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LITHIUM CHLORIDE RESCUES RETINAL GANGLION CELLS IN RAT MODEL OF GLUTAMATE EXCITOTOXICITY

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ABSTRACT: Retinal ganglion cell (RGC) death is distinctive of many ophthalmic disorders, such as glaucoma, optic neuropathies, and various retinovascular diseases. N-methyl-D-aspartate (NMDA)-type glutamate receptor (NMDAR)-mediated excitotoxicity is thought to be an important contributor to RGC death. Uninterrupted activation of a large number of NMDARs leads to increases in intracellular calcium loads, initiating a cascade of events that eventually result in apoptosis or necrosis. We aim to prevent or delay RGC death by an intervention involving lithium to afford neuroprotection of RGCs or neutralization of the deleterious effects of toxic factors. Existing reports highlight a range of lithium dosage applied from 7 to 21 days to promote the survival of RGCs damaged by optic nerve injury. Lithium was injected continuously for 7 days at a dose of 30, 50, and 70 mg/kg body weight (b.w.) to Wistar rats after injecting 20 mM NMDA intravitreally. Morphological changes observed by hematoxylin and eosin (H & E) staining along with measurement of the thickness of the inner retina (IR) , made up of ganglion cell complex (GCC), showed an increase in neuroprotection of RGCs in increasing order of dosage of lithium chloride after exposure to NMDA. Ultrastructure changes seen by transmission electron microscopy (TEM) showed necrosis, rupture of the membrane after NMDA insult, which was again overcome by increasing doses of lithium chloride with the best results observed at the highest dose of 70 mg/kg. Since early morphological changes of RGCs and their subsequent death are indicators of glaucoma, the present study indicates that the prevention of RGC loss by lithium in controlled dosage can lead to affordable neuroprotection against excitotoxicity.

INTRODUCTION: Loss of RGCs and their axons is a hallmark of several neurodegenerative diseases like glaucoma, retinal ischemia, and diabetic retinopathy [1, 2, 3].

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RGCs from a part of GCC which is made up of retinal nerve fiber layer (RNFL), RGCs, and inner plexiform layer (IPL) and get primarily damaged in glaucoma leading to loss of vision and hence prove to be important targets for developing neuro-protective therapies [4, 5, 6].

The most convenient animal model was found to be rats, for inducing damage of RGCs through optic nerve injury [5] followed by retrograde labeling with a fluorescent marker to identify the affected cells. Glutamate is an important neurotransmitter in the

retina and produces substantial excitotoxic damage by interacting with NMDAR on the RGCs. The characteristic patterns of lesions induced to RGCs in IR [6] are the possible targets of pathologic conditions in retinal ischemia and glaucoma. Exposure to glutamate agonist NMDA through intravitreal injection targets specific receptors on RGCs, components of GCC and IR within 3 to 7 days to produce changes in intrinsic and extrinsic pathways and hence considered as an important tool to study therapeutics aimed at promoting RGC survival [7, 8, 9, 10, 11].

Several efforts to develop therapeutics towards treating glaucomatous injury of NMDAR on RGCs with molecules like memantine, taurine, taurour-sodeoxycholic acid had shown significant effect [12, 13, 14, 15, 16]. Many neurodegenerative diseases of the CNS occur due to the binding of glutamate to NMDA and AMPA receptors [17]. Lithium is one of the major drugs which addresses complications arising from the degeneration of neural cells [18, 19]. In studies of optic nerve degeneration, damage of RGCs mediated through AMPA and NMDA receptors [20], citicholine, and lithium have been reported to support the survival of RGCs isolated from eyes of mice cultured *in-vitro* [21, 22]. When administered *in-vivo* in a rat model at 30 mg/kg for 21 days [23], it could produce a significant increase in survival of RGCs or at 60 mg/kg when administered for 7 to 14 days [24].

Another report describes the use of lithium aceto-acetate supplementing β-hydroxybutyrate to stimulate release of kyneurinic acid which renders neuro-protection against NMDA mediated hyper-activation of RGCs [25], but no conclusive data could be inferred regarding the involvement of lithium at high dose in the repair mechanism. Lithium is known to protect retinal neurocytes from ischemia-induced damage in rat retina following ischemia-reperfusion injury [26] and partially promoted the normalization of vasculature and visual function in Lrp5$^{-/-}$ eyes [27].

