

Original Article

Evaluation of the Protective Effect of NMDA/Non-NMDA Receptor Antagonists Against Ethambutol Induced Retinal Toxicity Using ERG in Wistar Rats

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Abstract

To study the protective effect of NMDA and non-NMDA receptor antagonists against ethambutol (EMB) induced retinal toxicity in *Wistar* rats using flash electroretinogram (ERG). Rats were randomized into four groups: Group-1 received vehicle. Group-2 received oral EMB (200 mg/kg/day). Group-3 and 4 were fed with oral EMB along with memantine (MEM) (1 mg/kg, ip) and trimetazidine (TMZ) (3mg/kg, ip) respectively. All treatments were continued up to 28 days. ERG was recorded at 0 and 21st day using green and white lights. Ethambutol and 2, 2' ethylene diimino dibutyric acid (EDBA) levels were quantified in rat body fluids and tissues using LC-MS/MS. A higher rate of rat mortality was observed between 21st and 28th day, 21st day considered for ERG recording among groups. Ethambutol did not cause any significant change in 'a'-wave amplitude of rat ERG but caused a predictable decrease in 'b'-wave amplitude of the rat ERG on the 21st day. Memantine treatment showed a significant ($P=0.029$) protection against the fall of 'b'-wave amplitude on 21st day. Interestingly, we found that plasma levels of EMB in memantine treated rats were significantly reduced when compared to the positive control group. Memantine reversed the effects of EMB on 'b'-wave of rat ERG suggests its protective role. We suggest MEM may be considered as a possible preventive treatment modality for EMB induced vision toxicity warranting further clinical investigations.

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Introduction

Ethambutol (EMB) was introduced in 1961 as a chemotherapeutic agent in tuberculosis (1, 2). It is active against genus of *Mycobacterium tubercule* as well as isoniazid and streptomycin-resistant strains of *M. tuberculosis* (3, 4). Chronic EMB therapy is known to cause retinal toxicity and mild to moderate amblyopia. EMB induced optic neuritis (both early and late onset) has been well documented. In addition, EMB is also known to damage the optic nerve that leads to a permanent decrease in visual acuity, color vision deficit and central or ceco-central scotomas (5). Both ethambutol and its metabolite 2, 2' ethylene diimino dibutyric acid (EDBA) are reported to be toxic to retinal ganglion cells *in-vitro* and *in-vivo* (6).

EMB and EDBA reduce mitochondrial activity by making ganglion cells more sensitive to normally tolerated external levels of glutamate by a mechanism involving mitochondrial energy homeostasis. This leads to a release of excitatory amino acids, which results in a rapid cellular efflux of K^+ ion and an increased influx of Ca^{2+} and Na^{2+} ions through *N*-methyl-D-aspartate (NMDA) and non-NMDA receptor channels. After this process, secondary influx activated by voltage changes or by intracellular messenger system takes place through other Ca^{2+} channels. The resulting intracellular Ca^{2+} accumulation is assumed to cause cell death by a number of injurious processes. These processes include activation of nitric oxide synthase and generation of free radicals (7). Therefore, glutamate antagonists are expected to limit the side effects seen with ethambutol administration to rat retina.

Excitatory amino acid pathway is activated by NMDA and alpha amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors. NMDA channels operate for millisecond periods of time due to release of Mg^{2+} ions blockade at normal neurotransmission (8). This blockage occurs after the cation influx into the neuron *via* AMPA-sensitive glutamate receptor channels. In pathological condition, NMDA receptor over activation causes the excessive Ca^{2+} influx into the nerve cell, thus leading to cellular damage and death (9, 10, 11).

The electroretinogram offers a quantitative, objective and noninvasive method to examine light evoked neuronal activity. It is commonly used to study the functional integrity of normal and diseased retina (4, 12, 13, 14, 15). Among the several different ERG components, the 'a'-wave is of negative polarity in nature and is the first major component of the ERG. Both photopic and scotopic states of retinal adaptation, either cones alone or the cones and rods will contribute to the genesis of the 'a'-wave from photoreceptor of the retina. Another most sensitive component is 'b'-wave, which is of positive polarity and it is generated from the bipolar cells of retina (16).

The systemic administration of ethambutol causing optic neuritis and color vision disturbance are still a puzzle. Therefore, the objective of the present study was to explore the possible neuroprotective effect of NMDA and non-NMDA receptor antagonists on ethambutol induced retinal toxicity in *Wistar* rats. This study was also extended to quantify the ethambutol and its major metabolite EDBA in various tissues of rats after chronic oral administration of ethambutol.

Materials and Methods

Ethambutol, memantine and trimetazidine were procured from Sigma Chemicals, USA; MO. EDBA was procured from Santa Cruz Biotechnology, USA. All other analytical grade chemicals and reagents were obtained from their respective commercial sources. The study protocol was approved by the Institutional Animal Ethics Committee. All experimental procedures were followed as per the Association for Research in Vision and Ophthalmology (ARVO) guidelines and standing Institutional Animal Ethics Committee (IAEC) of AIIMS.

Electroretinogram (ERG) procedure

Light exposure set up

A customized light probe was built in-house to deliver a constant amount of light to rat retina. The probe consisted of customized corneal gold electrode

mounted on the surface of light emitting diode (LED). The gold wire was connected to an amplifier and simultaneous acquisition of evoked responses was made possible. Standard silver/silver chloride (Ag/AgCl) (powder coated, diameter 10cm) electrode was used for recording from the bare skin surface of the animal. The reference electrode was placed into the buccal cavity and a ground electrode needle was inserted into tail region of animal for recording biopotentials.

ERG recording

Flash ERGs were recorded against saturating white (445 nm) and green (510 nm) light. The intensity of white light was $0.77 \text{ Cd}\cdot\text{s}/\text{m}^2$ and that of green light was $678.3 \text{ Cd}\cdot\text{s}/\text{m}^2$. Luminosity of all the LEDs was standardized using photoradiometer (Model # HD 2102.2., Hotek Technologies, USA. Probe: LP 471 PHOT). The stimulus device was held on the fiber optic recorder at a perpendicular position in order to record the luminous intensity of the LED source. Light irradiance was measured in $\mu\text{W}/\text{cm}^2$ and total illuminance was measured in $\text{Cd}\cdot\text{s}/\text{m}^2$ (SI unit of light intensity). White saturating light flash was employed to ensure recording of maximal 'a'-wave (and therefore corresponding 'b'-wave) amplitudes from dark-adapted rat retina. Thereafter retinal response was measured against green flash.

ERG was recorded by means of an instrument provided by Nicolet Biomedical Systems (Madison, USA). It consists of a two channel amplifier, which is supported by Bravo NT Hardware and Frontier Master Software Ver. 6.0. The light stimulus was delivered through a specially designed LED probe, which contained an inbuilt circular gold ring (test electrode) (4.5 mm complementary to rat corneal size) at its apex to simultaneously record ERG from rat cornea. The stimulus probe was placed directly over the rat cornea, parallel to the visual axis with the help of a micromanipulator (Narshige, Germany). Reference electrode (Ag-AgCl) was placed into the rat mouth while the ground electrode was needle inserted into the tail skin. Ground and reference electrode contacts were maintained with the use of normal saline, while the contact of active electrode was maintained through the use of artificial tear. The

impedance between the electrodes was always below 5 KW throughout the study.

