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PHENYTOIN INDUCED DIFFERENTIALLY REGULATED BINDING GENE EXPRESSION IN ALBINO RAT TESTIS - GENE MICROARRAY ANALYSIS

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ABSTRACT

Phenytoin alters brain cell sodium channels, which has the effect of limiting rapid firing of the brain cells Phenytoin may influence the secretion and functions of different hormones which may contribute to sexual dysfunction .Binding genes are involved in the regulation of gene expression by their molecular functions. They play many important roles like Basal transcription regulation, Differential enhancement of transcription, Development, Response to intercellular signals, Response to environment, Cell cycle control and Pathogenesis. The objective of the present study was to evaluate effect of phenytoin on the testicular binding genes of albino rat testis. The albino rats of test groups were treated with phenytoin for 45 consecutive days by oral gavage and control group was given equal amount of normal saline. After final day of exposure the testis were dissected out from both the groups and subjected for gene microarray analysis which revealed around 3373 genes were up regulated and 4430 genes were down regulated out of 44000 genes analysed further gene cluster analysis was performed to identify the specific binding genes.

KEYWORDS: Gene microarray analysis, Phenytoin, Epilepsy, Seizures

INTRODUCTION

Epilepsy is a disease characterized by spontaneous recurrent seizures neurological disorders characterized by sudden recurring attacks of motor, sensory, or psychic malfunction with or without loss of consciousness or convulsive seizures. Seizure is the clinical manifestation of an abnormal and excessive excitation and synchronization of a population of cortical neurons. A drug which decreases the frequency and/or severity of seizures in people with epilepsy treats the symptom of seizures, not the underlying epileptic condition by maximizing quality of life by minimizing seizures and adverse drug effects. Phenytoin is an anti-epileptic drug, also called an anticonvulsant. It works by slowing down impulses in the brain that cause seizures.

Phenytoin exerts a stabilizing effect on excitable membranes of a variety of cells, including neuron sand cardiac myocytes. It can decrease resting fluxes of sodium as well as sodium currents that flow during action potentials or chemically induced depolarisations. It is therefore used to control tonic-clonic (grand mal) and partial (focal) seizures; it has also been used for the prophylactic control of seizures developing during and after neurosurgery or following severe traumatic injury to the head. It is believed to stabilize rather th an elevate the seizure threshold and to limit the spread of seizure activity. Phenytoin also has antiarrhythmic properties.

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 in polypropylene cages and maintained at 25℃ ± 2℃ under 12 hours light/dark cycle and animals were fed with pelleted food and tap water ad libitum. 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 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. DNA-binding genes with functions involving DNA structure have biological roles in the replication, repair, storage, and modification of DNA, such as methylation. Many proteins involved in the regulation of gene expression contain DNA-binding domains. Proteins that regulate transcription by binding DNA are called transcription factors. The final output of most cellular signaling cascades is gene regulation.

The DNA binding genes interacts with the nucleotides of DNA in a DNA sequence specific or non-sequence-specific manner, but even non-sequence-specific recognition involves some sort of molecular complementarity between protein and DNA.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.

Up regulated binding genes and their functions: Scd1 gene codes for Stearoyl-CoA which is required for the acrosome reaction The focus of this study was to characterize the expression and regulation of stearoyl-CoA desaturase 1 (SCD1), stearoyl-CoA desaturase 2 (SCD2), and Delta5- and Delta6-desaturase in rat testis. Desaturase gene expression was detected in testis, epididymis, and separated cells from seminiferous tubulus. SCD1 and SCD2 expression is demonstrated in rat testis and epididymis .In the present study the Scd1 gene is up regulated due to the deficiency of stearoryl –CoA due to phenytoin action.

LPLThe lipoprotein lipase encoded by LPLgenes, The LPL is secreted by adipose tissues. The testis is covered by connective tissue capsule called tunica albuginea,internal to which is a vascular layer of loose connective tissue, called the tunica vasculosa .The connective tissue extends inward from the tunica vasculosa into the testis to form interstitial connective tissue ,which surrounds ,binds and supports seminiferous tubules .It contains blood vessels loose connective tissue containing adipocytes which secretes LPL .The The endothelium of testicular blood vessels also secrete LPL In the present study phenytoin treated test group showed 3.2 fold change of LPL gene expression which possibly reveals the reciprocal relationship between testosterone and LPL.

FABP: The fatty-acid-binding proteins are a family of carrier proteins for fatty acids and other lipophilic substances such as eicosanoids and retinoids.These proteins are thought to facilitate the transfer of fatty acids between extra and intracellular membranes abundantly expressed in brown and white adipose tissue of Testis.

Car3: Carbonic anhydrase III is a cytosolic protein which is particularly abundant in skeletal muscle, adipocytes and also in rat testis.

Irx1: This gene encodes a member of the Iroquois homeobox protein family. Homeobox genes in this family are involved in pattern formation in the embryo and also plays important role in development of testis.

