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Differentially Regulated Extracellular Genes Expression In Phenytoin Treated Albino Rat Testis - Gene Microarray Analysis

KEYWORDS

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ABSTRACT *Phenytoin (or diphenylhydantoin) is a primary drug for all types of epilepsy except absence seizures, and in ordinary doses, its anticonvulsant action is without sedation. Many research studies proved that reproductive and sexual dysfunction in male epileptic patients have been attributed to androgen deficiency and also the antiepileptic drugs may alter the release and action of different hormones..The present study is aimed at the effect of phenytoin induced differential regulation Extracellular genes in albino rat testis .The albino rats were divided into two groups ,control and test .The test group was given 120mgs/kg /body weight of phenytoin orally and equal amount of normal saline was given for the control group. After 45 days with the rat under deep anaesthesia ,The testis were removed from the scrotum and stored in liquid nitrogen .The stored specimens of testis of control and tests group were subjected to cDNA microarray analysis. This study showed the differential expression of extracellular genes in test group when compared with the control group.*

INTRODUCTION

Phenytoin causes stabilization of neuronal membranes and a decrease in post-tetanic potentiation. It is due to its effect on movement of ions across the membranes. There is inhibition of the influx of Na+ ions across during depolarization. The effect is mediated by a slowing of the rate of recovery of voltage-activated Na+ channels from inactivation. As a result , phenytoin suppresses episodes of repetitive neuronal firing induced by passage of intracellular current and also limits the spread of seizure activity. The anti convulsing drug phenytoin treated patients commonly complain of diminished libido or impotence. Reduced plasma concentration of free testosterone levels have been detected in male epileptic patients receiving phenytoin. It was observed to cause mutagenic effect on human sperm cells. I t also causes chromosomal anomalies in toxic doses can modify cytoskeletal components and extracellular matrix-cell adhesion, with consequent effects on gene expression. The extracellular matrix (ECM) is a collection of extracellular molecules secreted by cells that provides structural and biochemical support to the surrounding cells. The common functions of extracellular matrix are cell adhesion, cell-to-cell communication and differentiation. The testicular extracellular matrix relation plays a role in cell activity and in gene expression required for normal spermato genesis

MATERIALS AND METHODS

Animal treatment and sample collection: Male albino rats weighing 150-175 grams were maintained in the animal house with standard facilities. The animals were housed under 12 hours light/dark cycle and animals were fed with pelleted food and tap water ad labium. The animals were acclimatized under standard conditions, and were divided in to control and Test groups. The test group was given120 mgs/kg/body weight of phenytoin by oral gavage and equal amount of normal saline was given for control group for 45 days in life study protocols, including animal housing, dosage, sacrifice and tissue harvesting were as per IAEC guidelines. After 45 days the tissue samples from test and control collected in Rnase free tubes and snap frozen in liquid nitrogen. Frozen tissues were stored in RNA later at-70 c until processed for RNA extraction RNA Isolation and DNA Microarray Hybridization and Analysis.

RNA Isolation and RNA extraction was performed from the testis by Trizol method, the extracted RNA was preserved in RNA later solution and checked for purity and concentration using spectrophotometer. In order to determine gene expression profiles after phenytoin exposure of testis. The extracted testis total RNA was pooled following the specified dose and period of phenytoin exposure. To reduce variability in the level of gene expression to reduce variability in the level of gene expressions of control and test groups, have been pooled and used for hybridizations. The mRNA isolated from test and control samples were converted cDNAs by reverse PCR separately. The cDNAs of control samples were labeled with green fluorescent dye (CY3) and the cDNAs of test samples were labeled with red fluorescent dye (CY5). These two preparations were mixed together before hybridization

Microarray hybridization: Hybridization was carried out performed in a hybridization cassette. Base pairing takes place between the fluorescent complementary strands in the sample with probe DNAs to from duplex DNA which are called Hybrid DNAs. These Hybrid DNAs emitted fluorescence. Microarray was washed with a wash buffer to remove fluorescent DNAs which did not take part in hybridization after hybridization process.

