Effect of NMDA and AMPA/Kainate Receptor Antagonists Against Ethambutol Induced Retinal Toxicity Using Optomotor Response (OMR) in Goldfish

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Abstract

Objective: Ethambutol (EMB) is known to cause ocular toxicity on prolonged use. The present study evaluated the effect of NMDA and AMPA/Kainate receptor antagonists (memantine & trimetazidine) against ethambutol induced ocular toxicity using Optomotor response (OMR) in goldfish.

Materials and Methods: Either sex of goldfishes randomized into three groups (n=8 each group) and were exposed to daily dose of ethambutol (1 mg/ml for one hour) for 26 days. Group 1 fishes received an intravitreal injection of 1 µl of normal saline. Group 2 and 3 fishes were given intravitreal injections of 20 µg memantine (MEM) and 10 µg trimetazidine (TMZ) respectively at 10, 15, 20th and 25th day following anesthesia. After drug exposure, fishes OMR was evaluated, and pattern velocity was recorded (on 11, 16, 21st and 26th day) at 5 rpm in different light condition (blue, green and red).

Results: Upon chronic exposure (1 hr in bathing solution / day) of ethambutol, at the dose of 1 mg/ml fishes showed statistically significant decrease in percentage relative frequency (PRF) at 7th day upon comparison to their baseline values on day 0. Significant decrease in PRF was observed in the green color (550 nm, p=0.002) and red color (605 nm, p=0.001) and this effect persisted up to 21st day. Both
memantine and trimetazidine showed varying degrees of protection on 16th days against EMB induced ocular toxicity.

**Conclusion:** Intravitreal administration of trimetazidine and memantine provide significant protection in the PRF-OMR, indicating the possibility of their use as a therapeutic intervention in the patients developing ocular toxicity during antitubercular therapy (ATT).

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**Introduction**

Tuberculosis (TB) is globally the top tenth leading cause of death worldwide. The World Health Organization (WHO) global tuberculosis report for 2019 indicates that, approximately 10.0 million (range, 9.0 to 11.1 million) people were affected by TB in 2018 and this number that has been relatively stable in recent years. The burden of TB varies enormously among the countries, from less than 5 to more than 500 new cases per 100 000 population every year, with a global average being around 130. The report showed that, 1.2 million tuberculosis deaths among HIV-negative people (range, 1.1 to 1.3 million) in 2018 (a 27% reduction from 1.7 million in 2000) and 0.251 million deaths from TB (range, 0.223 to 0.281 million) among HIV-positive people (a 60% reduction from 0.620 million in 2000). According to the WHO (2019) report, there are 44% TB cases geographically in WHO regions of South-East Asia. In India, alone 27% of TB cases among the eight countries accounted for two-thirds of global cases (1).

In India, among 21.5 lakh TB cases, 25% were notified from the private sector. TB cases between 15 to 69 year age groups are most affected (89%) and about two-third of the cases are males as reported by the Government of India’s Revised National Tuberculosis Control Program (RNTCP, 2019) (2).

Ethambutol has been reported to inhibit arabinosyl transferase III, which in turn disrupting the transfer of arabinose into arabinoglactan biosynthesis which involved in the process of mycobacterial cell wall synthesis. Ethambutol and its metabolite 2, 2' (ethylenediamino)-dibutyric acid (EDBA) are excitotoxic to the ganglion cell layer, through the stimulation of the NMDA receptor by glutamate. It is expected to induce hyperpolarization of the horizontal cell membrane which is associated with a reduction of the receptive field size and a stronger reduction of the sensitivity to red to green stimuli (3). The polarization status of the horizontal cells has been associated with changes in relative sensitivity to red, green or blue light stimuli, as hyperpolarization of the horizontal cells results in an influx of intracellular calcium in cones causing further depolarization. This horizontal cell - mediated changes in cones are recognized as negative feedback (3). Chemical like cobalt ions, glutamate antagonist and L-2-amino-4-phosphonobutyrate (APB) hyperpolarizes the horizontal cells and were found to decrease the response to red to green light (4, 5, 6). As this feedback has been reported to be more pronounced in the green part of the spectrum, reduction of feedback strength during hyperpolarization resulted in a smaller reduction in green to red color stimuli (4).

