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Research Article

Testicular corticotrophin releasing hormone gene expression of phenytoin treated Albino rats using qRT PCR Analysis

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ABSTRACT

The present study is aimed at the effect of phenytoin induced differential regulation Crh gene 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 was 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 test group when compared with the control group. The expression patterns of several genes differentially regulated which can be confirmed by qRT PCR to verify the results of the microarray analysis.

Keywords: Crh gene; epileptic seizure; qRT PCR; microarray analysis.

INTRODUCTION

An epileptic seizure is a transient symptom of "abnormal excessive or synchronous neuronal activity in the brain". The outward effect can be as dramatic as a wild thrashing movement or as mild as a brief loss of awareness (absence seizure). It can manifest as an alteration in mental state, tonic or clonic movements, convulsions, and various other psychic symptoms phenytoin antiepileptic activity without causing general CNS depression; stabilizes neuronal membranes and prevents hyper excitability caused by excessive stimulation; limits the spread of seizure activity from an active focus; also effective in treating cardiac arrhythmias. Studies show that phenytoin directly affect brain regions that mediate sexuality. Phenytoin may cause sexual dysfunction by inducing secondary effects on reproductive hormones. phenytoin change the concentrations of sex steroid hormones.

Research suggests that the phenytoin, adversely affect hormone levels by reducing the level of free testosterone which, in turn, reduces sexual desire. Phenytoin is an anticonvulsant used to control grandmal and psychomotor seizures. It produces chromosomal anomalies. Phenytoin is excreted in human semen in small quantities and this may possibly affect testosterone levels. (Duncan S et al, 1999) Reduced plasma concentration of free testosterone has been detected in male epileptic patients receiving phenytoin. (Meng H et al, 2001) observed possible mutagenic effect on human sperm cells. According to (Bauer J et al, 2004) and (Kuhn-Velten WN et al, 1990) phenytoin acts directly on the testis to inhibit testosterone synthesis by leydig cells. Abnormal Spermatogenesis is due to the differential expression of genes that codes for enzymes, receptors, cell apoptosis, transcription regulation, and so on.

The genetic expression ability of these genes, infection, and environment jointly contribute to non-obstructive azoospermia and oligozoospermia in males. 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 anti reproductive effects at the central level (inhibition of sexual behavior and LH secretion).

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. The analysis of the single nucleotide polymorphism (SNP) of abnormal spermatogenesis impairment related genes helps explain the possible molecular mechanism of pathogenesis.

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MATERIALS AND METHODS

Animal treatment and sample collection

Male adult albino rats were segregated into control and test groups. The test groups were treated with phenytoin 120mgs/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. To investigate the molecular consequence of phenytoin induced differential gene expression of albino rat testis a large-scale gene expression cDNA Microarray analysis was conducted. Cluster analysis were performed to identify testis specific differentially regulated genes following phenytoin exposure to pick the candidates for RT-PCR analysis, which also verifies the results of cDNA Microarray analysis, as it is subjected for qRT PCR study.

RNA Isolation and DNA Microarray Hybridization and Analysis

RNA was extracted from the testis preserved in RNA later using QIagen's RN easy mini kit 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.

Comprehensive Data Analysis

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 GXv1o==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, semi quantitatively, 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/ul of cDNA.