Experimental animals

Wistar rats weighing 250 to 300g body weight were maintained in regular 12h light/dark cycle with a room illumination of 20 Lux and room temperature at $25\pm 2^\circ\text{C}$. A dark room was used to house the experimental animals before the initiation of each experiment with free access to food and water *ad libitum*. Animals were dark adapted at least 12h prior to the study. Rats were anesthetized with an intraperitoneal injection of a mixture of ketamine (70 mg/kg body weight) and xylazine (20 mg/kg body weight). Anesthetized rat was placed on restrainer and active electrode was placed on the corneal surface with the help of a micromanipulator. Ground and reference electrodes were secured in place with the help of a contact gel with slight pressure. Animal body temperature was maintained by using a heating pad that maintained temperature at $37\pm 2^\circ\text{C}$ (by using a hot water circulator). The pupils were dilated with topical 1% tropicamide solution. Before placing corneal electrode, the eye was anesthetized with proparacaine. The surface of the cornea was kept moist with artificial tear drops. LED probe was placed over the surface of the rat cornea to aid simultaneous stimulation with light and recording with customized corneal gold electrode. One eye of each animal was exposed to single flash stimuli of 1.2 Lux intensity for the duration of 5 ms and trigger rate was set at 1cps and the other eye was served as an unexposed control. Each ERG response consisted of a pre-stimulus baseline, which was recorded for 30 ms followed by a stimulus record of 200 ms. Recorded ERG was taken as an average of 50 sweeps. Signals were recorded with 0.1 Hz -1 KHz band pass. Notch filters were applied at 50 Hz.

Experimental study

Wistar rat of either sex (n=8) was randomized into four groups. Group 1 was considered as a vehicle control (distilled water, 10 mL/kg), group 2 was treated with an oral ethambutol (200 mg/kg), group 3 and 4 were fed with oral ethambutol along with memantine (1 mg/kg, ip) and trimetazidine (3 mg/

kg, ip), respectively. All treatments were continued for 28 days and individual rat ERG was recorded on 0 and 21st day after the initiation of experiment. The ERG was recorded by green and white light conditions.

Ethambutol and EDDBA concentration in plasma, humors, ocular and body tissues

Aqueous humor was aspirated at 1st, 7th, 21st and 28th day after treatment with ethambutol and other respective treatment group animals. At the end of 28th day, animals were sacrificed with an overdose of anesthesia and blood was collected from each animal by cardiac puncture in EDTA vials. Eyes were enucleated and ocular tissues such as cornea, sclera, lens and retina were separated by standard method (17). Vitreous humor was also collected. Body tissues such as liver, kidney, lungs, heart, brain and muscles were collected and were kept in -80°C until the analysis by LC-MS/MS.

Statistical analysis

The ERG values were expressed as mean ± SEM. Student paired ‘t’ test was applied to compare the

values between day 0 and 21st day. Conc. of EMB and EDDBA in ocular fluids was compared amongst 1st day 7th, 21st and 28th day. Data was analyzed by using both Student paired and unpaired ‘t’ test. Data was considered to be significant at the p value of <0.05. Jandel sigma stat statistical software programme (ver. 3.5) used for the analysis.

Results

Effect of EMB on the body weight of rats

After 200 mg/kg oral administration of EMB, rats of either sex showed a progressive weight loss (30%) in 4 weeks. In between 3 to 4 weeks, 50% mortality was observed in EMB group. Although, the loss of body weight was observed in MEM as well as TMZ treated groups. No animal mortality was observed within the study period. The % loss of body weight is shown in the Fig. 1.

ERG changes in presence of NMDA and non-NMDA receptor antagonists against green and white light

After the oral administration of ethambutol at the dose of 200 mg/kg to the *Wistar* rats, a predictable

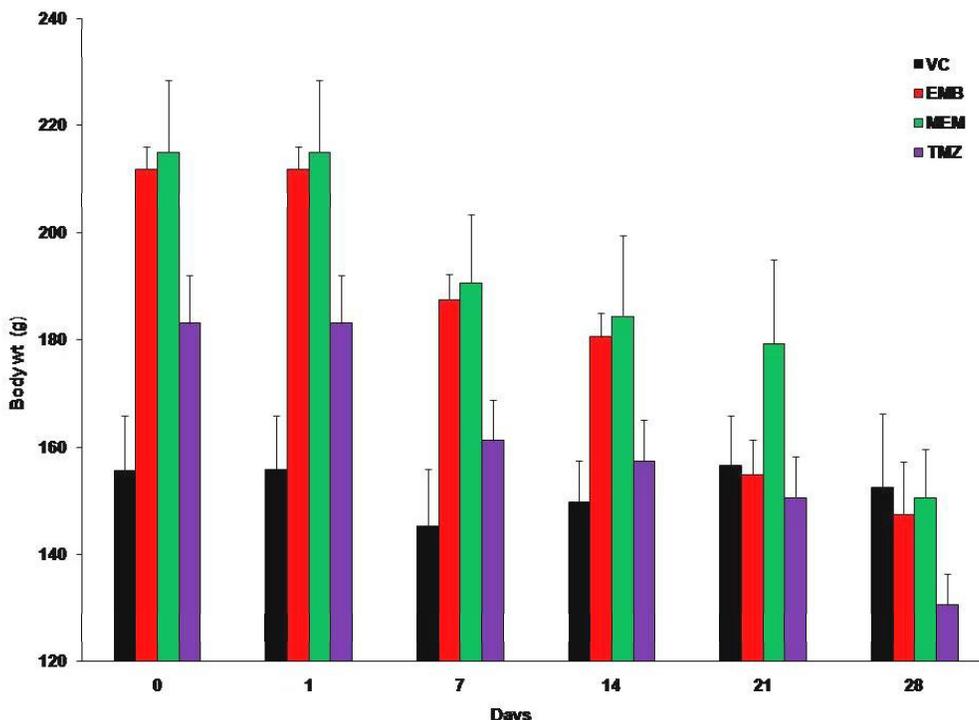


Fig. 1 : Percentage loss of body weight of the EMB treated rats (n=8).

decrease in 'b'-wave amplitude in ERG was observed on the 21st day. As a higher rate of rat mortality was observed on the 28th day, 21st day ERG values were taken for comparison among the groups. On the 21st day, EMB was found to significantly decrease the 'b'-wave amplitude of white (p=0.005) and green (p<0.001) light ERG. However, EMB did not show any predictable significant ERG change in 'a'-wave amplitude. Interestingly, NMDA antagonist memantine showed a significant (p=0.029) protection against the fall in 'b'-wave amplitude observed on the 21st day after EMB administration. Non-NMDA antagonist TMZ did not show any protection against the fall in 'b'-wave amplitude. The EMB induced ERG changes and effect of NMDA and non-NMDA antagonists in white and green light conditions are shown in the Fig. 2 and 3 respectively.

