CHAPTER 11

MATERIALS & METHODS
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The methodology of subchronic male reproductive toxicity testing was basically as that adopted for short duration spermatotoxicity study by Linder et al. (1992, 1994), and the WHO Protocol MB 50, according to Agnes and Akbarsha (2001).

2.1 Materials

2.1.1 Animals

Swiss strain male albino mice, Mus musculus (90 day old) weighing approximately 30 g, were used in the study. Female mice used in the fertility tests also belonged to the same strain and age. Mice were developed from a stock procured from Frederick Institute for Plant Protection and Toxicology (FIPPAT), Padappai, India. All mice were housed in polypropylene cages (8" x 12 " x 8 ") with metal grill tops. They were maintained under constant conditions of temperature and a photoperiod of 13h light: 11h dark. They were fed with standard pellet feed (Gold Mohur Laboratory Animal Feeds, Lipton India Ltd., Bangalore) and water ad libitum.

2.1.2 Glassware and chemicals

All the glassware used for analytical purposes were CORNING (R), VENSIL or BOROSIL (R) make. Chemicals used in the study were of high purity, commercially available and were obtained from SIGMA, RANBAXY, LOBA, or MERCK.

2.1.3 Toxin

The toxin, Aflatoxin B1 (AFB₁) used in the study, was obtained from SIGMA,(St. Louis, MO, USA).

2.2 Experimental protocol (Agnes and Akbarsha, 2001)

2.2.1 Toxin preparation

The AFB₁ was quantitatively prepared in corn oil and ethanol (95:5).

2.2.2 Experimental 1 (Dose standardization)

AFB₁ was administered to male mice at a dose of 50, 100, 150, 200 or 250 µg/Kg/day through intraperitoneal route for 15 days.

2.2.3 Experimental 2 (Duration response)

AFB₁ at a dose of 50 µg/Kg/day was administered to the experimental animals through intraperitoneal route for 7, 15, 35 and 45 days. Each experimental group
consisted of 45 animals. Respective control groups were maintained parallel to each experimental group, and consisted of 45 animals each. At the end of each experimental period, 5 animals from experimental and control groups were killed through cervical dislocation. They were dissected and the testis, epididymis and seminal vesicles were used for gravimetric and histological analysis adopting paraffin method. In another 5 mice from each of the experimental and control groups the reproductive system was perfused with Karnovsky’s (1965) fluid adopting the procedure of Hess and Moore (1993) and the animals were sacrificed using pentobarbital anesthesia. The right testis was removed and thin slices of the tissue were fixed for semithin sectioning and transmission electron microscopy. The remaining 5 mice in each of the experimental and control groups were tested for fertility adopting WHO protocol MB50. Each male mouse was housed with two female mice at estrus. 30 animals in each of the experimental and control groups were left for recovery. For these mice, treatment of AFB1 was discontinued after the respective days. 15 animals were sacrificed/subjected to tests as above, after 35 days of recovery (duration of one spermatogenic cycle) and the remaining 15 animals sacrificed/tested after 72 days (duration of two spermatogenic cycle). Testing in the recovery animals was the same as for AFB1-treatment.

2.3 Methods

2.3.1 Fertility test

Fertility test was carried out after withdrawal of the toxin treatment or after the period of recovery, by allowing the treated male mice to mate with female mice at estrus. Mounting was observed overnight. The following morning, vaginal smears were examined under the microscope for spermatozoa. Subsequently, the female mice were separated and allowed to go to term and the litter size was recorded. Thus, male fertility was evaluated indirectly by registering female fertility.

