Introduction

Clinically, the most prominent effects of anticonvulsant medications on endocrine function are those on sexuality and the reproductive function. The hepatic enzyme-inducing antiepileptic drugs (AEDs) - phenobarbital, primidone, phenytoin, and carbamazepine - may all contribute to or cause reproductive and sexual dysfunction in men with epilepsy.[1,2] Valproate, an enzyme inhibitor, has also been associated with altered androgen levels,[3] as well as altered reproductive parameters.[4,5] The maintenance of adult mammalian spermatogenesis is dependent upon the steroid hormone testosterone, which is produced by testicular leydig cells, in response to the secretion of pituitary luteinizing hormone.[6] Previous studies of spermatogenesis in rats have shown that the experimental reduction of intratesticular testosterone to low enough levels results in germ cell loss[7-9] and that the readministration of testosterone restores spermatogenesis.[10,11] Intratesticular testosterone is thought to play a very important role in spermatogenesis; however, it is very rarely measured in patients receiving AEDs.

Lactate dehydrogenase (LDH) is a ubiquitous enzyme present in both plants and animals. Eliasson and Virgi[12] suggested that quantitative analysis of the isoenzyme lactate dehydrogenase - C4 in semen from fertile and infertile men may provide a guide regarding the status of the seminiferous epithelium and the degree of germ cell degeneration. The estimation of LDH levels provides a quantitative basis for the loss of cell viability and its application in assessing the cytotoxicity of the cell.[13,14] Most of the studies on sodium valproate indicate that it is gonadotoxic and hence can affect fertility. However, all these reports are at one point sampling and there are no reports regarding how long the effects last after exposure to the drug. Hence, a study was planned to assess the effects of sodium valproate at different sampling time on the biochemical markers of testicular function in male Wistar rats.

Materials and Methods

Animals

Twelve-week-old male Wistar rats (150-200g), bred locally in the central animal house, were selected for the study. They were housed in propylene cages and were provided bedding with paddy husk. Temperature was maintained at 25 ± 1°C, with a humidity of 45 ±1%. The animals had free access to sterile food (animal chow) and water ad libitum. Animal care and handling was done as per the guidelines set by the Indian National Academy, New Delhi, India. The study was started after getting clearance from the Institutional Animal Ethics Committee.

A total of 108 rats were segregated to 18 groups of 6 animals each. Six groups each were treated with 0.1 ml of distilled water, sodium valproate 200 mg and sodium valproate 400mg for 60 days (n = 6/group/dose/sample time). The powdered form of sodium valproate was obtained from Knoll Pharmaceuticals Ltd, Mumbai. The sodium valproate was dissolved in distilled
water and administered orally. The median lethal dose of sodium valproate in rodents varies between 1100 and 3900 mg/kg body weight.\textsuperscript{[15]} The dose and route of administration was based on earlier reports.\textsuperscript{[16,17]} The rats were sacrificed by terminal anesthesia (pentobarbital sodium, 45mg/kg) at the end of the 2nd, 4th, 5th, 7th, 10th and 15th week, after the last exposure to sodium valproate.

Preparation of tissue homogenate

Testes were removed and weighed. The testes were then minced in phosphate buffer solution at a ratio of 1: 10, using pestle and mortar. The tissue homogenate obtained was cold centrifuged. The supernatant was taken for the estimation of intratesticular testosterone and intratesticular lactate dehydrogenase.

Estimation of intratesticular testosterone level

The testicular level of testosterone was analyzed in the homogenate by using a kit designed for ELISA (EIAgen Testosterone-Biochem Immunosystem, Italia S.P.A.). About 50µl of calibrators and tissue homogenate sample were added to appropriate wells of strips. About 200µl of horseradish peroxidase - testosterone conjugate was added to each well in sequence. The mixture was incubated for two hours at 37ºC, without covering the plate. Following this, the solution was discarded, the wells were rinsed thrice with washing solution (Tween 20) and amphotericin-B (2.5µg/ml in citrate- borate buffer) and the residual fluid was removed. Immediately, 100 µl of chromogen substrate mixture (0.26mg/ml of 3, 3’, 5, 5’-tetramethyl benzidine and 0.01% (w/v) of hydrogen peroxide in citrate buffer) was added to the wells and incubated for 15 min at room temperature, avoiding exposure to sunlight. Reaction was stopped by pipetting 100 µl of the stop solution (sulfuric acid-0.3mol/l) into the wells. Absorption was read in ELISA at 450 nm, within one hour from the addition of stop solution, as per the manufacturer’s instructions.

Estimation of intratesticular lactate dehydrogenase level

Testicular lactate dehydrogenase (LDH) was estimated by Optimized standard kit method (Roche/Hitachi), based on the principle that lactate dehydrogenase catalyses the conversion of pyruvate to lactate; reduced Nicotinamide Adenine dinucleotide (NADH) is oxidized to Nicotinamide Adenine dinucleotide (NAD) in the process. The rate of decrease in NADH is directly proportional to the LDH activity. The LDH activity was estimated by a kit using a spectrophotometer (Optimized Standard Kit; Roche/Hitachi).