Quantification of EMB and EDDBA in plasma, humors, body and ocular tissues:

Plasma

After 28th day of EMB administration, plasma EMB

levels in control group 12 h after the last dose on 28th day was found to be 65.53 μ g/mL. At this time point, TMZ co-ingested animals showed significant increase in EMB levels (72.53 μ g/mL) and was found to be statistically significant. Interestingly, MEM co administration decreased the plasma EMB levels to 46.83 μ g/mL and was found to be significant as compared to control animals ingested only with EMB (Fig. 4a). Although, MEM and TMZ co-administration decreased EMB's major metabolite EDDBA as compared to control, the decrease was found to be insignificant (Fig. 4b).

Aqueous humor

EMB and EDDBA levels were found to increase continuously along with the administration of oral ethambutol in rat aqueous humor. The level of EMB was found to be significantly increased during co-administered MEM and TMZ treated group at 21st and 28th day after the treatment. The level of EDDBA was found to be significantly decreased in co-administered MEM treated group at the 21st day after the treatment. The levels of EMB and EDDBA were

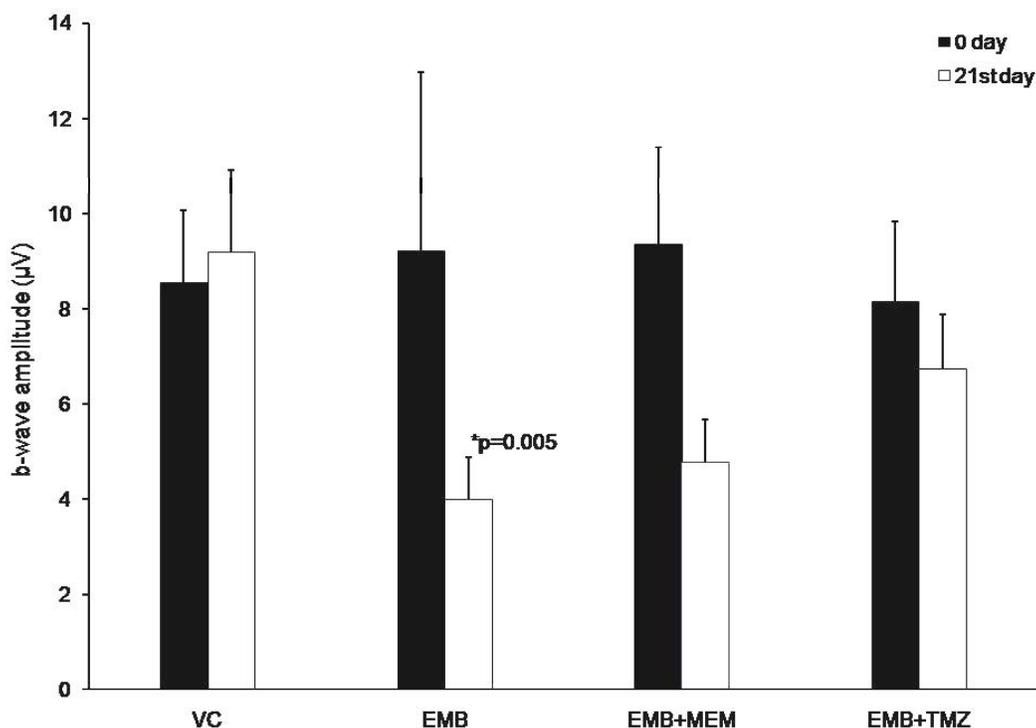


Fig. 2 : Protective effect of memantine (MEM) and trimetazidine (TMZ) against EMB induced changes in 'b'-wave amplitude in rat ERG with white flash light (n=8). The ERG values are expressed as mean \pm SEM. Note: *p< 0.05 (EMB treatment on 0 day vs 21st day).

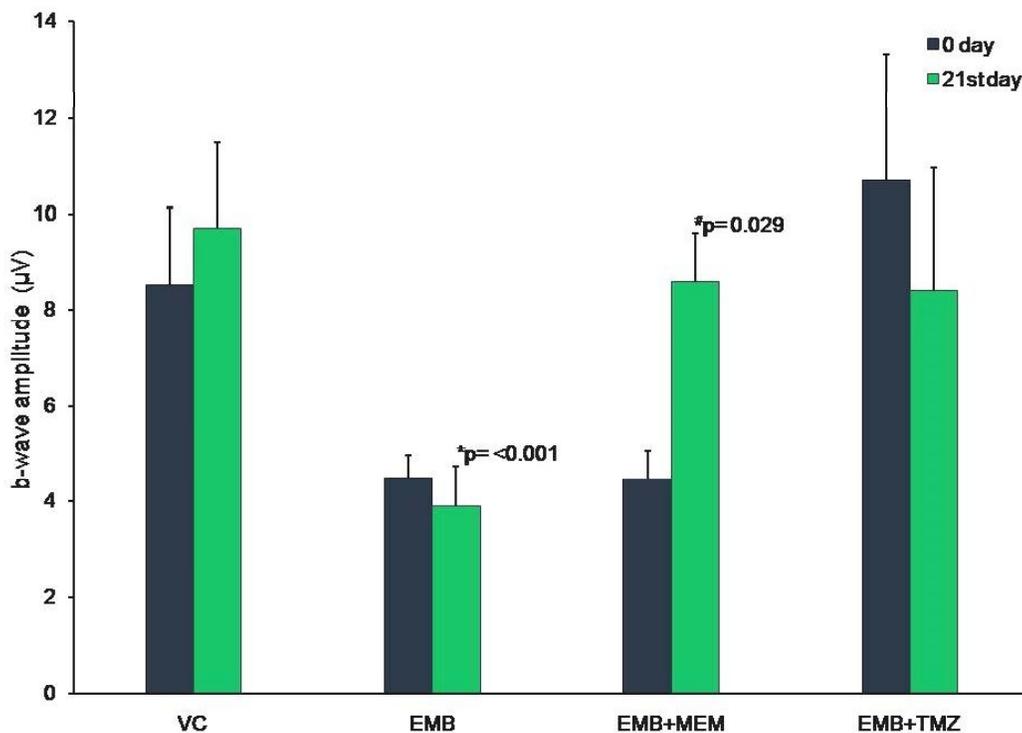


Fig. 3 : Protective effect of memantine (MEM) and trimetazidine (TMZ) against EMB induced changes in 'b'-wave amplitude in rat ERG with green flash light (n=8). The ERG values are expressed as mean±SEM. Note: *p<0.05 (EMB treatment on 0 day vs 21st day) and #p<0.05 (EMB+MEM treatment on 0 day vs 21st day).