2.3.2 Gravimetric analysis

In the case of gravimetric analysis the paired testicles and seminal vesicles from each animal were weighed separately. Data for each organ from each group were used to calculate the respective means and the standard deviations.
2.3.3 Histology – paraffin sections

The histology of the testis and epididymidis was studied adopting paraffin method (Humason, 1979). Reagents used in this method were as follows:

- Physiological saline (0.9%): 0.9 g of sodium chloride in 100 ml of distilled water.
- Bouin – Hollande fixative: Prepared by adding 2.5 g of copper acetate, 4 g of picric acid, 10 ml of formaldehyde and 1.5 ml of glacial acetic acid, and made up to 100 ml with distilled water.
- Ehrlich's haematoxylin: 10 mg of haematoxylin powder was dissolved in 100 ml of ethyl alcohol, to which 100 ml of glacial acetic acid, 100 ml of glycerine and 100 ml of distilled water were added. The solution was mixed well and allowed to ripen in sunlight for 2 months. After the solution turned deep red, 2 g of aluminium ammonium sulphate was added and used for staining.
- Eosin: 0.5 g of eosin powder was dissolved in 30 ml of distilled water and made up to 100 ml with 70 ml of absolute alcohol.
- Periodic acid, 1%
- Schiff's reagent: 1 g of basic Fuchsin was dissolved in 400 ml of boiling distilled water. It was then cooled to 50°C and filtered. To the filtrate 1 ml of thionyl chloride was added. It was left to stand in the dark for 12 hours after which it was cleared by shaking for 1 minute with 2 g of activated charcoal. After filtering the reagent was stored in the dark at 4°C.

Procedure:

The testicles and the epididymides dissected out from control and experimental mice, were blotted free of mucous, washed thoroughly in physiological saline, cut into pieces of desired size and fixed in Bouin-Holland fixative for 24 h. After fixation, the tissue was washed in 70% alcohol for two to three days to remove excess picric acid and dehydrated in graded series of alcohol. The tissue was dealcoholized using xylene. The dealcoholized cleared tissue was infiltrated with moulten paraffin at 58-60 °C through three changes (20-30 minutes each) and, finally, embedded in paraffin.

3-5 μm thick serial sections of all the tissues were obtained using a rotary microtome (Leica, Germany) and stained in Ehrlich's haematoxylin with eosin as
counter-stain or periodic acid-Schiff’s reagent (PAS) with haematoxylin as the counter stain. The slides were mounted using DPX mountant.

2.3.4 Light as well as transmission electron microscopic studies adopting resin embedding

Resin embedding of tissues was done according to Hayat (1981), using Karnovsky's fluid (Karnovsky, 1965) as the perfusate.

Reagents used

Perfusion fluid: Perfusate (Karnovsky's fluid) was prepared with the following composition: glutaraldehyde 1%, paraformaldehyde 1% and phosphate buffer 0.1 M (3.205 g of sodium dihydrogen phosphate and 20.65 g of disodium hydrogen phosphate) were added and made up to 1000 ml in double distilled water.

Phosphate buffer: (0.2 M): 6.41 g of sodium dihydrogen phosphate and 41.3 g disodium hydrogen phosphate were dissolved in 1000 ml of double distilled water.

Wash buffer: Wash buffer was prepared by dissolving 5 g of sucrose in 100 ml of 0.1 M phosphate buffer.

Glutaraldehyde: 1.78 g of sodium cacodylate was dissolved in 50 ml of double distilled water. To this solution, 10 ml of glutaraldehyde was added to form solution 'A'. 21 mg of calcium chloride was dissolved in 10 ml of double distilled water to form solution 'B'. Solutions 'A' and 'B' were mixed together and made up to 83.3 ml with double distilled water.

Osmium tetroxide: A stock solution of 2% osmium tetroxide (OsO₄) was prepared by dissolving 1 g in 50 ml of double distilled water and stored at 4°C. It was diluted 1:1 using water just before use.

Spurr's mixture: Prepared by adding 8 ml of 2-nonen-1-y1 succinic anhydride (NSA), 1ml of 4-vinylcyclohexane dioxide, 10 ml of resin and 0.1 ml of 2-dimethylaminoethanol (DMAE).