Statistical analysis

For each group, six animals were used and mean ± SD (standard deviation) was calculated. Results obtained from the present study were correlated and analyzed by One Way Analysis of Variance (ANOVA). Values of $P < 0.05$ were considered statistically significant.

**Results**

The intratesticular testosterone was significantly reduced in rats treated with 200mg/kg and in rats treated with the 400mg/kg. Significant difference was also seen in the levels of intratesticular testosterone between the treated groups. Significant difference between the treated groups was observed till the 7th week. Recovery period for both the doses took a longer time and reached normal values only by the 15th week [Figure 1].

![Figure 1: Time response relationship for sodium valproate induced changes in testosterone level. Each time at particular dose represents mean +SD from six animals. Significant values are: normal control vs. treated, $3 = P<0.001$; 200mg vs. 400mg, $c = P<0.001$](image1)

The decline in intratesticular testosterone level was the highest during the 5th and the 7th week, for both the dose levels.

The intratesticular LDH level was significantly increased by valproate, in a dose dependent manner in the 2nd week. A similar observation was made in the following sampling weeks and complete recovery to normal values was reached only by the 15th week. The elevation of LDH level was the highest in the 5th and 7th week. Significant differences between the groups were observed during the 5th and 7th week [Figure 2].

**Discussion**

Data generated clearly shows that the levels of intratesticular testosterone in rats treated with sodium valproate decreased significantly in the 2nd to 7th week sampling time. Bauer et al.\textsuperscript{[4]} and Kuhn-Velten et al.\textsuperscript{[18]} reported that valproate acts directly on the testis, to inhibit testosterone synthesis by the Leydig cells. In the present study, it is more than likely that a similar effect has been responsible for the low intratesticular testosterone level. The testosterone-to-LH ratio is a sensitive measure of testicular function. It is low in men with temporal lobe epilepsy not taking AEDs, but is even more abnormal in this same population with the use of valproate.\textsuperscript{[4]} However, the

![Figure 2: Time response relationship for sodium valproate induced changes in lactate dehydrogenase level. Each time at particular dose represents mean +SD from six animals. Significant values are: normal control vs. treated, $1 = P<0.05$, $3 = P<0.001$; 200mg vs. 400mg, $c = P<0.001$](image2)
latter possibility can be ruled out, since the present study was carried out on non-epileptic rats.

Although incompletely researched, it has been suggested that AEDs induce the production of the enzyme aromatase in the liver. This enzyme converts testosterone to estradiol (the final common path of all natural estradiol production). Induction of aromatase production leads to an elevated serum level of estradiol. By shunting free testosterone to estradiol, serum free testosterone level is further reduced. Thus, the ratio of free testosterone to estradiol is lower in men with epilepsy and hyposexuality than in sexually normal epilepsy patients or in normal controls. Estradiol may impair testes function by suppressing male luteinizing hormone secretion or by producing premature aging of the hypothalamic arcuate nucleus. However, it is not known whether the antiepileptic drugs can cause the conversion of testosterone to estradiol within the testes or whether it is a process only in the serum. So the decrease in the levels of intratesticular testosterone observed in the present study may be mainly due to the direct effect of these drugs on the Leydig cells and, at the same time, the possible conversion of intratesticular testosterone to estradiol by the enzyme aromatase cannot be ignored.

Lactate dehydrogenase (LDH) enzyme is widely distributed throughout the body, cellular damage causes an elevation of the total serum LDH. When disease or injury affects tissues containing LDH, the cells release LDH into the bloodstream, where it is identified in higher than normal levels. Eliasson and Virji suggested that quantitative analysis of the iso-enzyme lactate dehydrogenase -C4 in semen from fertile and infertile men may provide a guide regarding the status of the seminiferous epithelium and the degree of germ cell degeneration. LDH - C4 is a testes specific enzyme, however, in the present study, the estimation was done on the total LDH.

Goddard et al. showed that adult rats treated with flutamide (an antiandrogen) in utero induced altered spermatogenesis. However, the levels of LDH were decreased, which is contrary to the earlier reports. They further suggest that this decrease in LDH indicates that transport of lactate produced by sertoli cells to the germ cells could be altered. In the present study, it was observed that the LDH level was increased in a significant manner during the 2nd to 7th week sampling time. When the germ cells which possess LDH - C4 degenerates, some of the enzymes leak into the seminiferous tubule fluid and eventually find their way into the semen. The estimation of LDH levels provides a quantitative basis for the loss of cell viability and its application in assessing the cytotoxicity of the cell. According to Sinha et al. and Pant and Srivastava, the increase in LDH activity level has a direct effect on testicular functions such as sperm count and sperm production, as well as sperm morphology. This indicates the cytotoxicity of sodium valproate.

The present study concludes that even though valproate decreases the fertility by affecting the germ cells, somatic cells, intratesticular testosterone and LDH, these effects are not permanent and are reversed once the drug is withdrawn. This finding has a clinical relevance because of the widespread use of valproate in the management of epilepsies and also its use in bipolar disorders. Future studies should focus on the effect of this drug on other hormones and enzymes, as well as its effect on other reproductive parameters.

References