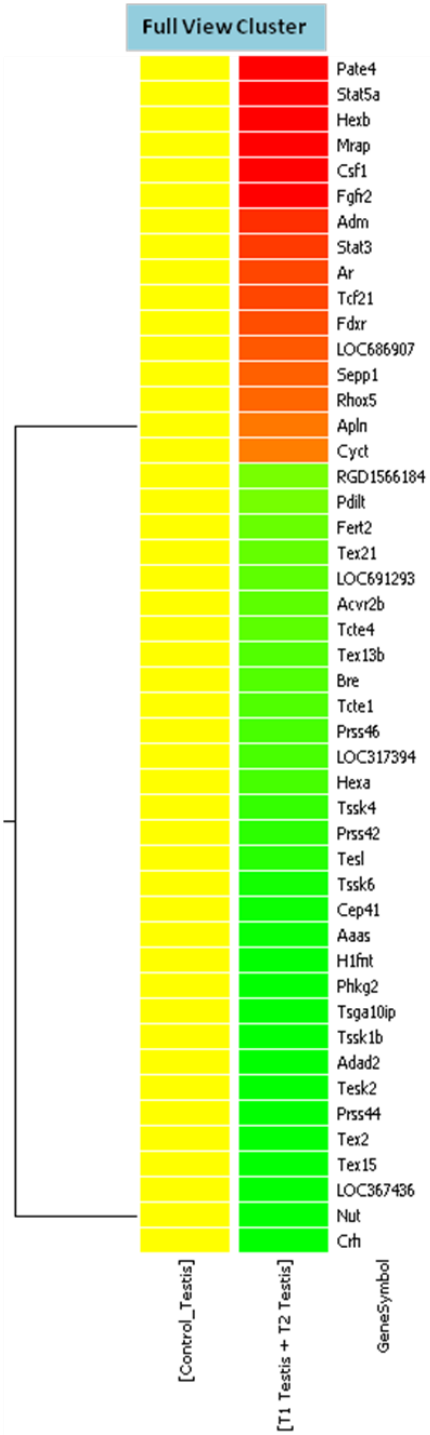

Figure 1: Gene Cluster Analysis – Testis Specfic Genes

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

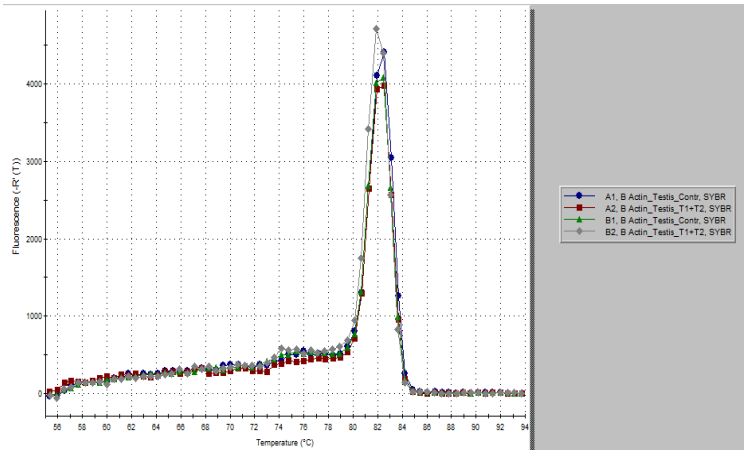

Figure 2: Dissociation Curve – Bactin Gene (Housekeeping Gene) – Test & Control

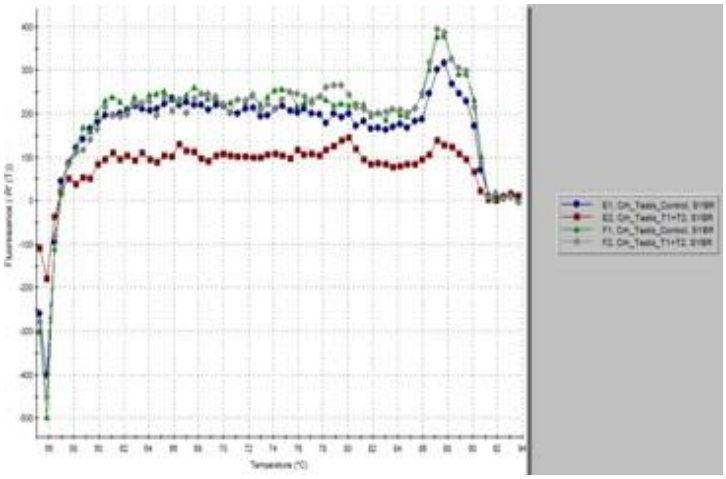

Figure 3: Dissociation Curve-CRH Gene Test and Control

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.

