

PHENYTOIN INDUCED DOWN REGULATION OF TESK2 GENE IN ALBINO RAT TESTIS GENE qRT PCR ANALYSIS

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ABSTRACT

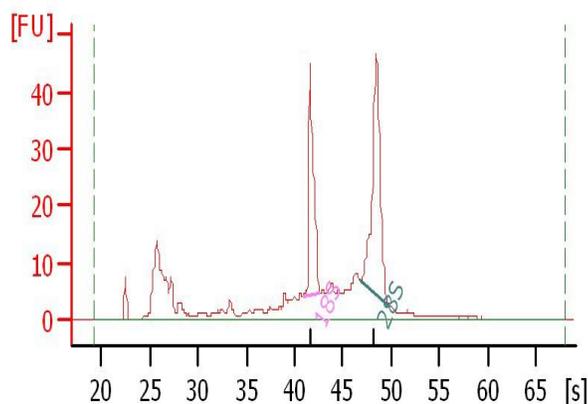
The abnormal, excessive or hyper synchronous neuronal activity in the brain causes epileptic seizures. About 50 million people worldwide have epilepsy and nearly 90% epilepsy occurs in developing countries. Male epileptic patients experience sexual dysfunction. Hormonal changes are observed in epileptic patients. 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 of *Tesk2* gene in albino rat testis. The albino rats were divided into two groups, control and test. The test group was given 120mg/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 test group were subjected to cDNA microarray analysis. This study showed the differential expression of *Tesk2* gene in test group when compared with the control group.

INTRODUCTION

The anti convulsant 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 produces possible mutagenic effect on human sperm cells. Phenytoin produces chromosomal anomalies. 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 tubules. The molecular mechanisms regulating spermatogenesis are to a large extent unknown, however, several kinases have been implicated in various stages of spermatogenesis. The recent DNA micro array technology facilitates to understand large number of gene expression profiling. The technology has potential possibility to comprehend mechanism of multiple genes were related to compounds which have toxicity in biological system. The toxicogenomics through this technology may be very powerful for understanding the effect of unknown toxic mechanisms in biological systems. qRT-PCR is commonly used in research methods to measure gene expression which can confirm the results of gene micro array analysis.

MATERIALS AND METHODS

Animal treatment and sample collection: Male adult albino rats were segregated into control and test groups. The test group were treated with phenytoin 120mg/kg body weight/day orally for 45 days. Similarly control groups were given equal amount of normal saline. 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.



Overall Results for sample : Control Testis

RNA Area: 392.1

RNA Concentration: 357 ng/μl

rRNA Ratio [28s / 18s]: 1.5

RNA Integrity Number (RIN): 8.3 (B.02.08)

Result Flagging Color:

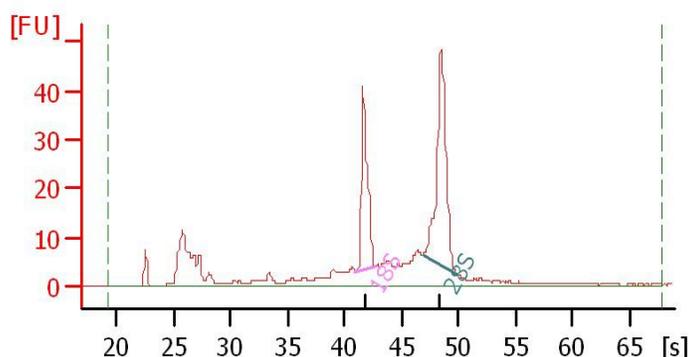
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Fragment table for sample 4 : Control Testis

Name	Start Time [s]	End Time [s]	Area % of total Area
18S	40.90	42.68	56.7
18S	42.68	56.7	14.5