Krt18: Keratin 18 is a type I cytokeratin. It is, together with its filament partner keratin 8, perhaps the most commonly found products of the intermediate filament gene family. They are expressed in single layer epithelial tissues of the body.

Thrsp: Thyroid hormone-inducible hepatic protein is a protein that in humans is encoded by the THRSP gene. The protein encoded by this gene is similar to the gene product of S14 Spot 14 (S14) is a small acidic protein with no sequence similarity to other mammalian gene products. Its biochemical function is elusive. Recent studies have shown that, in some cancers, human S14 (hS14) localizes to the nucleus and is amplified, suggesting that it plays a role in the regulation of lipogenic enzymes Spink3 modulates sperm physiology through a downstream reduction of endogenous Nitrogen oxide concentration and independently of SPINK3 trypsin inhibitory activity. Pou3f3 the class III POU transcription factor genes play an important role in the nervous system. Pou3f3 play an important role in the nervous system. Comparison of their entire amino acid sequences disclosed a remarkable feature of particular mammalian class III POU genes. Alanine, glycine, and proline repeats were present in the mammalian Brain-1 gene, whereas most of these repeats were absent in the non mammalian homologue

Down regulated binding genes and their functions:

Hgfac: Hepatocyte growth factor activator is a protein that in humans is encoded by the *HGFAC* gene. In mammalian testes Sertoli cells form tight junctions whose function is fundamental for the maintenance of a normal spermatogenesis. Hepatocyte growth factor (HGF) is a cytokine influencing the cellular tight junctions either in normal or in tumor cells. HGF is expressed in the rat testis and influences many functional activities of somatic and germ cells. HGF decreases the levels of testicular occludin and influences the position of the molecule in the tight junctions.

Tbx20: This gene encodes a T-box family member. The T-box family members share a common DNA binding domain, termed the T-box,which is a pseudogene. Pseudogenes are dysfunctional relatives of genes that have lost their protein-coding ability or are otherwise no longer expressed in the cell. Pseudogenes often result from the accumulation of multiple mutations within a gene whose product is not required for the survival of the organism. The DNA of pseudogenes is not functional Lars2Probable leucyl-tRNA synthetase, mitochondrial is an enzyme

encoded by the *LARS2* gene. This gene encodes a cytosolic leucine-tRNA synthetase, a member of the class I aminoacyl-tRNA synthetase family. The encoded enzyme catalyzes the ATP-dependent ligation of L-leucine to tRNA(Leu). It is found in the cytoplasm as part of a multisynthetase complex and interacts with the arginine tRNA synthetase through its C-terminal domain. Alternatively spliced transcript variants of this gene have been found; however, their full-length nature is not known.

Abp1 Catalyzes the degradation of compounds such as putrescine, histamine, spermine, and spermidine, substances involved in allergic and immune responses, cell proliferation, tissue differentiation, tumor formation which is requied for spermatogenic cell differentiation. Interleukin 17F The protein encoded by this gene is a cytokine that shares sequence similarity with IL17. This cytokine is expressed by activated T cells, and has been shown to stimulate the production of several other cytokines, including IL6, IL8, andCSF2/GM_CSF. This cytokine is also found to inhibit the angiogenesis of endothelial cells and induce endothelial cells

CrhGene: CRH gene acts as an antireproductive hormone and as a major local inhibitory regulator of Leydig cell function. Corticotropin-releasing hormone (CRH) also known as corticotropin-releasing factor (CRF). Adh7 Alcohol dehydrogenase class 4 mu/sigma chain is an enzyme that in humans is encoded by the *ADH7* gene. It may participate in the synthesis of retinoic acid, a hormone important for cellular differentiation. The expression of this gene makes it much more abundant in the stomach than the liver, thus it differs from the other known gene family members. CRH gene acts as an antireproductive hormone and as a major local inhibitory regulator of Leydig cell function. Corticotropin-releasing hormone (CRH) also known as corticotropin-releasing factor (CRF) or corticoliberinT Corticotropin-releasing factor (CRF), the key neuropeptide in the stress cascade, has major inhibitory actions on testicular function in addition to its known antireproductive effects at the central level. CRF is secreted by the Leydig cells of the testis and acts through high-affinity receptors at the Leydig cell membrane, as a potent negative regulator of LH action, inhibiting gonadotropin-induced cAMP generation and androgen production.

Lcn5: lipocalin 5This gene encodes a small secreted protein that is expressed in the epididymis and binds retinoic acid. The up and down regulated genes of binding genes are concerned with functions involving DNA structure which have biological roles in the replication, repair, storage, and modification of DNA, such as methylation.Which plays important role in production, maturation and apotosis of spermatogenic and sertoli cells and synthesis of testosterone which suggests phenytoin disturbs the functions of testicular tissues and hormones by disturbing the binding gene functions.

