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Effect of Carbamazepine Drug on the Expression of *miR373* and *miR222* Genes in the Testis of the MIC

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Abstract

Infertility is a major health problem worldwide, and it is estimated that infertility affects 8 to 12% of marriages worldwide. Infertility can be defined as pregnancy after at least 12 months of regular and unprotected gender. Approximately 15% of couples are infertile and in these couples the male factor usually accounts for 50% of the causes of infertility. Infertility in men is one of the several factors that play a role in disorders. Two groups of male Balb/C mice was involved in this study including control and treatment groups. Carbamazepine drug was used orally and mixed with rat food in the treatment group. Finally, the testis of treatment and control animals was dissected and subjected to a relative Real-time RT-PCR analysis for calculation of mi*R373 and miR222* genes expression changes analysis. Our results showed that carbamazepine can disrupt the normal growth of male gonads and a significant increasing rate in the expression of *miR373* gene compared with the control group (Sig.=0.000, P ≤ 0.05, CI=95%). There is no significant change in *miR222* gene expression. These findings are supported by previously reports about the role of these genes. Considering the result of this study, follow-up for infertility in men that are on carbamazepine diet is necessary.

Keywords: Carbamazepine; Infertility; Gene expression analysis; Pregnancy; Gene

Introduction

Infertility is a major health problem worldwide, and it is estimated that infertility affects 8 to 12% of marriages worldwide. Infertility can be defined as pregnancy after at least 12 months of regular and unprotected gender [1]. Approximately 15% of couples are infertile, and in these couples the male factor usually accounts for 50% of the causes of infertility. Infertility in men is one of the several factors that play a role in disorders [2]. About 40% of women's infertility problems are related to men. For 60 to 75% of men, the obvious cause of infertility is indistinguishable, resulting in the term "idiopathic male infertility" [3]. Different factors such as congenital factors including cryptorchidism, Y chromosome microdeletions, chromosomal or genetic abnormalities, klein-felter syndrome and its types kalman syndrome, Robertson's transmission, mild insensitivity syndrome Aadrogens, genetic endocrinopathy, congenital obstruction and acquired factors including varicocele, testicular trauma, testicular torsion, germ cell tumors, acquired hypogonadotrophic hypogonadism, recurrent urogenital tract infections, obstructive urogenital tract can cause infertility in men. Also, cancer chemotherapy, drugs, radiation, heat and idiopathic risk factors such as smoking, alcohol and obesity may induce some types of on fertility [4]. In men, genetic abnormalities can cause infertility by affecting sperm production or sperm transfer [5]. The most common genetic causes of infertility in men are single-gene mutations (such as cystic fibrosis), chromosomal abnormalities, and Y-chromosome microdeletions [6]. It is estimated that about 2,000 genes control this process, most of which are located on autosomal chromosomes, and about 30 genes are located on the Y chromosome. Several X-related genes are expressed in the testis and have been suggested to play a role in spermatogenesis. Mutation in some of the important genes including *CFTR, DAX1, GATA4, WNT4, WT1, AKAP82, HBB, KALIG-1, HAMP, HFE, HFE2, SLC40A1, TFR2, SOX9* can lead to male infertility. *SOX9* regulates maturation of sertoli cells. Sertoli cell immaturity and inability to support the final stages of spermatogenesis cause infertility. Therefore, decreased SOX9 expression can be one of the causes of male infertility [7]. Testicular growth and function are controlled by hormones, cytokines and growth factors [8]. TGFβ1 can modulate androgen synthesis in leydig cells by regulating the expression of human Chorionic Gonadotropin receptor (hCG) and modulating steroidogenic enzymes including P450 17α-hydroxylase/C17-C [9]. Mice lacking TGFβ1 have been shown to suffer from complete infertility due to impaired LH secretion, which leads to decreased testosterone levels and acts in coordination with testosterone-independent sexual dysfunction. Spermatogenesis depends on the epigenetic and genetic regulation of male germ cell. Recently, a number of microRNAs have been identified in human testicular tissues and are closely related to male fertility [9]. One of these molecules is *miR373* at chromosomal position 19q13. 4 that belongs to the *miR371-3* gene cluster. To date, the

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role of *miR373* in the processes of regulating cell proliferation, apoptosis, aging, mesododerm differentiation, migration and invasion, as well as in the miRNA response induced by hypoxia has been identified [10]. *miR373* performs its functions by pairing with 3 'Untranslated (UTR) regions of mRNA after transcription and reduces gene expression, or by binding to target DNA promoters, positively regulates mRNA expression [11]. microRNA through *SIRT1* gene and *TGFRB2* gene can reduce the expression of *SOX9* gene and play a role in infertility [12]. Upregulation of *miR222* represses cell cycle progression and induces the apoptosis of immature porcine sertoli cells, whereas inhibition of *miR222* lead to the cell proliferation. Also, both *miR222* overexpression represses the phosphorylation of PI3K and AKT and suppresses immature porcine sertoli cell growth by targeting the *GRB10* gene [13].

