0189350	<.001
	14D	.291388	.1748523 + .0713831	.010	.333727	.2721050 + .1110864	.030
	28D	.478668	.2726384 + .136319	.039	.671248	.2729234 + .1114205	.002
Brn3b	7D	.971327	.4919620 + .28403	.076	.415720	.0679446 + .02774	<.001
	14D	.432939	.2897533 + .11829	.015	1.135345	1.055326 + .47196	.074
	28D	.621289	.4941992 + .2471	.087	1.465695	.6634392 + .27085	.003
GFAP	7D	1.47267	.19378 + .13702	.059	2.290373	.55309 + .27655	.004
	14D	.26484	.11359 + .04637	.002	2.267443	.33967 + .13867	<.001
	28D	3.48528	1.29944 + .64972	.013	3.728287	.71831 + .32124	<.001
GDNF	7D	2.01358	.9668 + .3947	.004	1.056857	.117665 + .0480	<.001
	14D	.22600	.177815 + .0726	.026	3.740277	2.743927 + 1.12020	.021
	28D	3.71870	6.292648 + 3.6331	.414	3.812304	.903641 + .45182	.003

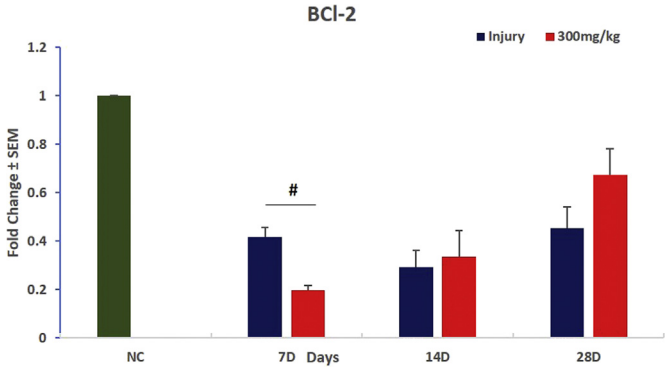

Fig. 3. Fold change in the BCl-2 mRNA expression for injury and 300 mg/kg A. *cepa* group estimated through Real Time PCR analysis at 7, 14, and 28 day after PPA surgery in ipsilateral eye. The values were normalized against the endogenous control (β-Actin) (n = 3). The data was found to be significant at P ≤ .05 (# = **<.001**).

effect induced by the I/R injury. However, it is interesting to note that at the later time points (14 and 28 day), *A. cepa* pretreatment group showed significantly increased expression (p value at 14 day- BCl-2: .030; GDNF: .021; GFAP: < .001, and at 28 day- BCl-2: .002; GDNF: .003; GFAP: <.001).

RGCs express certain specific markers like Thy1, and Brn3b, which are used as a marker for RGC injury [28]. Brn3b is a major transcription factor that belongs to mammalian POU family. It is a key regulatory marker of RGCs and is known to play an important role in RGC development and survival [29]. BCl-2 and Brn3b are neuroprotective in their action, as there is a binding locus for Brn3b present at the upstream site of BCl-2 [29]. A study by Buckingham et al. suggests that the injury decreases the number of cells expressing these markers [30]. However, the reduced Brn3b expression could indicate either loss of RGCs or decrease in the optic nerve fibers [31]. In our study, the expression of Brn3b declined after the injury and we observed a reduced expression at day 14 and day 28. And after the *A. cepa* pretreatment, we found that at a later time point i.e., 14 and 28 day, there was significant increase in the expression of Brn3b.

Next, we investigated the role of *A. cepa* pretreatment on the cell apoptosis during retinal I/R injury. BCl-2, an anti-apoptotic gene critical for the regulation of programmed cell death [32], is known as an essential controller of photoreceptors (rods, cones) and RGCs death in degenerated retinas [33]. In the current study, there was a significant reduction in the expression of BCl-2 at 7 day in both injury and pretreatment groups as compared to the control. There was a trend of increase in BCl-2 expression in treatment group analyzed at 14th and 28th-day post treatment, as compared to respective injury groups, though the difference was not significant.