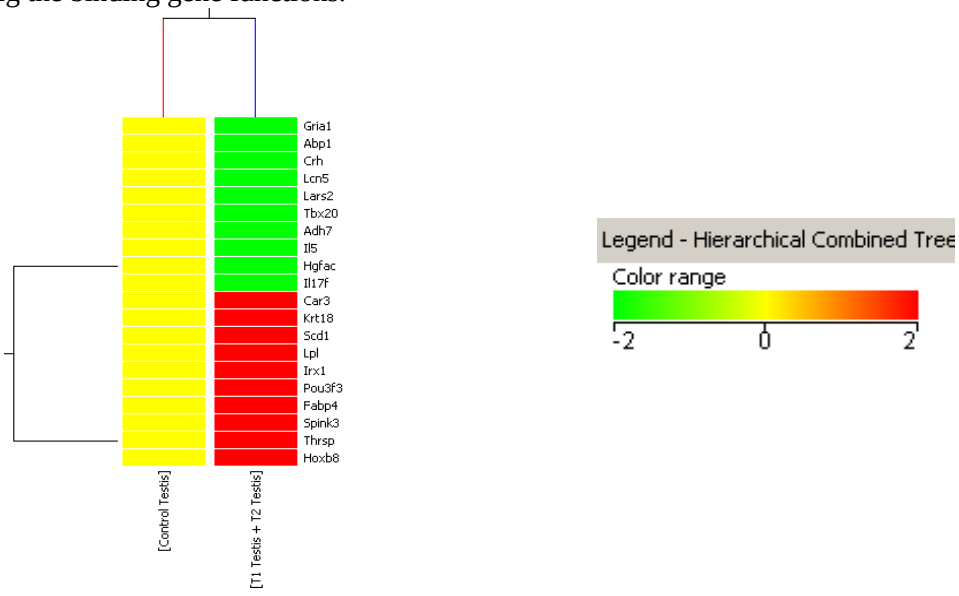

Figure.1.Binding Gene Cluster Analysis

Table.1.Binding Gene clusters Expression pattern

ProbeName	Regulation	Fold_ Control Testis	Flag_Control Testis	Fold_T1 Testis + T2 Testis	Flag_T1 Testis + T2 Testis	gProcessed Signal_ Control Testis	gProcessed Signal_ T1 Testis + T2 Testis	Gene Symbol
A_64_P085530	**Up**	**0.00**	Detected	**9.36**	Detected	148.03	61262.58	Scd1
A_44_P1016480	**Up**	**0.00**	Compromised	**9.23**	Detected	3.22	1218.93	Lpl
A_43_P12786	**Up**	**0.00**	Detected	**8.69**	Detected	19.54	5077.84	Fabp4
A_44_P244851	**Up**	**0.00**	Compromised	**8.25**	Detected	2.68	513.27	Car3
A_64_P080379	**Up**	**0.00**	Compromised	**7.93**	Detected	6.83	1049.53	TC599202
A_64_P086235	**Up**	**0.00**	Compromised	**7.46**	Detected	2.52	278.15	Irx1
A_64_P115506	**Up**	**0.00**	Detected	**7.45**	Detected	30.96	3415.21	Krt18
A_43_P11520	**Up**	**0.00**	Detected	**7.44**	Detected	54.62	5953.05	Thrsp
A_44_P360767	**Up**	**0.00**	Compromised	**7.26**	Detected	2.54	244.72	Spink3
A_44_P311106	**Up**	**0.00**	Compromised	**7.03**	Detected	2.37	194.95	Pou3f3
A_42_P511187	**Down**	**0.00**	Detected	**-4.53**	Compromised	300.86	8.22	Hgfac
A_64_P102783	**Down**	**0.00**	Detected	**-4.56**	Detected	362.23	9.65	Il5
A_64_P007767	**Down**	**0.00**	Detected	**-4.81**	Compromised	90.33	2.02	Tbx20
A_43_P20717	**Down**	**0.00**	Detected	**-4.82**	Compromised	147.96	3.29	Lars2
A_42_P592385	**Down**	**0.00**	Detected	**-4.97**	Compromised	96.34	1.94	Abp1
A_43_P15761	**Down**	**0.00**	Detected	**-5.09**	Compromised	104.13	1.93	Gria1
A_64_P103025	**Down**	**0.00**	Detected	**-5.75**	Compromised	280.36	3.29	Il17f
A_43_P12478	**Down**	**0.00**	Detected	**-5.87**	Compromised	159.44	1.71	Crh
A_44_P166161	**Down**	**0.00**	Detected	**-6.02**	Compromised	162.22	1.57	Adh7
A_64_P130324	**Down**	**0.00**	Detected	**-8.14**	Compromised	1136.89	2.54	Lcn5

CONCLUSION

Binding genes are very large, and enormously complex, molecules consisting of chains of amino acids by peptide bonds, and they can take on a variety of complicated shapes. They can bond with molecules, including other proteins, at particular places known as binding sites, which often consist of indentations into which other molecules, or parts of them, can neatly fit. The chemical properties of the binding site, and the other molecule, are also important: bonding will only take place if it is chemically feasible. A single protein may have more than one binding site.The drug phenytoi disturbs the normal functioning of testicular binding genes and causes infertility and diminished libido.

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