Biomedical Research and Therapy 2023, 10(9):5884-5895

Open Access Full Text Article

Original Research

Assessment of circulating miRNA-218, miRNA-222, and miRNA-146 as biomarkers of polycystic ovary syndrome in epileptic patients receiving valproic acid

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History

- Received: Jul 05, 2023
- Accepted: Sep 24, 2023
- Published Online: Sep 30, 2023

DOI : 10.15419/bmrat.v10i9.833

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ABSTRACT

Introduction: This study was designed to evaluate the relationship between taking sodium valproate (VPA) and the onset of polycystic ovarian syndrome (PCOS) in women with epilepsy (WWE) and analyze the biochemical factors and expression levels of three miRNAs as diagnostic or predictive biomarkers. These miRNAs target numerous genes and molecular pathways involved in hyperandrogenism, insulin resistance, and obesity in PCOS patients. **Methods**: The study was conducted on 120 WWE aged 18–35 years before and after monotherapy with VPA and 120 women with PCOS (WWP). After collecting the plasma samples of patients, the total RNA was extracted. Then, the miRNA-218, miRNA-222, and miRNA-146 expression levels were determined via qRT–PCR. **Results**: In this study, the relative expression levels of miRNA-146 and miRNA-218 showed a significant increase in the WWP and treated epileptic patients ($P \leq 0.01$). However, the expression level of miRNA-222 significantly ($P \leq 0.05$) reduced. Our finding showed a significant increase in the concentrations of anti-Müllerian hormone (AMH), free testosterone, and estradiol and an increased LH/FSH ratio after treatment compared with pre-treatment with VPA ($P \leq 0.05$). **Conclusion**: Significant changes were observed in the expression of the examined miRNAs after receiving VPA, especially miRNA-218. In addition, a significant correlation was found between PCOS and AMH, free testosterone, estradiol, and the LH/FSH ratio. Therefore, the miRNA-218 expression and these biochemical factors are valuable biomarkers for predicting PCOS symptoms. They are cost-effective for controlling side effect and timely medication adjustments in patients receiving VPA.

Key words: Anti-seizure medication, Sexual disorders, miRNA-146, miRNA-222, miRNA-218

INTRODUCTION

Epilepsy is a common neurological disease affecting both genders, and it is estimated that approximately 50 million individuals are afflicted with this disorder worldwide[1]. According to epidemiological studies conducted on Iranian epileptic patients, the incidence of epilepsy is likely 5 per 1000 people[2], and its prevalence is higher in women than men[3]. Epilepsy has been linked to several reproductive endocrine disorders depending on the type, duration, and dose of anticonvulsant drugs. Hormonal changes, irregular menstrual cycles, and symptoms resembling polycystic ovarian syndrome (PCOS) are some of the most frequently described signs[4]. There are various antiepileptic medicines (AEDs) available for therapy, but valproate (VPA) is regarded as the top option[5]. VPA has mood-stabilizing properties and is prescribed for treating focal and generalized epilepsy. It is also effective for partial seizures, bipolar disorder, migraines, and neuralgia[6]. It has been shown that VPA inhibits liver enzymes[7] and can increase the concentration of sex hormone-binding globulin (SHBG) in both sexes. It has been associated with an increased free androgen index and increased levels of dehydroepiandrosterone sulfate, androstenedione, and testosterone, as well as the development of ovarian cysts, sexual problems, subfertility, and irregularities in the menstrual cycle[8]. Finally, a higher prevalence of PCOS-like symptoms in epileptic women who use VPA indicates that it may impair androgen synthesis and ovarian function. This is probably due to various effects on the hypothalamic–pituitary–ovarian axis[9]. Recent research has shown that using AEDs while having epilepsy causes several issues, with PCOS and its consequences having been considered[10].

MicroRNAs (miRNAs) are small, non-coding RNAs with a length of 18 to 25 nucleotides that significantly affect cell development, proliferation, differentiation, inflammation, apoptosis, stress response, and the spread of malignant tumors via post-transcriptional

Cite this article : Rajabi M, Kalantar S M, Mojodi E, Salehi M, Firouzabadi R D, Etemadifar S M, Montazeri F. **Assessment of circulating miRNA-218, miRNA-222, and miRNA-146 as biomarkers of polycystic ovary syndrome in epileptic patients receiving valproic acid**. *Biomed. Res. Ther.* 2023; 10(9):5884-5895.