Scanning: The Microarray was inserted into the slide port of the Microarray Scanner. The scanned image of the microarray was captured in the computer screen. Fluroscent spots indicated the presence of corresponding DNA in the sample, where as no fluorescence indicated that the particular gene was not expressed in the samples. The level of gene expression was analysed from the individual spots. The intensity of the fluorescence corresponds to rate of expression of the gene.

Data Analysis: The signals emitted from green dye (control) and red dye (Test) were measured and recorded automatically. The ratio of green to red dye was calculated by the computer. If the signals of green and red dyes are in equal proportions, the ratio is 1:1 and the expression of the gene is equal in both test and control samples. If the signals of green dye exceed red dye, the expression of the gene in the control is relatively higher than that in the test

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sample. If the signals of red dye exceed the green dye, the expression of the gene in the test sample is relatively higher than that in the control sample.

Data analysis includes automated feature extraction using Agilent feature extraction Software.

Data analysis has been done using Gene Spring GX version 12.0 and Microsoft Excel.

Normalization and statistical analysis:
Normalization: The normalization was done using Gene Spring GX 12.0 Software.

Normalization Method Used: Percentile Shift Normalization

Percentile shift normalization is a global normalization, where the locations of all the spot intensities in an array are adjusted. This normalization takes each column in an experiment independently, and computes the percentile of the expression values for this array, across all spots (where n has a range from 0-100 and n=75 is the median). It subtracts this value from the expression value of each entity. Analysis is done with respect to control samples.

Biointerpreter: Biological Annotation Report

Significant pathways for differentially regulated genes were generated using Biointerpreter.

RESULTS AND DISCUSSION

On the Agilent's custom 44k cDNA microarray which contains 44000 rat genes. The results obtained from microarray analysis revealed significant gene expression changes and brought out expression changes and biological function relationships. Out of 44000 genes analysed 2273 genes were up regulated and 4430 genes were down regulated in test group of rat testis when compared with control group of rat testis samples. Gene cluster analysis was performed to group binding gene specific clusters which revealed 20 genes were binding specific out of which 10 genes were up regulated and 10 genes were down regulated.

Samples hybridized	Up	Down
Testis	2273	4430

Microarray technology has been used to examine the effects of phenytoin exposure in a variety of organisms. Although phenytoin regulated gene expression has been documented, in many organ system there is inadequate information regarding the genome response to this antiepileptic drug in testis. In the present study out of 44000 genes analysed 2273 genes were up regulated and 4430 genes were down regulated in test group of rat testis when compared with control group of rat testis samples. After identifying the group of genes involved in differential expression of phenytoin treated group Cluster analysis was performed to identify testis specific differentially regulated binding genes following phenytoin exposure to pick the candidates for RT-PCR analysis

Clusters for differentials: Genes with similar expression patterns functions clustered together, which helps in further understanding of the genes. Algorithm used is Hierarchical: The most similar expression profiles are joined together to form a group. These are further joined in a tree structure, until all data forms a single group. Spermatogenesis is a complex process involving specific interactions between the developing germ cells and their support cells, the Sertoli cells, within the seminiferous tubules .This process is regulated by the androgen-producing Leydig cells which are found in the interstitial tissue surrounding the seminiferous tubule.

Extracellular matrix is the substrate for cell adhesion, growth, and differentiation, and it provides mechanical support to tissues which are essential for normal spermatogenesis which is regulated by extracellular genes.There are about 20 extracellular genes which are markedly differentially regulated out of which about 10 genes were up regulated and about 10 genes are down regulated.

CONCLUSION:

Most testicular cells require adhering to the extracellular matrix to grow and proliferate and, in majority of the cases, even to stay alive. This dependence of cell growth, proliferation, and survival on attachment to a substratum is known as anchorage dependence, and it is facilitated predominantly by integrins and the intracellular signals they evoke. The physical spreading of a cell on the matrix also has a strong influence on intracellular events which is required for normal spermatogenesis. phenytoin induces differential regulation of extracellular genes which in turn disturbs the cell anchorage required to form various generations of spermatogenic cells and also the secretions of Testoserone which leads to diminished libido and infertility.