In the animal visual systems, perception of motion is one of the major ability factors and most of the animal species are capable of having visual discrimination. The color vision in vertebrates is generally assumed to be mediated through neural pathways that uses gamma amino butyric acid (GABA) and glutamate as neurotransmitters (7, 8). Spekreijse et al., (1991) reported that ethambutol (EMB) does not affect photoreceptors in goldfish because the electroretinogram (ERG) and the behaviorally determined absolute light detection thresholds remain unaffected (3). The spectral sensitivity function of ethambutol fed goldfish resembles the spectral sensitivity function obtained under mesopic background illumination (9). Therefore, Spekreijse et al., (1991) suggested that EMB arrests the retina in a dark-adapted state (3).
Goldfishes not only respond readily to moving stimuli, but also have a very well developed color vision (10). Goldfishes have tetrachromatic color vision, especially ultraviolet (360 NM-UV), small (450 NM-S), middle (540 NM-M) and long (625 NM-L) cones (11, 12). Among the four types, only long type (L) of cones contributes to the optomotor response (9). Goldfishes cones contain GABA$_A$ gated chloride channel. When cones were exposed to green / blue color light at dark condition the GABA uptake by Ab pyriform amacrine cells was found to be increased. However, the reverse phenomena happened when cones were exposed to red light. In light condition, the GABA uptake by horizontal cone cells was found to be increased, which was reported to be decreased in dark condition. Similarly, in dark conditions, horizontal cone cells got depolarized and led to the release of GABA (13).

The various experimental studies showed the release of glutamate by cones in goldfishes during the dark condition in a Ca$^{2+}$ dependent manner (14). In the presence of light cones were found to be hyperpolarized to cause a decrease in glutamate release. Color and motion were reported to have separate pathways in human. In isoluminance condition, when the stimuli contains only contrast in color, there will be disappearance of motion perception, but not in luminance (15).

GABA antagonist, bicuculline affected the wavelength discrimination ability of goldfish in red-green, but not in blue-green part of the spectrum respectively (6). Bicuculline had the same effect on wavelength discrimination ability on goldfishes comparable to ethambutol (3). Though many studies have reported the involvement of glutamate in the causation of ethambutol induced ocular side effects, yet the exact mechanism of retinal toxicity is unclear (16).

The pretreatment with N-methyl-D-aspartate (NMDA) antagonist (memantine) has been found to restore the fall of ‘b’-wave amplitude of the ERG, after chronic EMB administration in rats at a dose of 200 mg/kg of body weight (17). Therefore, the present study has been conducted to reinvestigate the protective effect of both NMDA and AMPA/Kainate receptor antagonists against EMB induced ocular toxicity using the Gyro-dotted-OMR in goldfish.

Materials and Methods

Chemicals

Ethambutol, memantine, trimetazidine and ethyl-3-aminobenzoate methane sulfonate (MS-222) were procured from Sigma-Aldrich, Co., St. Louis, MO, USA. All other analytical grade chemicals and reagents were obtained from their respective commercial sources and were used without any further purification.

Animals

Adult goldfishes (Carassius auratus) were obtained from local dealers and fishes were well maintained at 12-hour light/dark cycle in fish tanks. Aeration was supplied by using an electrical aerator for entire day to the fish tank and fish tank water was replaced twice weekly. The tank temperature was maintained between 22 to 25°C. Fishes with a body length ranging between 4-7 cm and weighing around the 10 to15 g were used for this study. All goldfishes were fed twice daily with standard dry sea food (Tokyu®, Japan). The study protocol was approved by the Institutional Animal Ethics Committee (IAEC). All experimental procedures were followed as per the Association for Research in Vision and Ophthalmology (ARVO) guidelines and Standing IAEC of the Institute. In order to develop a recordable ocular toxicity in goldfishes, Gyro-dot-OMR was developed and standardized in our laboratory (18). Goldfishes were selected as a tool, to study the ocular toxicity of ethambutol since they are reported to possess 4 types of cones viz, small (S), middle (M), large (L) and ultraviolet (UV) (9).

