Effect of Phenytoin Sodium on the Biochemical Parameters of Reproductive Function in Male Albino Wistar Rats

P Vijay, R Yeshwanth, KL Bairy

Abstract

To assess the effects of phenytoin sodium on biochemical markers of testicular function. Male Wistar rats (12 weeks old) were treated with phenytoin and sacrificed at the end of 2nd, 4th, 5th, 7th, 10th and 15th week after the last exposure to phenytoin sodium. The testes were removed, weighed and processed for biochemical analysis. The intratesticular testosterone level was significantly (P<0.001) reduced with 50 mg/kg and 100mg/kg treated rats. The intratesticular lactate dehydrogenase (LDH) level was significantly (P<0.001) increased by phenytoin in a time dependent manner regardless of dose. Phenytoin causes reversible change in testosterone and LDH level.

Key Words: *phenytoin sodium, testosterone, lactate dehydrogenase*

In our society sterility is a taboo subject till today. In 60% of all couples experiencing infertility, a male factor is involved. It is primarily a male factor in 40% of these couples and in an additional 20% of these couples; it is a combination of male and female factors. Therefore, when a couple is having trouble conceiving it makes sense not only to evaluate the woman but to evaluate the man as well.[1] Couples tend to delay seeking professional help and, in particular, males think it to be linked to the question of potency. Phenytoin sodium is an anticonvulsant used to control grandmal and psychomotor seizures. It can cause gingival hyperplasia, agranulocytosis, aplastic anemia, leukemia and various neurological deficits when given for long time. It produces chromosomal anomalies and increased incidence of malignant melanoma.[2,3] Phenytoin is excreted in human semen in small quantities and this may possibly affect the testosterone levels. Reduced plasma concentrations of free testosterone have been detected in male epileptic patients receiving phenytoin. Meng et al.[4] observed possible mutagenic effect of phenytoin on human sperm cells.

Epilepsy is a curable disease, which necessitates continuous treatment with optimal dose. The available data suggests that antiepileptic drugs can induce gonadotoxicity.[5-11] Though we may not be able to stop this totally, the outcome of this study may help in using the antiepileptics carefully, namely to control side effects without compromising the efficacy.

Hence a study was planned to assess the effects of phenytoin sodium on biochemical markers of testicular functions in male albino Wistar rats.

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Methods

Twelve week old healthy male Wistar rats (150-200 g) bred locally in the central animal house were selected for the study. They were housed under controlled conditions of temperature of 23±2 ºC, humidity of 50±5% and 10-14 h of light and dark cycles respectively. The animals were housed individually in polypropylene cages containing sterile paddy husk (procured locally) as bedding throughout the experiment and 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 Science Academy New Delhi, India. The study was undertaken after obtaining the approval of Institutional Animal Ethics Committee (IEAC approval letter No. IEAC/KMC/05/2002-2003 dated Dec 30th, 2002.

A total of 144 rats were segregated to 24 groups of 6 animals each. Six groups each were treated with 0.1 ml of distilled water, gum acacia control, phenytoin sodium 50 mg/kg and phenytoin sodium 100 mg/kg for 60 days. The powdered form of phenytoin sodium was obtained from Cadila Health care Ltd. The dose and route of administration was based on earlier studies.[8,10] Powdered form of phenytoin was weighed in an electronic weighing balance and was dissolved in 10 ml of gum acacia (0.2 g gum acacia dissolved in 10 ml of distilled water) and administered orally. Animals were sacrificed by terminal anesthesia (pentobarbital sodium, 45 mg/kg) at the end of 2nd, 4th, 5th, 7th, 10th and 15th week after the last exposure to phenytoin sodium.

Preparation of tissue homogenate

Testes was 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

(ElAgen Testosterone-Biochem immunosystem, Italia S.P.A.) About 50 µl of calibrators and tissue homogenate sample were added into appropriate wells of strips. About 200 µl of horseradish peroxidase–testosterone conjugate was added to each well in sequence. Mixture was incubated for 2 h at 37 ºC without covering the plate. Following this, the solution was discarded, wells were rinsed thrice with washing solution (Tween 20) and amphotericin–B (2.5 µg/ml) in citrate-borate buffer and the residual fluid was removed. Immediately, 100 µl of chromogen substrate mixture (0.26 mg/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-0.3 mol/l) into the wells. Absorption was read in ELISA at 450 nm within 1 h from the addition of stop solution, as per manufacturer's instructions.

Estimation of intratesticular Lactate dehydrogenase level (LDH)

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; NADH is oxidized to NAD in the process. The rate of decrease in NADH is directly proportional to the LDH activity. The LDH level 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 50 mg/kg and in rats treated with the 100 mg/kg. Significant difference was also seen in the levels of intratesticular testosterone between the treated groups. Significance between the treated groups was observed till the 7th week. Recovery period for both the doses took longer time and reached normal values only by the 15th week. The decline in intra testicular testosterone level was highest during the 5th week at the both the dose levels (Figure 1). Thereafter, it showed a progressive increase in the testosterone level in the following week sampling times till it reached the control levels in the 15th week (Table 1; Figure 1).