Figure.1. RNA concentration testis-control Sample

RNA Isolation and DNA Microarray Hybridization and Analysis: RNA was extracted from the testis preserved in RNA later using QIAGEN's RNeasy minikit Cat#74104 and checked for purity and concentration. The extracted mRNA labeled with Agilent's Quick-Amp labeling kit (p/n5190-0442) Hybridised with Agilent's in situ Hybridisation kit5188-5242 and scanned using high throughput Agilent scanner with "Surescan" technology



Overall Results for sample 2 : 1_Testis -TEST

RNA Area: 354.0

RNA Concentration: 322 ng/μl

rRNA Ratio [28s / 18s]: 1.7

RNA Integrity Number (RIN): 8.7 (B.02.08)

Result Flagging Color:

Result Flagging Label: RIN: 8.70

Fragment table for sample 2 : T1_Testis

Name	Start Time [s]	End Time [s]	Area % of total Area
18S	40.96	42.64	52.7
28S	46.82	49.92	87.1

Figure.2. RNA concentration test sample

Data analysis includes automated feature extraction using Agilent feature extraction Software, Normalisation and statistical analysis and pathway and gene ontology analysis using Agilent's Genespring GXv10==10.0 Biological interpretation of significant gene using Genotypics Biointerpreter Tool with literature curated information.

RT-PCR: The exponential amplification via reverse transcription polymerase chain reaction provides for a highly sensitive technique in which a very low copy number of RNA molecules can be detected. RT-PCR is widely used in the diagnosis of genetic diseases and, semiquantitatively, in the determination of the abundance of specific different RNA molecules within a cell or tissue as a measure of gene expression.

PCR primers: The Rat primers were manually designed using Gene Runner version 3.05. The primers were validated using one of the samples and amplicon sizes were confirmed using the Bioanalyzer.

PCR Assay: Using the Affinity Script QPCR cDNA synthesis kit (Agilent - Lot# 6144678), 200ng of DNase treated RNA was reverse transcribed to make 25ng/μl of cDNA.

Relative quantification by qPCR was then done using Brilliant II SYBR Green qPCR Master mix (Lot # 6127067). Each sample was run in duplicates for each gene using 25ng input per reaction. The experiment was conducted using Stratagene Mx3005P (Agilent technologies) platform. The relative expression levels of the genes were determined after normalizing with beta Actin (ACTB) as the reference gene by using Delta Ct method. The sequences and length of the primers used are as shown in the Table given below.

PCR Thermal Conditions: PCR consisted of initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 60°C for 1min, 72°C for 1 min. A melt curve was also performed after the assay to check for specificity of the reaction.

Steps for Calculation:

1. Each sample was run in duplicates for each gene. Ct values for each gene were averaged for replicates of each sample.
2. Delta Ct (DCT) was calculated by subtracting the average Ct value of the reference gene from the average Ct of the test gene. (Average Ct Gene - Average Ct reference gene)
3. The Delta Delta Ct (DDCT) was calculated by subtracting the DCT of the control group from the target group [DDCT=(DCT Target- DCT control)]
4. $2^{-(DDCT)}$ CALCULATION was done for each DDCT to yield absolute values. [Fold Change= (2^{-DDCT})]
5. The absolute values are converted into log base 2 values for comparison with microarray data.

Primer Name	Sequence	Tm	Length	Amplicon size (bp)
Rat_Bactin	AAGGCCAACCGTGAAAAGATGA	64C	22	101
Rat_Bactin	CCAGAGGCATACAGGGACAACA	68C	22	
Rat_Tesk2	CTATTACCAGCCACGAGATG	60C	20	143
Rat_Tesk2	GTGCATCCAGGTCAAATACAA	60C	21	