Toluidine blue O: Aqueous toluidine blue O (TBO) solution was prepared by dissolving 0.05% toluidine blue in benzoate buffer at pH 4.4 (benzoate buffer was prepared by adding 0.25 g of benzoic acid and 0.29 g of sodium benzoate in 200 ml of distilled water).
Uranyl acetate: This was prepared by adding 20 ml of filtered 50% ethanol to a saturated solution of uranyl acetate. After 2 minutes of centrifugation the excess uranyl acetate was allowed to settle and the clear supernatant was used as the stain (Hess and Thurston, 1977).

Lead citrate: One half pellet of sodium hydroxide was added to 12 ml of double distilled water and centrifuged. 50 mg of lead citrate was dissolved in it by thorough shaking followed by centrifugation (Hess and Thurston, 1977).

Procedure

In experimental animal studies, the best results are obtained using vascular perfusion fixation (Hess and Moore, 1973). Animals were subjected to mild ether anaesthesia and perfused by vascular perfusion. Vascular perfusion is generally carried out intracardially and offers a good preservation of organs. The procedure adopted for perfusion fixation was according to Hess and Moore (1973). After anaesthetization with ether, the mouse was laid on its back in a shallow tray and the heart was exposed. A vacuum system was used to remove the accumulating fluids into a holding container. The transfusion set consisted of a bottle with polythene catheter containing the perfusate, suspended at about 150 cm above the animal. The ascending aorta was cannulated with a polythene catheter. Care was taken not to let in air during this process. An incision was made in the right atrium to allow the perfusate to flush the blood out of the whole body. Initially, the perfusate was allowed to flow rapidly and then the rate of flow was reduced to a minimum.

Following perfusion, the right testis was removed and cut into 1mm cubes and immersed in 2.5% glutaraldehyde (primary fixative) overnight. The tissue was rinsed in wash buffer and post-fixed in 1% osmium tetroxide (secondary fixative) for 2-3 hrs. Subsequently, the tissue was washed thoroughly in wash buffer to remove the excess osmium tetroxide. Then the tissues were dehydrated gradually in increasing concentrations of ethyl alcohol and dealcoholized using propylene oxide. Infiltration was carried out with propylene oxide and Spurr's mixture (Sigma, USA) at increasing concentrations at room temperature, using a slow speed rotary shaker.

Embedding was done in a flat embedding mould with tissues oriented to obtain cross sections. Semithin sections were obtained in a Reichert Jung (Austria) ultratome.
The sections were stained with freshly filtered TBO. Areas were chosen to obtain ultra-thin sections (silver to gray, 60 nm – 90 nm) using LKB – Bromma ultracut (Germany). Sections were picked in copper grids and stained with uranyl acetate and lead citrate. TBO-stained sections were also used for light-microscopic observation of histopathological and histometric changes. Sections were observed under a Phillips 201C (Holland) transmission electron microscope.

2.3.5 Microscopy

Carl Zeiss Axioskop 2 plus (Carl Zeiss, Germany) research microscope which is connected to a Sony DxC-151a/151HP 2/3 inch color video camera with CCD (Charge Coupled Device) with a solid stage image sensor to a computer was used to record the pictures of paraffin/semithin sections using Axio Vision KS software (Carl Zeiss, Germany). The images were processed using the software Carl Zeiss Vision Imaging Systems. Phillips 201C (Holland) transmission electron microscope (TEM) was used to obtain electron micrographs at x 2000 to x 50000 magnifications. The photo prints were scanned into the computer using a HP (Hewlett Packard) color scanner and processed using the Carl Zeiss software already mentioned.

2.4 Identification of the stages in the cycle of the seminiferous epithelium

The methodology was as discussed by Oakberg (1956) for the identification of stages in the cycle of mouse spermatogenesis. This was supported by a software "Stages", graciously gifted by Dr. Rex A. Hess, Professor of veterinary Medicine, University of Illinois, Urbana, USA. The 12 stages were identified. Application of the periodic acid-Schiff technique to mouse testis revealed 16 steps in spermiogenesis. The characteristic cell associations at each stage of the cycle of the seminiferous epithelium are given in the Table1.

