Effect of metronidazole on spermatogenesis and FSH, LH and testosterone levels of pre-pubertal rats

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Metronidazole, a 5-nitroimidazole drug has been reported to decrease testicular weight, testicular and epididymal spermatid counts and causes abnormal sperm morphology with degeneration of seminiferous tubules with 6 weeks treatment of metronidazole (400 mg/kg, day). In contrast to DNA flow cytometry (FCM), the histological and gravimetric parameters do not allow a rapid, sensitive, objective and multiparametric evaluation of reproductive toxicants on spermatogenesis. Moreover, the exact mechanisms for such an effect are not entirely clear. The present study was therefore undertaken to assess the effects of intraperitoneal (i.p.) administration of metronidazole 400 mg/kg daily for 30 days on testicular germ cell changes assessed by DNA (FCM) and hormone levels of testosterone, FSH and LH in pre-pubertal rats. A significant reduction in the haploid cell population in metronidazole treated groups as compared to saline treated controls was observed. The mean serum FSH, LH and testosterone value were also lowered in treated animals. Thus, the spermatotoxic effects of metronidazole were probably mediated by decrease in the circulating hormones responsible for spermatogenesis.

Over the past 50 years, there has been an appreciable decrease in male fertility in human population¹ and since it is directly related to sperm count and morphology, the result may reflect an overall reduction in this process. Among the many factors that have been cited as possible causes of such an effect, environmental factors such as exposure to drugs and chemicals have particularly gained recognition in the past two decades². In addition, environmental presence of certain therapeutic drugs has been shown³.

Metronidazole, the first clinically effective 5-nitroimidazole drug has received widespread therapeutic use in the treatment of various infections especially anaerobes. Although in clinical use for many years, except metallic taste, headache, dry mouth, gastritis, glossitis and urticaria⁴ serious side effects with the use of this drug has not been reported. Less common side effects such as motor-sensory neuropathy⁵,⁶, ototoxicity⁷, esophagel ulcers⁸, pancreatitis⁹ and hepatotoxicity¹⁰ have also been described. Few of its metabolites have been shown to be mutagenic in certain bacterial test systems such as Ames test¹⁰.¹¹. In addition, genotoxic potential has also been shown in human lymphocyte cultures of the patients exposed to metronidazole¹².

Studies have also described the metronidazole as a reproductive toxicant¹³-¹⁵. However, there are no reports assessing the effects of metronidazole on maturating testes. Hence, this study was designed to assess the effects of metronidazole on pre-pubertal rats. In addition, the previous studies have assessed the effects of metronidazole on parameters like testicular histology, testicular sperm head counts, cauda sperm counts and testicular and epididymal weights. These parameters though adequate, do not allow a rapid, sensitive, objective and multiparametric evaluation of reproductive toxicants as compared to flow cytometric studies of spermatogenesis that allows identification of germ cell sub-populations undergoing both proliferative and maturative processes in normal and perturbed conditions¹⁶. In addition, FCM is better for assessing germ cell changes in testes¹⁷.

Therefore the present study was undertaken to assess the effects of administration (ip) of metronidazole 400 mg/kg daily for 30 days on testicular germ cell changes assessed by DNA FCM and hormone levels of testosterone, FSH and LH.

Experimental design—A total of 40 pre-pubertal male rats (30 days old) were procured from the experimental animal facility of the Institute. The rats were housed in cages in the centrally air-conditioned animal laboratory. Before the start and during the experiment, rats were fed standard chow diet and tap water ad libitum. After acclimatization period of 3
days, they were randomly divided into two groups A and B. While group A received metronidazole 400 mg/kg.day daily for 30 days as i.p. injection, rats in group B received saline 1 ml daily as i.p. injection. At the end of study, the animals were sacrificed under light ether anesthesia; their blood was collected, centrifuged and the serum was stored at -20°C for assessment of FSH, LH and testosterone levels. The testicles were harvested and transported in Rosewell Park Memorial Institute solution for DNA FCM.

**DNA FCM**—Freshly harvested testes were separated from the tunica albuginea and minced in phosphate buffer saline (PBS). The resultant single cell suspension was washed twice in PBS and a 100-

μl aliquot of the suspension was fixed in 70% ethanol in PBS. After centrifugation, the pellet was re-suspended in propidium iodide and subjected to RNA digestion by RNase A (Sigma, USA). DNA histograms were then obtained on a flowcytometer (Becton-Dickinson FAC Scan). The data then obtained were analyzed by Cell Software.