suppression. Circulating miRNA has recently been linked to the development of cardiovascular disorders, endometriosis, poor ovarian response, and cervical cancer[11]. Recent research demonstrates that the miRNA expression is altered in the serum, plasma, follicular fluid, and granulosa cells of PCOS patients compared with healthy controls, making them potential biomarkers for diagnosing, monitoring, and treating this condition[12,13]. Furthermore, miRNAs may indicate PCOS-related aberrant metabolism, impaired oocyte quality, and decreased endometrial receptivity. PCOS patients have considerably higher levels of miR-146a and miR-222 than healthy women[14]. In addition, miR-146a is differentially expressed in PCOS ovarian tissue. Bioinformatics research has revealed that miR-146a and miR-222 target genes are implicated in the cell cycle, apoptosis, and endocrine pathways, such as Wnt, MAPK, and Jak-STAT signaling. These findings suggest that miR-222 and miR-146a may play a role in the etiology of PCOS[15]. Mature miR-218 has been identified to regulate mRNA expression through more than 900 target genes, including RICTOR, LAMB3, BIRC5, and ROBO1, which may be crucial in the development of cervical cancer, according to the Microcosm Targets software program (http://www.ebi.ac.uk/enright-srv/microcosm/). Several of these genes are involved in various cancer signaling pathways, including the Wnt/β-catenin, ERK/MAPK, and Notch pathways[16]. One study showed that higher miRNA-218 levels are related to adiponectin suppression in obesity and PCOS by binding to AMP-activated protein kinase and p38 mitogen-activated protein kinase and acting on the adiponectin receptor (AdipoR2)[17]. Numerous studies have linked the markers miR-135b, miR-190, miR-217, miR-218, miR-299, and miR-342 to the presence of estrogen receptors in breast cancer[18]. Notably, the expression of these miRNAs regulated by estrogen receptors (ERs) exerts indirect steroidal effects on follicular cells or directly on the androgen receptor pathway to modulate androgen metabolism and related disorders, such as breast cancer and PCOS[19,20]. Because PCOS is diagnosed early, the severity and progression of its problems can be treated[2]. As a result, this study was established to identify a valid and non-invasive biological marker for PCOS prediction. Some miRNAs regulated in PCOS have been confirmed, while others have been only predicted[21]. In this study, miR-146a, miR-222, and miR-218 expression levels were investigated as potential indicators for developing PCOS-like symptoms in epileptic patients following VPA administration.

METHODS

Patient groups and study design

This study was performed at the Abortion Research Center, Yazd Reproductive Sciences Institute of Shahid Sadoughi University of Medical Sciences, Yazd, Iran, with the approval of the local ethics committee. All participants signed informed consent forms to use both their samples and information documented in their medical records.

All epileptic patients were referred from the neurology and epilepsy clinics of Maseh Epilepsy Clinic Isfahan and Isfahan Epilepsy Society. An expert neurologist diagnosed all epileptic patients (generalized or focal epilepsy) according to the criteria of the International League Against Epilepsy (ILAE) (2017). Samples from PCOS women were collected from the Abortion Research Center, Yazd Reproductive Sciences Institute of Shahid Sadoughi University of Medical Sciences, Iran, and the diagnosis was made by a gynecologist according to Rotterdam criteria. In the epileptic group, patients with a history of endocrine disorders, diabetes, liver disease, kidney disease, pituitary abnormality, depression, or any CNS disease were excluded. Other exclusion criteria were abnormal thyroid function tests and body mass index (BMI) > 30 kg/m^2 in women with epilepsy before VPA treatment (WWEb). Patients were asked to report any unwanted pregnancies or if they discontinued treatment and were excluded from the study. Patients with excessive body hair, acne, or lactation problems were screened thoroughly. The study also included 120 Iranian women with PCOS aged 18 to 35 years (WWP) with exclusion and inclusion criteria similar to the epileptic group. None in the WWP group had a history of seizures, neurologic or other endocrine disorders. All WWP and WWE patients were newly diagnosed or untreated.

Drug dosage and sample collection

All epileptic patients received VPA (RAHA DRUG) in a dosage of 500 mg/day (250 mg twice a day) for the first week, which was increased to 1000 mg/day the following week. The target maintenance dosage for VPA was 1000–2000 mg/day for at least 3 months. In this study, 5 mL of peripheral blood was drawn from each patient before and after treatment in the epileptic women. The whole blood was centrifuged twice: once at 4000 rpm for 10 min and then for 15 min at 1200 rpm to remove cell particles completely. Then, the serum was stored at -80°C for further analysis.

Demographic and hormonal information

Some of the required information for the study was extracted from patient records or questionnaires filled out by specialists. The demographic information and clinical findings included BMI, age, irregular menstruation, and hirsutism (F–G score). Furthermore, some information about the classification of epilepsy was recorded: length, frequency, family history, and age at symptom onset of seizures. Moreover, information on hormonal and biochemical parameters was extracted from the medical records. These parameters included luteinizing hormone (LH), follicle-stimulating hormone (FSH), LH/FSH ratio, anti-Müllerian hormone (AMH), estradiol (E), free testosterone, dehydroepiandrosterone (DHEA-S), estradiol, hydroxyl progesterone, prolactin, SHBG, fasting insulin, and fasting glucose levels.