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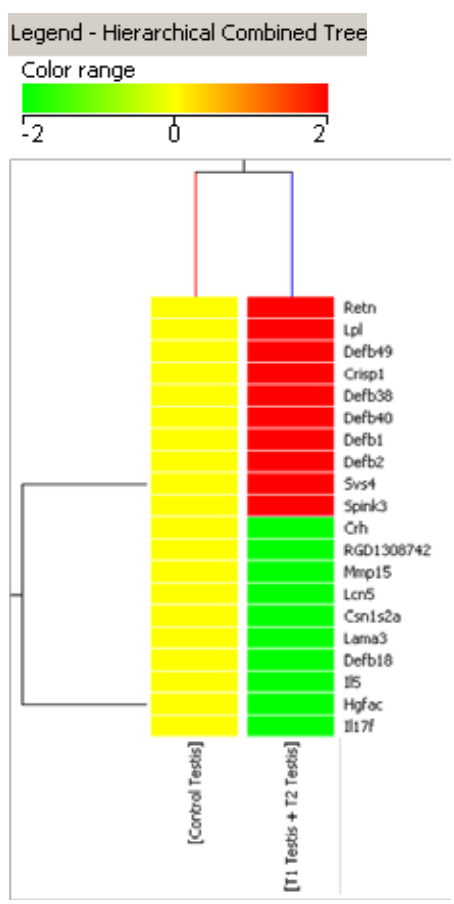
EXTRACELLULAR GENE CLUSTERS



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EXTRACELLULAR GENE CLUSTERS

Sl. No	Probe Name	Regu lation	Fold_ Control Testis	Flag_ Control Testis	Fold_T1 Testis + T2 Testis	Flag_T1 Testis + T2 Testis	gProcessed Sig-nal_ Control Testis	gProcessed Sig-nal_ T1 Testis + T2 Testis	Gene Symbol
1	A_64_P122462	Up	0.00	Compromised	12.02	Detected	2.59	6778.48	Defb2
2	A_43_P12689	Up	0.00	Compromised	10.97	Detected	2.41	3045.16	Defb1
3	A_44_P1016480	Up	0.00	Compromised	9.23	Detected	3.22	1218.93	Lpl
4	A_64_P002546	Up	0.00	Detected	8.90	Detected	31.35	9436.00	Retn
5	A_64_P109541	Up	0.00	Compromised	8.54	Detected	2.40	562.40	Defb38
6	A_64_P040605	Up	0.00	Compromised	7.80	Detected	2.48	346.85	Svs4
7	A_44_P438272	Up	0.00	Detected	7.45	Detected	399.63	43997.58	Crisp1
8	A_64_P150607	Up	0.00	Compromised	7.30	Detected	2.34	233.15	Defb40
9	A_64_P026815	Up	0.00	Compromised	7.27	Detected	2.54	247.24	Defb49
10	A_44_P360767	Up	0.00	Compromised	7.26	Detected	2.54	244.72	Spink3
11	A_44_P386579	Down	0.00	Detected	-3.94	Compro-mised	90.07	3.69	ENSRNOT00000052028
12	A_43_P16222	Down	0.00	Detected	-4.26	Compro-mised	46.44	1.52	Csn1s2a
13	A_43_P16675	Down	0.00	Detected	-4.36	Detected	17341.48	529.84	Mmp15
14	A_42_P511187	Down	0.00	Detected	-4.53	Compro-mised	300.86	8.22	Hgfac
15	A_64_P102783	Down	0.00	Detected	-4.56	Detected	362.23	9.65	Il5
16	A_42_P670493	Down	0.00	Detected	-4.89	Compro-mised	85.77	1.82	RGD1308742
17	A_64_P103025	Down	0.00	Detected	-5.75	Compro-mised	280.36	3.29	Il17f
18	A_43_P12478	Down	0.00	Detected	-5.87	Compro-mised	159.44	1.71	Crh
19	A_64_P109549	Down	0.00	Detected	-6.35	Compro-mised	336.67	2.60	Defb18
20	A_64_P130324	Down	0.00	Detected	-8.14	Compro-mised	1136.89	2.54	Lcn5

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