Reproductive disorders are more common among men with epilepsy than the general population. Epilepsy is one of the most common neurological diseases, and researchers around the world have reported epilepsy as being associated with genital disorders and the possibility of endocrine and reproductive and sexual endocrine disorders [14]. According to current results, men with epilepsy may experience changes in serum gonadotropin and testosterone levels that may affect sperm quality. Epilepsy with adverse effects on the nervous system, as well as on the production of male hormones and the psychological stress that follows, as well as the adverse effects of drugs used to control epilepsy cause sexual dysfunction in men. Epilepsy disrupts the secretion of hormones from the hypothalamus and pituitary gland and the production of hormones by the testes [15]. Taking medications prescribed for these patients, including carbamazepine, increases the concentration of serum Gender Hormone Binding Globulin (GHBG) in men and women with epilepsy. Over time, elevated Serum levels (SHBG) lead to decreased biological activity of testosterone and estradiol, which may lead to decreased sexual potency in men and menstruation in women as a result of reduced fertility [16]. Carbamazepine is the most widely used antiepileptic drug that is widely used in patients with epilepsy [17].

Material and Methods

Maintenance and preparation of mice

Ten the male BALB/c mice with 25 ± 5 gram and 1.5-2 months were prepared as model animals in this study. The animals were maintained at temperature 25℃ on a 12 h light/dark cycle. All animals received humane care in compliance with the guidelines of the animal care and use committee. The ethics committee of the Shahrekord university (approval ID. IR.SKU.REC.1399.012) approved the research. Two groups with 5 members randomly were selected. The first group was identified as the Treatment group (T) with CBZ and the second group as the Control group (C).

Calculation of CBZ dose

To evaluate the effect of CBZ, it was used orally and mixed with rat food. The dose of CBZ was calculated in two doses over two 7 day periods (14 days in total) (Table 1).

Table 1: Time and dose of CBZ.

Period	Dose	Time
First	0.057 mg/Kg	7 day
Second	0.114 mg/Kg	7 day

Tissue sampling, RNA isolation, and cDNA synthesis

After 14 days, testes were dissected from the studied groups. Tissue samples were transferred to a -70 freezer. Total RNA was isolated from the testis of mice model using the TriZol total RNA extraction kit (Fermentas, USA) according to the manufacturer's instructions. The purity of extraction RNA was measured with a NanoDrop™ 1000 Spectrophotometer (Thermo fisher scientific, USA). Then cDNA was synthesized using a cDNA synthesis kit (TaKaRa, Japan) according to the manufacturer's instructions.

Real-Time RT-PCR

Subsequently, Real-time RT-PCR was performed in order to the expression of genes in the testes. U6 noncoding RNA gene was selected as an interior control. Primers used are shown in Table 2. Amplification for all reactions in this study was performed under this condition.

An initial denaturation at 95℃ for 15 seconds, followed by 40 amplification cycles including denaturation at 95℃ for 20 s, annealing for 30 s dependent on primer TM for all genes and 25 s at 72℃ for extension. The quality of fragments was confirmed by agarose gel electrophoresis.

Table 2: Primer sequences used for amplification of the target genes.

Primer name	Sequence	Target length	Target gene	Accession no.
RT-Primer	AATATGGAACGCTTCACGAATTTG	104 bp	*U6*	XR_008330775.1
F-Primer	GTGCTCACTTCGGCAGCAC			
R-Primer	AATATGGAACGCTTCACGAATTTG			
RT-Primer	CCGACGAATACCCTCTGGTACAGCTAGAAGATGCCATCAG	131 bp	*miR222*	MIPF0000051
F-Primer	GCTGCTGGAAGGTGTAGGTAC			
R-Primer	CCGACGAATACCCTCTGGTAC			
RT-Primer	CTTCCATCCTGAGCTAGTACGGGACACCCCAAAATCGAAGC	89 bp	*miR373*	MI0000781
F-Primer	GGGATACTCAAAATGGGG			
R-Primer	CTTCCATCCTGAGCTAGTAC			

Gene expression and statistical analyses

Expression changes of studied genes was calculated based on $2^{-\Delta\Delta Ct}$ method [18]. All results are given as the mean ± SEM. Kolmograph-Smirnov method was used for normality assay of given data. Probabilities of chance differences between two groups were calculated using an independent t-test.

Results

As described in material and method, total RNA was extracted by using RNXplus solution and qualified in gel electrophoresis (Figure 1). 28s and 18s rRNA shows an optimal quality of the extracted RNA.

