Original Article

Ethambutol induced Histological Changes in Optic Nerve of Rabbit

Muhammad Zubair, Imran Ahmad, Muhammad Habib ur Rahman, Khalid Mahmmod Akhtar

ABSTRACT

Objectives: Ethambutol (EMB) induced ocular toxicity The current study was designed to check the ethambutol (EMB) induced histological changes in optic nerve of rabbit. **Study Design:** An experimental Randomized Control Trial (RCT). **Place and Duration:** The study was conducted in the experimental research Laboratory of University of Health Sciences, Lahore from January 2009 to December 2009. **Material & Method:** Ten, New Zealand white, albino rabbits of either sex, 6-12 month old, weighing 1- 2 kg were randomly divided in to two groups comprising five animals in each. Group A served as control, while Group B was given ethambutol 100 mg/kg/day for four weeks. At the end of experimental period, each animal was sacrificed under chloroform and both optic nerves along with chiasma were taken out and preserved. In this way, twenty optic nerve specimens (ten for each group) were collected from ten albino rabbits.

Results: The histological study showed that optic nerves of group A were quite normal, while the histological preparation of group B showed decrease diameter of optic nerves and cytoplasmic vacuolations. Statistical analysis was done using Independent "t" test for measurement of diameter of optic nerve and counting the number of vacuoles. The results showed an immense vacuolations ($p < 0.05$) and decrease in diameter of optic nerve ($p > 0.05$) in the sections from group B as compared with that in the group A. However, there was no evidence of inflammatory changes and myelin-like structure in any preparation from the two groups **Conclusion:** Ethambutol(EMB) induced histological changes produce vacuoles, representing damage to the optic nerve.

Key Words: Ethambutol (EMB), optic nerve, optic chiasma, cytoplasmic vacuolations.

INTRODUCTION

EMB is a water soluble heat stable compound and one of the first line synthetic antimycobacterial drug.[1,2,3] It has lower incidence of toxic side effects.[10], with no evidence of teratogenecity.[4] its antimycobacterial potential is due to its chelating property that disrupts the essential metal containing enzyme systems that causes bacterial cell death.[5] Its bacteriostatic and bactericidal effects are achieved at daily doses of 15 mg/kg and 25 mg/kg, respectively.[6,7] EMB has been reported to cause bulbar and retrobulbar neuritis, latter being more common, is manifested as painless, subacute, symmetrical, progressive visual loss, reduced visual acuity, central or caecocentral scotoma and dyschromatopsia.[8, 9,10] EMB induced ocular toxicity depends on dose and duration pertaining to the individual susceptibility, however, it may prove to be toxic at any dose even as low as 12.3 mg/kg/day.[10, 11] It has been observed that EMB induced ocular toxicity is reversible on discontinuation of the drug, however, a permanent damage has been reported in some cases at the therapeutic doses of 15-25 mg/kg/day.[8, 9] Several factors have been reported to be associated with irreversible damage such as old age, delayed diagnosis, alcoholism, diabetes mellitus, anemia and renal failure.[12] Human optic nerve is composed of 1,100,000 to 1,200,000 myelinated axons with 53:47 ratio of crossed to uncrossed nerve fibers. 90 % of these are of small diameter (1μm) and the remaining 10 % have a diameter of 2 to 10 μm.[28] EMB-induced optic neuropathy, results in loss of small diameter axons.[3] Different experimental animals treated with toxic doses of EMB revealed multiple cystic lesions,

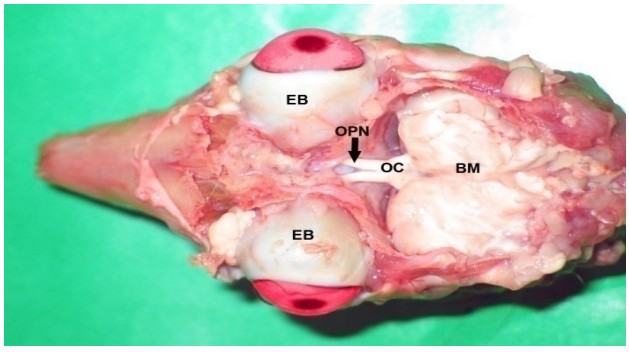
mild demyelination, axonal fragmentation, myelin like structure in the axoplasm and central necrosis with inflammatory changes in their optic nerve.[14,17]Aim of this study was to verify EMB induced histological changes in optic nerve of rabbit.