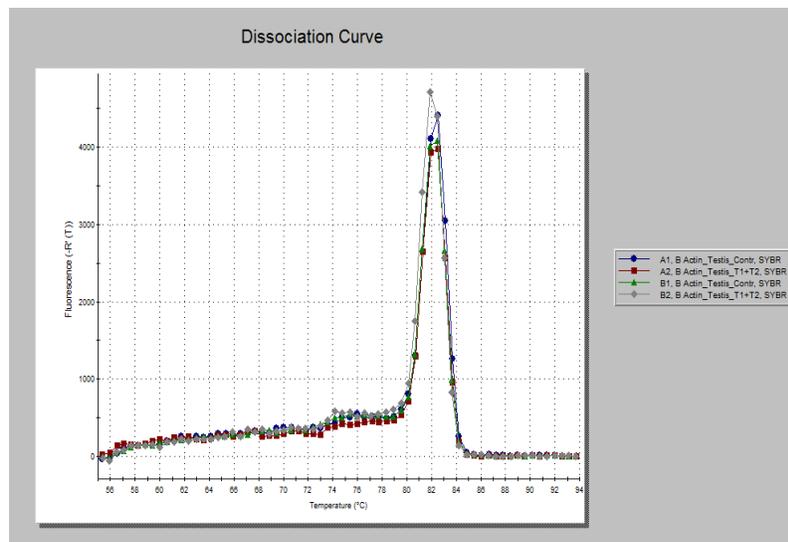


Figure 3 : Dissociation Curve – Bactin Gene (Housekeeping Gene) – Test & Control

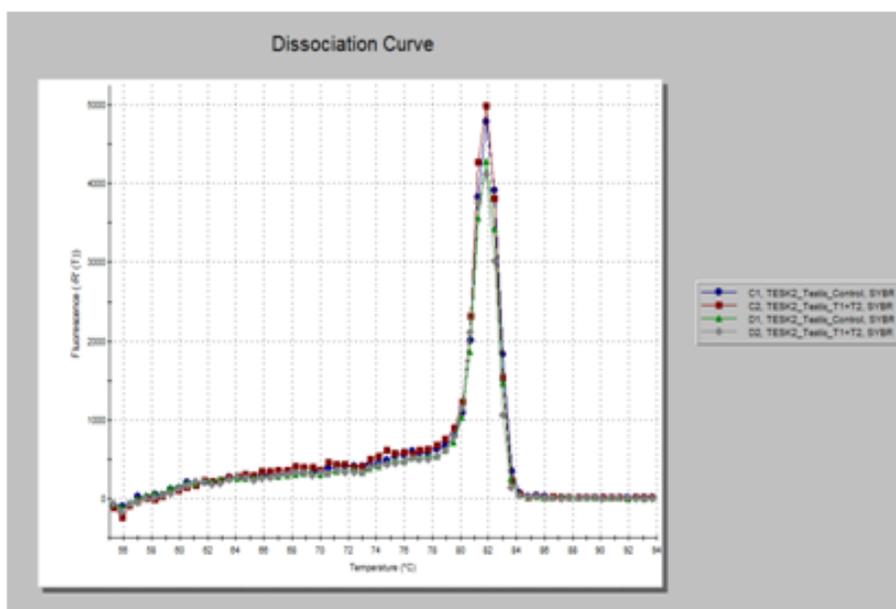
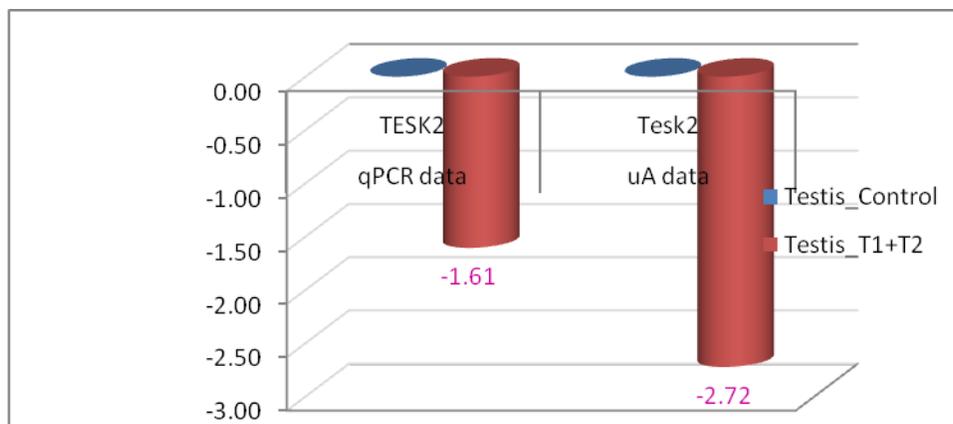