The intratesticular lactate dehydrogenase level was significantly increased by phenytoin sodium in a time dependent manner regardless of the dose. In both the doses recovery period was similar and reached nearer to control levels by the 10th week. The elevation of lactate dehydrogenase level was highest in the 5th and 7th week. Significance between the treated groups was observed during the 4th, 5th and 6th week (Table 2; Figure 2).

Table 1. Effect of phenytoin sodium on intratesticular testosterone level (levels in ng/ml), each dose from particular time represents mean ± SD from 6 animals

Dose	Sampling Time					
	2w	4w	5w	7w	10w	15w
Normal Control	8.65±0.44	8.85±0.36	8.63±0.29	8.53±0.39	8.66±0.21	8.41±0.30
Gum acacia control	8.20±0.32	8.90±0.33	8.18±0.19	8.55±0.24	8.33±0.31	8.45±0.43
50 mg/kg	6.90±0.40*	6.55±0.38*	5.98±0.27*	6.15±0.20*	8.16±0.39	8.70±0.24
100 mg/kg	6.18±0.24*†	4.95±0.52*††	4.03±0.34*††	4.95±0.37*††	8.01±0.37	8.90±0.28

Significant values are; normal control vs. treated * $P<0.001$; 50 mg/kg vs. 100 mg/kg † $P<0.05$, †† $P<0.001$; w=weeks.

Table 2. Effect of phenytoin sodium on intratesticular lactate dehydrogenase level (level in IU/L), each dose from particular time represents mean ± SD from 6 animals

Dose	Sampling Time					
	2w	4w	5w	7w	10w	15w
Normal Control	2317.66±235.65	2435.66±217.9	2515±109.04	2440±147.06	2482.33±103.17	2426.83±150.59
Gum acacia control	2422±192.72	2451.33±112.01	2483.83±124.99	2473.67±178.32	2517.167±126.42	2471.66±132.96
50 mg/kg	2877±136.69*	2996.66±99.3**	3548.5±137.36**	3085.83±130.27**	2633.66±111.15	2389.83±168.3
100 mg/kg	3187±103.75**	3317±124.41**†	4142±118.06**††	4165.5±138.87**††	2672.83±91.32	2410.83±98.05

Significant values are; normal control vs. treated * $P<0.01$, ** $P<0.001$; 50 mg/kg vs. 100 mg/kg † $P<0.05$, †† $P<0.001$; w =weeks.

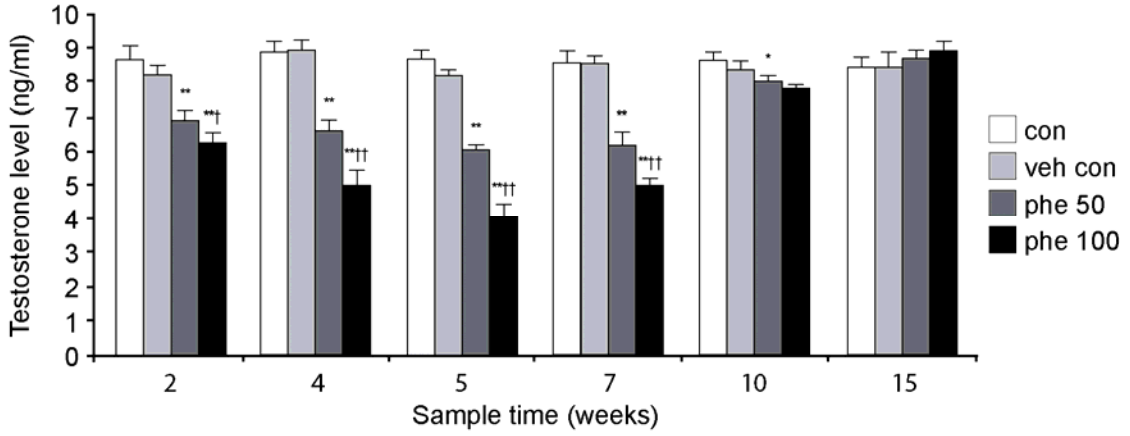

Figure 1. Time response relationship for phenytoin sodium induced changes in testosterone level. Each time at particular dose represents mean + SD from 6 animals. Significant values are; normal control vs treated, * $P<0.05$, ** $P<0.001$; 50 mg vs 100 mg, † $P<0.05$, †† $P<0.001$.