MATERIAL AND METHODS

Ten, New Zealand white, albino rabbits of either sex, 6-12 month old, weighing 1- 2 kg were procured from the Veterinary Research Institute, Lahore. Each rabbit was housed in a separate cage under controlled conditions of temperature 20 ± 0.5º C, humidity (50 ± 10%) and 12 hours light and dark cycle. They were fed on standardized diet and water ad libitum. The experiment was started by randomly dividing the animals into two groups. All interventions were done through feeding tube once daily for 4-weeks. Group A served as a control, and was fed with distilled water equal in volume given to experimental group B. Group B was treated with 100 mg / kg body weight / day of EMB. At the end of the experimental period, each animal was scarified under anesthesia, the scalp was removed, skull cap detached free from the dura mater and the brain with its meninges was exposed by lifting the calvarium. Falx cerebri was detached from the crista galli and pulled posteriorly. The frontal lobes were lifted from the anterior cranial fossa and incised to expose the orbital plates before removing them. Both optic nerves along with chiasma were thus exposed (Fig. 1) and the nerves were severed from the eye ball.[18]

Figure-1

Photograph of rabbit showing optic nerve in situ (OPN), optic chiasma (OC), eye ball (EB) and brain matter (BM), after removing frontal lobe of cerebrum and the floor of anterior cranial fossa



Optic nerve was taken as a single piece by incising it proximal to the optic chiasma, washed with normal saline and fixed in 10 % formal saline for 24 hours. The tissue pieces were processed, dehydrated by passing through ascending grades of alcohol using 50%, 70%, 90% and 100% ethanol, cleared in xylene and infiltrated with molten paraffin wax (melting point 56-58°C). The tissue pieces were placed vertically in Leuckhart's moulds, to obtain transverse sections of the intracranial part of optic nerve, embedded in molten paraffin. Tissue blocks were solidified, trimmed at chiasmatic end, sections cut at 5 µm thickness, were shifted to water bath at 45-50°C. Sections were then transferred on the surface of albumenized glass slide, dewaxed in xylene, hydrated and stained with hematoxylin and eosin (H & E) in a usual way. To demonstrate the myelin sheath, luxol fast blue and cresyl violet stains (LFB & CV) were used in separate preparations.

The prepared slides were examined under the light microscope (Leica, DM 1000) at different magnifications and observations were recorded in term of measurement of diameter, vacuolation, inflammatory changes and myelin- like structure in axoplasm. Vacuoles were counted per 0.50 mm^2 randomly selected area, after calibrating the ocular graticule with stage linear micrometer, using X40 objective[19]. Diameter of the optic nerve was measured after calibrating ocular micrometer with stage linear micrometer, using X10 objective.

STATISTICAL ANALYSIS

Statistical analysis was conducted using the computer software, statistical package for social sciences (SPSS version 13.0). The difference in number of vacuoles and diameter of the optic nerve, between control and the experimental group was analyzed by using the independent sample t-test. The significance level was set at $p< 0.05$.

RESULTS

Light microscopic examination revealed that the optic nerve of rabbit was surrounded by the three meninges with weak, branching septa arising from pia matter, extending transversely across the nerve. The nerve itself comprised of axons of ganglion cells, supported by astrocytes and oligodendrocytes. Invariably all sections showed branches of central

retinal artery inside the pial septa of the optic nerve (Fig. 2). There was no evidence of inflammatory changes and myelin like structure in the axoplasm of optic nerve fibers in control and experimental groups.