**Radio-immunoassay**—FSH, LH, and testosterone levels were determined by radio-immunoassay. FSH and LH serum levels were determined by RIA following the instructions given with the reagents generously provided by the National Hormone and Pituitary Program of NIDDK (Baltimore, MD). The LH reference preparation was LH-RP-2 and the antiserum was anti-rat LH-RIA-II. The rFSH reference preparation was rFSH-RP-2 and the antiserum was anti-rat FSH-S-11.

**Statistical analysis**—Student's T test (GraphPad, Instat, Version 1.14, INSERM 920666S, India) was used for comparing the differences between two groups and a probability of less than 0.05 was considered statistically significant.

The mean percentage of haploid, diploid and tetraploid cells is shown in Table 1 along with serum levels of FSH, LH and testosterone. A significant reduction ($P<0.001$) in haploid cell population in metronidazole treated groups as compared to saline treated controls was observed. In proportion, the percentage of diploid and tetraploid cells was increased in metronidazole treated group. Also observed a significant reduction in mean serum FSH, LH and testosterone ($P<0.001$) levels in treated groups.

Dose of metronidazole was selected on the basis of a previous study in which metronidazole (400 mg/kg.day daily for 6 weeks) resulted in adverse effects on the mating performance and fertility parameters of rats.

| Table 1 — Effect of intra-peritoneal administration of metronidazole (400 mg/kg.day) on DNA histograms of rat testis and hormonal levels of LH, FSH and testosterone |
|-----------|-----------|-----------|-----------|
|           | Saline treated | Metronidazole Treated (400 mg/kg) |
| **DNA Histograms** |          |          |
| Haploid cells (%) | 73.2±2.04 | 42.73±3.70 |
| Diploid cells (%) | 17.5±1.34 | 44.12±2.39 |
| Tetraploid cells (%) | 9.03±1.17 | 13.07±4.77 |
| **Hormonal levels** |          |          |
| FSH (ng/ml) | 15.36±1.09 | 11.65±0.90 |
| LH (ng/ml) | 0.461±0.0547 | 0.254±0.0463 |
| Testosterone (nmol/L) | 1.44±0.221 | 0.735±0.189 |

Treated group was compared with saline controls.

All values are statistically significant at $P<0.0001$.

Seminiferous epithelium cycle in the rat testes has 14 stages and identification of various stages of development is a tedious process, which is prone to inter and intra observer variations. The usual gold standard, the Johnsen testicular maturation score may thus not be accurate. DNA FCM analysis correlates strongly with the current standard of quantitative spermatogenic assessment and is a simplified and highly objective method of determining spermatogenesis as a statistically significant correlation has been shown between the percentage of haploid cells and tubular concentration of late spermatids and tubular spermatid-to-Sertoli cell ratio.

Testicular suspension contains 3 distinct populations of cells each having a different DNA content, the tetraploid peak mainly represents primary spermatocytes, spermatogonial G2 cells and spermatogonial G1 cells while secondary spermatocytes constitute the diploid peak; the spermatids and spermatooza are haploid. Thus, the percentage of haploid cells is directly proportional to sperm maturation. In this study, metronidazole caused dramatic fall in the haploid cell percentage in haploid cell population around 60 days post-natally implying delayed testicular maturation. The present study supports the previous findings of McClain et al.

Metronidazole significantly ($P<0.0001$) lowered the levels of FSH, LH and testosterone. Thus, the toxic effects of metronidazole on spermatogenesis were probably mediated by decrease in the circulating hormones responsible for spermatogenesis. However, oral metronidazole treatment (500 mg/day) for 4 weeks in humans failed to alter the levels of total testosterone, free testosterone and dihydrotestosterone...
while the levels of estradiol and dehydroepiandrosterone sulfate were decreased significantly. Neither did metronidazole inhibited in vitro testicular 17 alpha-hydroxylase and 17,20-lyase activity or testosterone biosynthesis in in vitro mouse Leydig cells cultures, though other imidazole drugs such as ketoconazole, bifonazole, clotrimazole, miconazole and isocazole have been shown to inhibit biosynthesis of testicular 17 alpha-hydroxylase activity.

To our knowledge this is the first study which has evaluated the in vivo effect of metronidazole on reproductive hormones in rats. It needs to be seen whether the lower doses of this drug have the same effects on reproductive hormones and testes and whether this process is reversible.

References