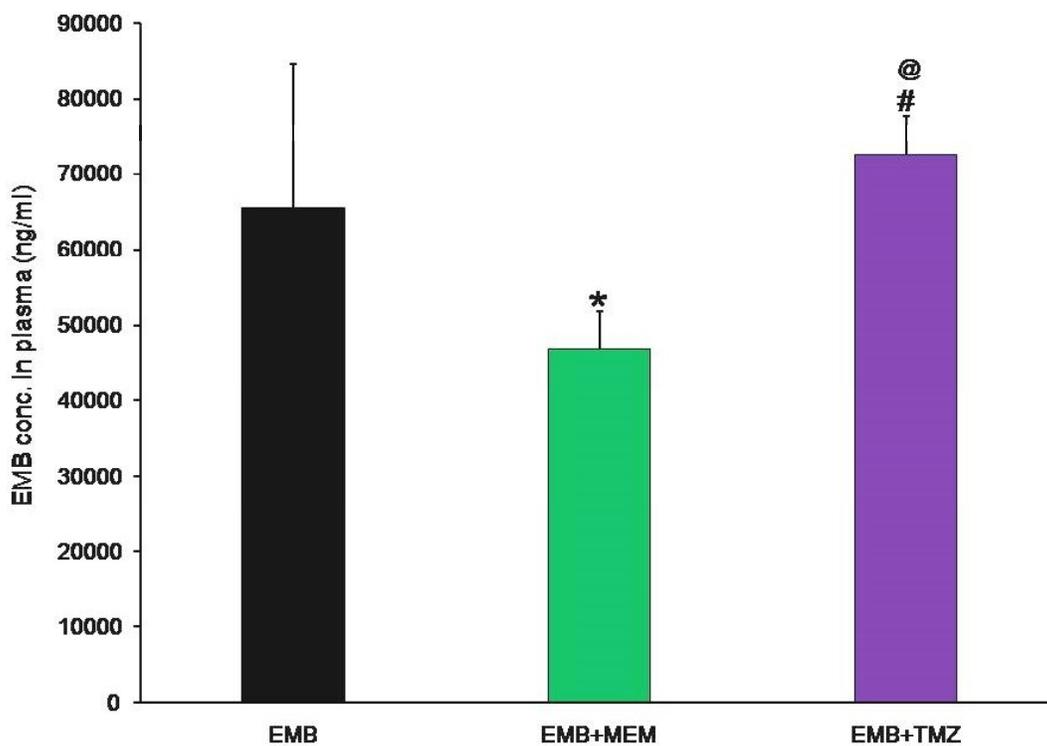


Fig. 4a : Mean EMB concentration in rat plasma after 28th day EMB treatment (n=4). The values are expressed as mean±SEM. Note: * = EMB vs EMB+ MEM, unpaired t-test (p<0.05); # = EMB vs EMB+TMZ, unpaired t-test (p<0.05) and @ = EMB+MEM vs. EMB+TMZ (p<0.05).

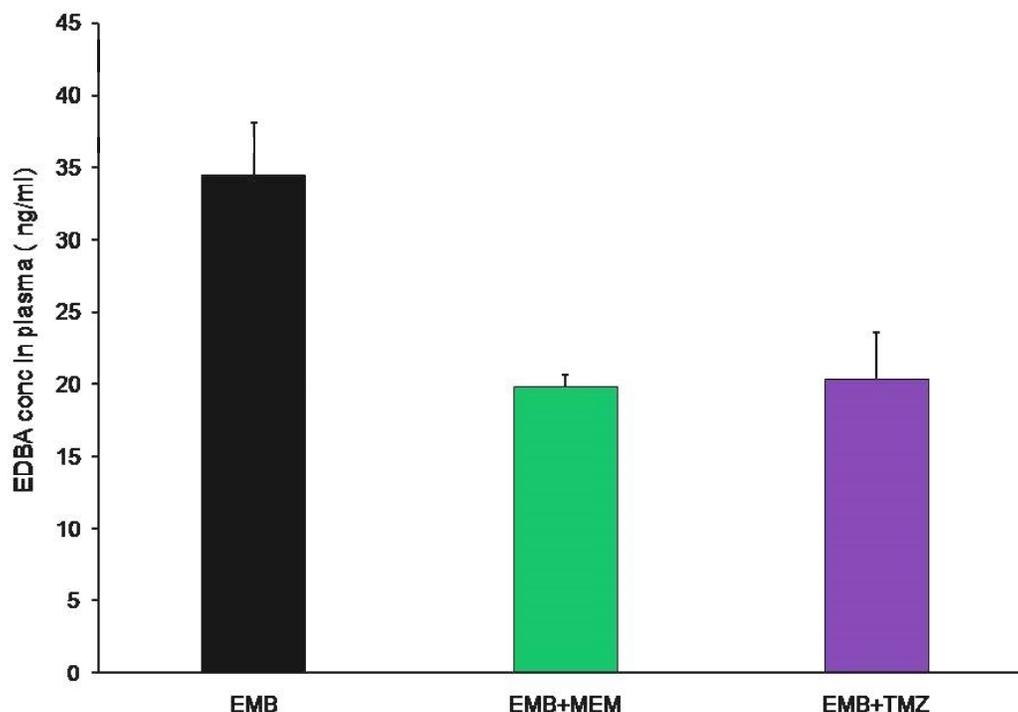


Fig. 4b : Mean EDDBA concentration in rat plasma after 28th day EMB treatment (n=4). The values are expressed as mean±SEM. Note: * = EMB vs EMB+ MEM, unpaired t-test (p<0.05); # = EMB vs EMB+TMZ, unpaired t-test (p<0.05) and @ = EMB+MEM vs EMB+TMZ (p<0.05).

shown in the figure 5a and 5b respectively.

Vitreous humor

EMB and EDDBA levels were found to be increased after administration of oral ethambutol in rat vitreous humor. The level of EMB was found to be significantly increased during co-administered MEM and TMZ treated group at 21st and 28th day after the treatment. The level of EDDBA was found to be significantly decreased in co-administered MEM treated group at the 21st day after the treatment. The levels of EMB and EDDBA were shown in the figure 6a and 6b respectively.

Ocular tissues

EMB and EDDBA levels in TMZ and MEM co-administered animal showed increase in retina. EDDBA level in MEM treated group showed increase in cornea, lens and sclera (Fig. 7a and Fig. 7b respectively).

Body tissues

EMB level was found to be decreased in liver, lung and heart, whereas the level was found to be increased in the kidney and muscles in MEM treated group animals. However, the EMB level in brain tissues was same in all the treatment groups. EMB level in TMZ co-administered group showed increased in all body tissues except muscles. EDDBA level in MEM and TMZ treated group showed increase in liver, kidney, heart and muscles (Table I and II).