Experimental setup of Gyro-dot-Optomotor response (OMR)

A digital light processing aided gyrating polychromatic dotted pattern - OMR (Gyro-dot-OMR) analyzer was used in this study (18). The fish bowl water level was maintained at 7 cm from the bottom
(holding 1.5 L of water) so that the fishes can swim freely in the bowl. A black velvet curtain was fixed around the experimental setup to provide optimal contrast and prevent the entry of stray light to fishbowl. The fish and dotted pattern cylinder movement were continuously recorded using a video camera fixed above the fish bowl. The video signal was stored simultaneously on a time-lapse recorder and observed on a monitor.

Measurement of the optomotor response (OMR)

For OMR measurement, the identical procedure was followed as reported by Schaerer and Neumeyer (1996) (19). Goldfishes were tested repeatedly before the injection of either drug or solvent until stable optomotor response were achieved for 5 rpm. Before all measurements, fishes were allowed to adapt for 5 min to the OMR setup and stimuli light intensity. After adaptation, fishes were allowed to swim for 1 min in clockwise and 1 min anticlockwise rotation, which were separated by 1 min with no rotation. For a testing session, pattern velocity (5 rpm) was repeated 4-5 times in each direction. Minimum two experimental sessions were performed with each fish before the injection of the standard or test drugs. After induction of EMB toxicity, evaluation of the effect of intravitreal injection of NMDA and AMPA/Kainate receptor antagonists was done.

Either sex of goldfishes was randomized into three groups, each group consisting of eight goldfishes (n=8). Group 1, 2 and 3 fishes were exposed to daily doses of ethambutol (1 mg/ml for one hour) up to 26 days. Group 1, 2 and 3 of fishes were given intravitreal injections of 1 µl of normal saline, 1 µl of a solution of 20 µg memantine (MEM) /ml and 1 µl of a solution of 10 µg trimetazidine (TMZ) /ml respectively on 10, 15, 20th and 25th day. After drug exposure, fishes were allowed to the OMR setup and fish pattern velocity was recorded (on 11, 16, 21st and 26th day) at 5 rpm in different light conditions (Blue, Green and Red). All intravitreal injections were performed by using 31-gauge needle attached to a Hamilton syringe (Hamilton Co., Reno, Nevada. USA) under the methanesulphonate (MS-222, 150 mg/L) anesthesia condition.

Percentage relative frequency (PRF) Calculation:

The percentage relative frequency (PRF) for a particular wavelength calculated using the following formula:

\[
PRF = \frac{PV \text{ at } Wx \text{ at } Tx}{PV \text{ at white light at } Tx} \times 100
\]

\[
PRF = \frac{PV \text{ at } Xw \text{ at } Xt}{PV \text{ at white light at } Xt} \times 100
\]

Where,

- \(PV\) = Pattern velocity
- \(Wx\) = Particular wavelength (\(x = 440\) or \(550\) or \(605\) nm)
- \(XW\) = Particular wavelength (\(w = 440\) or \(550\) or \(604\) nm)
- \(TX\) = Time point after the injection of intravitreal memantine / trimetazidine
  - (\(x = 10^{th}\) or \(15^{th}\) or \(20^{th}\) or \(25^{th}\) day).
- \(Xt\) = Time point after the injection of intravitreal memantine / trimetazidine
  - (\(x = 10^{th}\) or \(15^{th}\) or \(20^{th}\) or \(25^{th}\) day).

Data acquisition and statistical analysis

The pattern velocity of a goldfish was used for the calculation of percentage relative frequency (PRF). The values were represented as mean±SEM. Fish percentage relative frequency data were analyzed by using Students paired ‘t’ test within treatment group fishes and using Students unpaired ‘t’ test between the control and treatment groups. The results were considered to be statistically significant if the p value was <0.05. SigmaStat statistical software program (ver. 4.0) was used for the analysis.

Results

After chronic exposure (1 hr in bathing solution/day) of ethambutol at the dose of 1 mg/ml, fishes showed a statistically significant reduction in the percentage
of the relative frequency in the green (550 NM) and red (605 NM) wavelength on the 7th day as compared to their baseline values on day 0. A slight insignificant increase has been observed in the percentage frequency of blue wavelength at the consecutive days. Persistent significant decrease in PRF of green and red color wavelength observed in the studied time period following ethambutol exposure (Fig. 1).