RESULTS

1. DNA Microarray

Phenytoin induced -2.72 folds down regulated Crh gene expression was observed in phenytoin treated group when compared with untreated control group qRT PCR Analysis.

Phenytoin induced -1.61 folds down regulated Crh gene expression was observed in phenytoin treated group when compared with untreated control group.

Androgens are important in regulating potency and libido. Testosterone is the most important androgen. Its serum concentration-specifically the concentration of its free, bioactive form-is affected by Phenytoin.

DISCUSSION

Androgens are important in regulating potency and libido. Testosterone is the most important androgen. Its serum concentration-specifically the concentration of its free, bioactive form-is affected by Phenytoin

Serum testosterone exists in three forms:

- free testosterone (FT, 2% to 3% of total)
- albumin-bound testosterone (55%)
- sex hormone-binding globulin (SHBG)-bound (43% to 45%)

Table 1: Average Ct

Gene/Sample	Testis_Control	Testis_T1+T2
B Actin	17.39	18.84
Crh	37.74	45.00

Table 2: Delta Ct (Average Ct Gene – Average Ct B Actin)

Gene/Sample	Testis_Control	Testis_T1+T2
B Actin	0.00	0.00
Crh	20.35	26.17

Table 3: Delta Delta Ct (Average Ct Test – Average Ct Control)

Gene/Sample	Testis_Control	Testis_T1+T2
B Actin	0.00	0.00
Crh	0.00	5.82

Table 4: Fold Change (2^-DDCt)

Gene/Sample	Testis_Control	Testis_T1+T2
B Actin	1.00	1.00
Crh	1.00	0.02

Table 5: qPCR data

Gene/Sample	Testis_Control	Testis_T1+T2
B Actin	**0.00**	**0.00**
Crh	**0.00**	**-5.82**

Table 6: µA data

Gene Symbol	Fold_ Control Testis	Fold_T1 Testis + T2 Testis
Crh	**0.00**	**-5.87**

The sex hormone-binding globulin bound fraction is not biologically active, but the albumin-bound and free fractions are. biologically active. Reduction in free but not total testosterone is associated with diminished libido and potency. Testosterone increases potency and libido, whereas estradiol lowers it. Although estradiol constitutes only 1% of male gonadal steroids, it exerts almost 50% of the negative feedback on male LH secretion. Hepatic enzyme-inducing Phenytoin. (Heroz et al, 1991) Phenytoin lower the amount of free or biologically active testosterone available to stimulate sexual function; at the same time they increase the serum level of estradiol, which actively inhibits it. Phenytoin affect serum testosterone and estradiol levels by at least four distinct mechanisms. Direct action on the testes: Phenytoin act directly on the testis to inhibit testosterone synthesis by the Leydig cells. 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. This impaired feedback mechanism is probably an epilepsy-related effect, and not an Anti epileptic drug effect. The testosterone-to-LH ratio derived from these levels is a sensitive measure of testicular function. Enzyme induction and phenytoin induce the hepatic p-450 enzymes that catabolize both phenytoin and testosterone. Induction of those enzymes may lead to increased clearance of testosterone from the body and lowering of its level. Inducing liver production of sex hormone-binding globulin When sex hormone-binding globulin is elevated, more of the total testosterone gets bound to sex hormone-binding globulin, 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. Sex hormone-binding globulin levels may increase progressively during chronic treatment with Phenytoin, so that clinically manifest hyposexuality is more likely to occur after prolonged treatment The mechanism by which sex hormone-binding globulin production is induced is unknown. It may be via increased levels of serum estradiol in the presence of Phenytoin use; estradiol is a potent inducer of hepatic sex hormone-binding globulin production. Elevating serum estradiol: Although incompletely researched, it has been suggested that Phenytoin induce the production in the liver of the enzyme aromatase. 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 (FT) to estradiol, serum FT is further reduced. Thus, the ratio of FT to estradiol (FT/E2) is lower in men with epilepsy and hyposexuality than in sexually normal epilepsy patients or in normal controls. Estradiol may impair testosterone secretion in two ways:

