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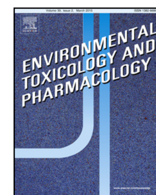

# Ethambutol induces testicular damage and decreases the sperm functional competence in Swiss albino mice

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ABSTRACT

The present study reports the effect of ethambutol (EMB) on testicular function. Prepubertal and adult male Swiss albino mice were treated with 40, 80, 160 mg/kg body weight of EMB, intraperitoneally, every alternate day for 4 weeks. After 2 weeks gap, mice were sacrificed to collect caudal spermatozoa. EMB treatment resulted in a dose-dependent decrease in the testicular weight, sperm count and motility while the percentage of sperm with head abnormalities, immature chromatin ($P < 0.001$) and DNA damage increased ($P < 0.01$). In addition, EMB treatment resulted in significant depletion of glutathione ($P < 0.05$–$P < 0.01$) and histopathological abnormalities such as large cells, vacuolation of tubules and isolated colonies of spermatogenic cells were observed. Oct4, 17β-Hsd and c-Kit mRNA was marginally elevated in EMB treated testes at the highest dose studied. In conclusion, the result of the present study indicates that EMB has adverse effect on testicular function and impairs the sperm functional competence.
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## 1. Introduction

Tuberculosis (TB) is the most common infectious disease caused by single infectious agent *Mycobacterium tuberculosis.* It remains one of the world's deadliest communicable diseases and second leading cause of death. According to the recent statistics, approximately one-third of the world's population is infected by TB in which 95% of the cases are reported from the developing countries every year. The incidence of this disease in India is very high especially in areas of dense population, poor nutrition and sanitation (Kamal et al., 2008).

Ethambutol (EMB) is a synthetic aminoalcohol and orally effective bacteriostatic drug used to treat pulmonary TB, usually in combination with other antituberculosis drugs. The compound is active against mycobacterium, including strains of *M. tuberculosis* that are resistant to isoniazid and streptomycin. It is indicated for prophylaxis in cases of inactive TB. The site of action of EMB ranges from interfering with trehalose dimycolate, mycolate and glucose metabolism to spermidine biosynthesis. However, the primary site of action seems to be arabinan biosynthesis. Activity of EMB is stereospecific and the dextro isomer exhibited maximum anti-tubercular activity (Kamal et al., 2008). A recent study by Fatima et al. (2013) using *in vitro* system has shown that EMB has significant cytotoxic and mutagenic action. *In vivo* studies have ascertained its toxic effects on liver, kidney, optic nerves (Younossian et al., 2005; Jaswal et al., 2013; Huang et al., 2015).

Studies pertaining to the effect of antituberculus drugs, especially EMB, on the testicular function are very few. An earlier study by Trentini et al. (1974) have reported that chronic administration of low doses of EMB to rats leads to testicular lesions characterized by regressive changes, parenchymal atrophy and arrest of spermatogenesis. Shayakhmetova and coworkers have recently shown that EMB can cause testicular damage and reduce the reproductive capacity of the male rats when injected alone (Shayakhmetova et al., 2016) or when co-administered with rifampicin, isoniazid and pyrazinamide (Shayakhmetova et al., 2012). Since the incidence of TB in children of prepubertal age group is very high in countries like India (Velayutham et al., 2015; Israni et al., 2016), it is important to know the consequences of commonly used drug EMB on the sperm functional competence when exposed at prepubertal stage. Hence,

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the present study was aimed to assess the testicular toxicity of EMB in both prepubertal and adult mice, in relation to sperm functional competence, endocrine function and gene expression pattern in testes.

## 2. Materials and methods

### 2.1. Animal model

Swiss albino male mice maintained in Central Animal Research Facility, Kasturba Medical College, Manipal University, Manipal, were used for the study. The animals were maintained under standard conditions of temperature (25 ± 2 °C), humidity (45–55%) and light (12:12 h of light and dark). Food and water were available to the animals *ad libitum*. The experiment was carried out with the approval of Institutional Animal Ethical Committee (IAEC/KMC/43/2010–2011).

### 2.2. Preparation of the drug

Ethambutol dihydrochloride, was purchased from Sigma-Aldrich, St. Louis, MO, USA (Cat. No. E4630). The drug was freshly prepared each time just before the injection by dissolving it in Phosphate-Buffered Saline (PBS, pH 7.4).

### 2.3. Animal treatment

The pre-pubertal (2 weeks) and adult (8–12 weeks) male mice were divided into 4 groups, with six animals in each group. The mice were injected intraperitoneally (i.p.) with 40, 80 and 160 mg/kg body weight of EMB, once in two days for 4 weeks. The doses selected in this study are based on the human dose used (1200 mg/kg body weight) which is extrapolated for mouse model (Ghosh, 1984). In addition, the previous reports have used similar doses in experimental models for *in vivo* studies (Trentini et al., 1974; Shayakhmetova et al., 2012, 2016). The mice were humanely sacrificed at two weeks after completion of the treatment to study the effect on testicular and epididymal sperm functions.