Long-term lithium treatment results in the attenu-ation of glutamatergic signaling at the cellular level, rendering neuroprotection to the cells of the CNS [28]. The present study was undertaken to neutralize the deleterious effects caused by NMDA induced excitotoxic injury while subsequently evaluating the effect of increasing doses of lithium resulting in maximum RGC survival.

MATERIALS AND METHOD:

Animal Maintenance: The study was conducted on male adult Wistar rats, weighing around 200g, approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) designated institutional animal ethics committee at Amity University, Noida, India (Proposal no. CPCSEA/IAEC/AIP/017/03/26). Animals were housed in the animal house for 7 days with food and water available *ad libitum* and were sorted into five groups (3 animals/group).

Dosing of Animals: Lithium chloride (LiCl) prepared at a concentration of 50 mg/ml in 0.9% normal saline was delivered to all groups at 30, 50, and 70 mg/kg body weight (b.w) intraperitoneally from day 1 to day 7, in order to maintain its concentration in the intravitreal space. On 3rd day, Group I (positive control) received 0.1M phosphate-buffered saline (PBS) through the intravitreal route. Group II (NMDA-treated only), Group III (NMDA-treated-LiCl-treated at 30 mg/kg), IV (NMDA-treated-LiCl-treated at 50 mg/kg), and V (NMDA-treated-LiCl-treated at 70 mg/kg) received an intravitreal injection of 2 µl of 20 mM NMDA through the dorsal limbus of the eye. The animals were sacrificed on day 8 after cervical dislocation, eyes enucleated, cornea excised to expose the lens, and retina extracted.

Hematoxyline and Eosin Staining: For morphometric analysis, H & E staining was done as per the standard protocol [29] but with slight modifications. Eyecups fixed in 10% formalin for 24 h, dehydrated through graded ethanol and xylene, 3 µm serial sections cut in microtome parallel to the maximal circumference of the eyeball through the optic disc taken from four eyeballs. Ten stained sections, lying midway from the optic disc to the outer periphery of the retina, from each eyeball were selected, and images acquired at 20X for morphological studies.

We estimated the thickness of the IR (ganglion cell layer + inner plexiform layer) defined as the total width between the inner limiting membrane and the interface of the inner nuclear layer using image analysis software Image J/Fiji [30].

Fixation of Eyeballs for TEM: The eyeballs were fixed in 4% paraformaldehyde (PFA) and 1% glutaraldehyde for 2h. After fixation, the retina was separated, the central retina cut into pieces, washed, and osmicated. The samples were embedded in Araldite CY 212. Semithin sections (1 µm) were stained with Toluidine Blue for light microscopy. Ultra-thin sections (80 nm) were contrasted with uranyl acetate and lead citrate and viewed under a Tecnai G2-20S twin transmission electron microscope (Fei Company, the Nether-lands).

Statistical Analysis: Morphometric data from different regions in each eye (n=4 eyes) were averaged to provide one value per section per eye for each condition. The mean and standard deviation (SD) for these measurements were calculated for each group, and comparisons between groups were made by using the Mann Whitney U method using Graphpad Prism 8.0. A p-value of less than 0.05 was considered statistically significant.

RESULTS:

Morphological Changes and Morphometry: GCL- ganglion cell layer IPL- Inner plexiform layer INL-Inner nuclear layer OPL-Outer plexiform layer ONL-Outer nuclear layer. General morphology of retina is observed in PBS treated sections of retina **Fig. 1A**. After NMDA insults, a reduced number of RGC with delocalized nuclei and diffused vacuolization in the RNFL was observed in **Fig. 1 B**.

On treatment with 30, 50 and particularly 70 mg/kg b.w of LiCl, the RNFL normalizes and attains intact integrity with centralization of nuclei in the RGC layer with the increasing order of the dosage **Fig. 1C, D** & **E**. For morphometry, the thickness of the IR (GCL + IPL) was measured which was taken to be the total width between the inner limiting membrane and the interface of the inner nuclear layer. The mean thickness of IR on exposure to 20 mM NMDA was found to decrease from 20.13 ± 5.9 in control to 13.2 ± 3 in NMDA group ($p=0.001$, $p<0.05$**) **Fig. 2**. The mean thickness (mean ± SD) in the treatment groups of 30, 50 and 70 mg/kg b. w. of LiCl increased in an ascending manner from 16.97 ± 1.8, 18.58±3.3 ($p < 0.0001$, $p < .05$**) and 19.79 ± 6.6 ($p = 0.0009$, $p<0.05$**) *vs* NMDA-treated respectively **Fig. 2.**