- by suppressing male leuteinizing hormone (LH) secretion, leading to hypogonadotropic hypogonadism
- by producing premature aging of the hypothalamic arcuate nucleus, causing hypothalamic hypogonadism

Estradiol also stimulates sex hormone-binding globulin synthesis, whereas testosterone inhibits it. Thus, Phenytoin -induced elevation of estradiol could have a downward-spiraling effect of decreased testosterone and testosterone/E2 ratio, stimulating sex hormone-binding globulin synthesis, resulting in further depression of bioactive testosterone over time.

Crh In Reproductive Tissues

Many investigators have shown that CRH is present in the testis of several animal species, including humans and is localized in Leydig and germ cells and in spermatozoa. (Mugila LJ et al, 1994) Determined the sequence of the mouse CRH gene using RT-PCR and found expression of CRH mRNA in adrenal, ovary, testis, gut, heart, anterior pituitary, lung and spleen in addition to cerebral cortex and hypothalamus. (Fabbri A et al,

1990). In the rat testis CRH acts as an antireproductive hormone and as a major local inhibitory regulator of Leydig cell function. Meanwhile, recently published data demonstrate that in mouse Leydig cells CRH exerts stimulatory effects on steroidogenesis (3 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 (inhibition of sexual behavior and LH secretion). 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. CRF is also a primary stimulus of beta-endorphin secretion by the Leydig cells, which in turn exerts paracrine inhibition of FSH action in the tubular compartment of the testis through high-affinity receptors in the Sertoli cells.

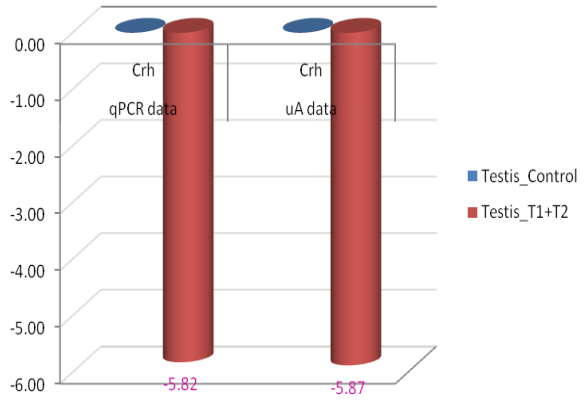

Figure 4: Graphical representation of qPCR & µArray Gene Analysis of CRH

(Tinajero JC et al, 1992) reported that 5HT mediates the stimulatory action of LH/hCG on CRF secretion from Leydig cells and, thus, participates in a negative autoregulatory loop to limit the testosterone response to the gonadotropic stimulus. Observations by (Ulisse S et al, 1990) demonstrate that CRF has a novel and potent antireproductive effect at the testicular level. Since CRF is synthesized in the testis and is present in Leydig cells, it is likely that locally produced CRF could exert negative autocrine modulation on the stimulatory action of luteinizing hormone on Leydig cell function.In the Leydig cell and that the inhibitory action of CRF on Leydig cell function is exerted mainly on the catalytic subunit of adenylate cyclase through a direct or indirect action of protein kinase (Budziszewska B *et al*, 2004) Inhibition of CRH gene promoter activity by antidepressant drugs may be a molecular mechanism by which these drugs inhibit the activity of Hypothalmo hypophyseal axis.

CONCLUSION

when the albino rat testicular cell is over stimulated by phenytoin for a prolonged period of time, and the expression of the CRH receptor protein is decreased in order to protect the cell. CRH represents a promising candidate owing to its expression pattern and anti reproductive property of different origins. Additional large scale studies are warranted to explore the anti reproductive property potential of CRH

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