VACUOLATION

All sections of the two groups showed variable number of vacuoles per 0.50 mm^2 area of each optic nerve preparation. Group A exhibited 2-4 number of vacuoles that was immensely increased (>200 per 0.50 mm^2) in each optic nerve preparation from four out of five rabbits of experimental group B (Fig. 3).The sections from each optic nerve of fifth animal showed 14 (mean number of vacuoles per 0.50 mm^2).

Figure-2

Optic nerve from group a showing a few small sized vacuoles (V), connective tissue septa (CTS), blood vessels (BV) and oligodendrocyte (OLG) H & E Stain, X 100

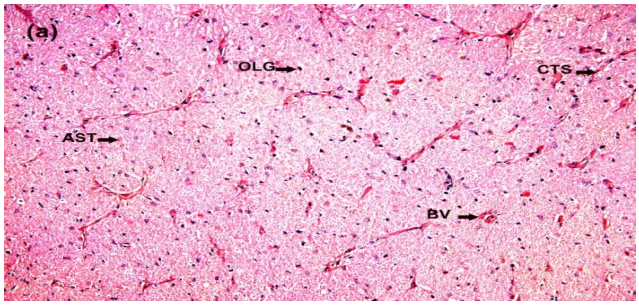
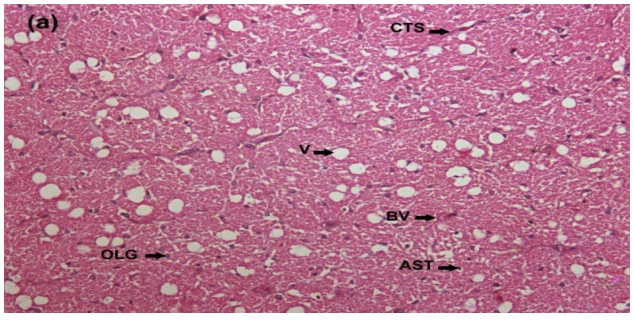


Figure-3

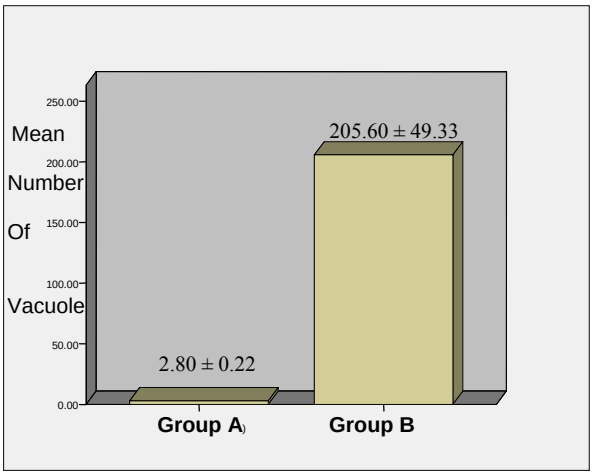
optic nerve section from group B, showing A large number of vacuoles of different sizes in the neurites of OPN (V), connective tissue septa (CTS), oligodendrocytes (OLG) and blood vessels (BV) H & E, stain, X 100



The statistical analysis of the changes in number of vacuoles, using independent sample t-test showed that mean number of vacuoles calculated in group B was 205.60 ± 49.33, that was significantly increased from 2.80 ± 0.22 that in group A ($p< 0.05$) as shown in Fig. 4.

Figure-4

Bargraph showing mean and standard error of number of vacuoles in groups A and B.

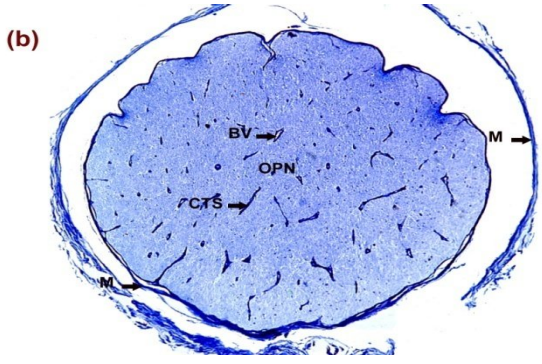


Diameter of Optic Nerve

Diameter of optic nerve from all sections of experimental group B was observed to have decreased as compared to those from group A, as shown in Fig. 5 and 6.