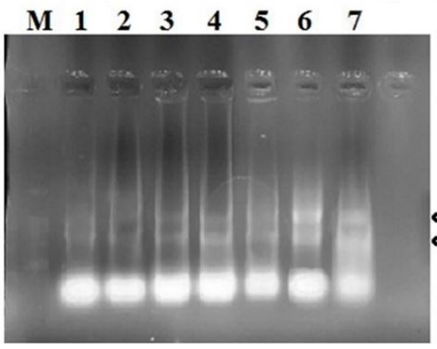


Figure 1: Separation and analysis of RNA. Green viewer-stained 1% TBE agarose gel showing RNA extracted from testis tissue of treated mice. M: 100 bp DNA size marker.

Figure 2 shows the melt curves for target genes amplicons. The single peak is typically interpreted as representing a pure, single amplicon. Also, amplification plot for every genes is showed.

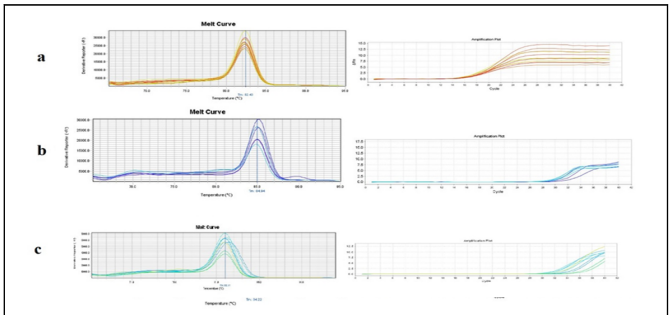


Figure 2: Melt curves and Amplification plots for the reference (ActB) and targeted amplicons of the studied genes. a) *U6*; b) *miR222* and; c) *miR373*.

As mentioned in this study, the expression changes of *AdipoQ*, *Glut 4*, *miR373* and *Mir373* genes as candidate genes involved in the metabolism of lipids and carbohydrates under the administration of carbamazepine were measured in testis by the $2^{-\Delta\Delta Ct}$ method [19]. Using graphpad prism 9.0.0 software, the statistical independent t-test method was carried out and the research hypothesis was tested and the results were presented. The distribution of data for the results of gene expression changes between control group and the animals treated with carbamazepine was performed by Simonov kolmograph test

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using SPSS version 26. Figures 3a and 3b, show the result of *MIR322*, *Mir373* and *Mir373* genes expression analysis.

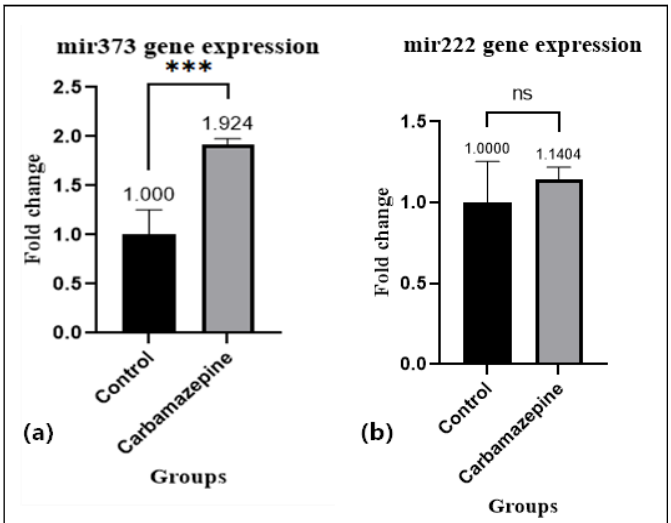


Figure 3: Gene expression changes between control and carbamazepine treated groups. a) *miR373* gene showed two fold increase in expression compared to the control group (P ≤ 0.001, Sig.=0.000); b) *miR222* gene expression with no significant change (P ≤ 0.05, Sig.=0.01, fold change=0.274) compared to the control group.

Discussion

Hormonal changes that can be caused by epilepsy include increased gender hormone bonding globulin, decreased free androgen index, impaired pulmonary gonadotropin secretion, and impaired dopamine secretion leading to hypogonadism, which can be associated with sexual dysfunction. Genetic factors may be important in sperm changes in men with epilepsy [20]. In addition, the number of people with normal sperm is low among people with epilepsy. If there are any abnormalities in the sperm, fertility can be compromised. In addition, men with epilepsy are five times more likely to have erectile dysfunction than men without epilepsy. The results of the first prospective study of the long-term effects of CBZ on serum androgen levels and sexual function in men with epilepsy showed that serum SHBG levels gradually increased during long-term CBZ treatment while serum testosterone levels remained unchanged. As a result, an increase in SHBG continuously decreases the free androgen release of Ramanger serum. The results of a study showed that patients treated with CBZ had significantly higher SHBG levels and lower FAI levels and DHEAS concentrations. Levels of LH, FSH, PRL or E2 were not different in male patients with epilepsy treated with CBZ and untreated with the control group. CBZ use alone does not significantly alter the serum balance of gender hormones. However, CBZ clearly affects serum SHBG and DHEAS levels. Many studies have shown changes in the testicles and the incidence of infertility and sperm deformity in people treated with carbamazepine.