Discussion

EMB therapeutic activity has been assessed in the experimental tuberculosis models of mice, guinea pig and monkey. Despite long standing evidence of the neurotoxic side effects such as toxic amblyopia, the underlying mechanism for the defects in color vision remained unclear for ethambutol (18). Several pathways and hypothesis were published to explain the toxicity. One of such explanations is that EMB

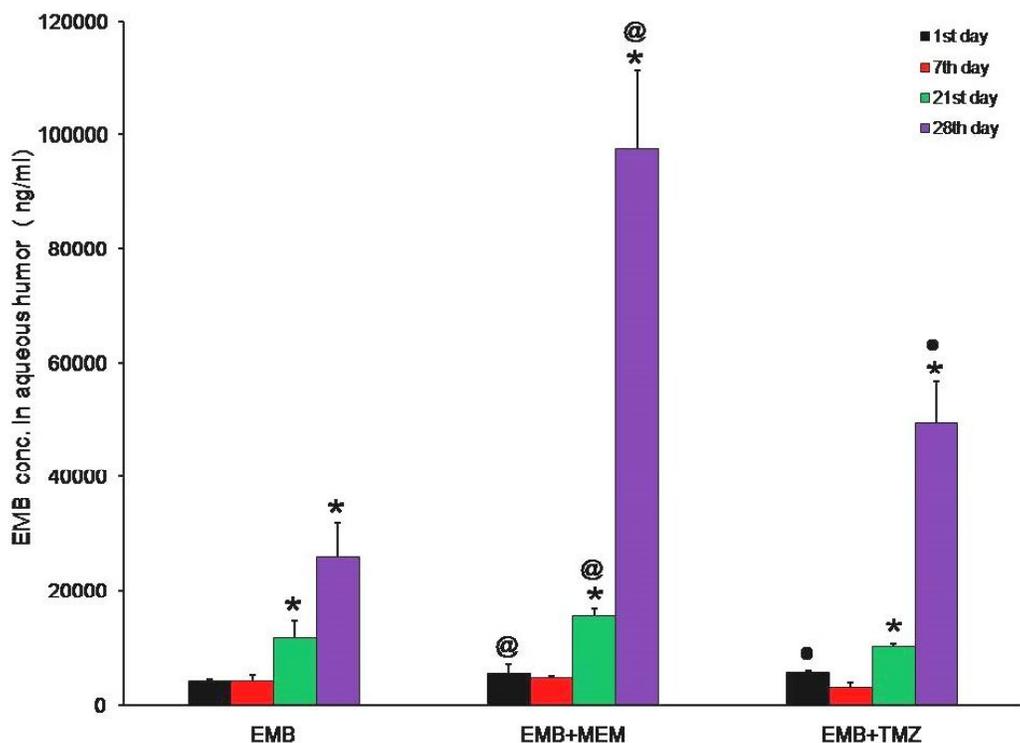


Fig. 5a : Mean EMB concentration in aqueous humor after EMB treatment (1, 7, 21 & 28th day) (n=4). The values are expressed as mean±SEM. Note: * = Conc. of EMB on 1st day vs 7, 21st & 28th day, paired t-test (p<0.05); @= EMB vs EMB+MEM, unpaired t-test (p<0.05); # = EMB vs EMB+TMZ unpaired t-test (p<0.05) and % = EMB+ MEM vs EMB+TMZ, unpaired t-test (p<0.05).

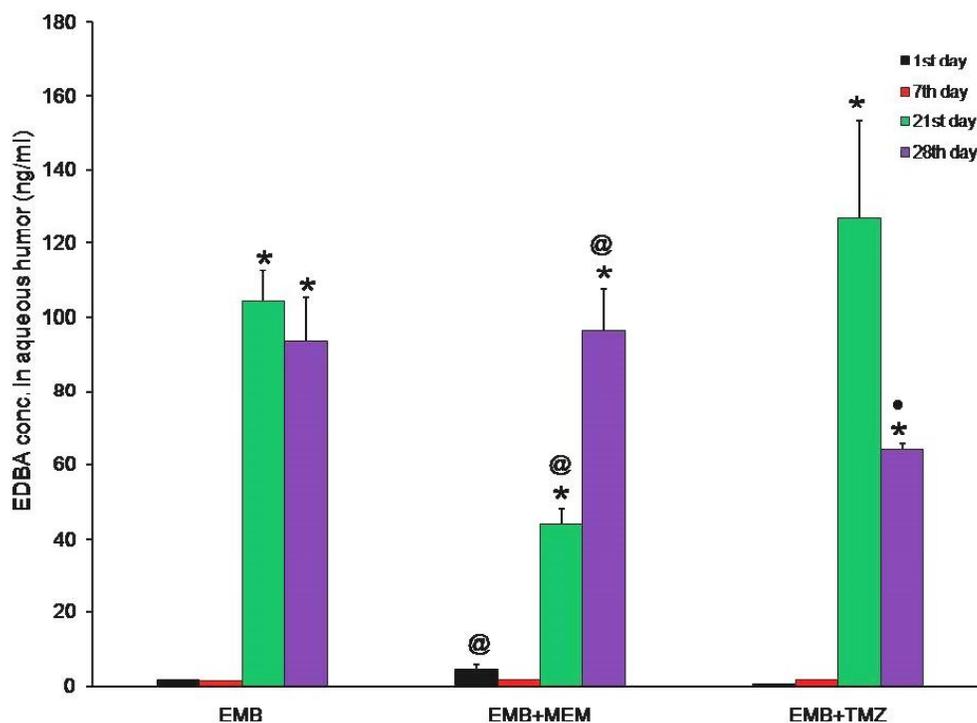


Fig. 5b : Mean EDDBA concentration in aqueous humor after EMB treatment (1, 7, 21 & 28th day) (n=4). The values are expressed as mean±SEM. Note: * = Conc. of EMB on 1st day vs 7, 21st & 28th day, paired t-test (p<0.05); @= EMB vs EMB+MEM, unpaired t-test (p<0.05); # = EMB vs EMB+TMZ unpaired t-test (p<0.05) and % = EMB+ MEM vs EMB+TMZ, unpaired t-test (p<0.05).