**Effect of NMDA receptor antagonist on ethambutol induced toxicity in goldfish**

After the induction of ocular toxicity following ethambutol exposure, in terms of decreased PRF, intravitreal injection (IVI) of NMDA receptor antagonist memantine was given on day 10, 15, 20 and 25. Following IVI, significant improvement in the green color PRF was observed from 21st day onwards (p=0.008). The NMDA receptor antagonist also increased the PRF at red wavelength in goldfishes from 16th day onwards, which was found to be significantly elevated at 21st and 26th days (p=0.028). Whereas PRF of blue color wavelength remains unaffected due to ethambutol and NMDA receptor antagonist exposure (Fig. 2).

**Effect of AMPA/ Kainate receptor antagonists on the ethambutol induced toxicity in goldfish**

Intravitreal injection AMPA/Kainate receptor antagonist trimetazidine was given at 10th, 15th, 20th and 25th days following ethambutol exposure. The IVI caused a significant increase in all three wavelengths namely blue (p=0.007), green and red on day 16th. The increase in PRF remained persistent throughout the study in all three wavelengths (Fig. 3).

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**Fig. 1**: Opto motor response (at different days) of ethambutol exposed goldfish (n=8). The values are expressed as mean±SEM. Note: The values are comparison between 0-day vs 7, 16, 21st and 26th day. Data are analyzed by using both Student paired and unpaired ‘t’ test (*p<0.05, **p<0.01, ***p<0.001).
Fig. 2: (A) Opto motor response (at different days) of memantine injected intravitreally in goldfish in presence of ethambutol (n=8). Arrow indicates the intravitreal injection at 10, 15, 20\textsuperscript{th} and 25\textsuperscript{th} day.  
(B-D) Shows the change in pattern at different wavelength. The values are expressed as mean±SEM. 
Note: The values are comparison between 7\textsuperscript{th} day vs 16, 21\textsuperscript{st} and 26\textsuperscript{th} day. 
Data are analyzed by using both Student paired and unpaired ‘t’ test (*p<0.05, **p<0.01, ***p<0.001).

Fig. 3: (A) Opto motor response (at different days) of trimetazidine injected intravitreally in goldfish in presence of ethambutol (n=8). Arrow indicates the intravitreal injection at 10, 15, 20\textsuperscript{th} and 25\textsuperscript{th} day.  
(B-D) Shows the change in pattern at different wavelength. The values are expressed as mean±SEM. 
Note: The values are comparison between 7\textsuperscript{th} day vs 16, 21\textsuperscript{st} and 26\textsuperscript{th} day. 
Data are analyzed by using both Student paired and unpaired ‘t’ test (*p<0.05, **p<0.01, ***p<0.001).
Discussion

The incidence of ethambutol induced ocular toxicity has been reported to be 1-18% in the patients undergoing ATT (20). The role of excitatory neurotransmitter like glutamate, playing a role in ethambutol induced ocular toxicity has been well documented in many *in-vitro* and *in-vivo* studies (21).

The studies were conducted to evaluate the ethambutol toxicity by ERG after chronic administration in Wistar rats, where ethambutol showed significant reduction in ‘b’-wave amplitude on the 21st day. Pretreatment with NMDA and AMPA/Kainate receptor antagonists were found to reduce the excitotoxicity. Pretreatment with memantine was found to protect excitotoxicity in ‘b’-wave in rat ERG (17). In addition, the present study was conducted to evaluate both NMDA and AMPA/Kainate receptor antagonists using Gyro-dot-OMR in goldfish after the chronic administration of ethambutol.

As ethambutol induced color toxicity has been reported in middle (green) and long (red) wavelengths (5), this study preferred goldfish as a tool over rats, as rats are reported to have only small (S) and middle (M) cones in the retina unlike human eyes.