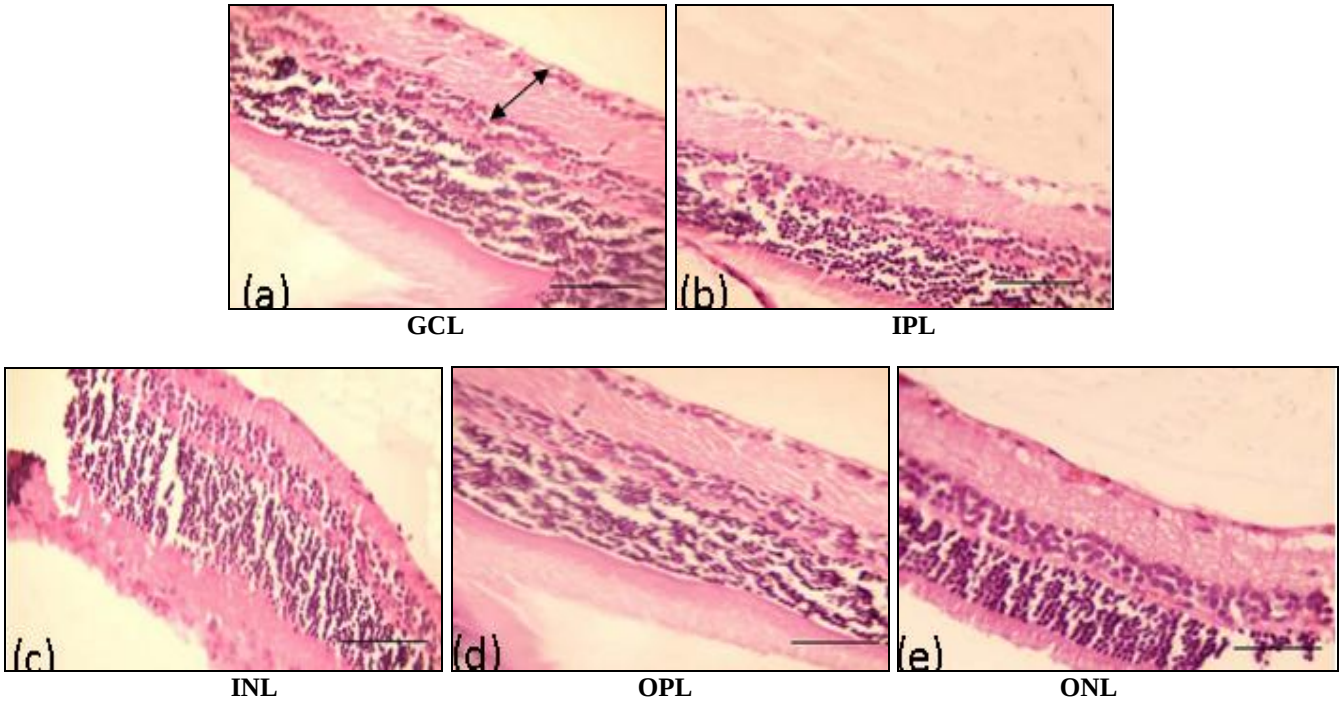

FIG. 1: REPRESENTATIVE IMAGES OF RETINAL HISTOLOGY. GENERAL RETINAL STRUCTURE IS SEEN IN CONTROL SECTION (A). CELL LOSS, NUCLEAR CONDENSATION, DIFFUSED VACUOLIZATION OF CELL COMPONENTS WITH THINNING OF RETINAL LAYERS IN NMDA-TREATED SECTIONS UNDERGOING NECROSIS IS OBSERVED (B). HOWEVER, THE RGC IN GANGLION CELL LAYER (GCL) REGAINS ITS STRUCTURE, AND NUMBER IN SUBSEQUENT LICL TREATED SECTIONS WITH OVERALL THICKNESS NORMALIZING PROGRESSIVELY IN SECTIONS TREATED WITH NMDA-LICL-TREATED AT 30 mg/kg, AT 50 mg/kg AND THOSE AT 70 mg/kg RESPECTIVELY. SCALE BAR EQUALS 50 µM. ARROW DEFINES AREA USED FOR THICKNESS MEASUREMENT