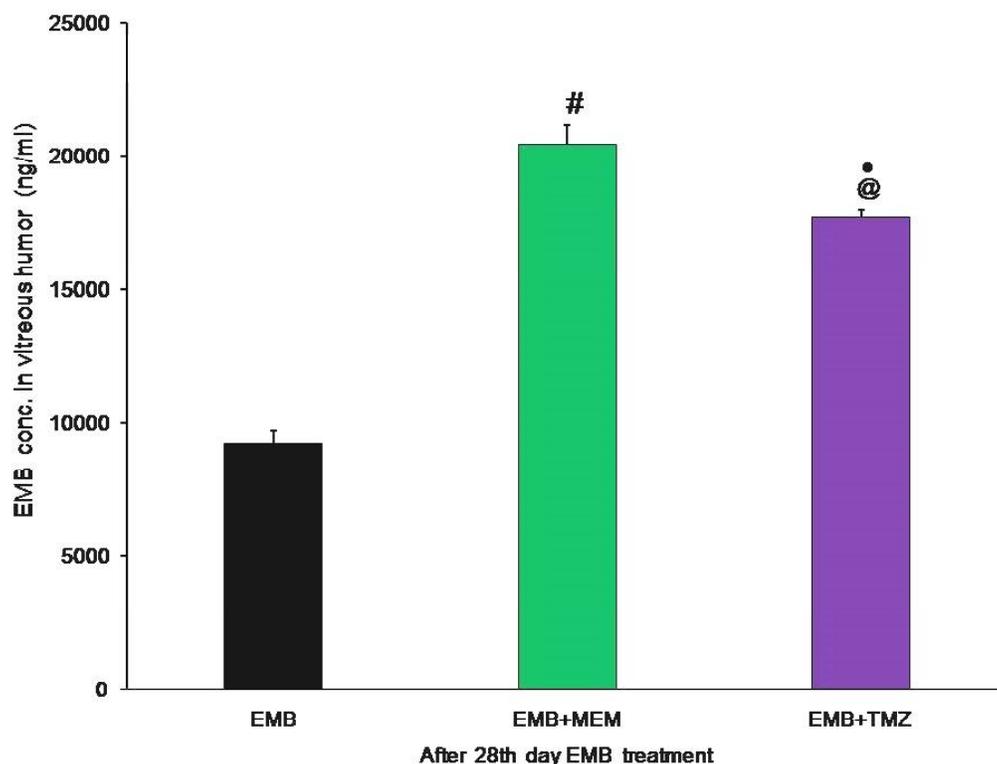


Fig. 6a : Mean EMB concentration in vitreous humor after 28th day EMB treatment (n=4). The values are expressed as mean±SEM. Note: # = EMB vs EMB+MEM, unpaired t-test (p<0.05); @ = EMB vs EMB+TMZ unpaired t-test (p<0.05) and % = EMB+ MEM vs EMB+TMZ, unpaired t-test (p<0.05).

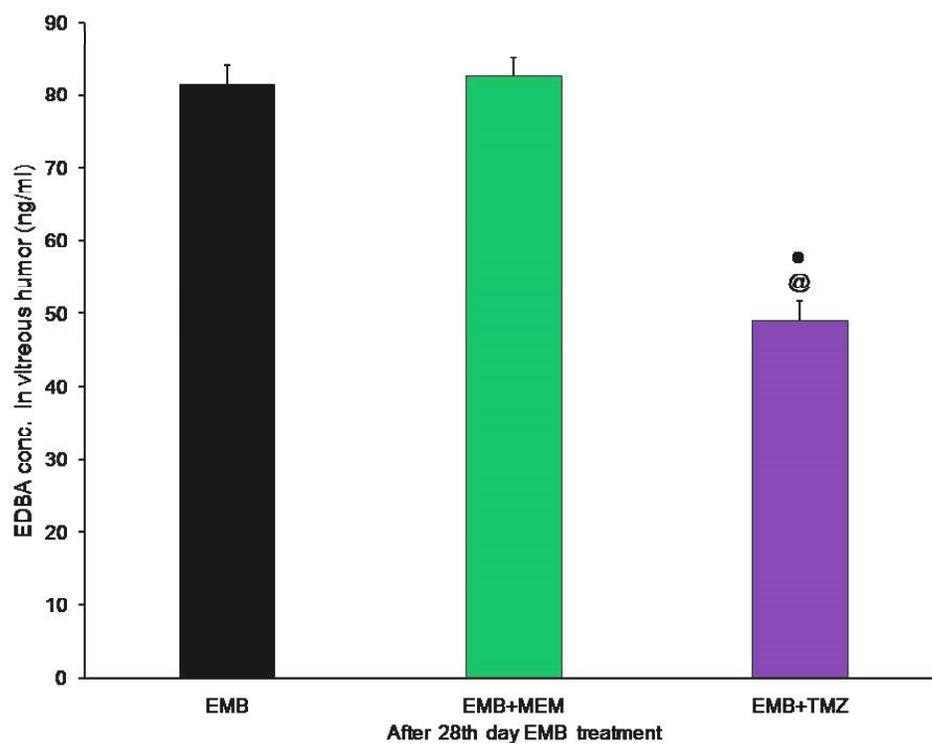


Fig. 6b : Mean EDDBA concentration in vitreous humor after 28th day EMB treatment (n=4). The values are expressed as mean±SEM. Note: # = EMB vs EMB+MEM, unpaired t-test (p<0.05); @ = EMB vs EMB+TMZ unpaired t-test (p<0.05) and % = EMB+ MEM vs EMB+TMZ, unpaired t-test (p<0.05).

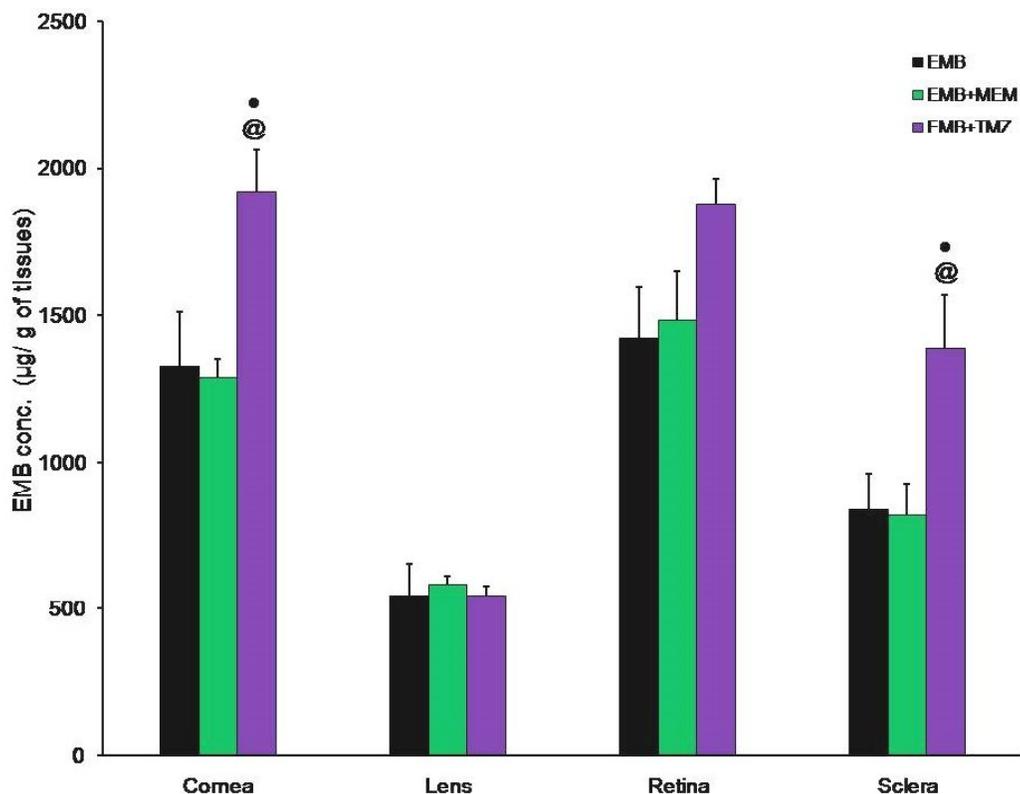


Fig. 7a: Mean EMB concentration in rat ocular tissues after 28th day EMB treatment (n=4). The values are expressed as mean±SEM. Note: # = EMB vs EMB+MEM, unpaired t-test (p< 0.05); @ = EMB vs EMB+TMZ unpaired t-test (p< 0.05) and % = EMB+MEM vs EMB+TMZ, unpaired t-test (p<0.05).