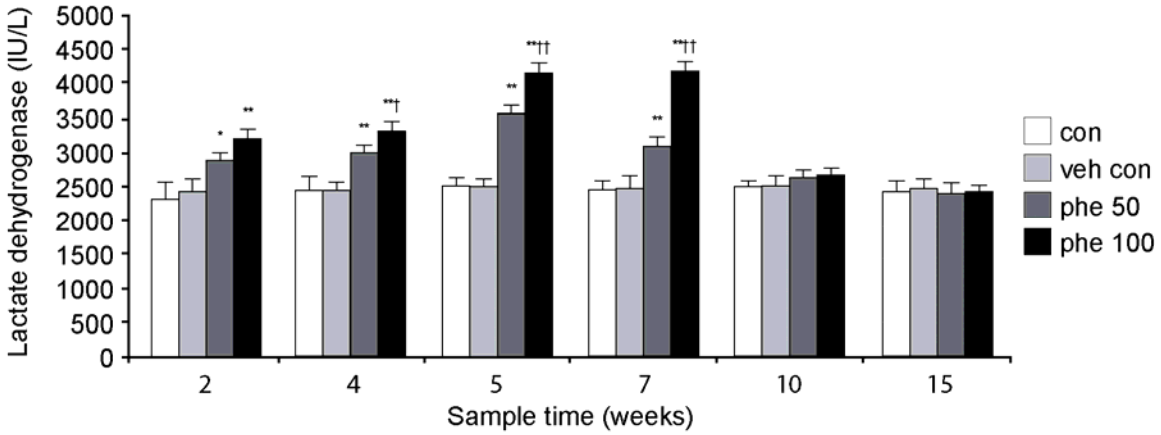

Figure 2. Time response relationship for phenytoin sodium induced changes in lactate dehydrogenase level. Each time at particular dose represent mean + SD from 6 animals. Significant values are; normal control vs treated, * $P<0.01$, ** $P<0.001$; 50 mg vs 100 mg, † $P<0.05$, †† $P<0.001$.

Discussion

Intratesticular testosterone is thought to play a very important role in spermatogenesis; however, it is very rarely measured. In this study the levels of intratesticular testosterone in rats treated with phenytoin sodium showed considerable decline in the 2nd to 7th week sampling time. According to Bauer et al and Kuhn-Velten et al phenytoin acts directly on the testis to inhibit testosterone synthesis by the leydig cells.[5,12] In the present study it is more than likely that a similar effect has been responsible for the low intratesticular testosterone level. In addition, epilepsy patients have an impaired central nervous system response to low testosterone production. Low circulating testosterone levels should trigger an increase in LH secretion from the pituitary, but the feedback mechanism appears to be impaired in men with temporal lobe epilepsy.[5] This impaired feedback mechanism is probably an epilepsy-related effect, and not an antiepileptic drugs (AEDs) effect. The testosterone-to-LH ratio derived from these levels 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.[5] However, this possibility can be ruled out since the present study was carried out on nonepileptic rats. It has been observed that when sex hormone binding globulin (SHBG) is elevated, more of the total testosterone gets bound to SHBG, and less of it remains available as free, or biologically active, testosterone. Thus levels of total testosterone may be normal or even elevated while the concentration of free, or bioactive, testosterone is reduced.[6,11]

SHBG levels may increase progressively during chronic treatment with phenytoin, so that clinically manifest hyposexuality is more likely to occur after 5 or more years of treatment.[13] Although incompletely researched, it has been suggested that antiepileptic drugs 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.[14] 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.[15] Estradiol may impair testosterone secretion 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 estrogen by the enzyme aromatase cannot be ignored.

Lactate dehydrogenase (LDH) is a ubiquitous enzyme present in both plants and animals. When disease or injury affects tissues containing LDH, the cells release LDH into the bloodstream, where it is identified in higher than normal levels. Quantitative analysis of the isoenzyme lactate dehydrogenase-C4 in semen from fertile and infertile men may provide a guide as to the status of the seminiferous epithelium and the degree of germ cell degeneration.[16] LDH-C4 is a membrane enzyme unique to primary spermatocytes, spermatids and spermatozoa and is the enzyme whereby the supply of lactate from the sertoli cell is utilized as an energy substrate. 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. When LDH-C4 activity is expressed in relation to the number of sperm in the semen, it appears to give a remarkably good guide to the efficiency of the seminiferous epithelium in an individual. 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.[17,18] LDH-C4 is a testes specific enzyme; however, in the present study the estimation was done on the total LDH. A study by Goddard et al showed that adult rats treated with flutamide in utero induced altered spermatogenesis.[19] However, the levels of LDH were decreased which is in contrary to the earlier reports. He further suggests that this decrease in LDH indicates 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 at the 2nd to 7th week sampling time. According to Sinha et al and Pant et al, the increase in LDH activity level has a direct effect on testicular functions such as sperm count, sperm production as well as sperm morphology.[20,21] This indicates the cytotoxicity of phenytoin sodium.

Most of the studies on phenytoin sodium indicate that they are gonadotoxic and hence can affect fertility. However, there are no reports regarding the reversal of these effects post exposure. The present study concludes that even though these antiepileptic drugs decrease the fertility by affecting the germ cells, somatic cells, intratesticular testosterone and LDH, these affects are not permanent and are reversed once the drugs are withdrawn. Future studies should address on the effect of these drugs on other hormones and enzymes as well as their effect on other reproductive parameters.

Conclusion

Phenytoin does affect testosterone and LDH level significantly, but this effect is reversible once the drug is withdrawn. This finding has clinical relevance because phenytoin is one of the premier antiepileptic drug that has been used over years and in recent years it is used in the management of other diseases also.

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