### 2.4. Sperm density assessment

The caudae epididymis was dissected out from the animal and it was placed in 2 mL of Earle's balanced salt solution (EBSS, Sigma-Aldrich, St.Louis, MO, USA Cat. No. M5017) supplemented with 0.1% bovine serum albumin (BSA, Sigma-Aldrich, St.Louis, MO, USA Cat. No. A3311). The epididymis was gently teased with the help of bent needle to release spermatozoa by observing under stereomicroscope. Sperm density was assessed using Makler's counting chamber by placing 10 μL of the sperm suspension and observing under light microscope (100×) and expressed in millions per mL (Nayak et al., 2016a).

### 2.5. Sperm motility assessment

The sperm motility is assessed by observing under the light microscope (400× magnification) by taking approximately 10 μL of the sperm suspension and placing it on a clean glass slide with coverslip on it. A total of 100 spermatozoa were observed in five random fields for progressive and non-progressive motility and expressed as percent motile spermatozoa as described by Nayak et al. (2016a).

### 2.6. Assessment of sperm head morphology

The sperm head morphology was assessed using Shorr staining technique as described earlier (Nayak et al., 2016a). Briefly, a thin smear of sperm sample was prepared on a clean glass slide by feathering technique. The smear was air dried and fixed in 75% ethanol for 1 min and later the smear was dipped in running water for 12–14 s followed by hematoxylin stain for 1 min and 30 s. The stained slides were again dipped in running water for 12–14 s followed by treatment with ammonium alcohol (25% ammonium hydroxide and 75% ethanol) for 5 s and washed with running water. Slides were immersed in 50% ethanol for 5 min and stained with Shorr's stain for 8 min. After staining the slides were passed through 50%, 75%, 95% for 5 min and absolute alcohol for 10 min in each followed by two dips through xylene for 5 min each. The slides were air dried. Approximately 2–3 drops of DPX (mixture of distyrene, a plasticiser and xylene) was placed to mount the slides. A total number of 300 spermatozoa were counted per slides and observed under light microscope (100× oil immersion objective). The spermatozoa were characterized into normal head, amorphous head, small head, vacuolated head, head without hook, banana shaped, large head and head containing cytoplasmic droplet. The number of spermatozoa with head defect was expressed in percentage.

### 2.7. Sperm DNA integrity assessment

The sperm DNA integrity was assessed by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay. The sperm suspension (~1 million/mL) were washed with PBS and smeared onto a clean coverslip. The cells were allowed to dry for few minutes and then fixed using 4% PFA at 4 °C for 30 min. The coverslips were washed with PBS twice for 5 min each on a dancing shaker. The cells were permeabilized for 1 h at room temperature by permeabilization buffer (0.5% Triton X100 and 0.1% sodium citrate) followed by 3 times washing with PBS with 0.1% BSA for 5 min each. The samples were incubated in TUNEL mixture (TUNEL reaction mixture: Labeling solution, 1:20, Roche Kit, Roche Diagnostics, Mannheim, Germany, Cat. No. 1215792910, TMR Red) at 37 °C for 1 h. Later the samples were washed 3 times with PBS with 0.1% BSA for 5 min each. The nucleus was counterstained with DAPI (4′,6-diamidino-2-phenylindole), coverslips were inverted carefully and placed on the slides containing DAPI. Spermatozoa were observed under fluorescent microscope and total of 500 cells were counted and expressed as TUNEL index.

### 2.8. Assessment of sperm chromatin maturity

The sperm maturity was assessed by Chromomycin A3 (CMA3)-assay as described by Lolis et al. (1996). Briefly, the sperm suspension was smeared on a coverslips treated with 100% ethanol and air dried. Samples were fixed in methanol and glacial acetic acid (3:1) at 4 °C for 20 min and kept in room temperature for air drying. The samples were treated with 10 μL of Chromomycin-A3 solution (0.4 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA, Cat. No. C2659) in McIlvain's buffer, (0.2 M disodium hydrogen phosphate and 0.1 M citric acid,) pH-7.0 for 20 min in dark conditions followed by thorough rinsing with McIlvain's buffer twice for 5 min each. Then the coverslips were air dried, inverted and placed on a clean glass slides having DAKO as a mounting media on it such a way that the smear comes in direct contact with mounting medium. Sperm were observed under fluorescent microscope and a total of 500 cells were counted. Evaluation of Chromomycin-A3 staining was done by distinguishing spermatozoa that stained bright yellow (Chromomycin-A3 +ve) from those that stained dull yellow (Chromomycin-A3 −ve). The maturity of sperm was expressed in terms of percentage.

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