2.5. Histo-cytometry

The histo-/cytometry were conducted using “Carl Zeiss Vision Imaging Systems”. It is a modular image processing and analysis system for use in modern microscopy. It includes basic functions for image acquisition, image processing, image archiving and image documentation.

a. Sections stained in H&E, PAS and H or TBO were viewed through the microscope and fields with sections of seminiferous tubules in transection were selected
at x100 magnification. Using the software, the boundary of the each of the seminiferous tubule was demarcated, and the total area of the seminiferous tubules was deducted from the total area of the field. The remaining area of the field gave the area of the interstitium. This was done for five different fields from the tissue of each mouse making 25 measurements for each sub-group.

b. The perimeter and the diameter of five different seminiferous tubules in perfect transections from each mouse were also measured at x400 magnification making 25 measurements of each parameter for five animals in each sub-group.

c. From the histological preparations of testis of each animal 20 sections were randomly selected at x200 magnification and the extent of degeneration and the number of such tubules were counted and recorded. Number of tubules not indicating any trace of epithelial degeneration were also counted and recorded. The data were used to calculate the percentage tubules without any trace of epithelial degeneration and those with epithelial degeneration.

d. At x400 magnifications the epithelium of the seminiferous tubules was analysed for the number of uninucleate giant cells and multinucleate giant cells. This was done in seminiferous tubules in semithin sections and a total of 25 tubules were assessed in each group. The data were used to find the mean ± SD of total number of cells in $10^4 \mu m^2$ area per tubule, and the number of uninucleate and multinucleate giant cells.

e. A similar count was made at x1000 magnification for cells in degeneration or nuclear pycnosis. From the total number of cells in the area and the number of cells in degeneration or nuclear pycnosis, the percentage of cells in degeneration/nuclear pycnosis was calculated. This was also done for a total of 25 tubules in transection in each group.

f. Seminiferous epithelium in semithin sections observed at x400 magnification were used to measure the area in the epithelium which was empty due to vacuolation consequent upon loss of germ cells. This was done by marking the empty areas within the purview of the total area of the seminiferous epithelium. With the values obtained from 25 seminiferous tubules from each sub-group, the mean ± SD of the total area of empty spaces and the empty space as the percentage of total area were calculated.
g. Using the same software, at x1000 magnification the number of Leydig cells per 10^3 \mu m^2 of the interstitial area was counted from five fields from each animal making it 25 counts for each sub-group. The values were used to calculate the mean ± SD. The perimeter of the Leydig cells and the diameter of the nucleus of the Leydig cells were also measured for 100 Leydig cells in the testis of each group and the data were used to calculate the mean ± SD.

2.6. Statistical analysis (Zar, 1974)

Data were subjected to analysis using the Carl Zeiss software as above. Mean and standard deviation were calculated for data from animals in each group. The formulae in the software adopted the following procedures.

**Mean**

$$\bar{X} = \frac{\sum x}{n}$$

Where \( \bar{X} \) - mean, \( \sum \) - summation and \( n \) - size of the population or individual

**Standard Deviation**

$$\delta = \sqrt{\frac{\sum (x - \bar{x})^2}{n-1}}$$

where

- \( \delta \) = Standard deviation,
- \( \sum \) = summation,
- \( n \) = size of the population,
- \( x, \bar{x} \) = mean values of variance

**Student's 't' test**

Data for each parameters for the respective control and treatment groups were used for application in paired sample 't' test. It was applied to test the level of significance of the results obtained for which the formula used in the software was as follows.

$$t = \frac{x_1 - x_2}{\sqrt{SE_1^2 - SE_2^2}}$$

where

- \( x_1, x_2 \) = difference of mean values
- \( SE_1, SE_2 \) = respective standard errors
- \( SE = SD / \sqrt{n-1} \)
The significant value for the t-test corresponding to the degrees of freedom (d.f. = \( n_1 + n_2 - 2 \)) at 5% level of significance was obtained from the probability table of t-distribution.