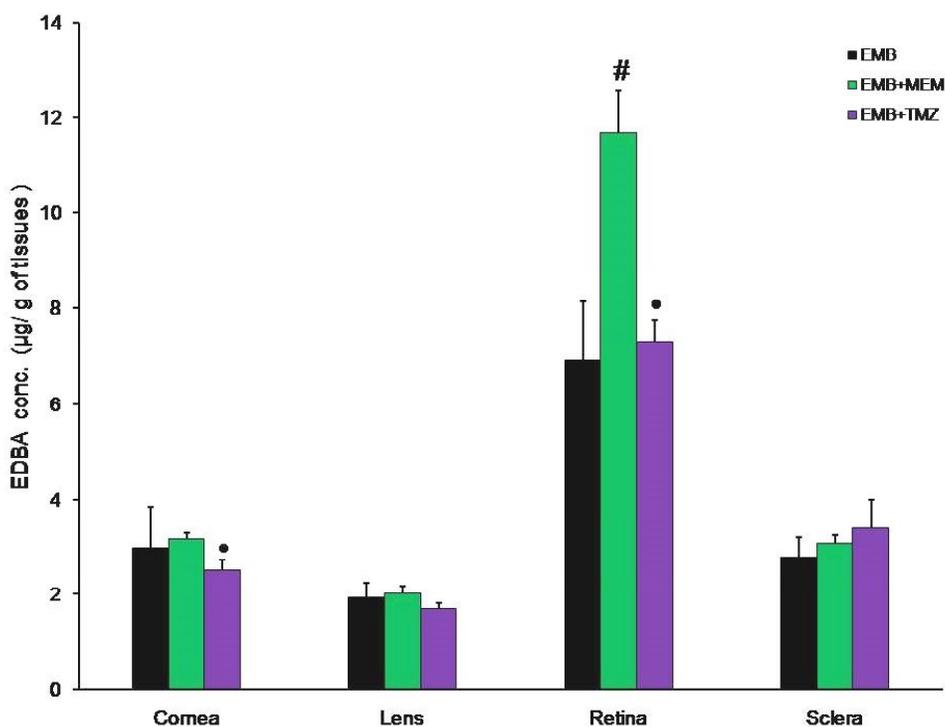


Fig. 7b: Mean EDDBA concentration in rat ocular tissues after 28th day EMB treatment (n=4). The values are expressed as mean±SEM. Note: # = EMB vs EMB+MEM, unpaired t-test (p<0.05); @ = EMB vs EMB+TMZ unpaired t-test (p<0.05) and % = EMB+MEM vs EMB+TMZ, unpaired t-test (p<0.05).

TABLE I: Mean EMB concentration in rat body tissues ($\mu\text{g/g}$) after 28th day EMB treatment (n=4). The values are expressed as mean \pm SEM.

Group	Liver	Kidney	Lungs	Brain	Heart	Muscles
EMB	24506.5 \pm 2681.2	11150 \pm 2731.8	4716.5 \pm 368.8	373.33 \pm 24.8	6563.33 \pm 401.5	5743.25 \pm 501.1
EMB+MEM	8400 \pm 1326.9	24236.5 \pm 1923.7*	3886.66 \pm 233	338.33 \pm 54	4026.66 \pm 47.8	5806.66 \pm 649.9
EMB+TMZ	24866.5 \pm 1449.3 [†]	20936.66 \pm 1780.3 [†]	5046.5 \pm 400.5 [†]	319.03 \pm 139.7	6866.6 \pm 395.3 [†]	4570 \pm 275.8 [†]

$\mu\text{g/g}$: Microgram/ gram, EMB: Ethambutol, MEM: Memantine, TMZ: Trimetazidine.

Note: * = EMB vs EMB+MEM, unpaired t-test ($p < 0.05$); [†] = EMB vs EMB+TMZ, unpaired t-test ($p < 0.05$) and [‡] = EMB+MEM vs EMB+TMZ, unpaired t-test ($p < 0.05$).

TABLE II: Mean EDDBA concentration in rat body tissues ($\mu\text{g/g}$) after 28th day EMB treatment (n=4). The values are expressed as mean \pm SEM.

Group	Liver	Kidney	Lungs	Brain	Heart	Muscles
EMB	2.81 \pm 0.31	4.61 \pm 0.82	1.64 \pm 0.14	1.10 \pm 0.11	2.25 \pm 0.40	2.05 \pm 0.07
EMB+MEM	3.18 \pm 0.31	19.73 \pm 1.99*	2.63 \pm 0.08*	1.07 \pm 0.05	3.62 \pm 0.28*	2.84 \pm 0.45
EMB+TMZ	4.60 \pm 0.06 [†]	7.40 \pm 0.56 [†]	1.27 \pm 0.06	5.31 \pm 1.35 [†]	4.09 \pm 0.34 [†]	2.18 \pm 0.17

EDDBA: 2, 2' ethylene diimino dibutyric acid, $\mu\text{g/g}$: Microgram/ gram, EMB: Ethambutol, MEM: Memantine, TMZ: Trimetazidine

Note: * = EMB vs EMB+MEM, unpaired t-test ($p < 0.05$); [†] = EMB vs EMB+TMZ, unpaired t-test ($p < 0.05$) and [‡] = EMB+MEM vs EMB+TMZ, unpaired t-test ($p < 0.05$).

chelates copper ion in the retinal ganglion cells (RGCs) and their fibers in the optic nerve. The metabolite of EMB, ethylene diimino dibutyric acid (EDDBA), is a strong chelator of copper ion. Copper ion is required as a cofactor for cytochrome C oxidase, a key enzyme in the electron transport chain and cellular oxidative metabolism within mitochondria. It is possible that EMB decreases the levels of EDDBA. In the present study, we found that EMB level in vitreous and retina chelates copper that is available for cytochrome C oxidase, and therefore the required energy for axonal transport around the optic nerve is compromised. In essence, mitochondrial insufficiency in the optic nerve fibers may underlie the impairment of axonal transport in the optic nerve leading to optic neuropathy (19, 20). Apart from the above findings, the possibility of EMB binding to the optic chiasma with high affinity causing bitemporal visual field a defect manifesting with toxicity has also been speculated (21, 22). Mitochondrial insufficiency, leads to excitotoxicity by rendering ganglion cells sensitive to normally tolerated levels of extracellular glutamate. This toxicity was reported to be due to decreased ATPase activity and mitochondrial energy homeostasis. Hence, the role of glutamate antagonists was justified by Heng et al., (1999) for limiting the side effects

seen with ethambutol. To evaluate the effect of NMDA and non-NMDA receptor antagonists in the ethambutol toxicity, the present study was conducted in rats and their preventive role was assessed using ERG.