Figure-5

Cross section of optic nerve from group A showing meninges (M), blood vessels (BV) and connective tissue septa (CTS) with a diameter (1.13 ± 0.03 Mm LFB & CV stain, X 50



Mean diameter of optic nerve calculated in group A was 1.13 ± 0.03 while mean diameter in group B was 1.03 ± 0.03. Statistical analysis using Independent sample t-test comparison showed that change in diameter is insignificant (p>0.05) as shown in Fig. 7

Figure-6
Photomicrographs of cross section of rabbit optic nerve from group B, showing A decrease in diameter (1.03mm ± 0.03) as compared with that in group a (1.13mm ± 0.03), H & E stain, X 50

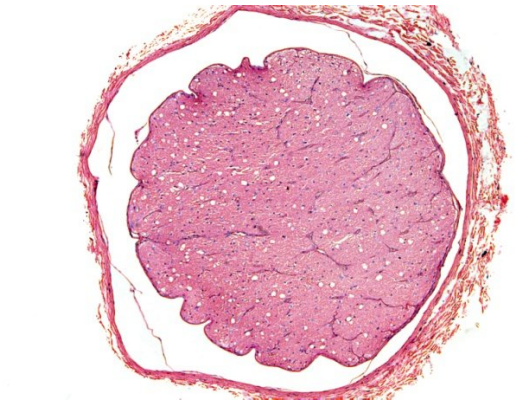
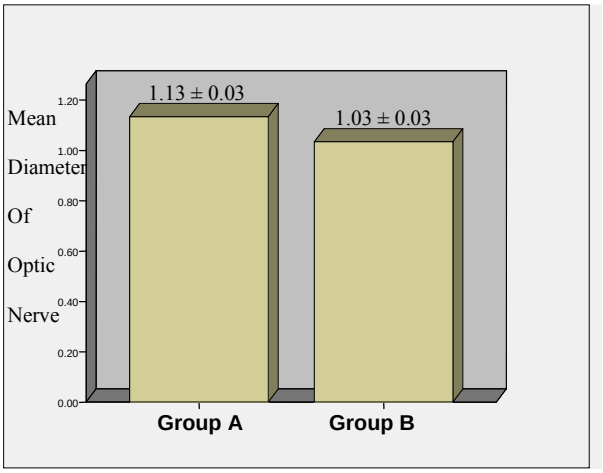


Figure-7
Bargraph showing mean and standard error of diameter of optic nerve in groups A and B



DISCUSSION

EMB produces histological changes in optic nerve by impairing the mitochondrial functions that disturb the physiology of axonal transport and axon-myelin interactions,this leads to a complex sequence of events that results in damage to the axons.[20, 21] The axonal damage may present itself as a change in the diameter of optic nerve therefore we investigated the same as a first parameter. It was observed that in group B, the diameter of the optic nerve was decreased, when compared with group A, the difference was statistically insignificant (p>0.05). Our observations seem to be pioneer in this respect since the search of literature did not reveal any comparable investigation. Demyelination, inflammatory changes, myelin like structure in the axoplasm and vacuolation had already been reported in rat dog, monkeys and rabbits.[14 – 17, 22] Our investigations revealed only prominent vacuolation in group B, other effects reported earlier were not seen in our preparations. The differences in our results could possibly be explained on account of a wide range of EMB dose, varying from 50 to 2500 mg and experimental period from two weeks to one year, observed in the previous work. In our study EMB was used in judicious dosage of 100 mg / kg / day for only four weeks to find its toxic effects.