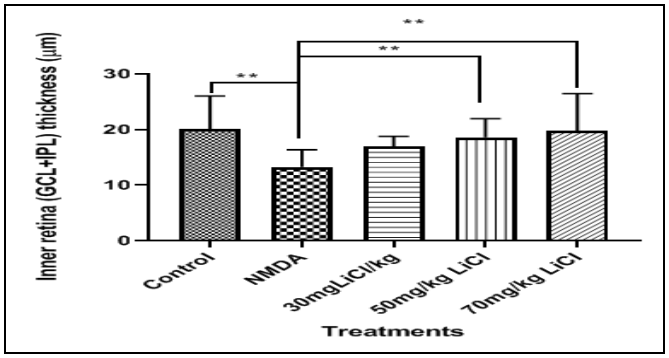

FIG. 2: QUANTITATIVE ANALYSIS OF RETINAL DAMAGE AS EVALUATED BY MEASURING MEAN THICKNESS OF IR (GCL+IPL) IN H&E STAINED SECTIONS. THE BARS (MEAN ± SD) INDICATE THE THICKNESS AS AVERAGED FROM TEN SECTIONS IN FIVE DIFFERENT CONDITIONS. SIGNIFICANT VARIATION (*P<0.05) SIGNIFYING LOSS OF THICKNESS IN IR WAS OBSERVED IN SECTIONS TREATED WITH NMDA WITH RESPECT TO CONTROL. AFTER TREATMENT WITH 30, 50 AND 70 mg/kg LICL A SIGNIFICANT PROGRESSIVE INCREASE IN IR IS OBSERVED WHEN COMPARED TO NMDA-TREATED SECTIONS

Morphological Assessment by Toluidine Staining: The semithin sections stained with toluidine blue were viewed under a light microscope to check the location of RGCs in the longitudinal sections. In PBS treated control, the intact cells along the RNFL with pale nuclei are RGCs alternating with darkly stained cells representing amacrine cell **Fig. 3A**.

After administering an intravitreal shot of 20 mM NMDA the structural integrity of RGCs is destroyed due to tissue death and necrosis, vacuoles appear in the cytoplasm, pushing the shrunken nucleus into the IPL **Fig. 3B**. On initiating treatment with 30 mg/kg b. w of LiCl, the nuclei partially maintain their position in the vacuolated cytoplasm of RGCs with remnants of necrosis **Fig. 3C**.

On increasing the concentration of LiCl to 50 mg/kg b. w. the necrotic spots disappear, and nuclei retain their position within the cytoplasm of RGCs but remain displaced from the RNFL **Fig. 3D**. Further, the increase of LiCl to 70 mg/kg b. w. showed RGCs with least damaged cytoplasm and nuclei placed along the RNFL and with least severe signs of necrosis **Fig. 3E**.