Hence, the levels of EMB and its metabolite EDDBA reaching eye were felt as important. Drug level analysis was carried out in various tissues in the same rats after the chronic administration of EMB for 28 days. Recording the ERG provides functional assessment of the retinal toxicity before, during and after EMB administration. Retinal Muller cells are reported to be involved in generating the 'b'-wave by a K⁺ ion regulated mechanism (23). The Muller cells possess glutamate receptors with a high permeability to Ca²⁺ ions (24). A single oral dose of 25 mg/kg of body weight of EMB has been reported to attain a peak of up to 5 $\mu\text{g/mL}$ in serum within 4 h of administration and is less than 1 $\mu\text{g/mL}$ by 24 h in human. When the drug is administered daily for longer periods of time at this dose, serum levels are reported to be similar. About 80% of an oral dose of ethambutol is absorbed from the gastrointestinal tract, and the remainder appears in the feces unchanged. Absorption is not significantly impaired by food (25). Prevalently, ethambutol at the dose of

15 mg/kg is used for the treatment of tuberculosis in human. The incidence of optic neuritis is reported with the regularly recommended dose of 15 mg/kg oral dose (26).

However, to study the role of NMDA and non-NMDA antagonists, the present study calculated human equivalent rat dose of 105 mg/kg based on the rodent metabolism (27). Therefore, oral dose in rats was doubled (200 mg/kg) than the therapeutic dose and subjected for chronic treatment in rats up to 4 weeks. Ethambutol was administered orally in rats similar to that in human studies rather than subcutaneous route.

In plasma EMB concentration of 65 µg/mL reached 7 times lower concentration of 9 µg/mL in the vitreous. In contrast, plasma EDDBA concentration was found to be 0.034 µg/mL, whereas in vitreous the same was 2.3 times higher (0.081 µg/mL) than plasma levels. However, the EDDBA level in the plasma was found to be 1900 times lower than its corresponding EMB levels, 12h after the oral dose of EMB. The EDDBA level in vitreous was found to be 1140 times lower than its corresponding EMB level. Hang et al., 1999 found the half maximal effect (EC_{50}) for the proposed excitotoxicity of EMB *in-vitro* was 2 µg/mL and 0.2 µg/mL (10 times lower than EMB) for EDDBA.

In the present study, we found that EMB level in vitreous and retina was found to be 9.2 µg/mL and 28.46 µg/g of tissue respectively. Whereas, the metabolite EDDBA, showed 0.08 µg/mL and 0.14 µg/g for vitreous and retina respectively. Based on the EC_{50} values of Heng et al (1999), the vitreous and retinal levels of EMB achieved after the chronic oral administration of EMB were multiple times higher than that of EC_{50} require causing excitotoxicity to retina in rats. However, the EDDBA levels were found to be lower than that of the EC_{50} values reported by Heng et al., (1999). Therefore, the excitotoxicity observed in the present study on 21st day ERG could be attributed to the elevated EMB levels. However, the possibility of lower levels of EDDBA marginally contributing to the toxicity of EMB cannot be ignored.

Chronic administration of EMB at the dose of 200

mg/kg caused detectable ERG changes on 21st day after the initiation of the study. As ethambutol has been reported to affect the color perception, this study also used different color stimulation to evaluate the possibility of recording the change in color perception. As rats have been reported to have the cone absorption spectra that peak at lower (359 nm) and middle (509 nm) wave lengths (28), this study used green light apart from standard white light flash ERG. The results of the present study showed that EMB induced significant ERG changes in the green light (510 nm) as compared to white light.

Memantine (MEM) is a noncompetitive, NMDA receptor open channel blocker which can enter the channel and block current flow only after the opening of the channel. It is a derivative of amantadine and is used in the effective treatment of Alzheimer's, Parkinson's and Huntington's diseases (29–32). Memantine is also under investigation as a potential treatment for other neurodegenerative disorders, including HIV associated dementia, neuropathic pain and glaucoma (33). It mainly binds at or near Mg^{2+} ion channel pore region of NMDA receptor associated channel (34–39). Therefore, it has been evaluated in the present study to prevent the excitotoxicity. Although, memantine did not show any protection in white light condition, in green light ERG it has shown significant ($p=0.029$) protection.

Trimetazidine (TMZ) (1 [2, 3, 4-trimethoxy- benzyl] piperazine) is one of the non-NMDA receptor antagonist and it is widely used in disease related to ischemia in cardiovascular conditions (40, 41), in otology (42) and in ophthalmology (43, 44). TMZ also inhibits platelet adhesion aggregation, neutrophil infiltration and the generation or activity of oxygen derived free radicals (45).

TMZ has been reported to inhibit anaerobic glycolysis, fatty acid metabolism and acidosis. Apart from this, it is also reported to inhibit free radical accumulation in the cell and cytoprotective properties. All these properties are promoting cell to restore the normal ionic condition and metabolic balance. It is frequently used as a prophylactic treatment for episodes of angina pectoris and ischemic chorioretinal disturbances. TMZ was blocked by calcium and

current response induced by AMPA / kainate receptors located on primary afferent nerve fibers (46, 47). Anti-ischemic effect of trimetazidine in rat inner retinal layers (48, 49) and an anti excitotoxic activity by diminishing extracellular glutamate accumulation during ischemia conditions have been well documented (50). Therefore, TMZ has been selected in the present study for the evaluation to reduce the excitotoxicity of ethambutol. The results of the present study for showing that among all the color used in ERGs studies, TMZ showed to decrease the excitotoxicity in white light condition however, the protection offered was found to be statistically insignificant. In the present study the EMB induced changes were observed only in 'b'-wave as compared to 'a'-wave indicating the possibility of toxicity occurring in horizontal cells of the retina rather than photoreceptors. This observation goes in concurrence with the earlier findings (51).

To conclude, the present study was conducted to evaluate the ethambutol toxicity by ERG after chronic administration in rats and the role of NMDA and non-NMDA antagonists were evaluated for their efficacy in reducing the excitotoxicity. This study was further extended to evaluate the levels of ethambutol, and its metabolite in body and ocular tissues and fluids. The results of this study indicated that NMDA receptor antagonist may be having a role in the prevention of ethambutol induced excitotoxicity.

Ethambutol level in rat plasma was found to be multiple times higher than the vitreous. In contrast, plasma EDDB level was lower than vitreous. Drug concentration measurement indicated that the levels achieved by ethambutol in the vitreous of the chronic EMB treated rats were multiple times above and EDDB levels were below the reported EC_{50} values in excitotoxicity induced by glutamate.

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