In this study, we investigated the effect of carbamazepine drug on the expression of four key genes in metabolism balance of lipids and carbohydrates. Several studies indicated that carbamazepine can promote hyperglycemia in human and animal. In a study in the rats, carbamazepine significantly increased fasting plasma glucose, insulin resistance index, total cholesterol and triglycerides compared with the control group. This phenomena is very important in about diabetic patients and the peoples that affected by obesity. We proposed here that this effects may resulted from an interference between the carbamazepine drug and gene expression profile in testis. As a simple view, adiponectin has been shown to enhance insulin sensitivity in several reports that result in entrance of glucose into the adipocytes and testis cells. In this situation, m*iR373* hormone acts in reverse direction in releasing of glucose into the blood stream. In our study gene expression showed 15 times increase (P ≤ 0.0001) compared to the control group. Adiponectin level elevates with hyperglycemia independent of body size. Optimization of blood glucose in the hyperglycemic patients that tolerate decrease in adiponectin levels is independent of diabetes type and/or body weight. Adiponectin played a critical role in regulating plasma glucose and reducing pancreatic islet apoptosis after gastric bypass surgery. So, increasing of adiponectin that we reported in this study, may be a secondary or compensation result of hyperglycemia. In this situation, the body may changes its metabolic source from glucose consumption to lipid catabolism. Other result of our study, decreasing of *miR322* gene expression to 0.4 times (P ≤ 0.001) compare to control group, is compatible with this hypothesis. Also, in this study, we investigated the expression change of Adiponectin and *miR373* genes under the carbamazepine diet. Adiponectin/*miR373* balance is one of the most important indexes for an optimal metabolic condition in the body. Results of this study showed decreasing of *miR373* to 0.76 (P ≤ 0.05) compare to control group. This decreasing of miR373 level after the carbamazepine consumption, may be a result of glucose level elevation in blood that can induce a satiety situation result in decrease of miR373 production.

Similar to our findings, some studies indicated that carbamazepine cause diminish in level of *miR373* in the patients' blood. Carbamazepine and carbamazepine is two common anti-epileptic drug. Decreasing of *miR322* gene expression that we reported here, must be considered beside of *miR373* diminish. Low level of *miR373* in blood can lead to decreasing of insulin secretion and decreased *miR322* gene expression. Previous researches showed that changes in *miR322* gene expression under the high level of cAMP is accompanied by similar alterations in miR322 protein expression and glucose uptake, suggesting a role of *miR322* gene expression in regulation of cellular insulin role in glucose transport. Based on this findings, we proposed that decreased *mir373* that resulted from carbamazepine drug may be act through a cAMP manner. As supportive reports, correlation analysis indicated that the decrease in *miR322* gene levels was only observed in hyperglycemic mice. On the other hand, the glucose catabolic related genes do not exhibit any clear coordinate expression. Agitated expression of glucose catabolic genes may contribute to hyperglycemia and muscle insulin resistance. Interestingly, a study showed that hyperglycemia correlate with increasing of GLUT1 protein, while, *miR322* gene level is decreased under hyperglycemic condition. It seems, because of a wide role of

miR373 hormone compared with restricted action of adiponectin, the expression changes in *miR373* gene shows lower variance.

Conclusion

Finally we analyzed the expression of *miR373* gene after treatment of animals with carbamazepine drug. Our result showed a more than 3 times increasing in expression for *miR373* gene compare to control group of mice ($P \leq 0.001$). *miR373* is expressed in many mammalian tissues. Specially, during adipocyte differentiation *in vitro*, *miR373* is expressed at a relatively high level in the early stages of adipogenesis, however its expression is gradually decreased at later stages. Thus, it is possible that *Mir373* is necessary for the early stages of adipogenesis. Also *miR373* has a well-defined role in phosphorylation and translocation of *miR322* protein. This role can explains increasing of *miR373* gene expression under carbamazepine consumption while *miR322* gene level has decreased. On the other word, we think that, *miR373* gene expression is increased to compensate the low level of *miR322* protein. Considering the finding of this study, it can be concluded that carbamazepine is a relatively high-risk antiepileptic drug for obesity and metabolic syndrome, but more studies are needed.

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Author Contributions

Laboratory work has done by Elaheh Raji. AMA participated as the group leader and the owner of the idea and the main author of the article. Marzieh akbari was collaborate as a part of laboratory work and animal treatments.

Conflict of Interests

The authors declare that they have no competing interests.

Ethical Declarations

The study has been approved by the ethical committee of Shahrekord University. Ethical code: IR.SKU.REC.1399.013.

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