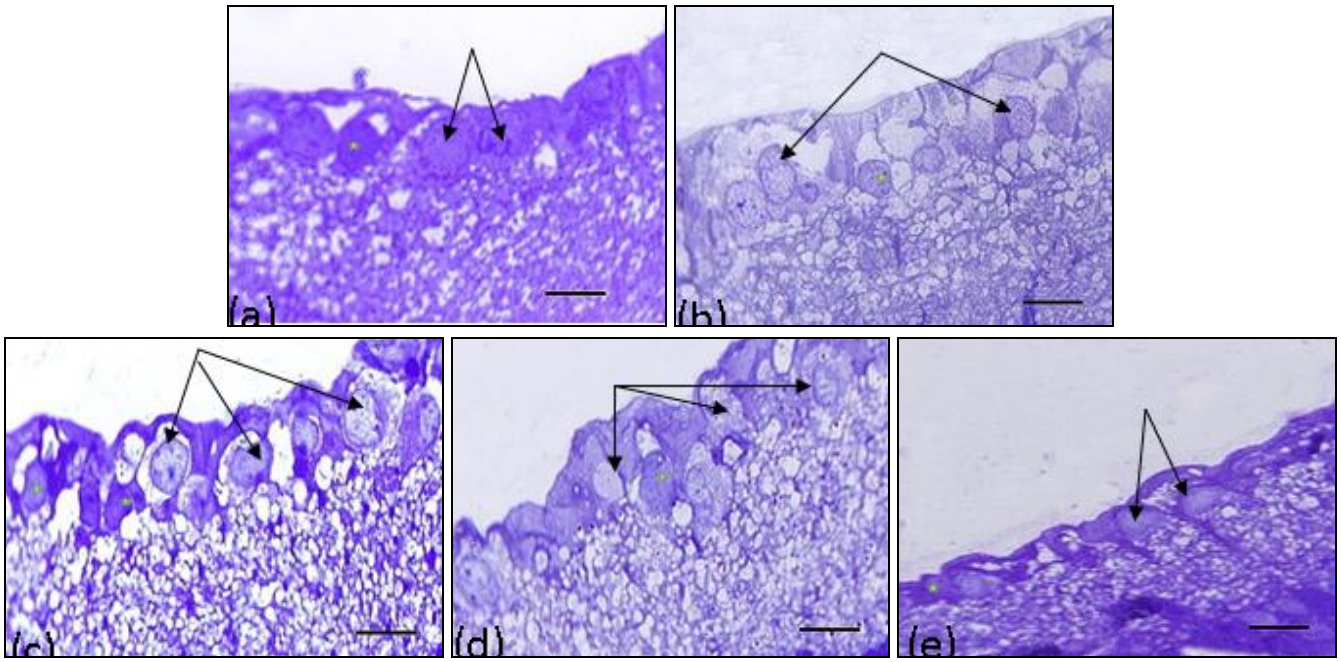

FIG. 3: REPRESENTATIVE IMAGES OF MORPHOLOGICAL CHANGES VISIBLE UNDER LIGHT MICROSCOPE OF RESIN EMBEDDED SEMITHIN SECTIONS. RGCS POSSESSING PALE NUCLEI ALTERNATE WITH DARK STAINED SMALLER AMACRINE CELL (STAR LABELLED) IN THE GCL OF CONTROL (A) NMDA TREATMENT DISPLACES THE NUCLEI WHILE DISINTEGRATING CELL CYTOPLASM (B). NMDA-LICL TREATED SECTIONS AT 30 mg/kg SHOW PARTIAL NECROSIS OF CELLS AND NUCLEI ALIGNED IN DISRUPTED GCL (C). NMDA-LICL-TREATED AT 50 mg/kg SHOWS REDUCED SIGNS OF NECROSIS (D). NMDA-LICL TREATED AT 70 mg/kg SHOWS INTACT GCL, RGCS WITH INTACT NUCLEI AND SMALLER AMACRINE CELLS WITH DENSE NUCLEI (E). ARROWS INDICATE RGCS AND STARS INDICATE AMACRINE CELLS. SCALE BAR EQUALS 50 µM.

Ultra-structure Analysis by TEM: The ultra-structure data of RGC obtained from TEM reveals the presence of intact cells with the plasma membrane and prominent nuclei in control, and the continuous nuclear membrane encircles homogenously dispersed karyoplasm with a small dark patch of the nucleolus (Fig.4a). When exposed to an insult of 20 mM NMDA, the GCL is disrupted due to the damage of nerve fibers just beneath the outer limiting membrane. The cytoplasm of RGC undergoes necrosis, and the nucleus shows chromatin condensation **Fig. 4B**. On treatment with 30 mg/kg LiCl after NMDA exposure, the nuclei show dispersed heterochromatin with intact nucleolus inside the nuclear membrane but with degenerated cytoplasm and plasma membrane **Fig. 4C.** At 50 mg/kg LiCl, the RGCs tend to retain their cellular integrity showing traces of clumped chromatin along the nuclear periphery **Fig 4D**. On increasing the dosage further up to 70 mg/kg LiCl, an almost normalized RGC containing dense cytoplasm, membrane-bound nucleus, nucleoplasm without fragmented chromatin, and a prominent nucleolus comparable to that seen in control **Fig. 4E.**