Figure 4 : Dissociation Curve – TESK2 Gene – Test & Control

RESULTS

DNA Microarray: Phenytoin induced -2.72 folds down regulated TESK2 gene expression was observed in phenytoin treated group when compared with untreated control group

qRT PCR Analysis: Phenytoin induced -1.61 folds down regulated TESK2 gene expression was observed in phenytoin treated group when compared with untreated control group



**Graph 1: Graphical representation of qPCR & μ Array Gene Analysis of – TESK2 Gene Fold changes
X-axis –Type of gene Analysis.Y-Axis – TESK2 Gene Fold changes**

CONCLUSION

TESK2 Gene and Protein kinase

Protein kinase 2 is an enzyme that is encoded by the TESK2 Gene. TESK2 is expressed predominantly in non germinal Sertoli cells. Thus, TESK2 seem to play distinct roles in spermatogenesis. TESK2 was localized mainly in the nucleus, which means that TESK2 likely have distinct cellular functions. This gene is predominantly expressed in testis and prostate. The developmental expression pattern of this rat gene in testis suggests an important role in meiotic stages and/or early stages of spermiogenesis.

Phenytoin and cell signaling molecules

Phenytoin directly affects brain regions that mediate sexuality. Phenytoin may cause sexual dysfunction by inducing secondary effects on reproductive hormones. It changes the concentrations of sex steroid hormones. It adversely affects hormone levels by reducing the level of free testosterone which, in turn, reduces sexual desire. It also affect calcium metabolism by inducing a decrease in the Ca^{2+} cell influx which plays important role in cell signalling.

Protein Kinases and cell signaling

Protein Kinases are key regulators of cell function that constitute one of the largest and most functionally diverse gene families. By the addition of phosphate groups to substrate protein they modulate the action, localization and comprehensive function of the wide range of proteins and play a crucial role in co ordinating almost all cellular functions. Kinases play a vital role in signal transduction and synchronise complicated functions such as cell cycle.

Cell signalling

Cell signalling is a comprehensive system of cell interaction which regulates the fundamental cellular activity that controls the cell function. The capacity of cells to perceive and react accordingly to their immediate environment plays an important role in development, tissue repair, immunity and normal tissue homeostasis. Errors in cellular signaling processing leads to cancer, autoimmunity and various metabolic disorders and diseases.

Signal transduction

Extracellular signaling molecule stimulates cell surface receptor which in turn alters intracellular molecules creating a response called signal transduction. In both steps signalling can be amplified by a signalling molecule and in this way signal transduction acts as a molecular switch.

Sertoli cell and protein kinase

Sertoli cells play vital role in modulating the cell signaling. They translate the signals of testosterone to developing germ cells by differential regulation of their own gene expressions (classical pathway) or testosterone itself can activate the protein kinases that play a role in regulating spermatogenesis. (Non-classical pathway).

These observations suggest that TESK2 plays an important role in spermatogenesis. In our study Phenytoin down-regulated the expression of TESK2 gene by -2.72 folds in our gene micro array analysis and -1.62 folds in our q RT-PCR analysis suggesting that phenytoin disturbs normal process of spermatogenesis.

ACKNOWLEDGEMENT

The authors are to acknowledge Dr. A.K. Munirajan and his Research scholars of university of Madras for sample preparation and. M/SGenotypic Technology (P) Ltd, Bangalore, India, for Gene microarray analysis and qRT-PCR analysis.

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