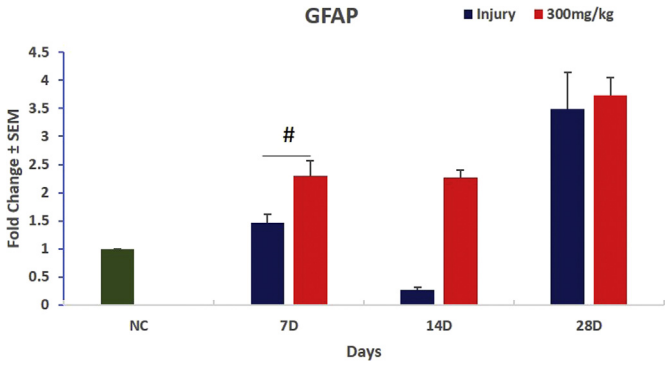

Fig. 4. Fold change in the gene expression of GFAP (Glial Fibrillary Acidic protein) for injury and 300 mg/kg A. *cepa* group estimated through Real Time PCR analysis for 7-day, 14-day, and 28-day after PPA surgery in ipsilateral eye. The values were normalized against the endogenous control (β-Actin) (n = 3). The data was found to be significant at P ≤ .05 (# = ≤**.001**).

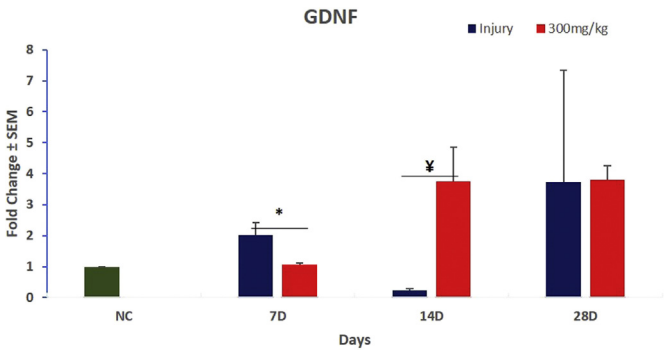

Fig. 5. Fold change in the gene expression of Glia derived Neurotrophic factor (GDNF) for injury and 300 mg/kg A. *cepa* group estimated through Real Time PCR analysis for 7-day, 14-day, and 28-day after PPA surgery in ipsilateral eye. The values were normalized against the endogenous control (β-Actin) (n = 3). The data was found to be significant at P ≤ .05 (* = **.037,** ¥ = **.011**).

Further study with different doses and time points is needed to understand the BCl-2 temporal expression profile.

GFAP, a class III intermediate filament protein mainly expressed in astrocytes, is known to play an important role during CNS development. It has been reported that astrocytes are activated post retinal injury, evident from the studies showing an increase in expression of GFAP [2,34,35]. Muller cells and RGCs are the two types of cells that express GFAP in retina. We report a similar pattern in our studies, where after I/R injury, the expression of GFAP was found to be elevated at 7-day ($p = .059$) and 28-day ($p = .013$) post treatment. However, in *A. cepa* treated group there was increased expression of GFAP at 7 day ($p \leq .001$) in comparison to the injury group. According to Lewis et al., the expression of intermediate filaments and Muller cells differs on the basis of detachment or reattachment of retina [35]. This indicates a differential expression (and an important role) of GFAP in both injury and pretreatment group.

Neurotrophic factors are known to enhance the cell survival mechanisms and hence, exert neuroprotective effect towards injury and degeneration. GDNF is a neurotrophic factor that has been shown to promote the survival of RGCs when introduced in different retinal injury rat models [36–39]. The effect of aqueous extract of *A. cepa* on the expression of GDNF was examined in I/R injury and it was found that the expression at 7 day was similar to that of normal control while it increased in the injury group, as it is known that an injury or degeneration can initiate protective strategies to prevent further damage. However, the expression was augmented at 14 day and 28 day with the *A. cepa* treatment. This data suggests that *A. cepa* extract is associated with the increase in the GDNF expression at later time points, which will further promote cell survival. The increased expression in response to *A. cepa* pretreatment at later time points suggests its potential role in recovery from I/R injury.

5. Conclusion

The current study explores the neuroprotective role of an herbal extract (*A. cepa*) in rescuing the damaged RGCs in I/R induced injury in mouse model of PPA ligation. The study investigated the expression of genes by RT-PCR, however, no dose dependent neuroprotective effect of *A. cepa* was observed. We plan to examine the related apoptotic pathway genes in our future study, which might have a role to play in the neuroprotective effect of *A. cepa*.

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Conflict of interest

None

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