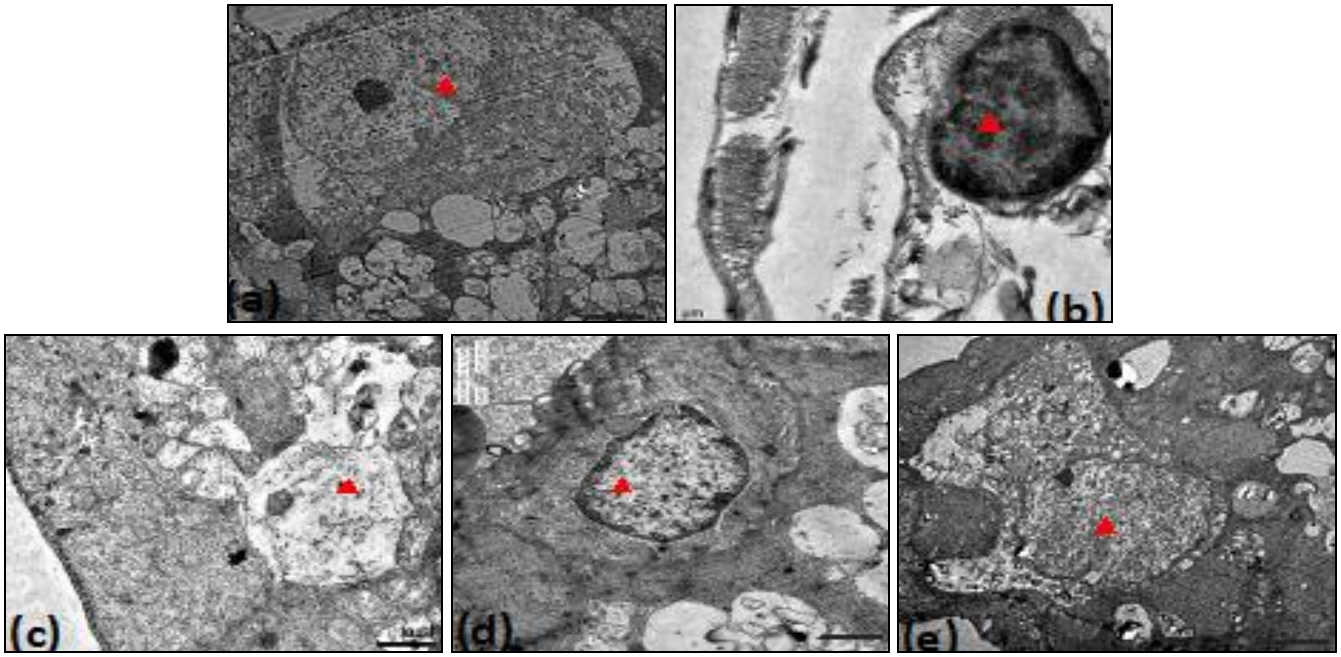

FIG. 4: ULTRA-STRUCTURE CHANGES AS OBSERVED IN RGCS. A TYPICAL RGC WITH DENSE CYTOPLASM, INTACT NUCLEI (RED TRIANGLE LABELLED) AND NUCLEOLUS IS SEEN IN CONTROL (A) AS COMPARED TO A NECROTIC CELL THAT IS SUBJECTED TO NMDA-INDUCED CHANGES, SHOWING DISINTEGRATED CYTOPLASM, NUCLEI WITH FRAGMENTED CHROMATIN (B). CELL BODY DISINTEGRATION WITH NUCLEUS CONTAINING DISPERSED HETEROCHROMATIN AND NUCLEOLUS IS OBSERVED IN EYES TREATED WITH NMDA-LICL-TREATED 30 mg/kg (C). AN INTACT RGC WITH NUCLEUS CONTAINING DISPERSED HETEROCHROMATIN ALONG NUCLEAR MEMBRANE IS OBSERVED IN EYES TREATED WITH NMDA-LICL- 50 mg/kg (D). THE CELLULAR INTEGRITY OF RGC WITH NUCLEUS AND NUCLEOLUS RETAINS IN EYES TREATED WITH NMDA-LICL- 70 mg/kg (E) SCALE BAR EQUALS 1 µM

DISCUSSION: RGCs constitute only 1% of all retinal cell types, but they are heterogeneous on the basis of the type of receptors that respond to external stimulus [31, 32]. The possession of tropic glutamate receptors, that are highly susceptible to hyperactivation by NMDA mediated injury and affect the IR more intensely when compared with other methods like an injection of paramagnetic microbeads in the anterior chamber which causing lesion in the episcleral vein of the eye [33]. Lithium exerts a neuroprotective effect on the central nervous system by preventing calcium influx resulting from activation of NMDARs in brain cerebellar cortical cultures stimulated by excitatory amino acid, glutamate [34]. There is a decrease in the number of RGCs and thickness of IR either with optic nerve crush [35] or intravitreal NMDA administration [36, 37]. An ideal intermediate concentration of 20 mM was chosen to be delivered through an intravitreous route in our rat model as found effective enough to induce injury and produce early symptoms of glutamate-induced damage even in the retina of adult zebrafish model [38].