To mimic ethambutol induced ocular toxicity in human, chronic exposure model was preferred. The goldfishes were exposed to the concentration of 1 mg/ml for 1 hour period every day. Various EMB exposures strategies like, drug containing food pellet, intramuscular injection, etc. have been used in the goldfish model (21, 22). The present study opted for pan exposure of the drug to the organism through bathing solution. After using various time intervals of exposure, this study found that 1 mg/ml solution for 1 hour was appropriate to cause a fall in percentage relative frequency OMR (PRF-OMR) on the 7th day. The initial acute toxicity studies revealed (17) a fall in PRF-OMR after 12 hours following the intravitreal administration of ethambutol at the dose of 2 µg/ml (2 µl) in each eye (23).

As an acute exposure of ethambutol through bathing solution showed a predictable excitation and depression as evidenced by the increase and decrease in OMR, the similar strategy was adopted to study the chronic toxicity of ethambutol in goldfish (18). In order to analyze ethambutol ocular toxicity, goldfishes were subjected to the gyrating polychromatic dotted pattern-OMR on 7, 16, 21st and the 26th day, after the initiation of ethambutol exposure. The results of this chronic study showed a significant fall in PRF-OMR on day 7, after exposure of ethambutol every day. The fall in PRF-OMR was significant from baseline for both green and red colors on day 7 and day 26. Intravitreal administration of memantine and trimetazidine treatments at 10, 15, 20th and 25th day, after the initiation of ethambutol exposure was found to restore PRF-OMR significantly in red and green wavelength.

Memantine is an analogue of amantadine and clinically used in the treatment of parkinsonism and epilepsy diseases. It mainly binds to non-competitive NMDA receptor site and thus block the effect of glutamate. Memantine has been found to be protective against the acute excitotoxicity insult in both *in-vivo* and *in-vitro* experimental studies (24, 25). Memantine has been reported and approved as a neuroprotectant in human for Alzheimer’s disease (26). It has also been reported in experimental models as a neuroprotectant in glaucoma, but subsequent clinical trials failed to show any efficacy (27). This could be due to the poor levels of memantine reaching the human eye across blood ocular barriers (28). In the current study, intravitreal injection of memantine showed improvement in the green and red wavelength in the ethambutol induced color toxicity from the 21st day onwards.

AMPA/Kainate receptor antagonist trimetazidine has shown a significant improvement in the PRF of ocular toxicity affected goldfishes from the day 16th onwards. The significant protection shown by the trimetazidine may be due to its ability to inhibit the extracellular glutamate accumulation, which represents the first step of the excitotoxicity phenomenon (10). Being a vasodilator, trimetazidine has been reported to be effective in reducing ischemic chorioretinal disturbances (29). However, its intraocular penetration is yet to be determined in animal models or in human. As intraocular penetration of any neuro-protectant
across ocular barriers is a matter of concern (28), intravitreal route was opted in the present study.

Exposure of the ethambutol did not cause any significant change in the PRF-OMR of the blue wavelength (440 nm). As literature suggests that glutamate induced hyperpolarization in the horizontal cells causes decreased response in red to green light, our previous finding results were found to be consistent with it (4, 5, 6). Surprisingly, an increase in the PRF-OMR of the blue wavelength was observed following TMZ intravitreal injection. The increase remained sustained over the study period, although the justification for this increase needs to be validated by further experiments.

The results of this study showed the possibility of the involvement of excitatory amino acid pathway in the EMB induced ocular toxicity and predicted the usage of NMDA and AMPA/Kainate receptor blockers in reducing the excitotoxicity leading to permanent retinal damage in patients undergoing anti tuberculosis therapy.

Further, studies are in progress in evaluating the suitability of various NMDA antagonists across the blood retinal barrier in experimental models for their suitability for neuroprotection in conditions like glaucoma and ethambutol induced retinal toxicity.

To conclude, this study has been conducted to evaluate the protective effects of both NMDA and AMPA/Kainate receptor antagonists against EMB induced ocular toxicity by the newly developed model of Gyro-dot-OMR in goldfish. Chronic administration of ethambutol at the dose of 1 mg/ml/day for the period of one hour caused significant decrease in PRF-OMR in red and green color. Intravitreal administration of trimetazidine and memantine offered significant protection in the PRF-OMR indicating the possibility of using them as a therapeutic intervention in patients developing ocular toxicity during antitubercular therapy. However, further studies are required to evaluate their intraocular penetration before initiating clinical studies.

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