In the current study, vacuoles were observed in almost all preparations obtained from experimental and control groups, however these were small in number in the latter. In the control, these vacuoles were presumably produced on account of dissolution of lipid constituents by the lipid solvent used in processing the preparations.[23] EMB produced marked vacuolation in four out of five rabbits of the experimental group B (mean = 205.60 ± 49.33) that was significantly greater than control group A (2.80 ± 0.22) (p<0.05).The results of our investigation are supported by the earlier observations reported in many studiess.[14-22, 17] In a study conducted in 1976, EMB was observed to cause a bilateral stereotyped clustered or scattered vacuoles in the distal part of optic nerve and optic chiasma without any evidence of demyelination.[14] This observation was also supported by additional experimental studies,[15-17] using different experimental models. Their authors established a predilection for EMB induced lesion to develop in optic chiasma and its adjoining parts in the anterior visual pathway. Different hypothesis

were suggested to justify the reasons for specific distribution of these lesions in the optic nerve, including the selective effect of EMB in these parts, or it could be due to variations in the blood vasculature, or through physical contact with the cerebrospinal fluid.[14] EMB induced vacuolation was contradicted by Cappiello VP and Layton WM (1964). They reported multiple histological changes in brain, heart, liver, lung and spleen produced by EMB but could not detect vacuolation in optic nerve or chiasma of dogs.[29] The authors could not however, explain the reasons for this difference. Toxic effects of EMB had also been reported in form of distinct cytoplasmic vacuolar degeneration and neuronal loss in the rat's retinal cell culture.[24]

Vacuolation forms the histological base of ocular toxicity produced by EMB by its indirect stimulation of N -methyl-D-aspartic acid (NMDA) channel, present in the retinal ganglion cells by the endogenous glutamates. Ganglion cells become more sensitive to glutamate due to an increased concentration of extracellular calcium[25] and disturbance of energy balance in mitochondria. EMB is also reported to chelate intracellular Zinc which is essential for modulation of endonuclease. This enzyme becomes dysregulated in the absence of Zn, resulting in DNA cleavage and cell death. Other target of Zn chelation by EMB is the ATPase inhibitory protein IF1 which itself is inhibited by Zn. Chelation of Zn allows the inhibition of ATPase acitivity by IF1 that results in decreased ATP synthesis.This inhibits mitochondrial dehydrogenase activity, resulting in death of ganglion cells. EMB also chelates iron and copper which are essential for complex I and IV function respectively in the electron transport chain. It interferes with the process of oxidative phosphorylation and causes a decrease in ATP production and compromises the axonal transport, thus initiating the cascade of events leading to cell death. [21, 25, 26, 27, 28]

The exact mechanism, underlying the formation of vacuoles is not known, however, two possibilities are proposed; EMB mediated increased Ca^{+2} influx activating certain intracellular enzymes including phospholipases, resulting in cell membrane damage,proteases promoting digestion of membrane and cytoskeletal proteins endonucleases associated with chromatin fragmentation and ATPases resulting in ATP depletion. The result is the failure of $Na^{+}K^{+}$ pump leading to cellular swelling, anaerobic glycolysis, impaired protein synthesis and accumulation of lipids inside the cell that microscopically appear like vacuoles due to processing techniques. In other mechanism, the metabolites of EMB form a complex with Zn and Cu which enters the axons and enhance the axonal dilatation (vacuolation) process.[27]

CONCLUSION

Present data in this study, concludes that EMB (100 mg / kg / day for four weeks) given to an experimental group of rabbits, results in different levels in the ureter. One should be very careful in marked vacuolation in the intracranial part of their optic nerve and an insignificant decrease in diameter of the optic nerve, indicating the toxic effects of EMB.

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AUTHORS

- **Dr. Muhammad Zubair**
  Assistant Professor Anatomy
  Quaid e Azam Medical College, Bahawalpur
  zubairuhs@gmail.com
- **Dr. Imran Ahmad**
  Associate Professor of Anatomy, Quaid e Azam Medical College, Bahawalpur
- **Dr. Muhammad Habib ur Rehman**
  Assistant Professor Anatomy
  Nawaz Sharif Medical College, University of Gujrat
- **Dr. Khalid Mahmood Akhtar**
  Associate Professor of Anatomy
  Punjab Medical College, Faisalabad