The enhanced level of glutamate released from NMDA brings about characteristic changes in the IR extending up to the junction of IPL and INL [6]. The regions mentioned as GCC or IR encompass RNFL, RGC, and IPL and is most susceptible to NMDA induced excitotoxicity, which was also evident in our study as seen by the destruction of nerve fibers and apparent necrosis of RGC bodies undergoing progressive degeneration in RNFL when assessed by H & E. The loss of cellular integrity leads to pyknosis marked by the presence of shrunken nuclei and appearance of vacuoles after NMDA insult as compared to control **Fig. 1A** & **Fig. 1B** and these symptoms are good diagnostic indicators of glaucoma [39] ultimately leading to irreversible loss of vision. The neuroprotective potential of lithium selectively on RGCs is established in models of optic nerve degeneration, which is explored to treat the targeted disruption of NMDARs present in the IR.

Previous studies reported prolonged exposure of lithium at 30 mg/kg b.w. for 21 days [23] or at 60 mg/kg b. w. for 14 days [24], caused an increase in the density of RGCs. An appropriate highest possible lithium dose is required to attenuate morphological aberrations of RGCs like pyknotic nuclei, disintegration of nuclear chromatin, and appearance of cytoplasmic vacuoles due to initiation of necrosis coherently with morphometric alterations associated with a reduction in thickness of IR induced by glutamate analog, NMDA [30, 31].

When exposed to selected three intraperitoneal dosages of 30, 50, and 70 mg/kg b.w. of lithium, a concentration-dependent effect revealed that LiCl at 70 mg/kg proved to be the most effective in significantly increasing RGC survival and regularizing the thickness of IR while protecting the cytoplasm from necrosis and nucleus of RGCs from pyknotic death **Fig. 1C, D** & **E**.

The ultrastructural study of an individual RGC placed at the junctions of GCL and IPL under normal condition (control) clearly distinguishes the integrity of plasma membrane and nuclear membrane **Fig. 4A**. The early signs of damage by NMDA show extreme cytolysis, causing loss of cellular architecture, disruption of nerve fibers, with neuronal debris and clumps of chromatin condensation **Fig. 4B** inside the nucleus, which is in coherence with previous studies [40].

Lithium at lower concentration protected the nuclear organization of RGCs with a prominent nucleolus and at the highest effective dose; the nucleolus and less disturbed cell plasma membrane are distinguishable **Fig. 4C, D,** and **E.** This data further supports the concept of lithium-induced RGC protection and survival [41]. Glutamate binding to glutamate receptors (iGluRs) is abundantly distributed on the RGCs in the retina [42] and classified into GluN1 (NR1) and GluN2 (NR2) subunits. The modulation in the expression of these receptors is expected by lithium, which is known to decrease the level of GSK-3β and consequently prevent β-catenin degradation through Wnt signaling pathway in the brain [43, 44]. Lithium supplementation in psychiatric disorders augments the levels of GluN2A in the prefrontal cortex of the brain [45]. A decrease in the production of RGCs is associated with activation of Wnt signaling pathway [46]. The involvement of GSK-3β and β-catenin axis during activation of NMDA receptors or optic nerve degeneration in RGCs has been established recently [47, 48] but unlike the chronic effect of lithium on the cascade of events of Wnt signaling pathway [49] in rats, its short term effect demands more extensive molecular studies of the receptors (GluN2A through 2D) on RGCs to reiterate lithium at its therapeutic doses against retinal pathologies.

CONCLUSION: The neuroprotective effect of LiCl post optic nerve injury as per previous studies was restricted to RGCs at 60 mg/kg[2] [4]. Several pathological conditions of the retina like glaucoma, diabetic retinopathy, *etc*. are associated with degeneration of NMDAR present on RGCs and amacrine cells in RNFL as well as those displaced to IPL and INL after NMDA insult. The use of lithium to treat NMDA-induced excitotoxic damage of cells across the inner retina is reported with a standardized dose of up to 70 mg/kg b.w of LiCl administered intraperitoneally for 7 days. This ensures retention of enough concentration of the drug in the microenvironment of the retina that prevents cytolysis due to necrosis, apoptosis, and morphometric alterations like the variation in thickness of IR. A dose-dependent effect of lithium was evident in repairing the cytoplasmic and nuclear degeneration of RGCs with 70 mg/kg evolving as the optimum dosage to attenuate cytotoxicity induced by glutamate analog, NMDA.

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