microRNA extraction and quantification

Circulating miRNA from plasma was extracted using a miRNA extraction kit (Roche) according to the manufacturer's instructions. A NanoDrop 2000c from Thermo Scientific was used to evaluate the concentration of the extracted RNA and its purity by determining the A260/A280 ratio, and then, it was stored at −80°C. cDNA synthesis was achieved using the BON-miR 1st-strand cDNA synthesis miRNA kit (catalog #BON209001). qRT–PCR was performed using RealQ Plus 2x Master Mix Green with high ROX^{TM} (Ampliqon, cat. No: A325402) and StepOne software v2.3 on the qRT–PCR ABI system (Applied Biosystems, Carlsbad, CA, USA). SNORD was used as an endogenous control due to its stable expression in all samples in the profiling experiments for all miR-146, miR-218, and miR-222. qRT–PCR was performed to evaluate the expressions of the miRNAs using a specific primer (**Table 1**) in a total volume of 25 μL by following the thermal profile of initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 45 s. All PCR experiments were performed in duplicate. The relative expression values of all miRNAs were evaluated using the relative quantification $2^{-\Delta CT}$ method.

Statistical analysis

Data processing and statistical analysis were performed using the SPSS software version 26. The parameters are reported as means ± SD between the PCOS and epileptic patients before and after treatment. Multiple comparisons were made using Pearson correlations and ANOVA test, discrete variables were compared with Pearson's test, and $P < 0.05$ was considered significant. For comparing the mean differences in miRNA-146, miRNA-222, and miRNA-218 expression levels between epileptic patients before and after treatment, $P < 0.01$ was considered significant.

RESULTS

A total of 120 women (mean age, 26.5 years; range, 18–35 years) before and after monotherapy with VPA were compared to women who only had PCOS (mean age, 27.5 years; range, 18–35 years). There was no significant difference in BMI between groups 1 and 2, but women in the VPA-treated group became more obese (BMI > 25) after treatment (**Table 2**). The hirsutism score was significantly different between groups ($P \leq 0.05$) with an average of 5.83 ± 0.314 in WWEb, 15.06 ± 0.480 in WWEa, and 19.13 ± 0.980 in WWP (F–G score in **Table 2**). The information about clinical menstrual disturbances summarized in **Table 1** includes irregular menstruation (amenorrhea, dysmenorrhea, and oligomenorrhea) or the time of irregular menses. The pattern of menstrual cycle irregularities in WWEa was very similar to that of WWP and significantly different from WWEb.

The comparison of biochemical parameters, including target hormones, between the three groups, WWEa, WWEb, and WWP, is summarized in **Table 3**. The women had significantly lower free testosterone levels before taking the VPA than the control (WWP) and VPA groups (WWEb) ($P \leq 0.05$). Next, we examined DHEA-S, and its serum level increased after treatment. However, the DHEA-S mean was not significantly different after VPA treatment ($P \leq 0.05$). Concerning PCOS, serum levels of LH, FSH, and AMH are important for both early diagnosis and prognosis. Our findings showed increased levels of all three hormones after VPA treatment ($P \leq 0.05$). The increases in serum levels of AMH and LH were significant, and the increase in the serum level of AMH was high. No difference was observed compared to the WWP group ($P > 0.05$). However, in the case of FSH, the increase showed an insignificant difference after treatment. Moreover, the LH/FSH ratio significantly increased after treatment, reaching the amount found in the PCOS patients (WWP) (**Table 3**).

Regarding the increase in estradiol hormone, there should be a significant difference after treatment with VPA, but not to the extent that it reaches the WWP group level. In contrast to the increasing trend of estradiol, a significant decrease in the serum level of SHBG was observed after treatment (**Table 3**). Moreover, in the women after VPA treatment, higher serum levels of PRL and hydroxy progesterone than

Table 1: Tm and forward primer sequence of miR-146, miR-218, miR-222 and hsa- snord47 as endogenous control primer

miRNA	Tm	Primer (stem loop) (5'-3')
hsa-mir-222-F	58/5	AACTACATCTGGCTACTGGGT
hsa- snord47-F	60	ATC ACT GTA AAA CCG TTC CA
hsa-miR-218-5p-F	58	TTG GGC TTG ATC TAA CCA T
hsa-miR-146a-F	58/3	TGG AAG GTT GAG AAC TGA AT

Table 2: Comparison of demographic information and clinical findings of WWE patients group before and after treatment with VPA and WWP patients group were participated in this study

	WWEb[1] (n = 120) (mean ± std. Error)*	WWEa[2] (n = 120) (mean ± std. Error)*	WWP[3] (n = 120) (mean ± Std. Error)*	*P* value (between groups)		
				1-2	2-3	1-3
BMI (kg/m^2)	24.64 ± 0.580	25.70 ± 0.499	27.90 ± 0.53	0.51	0.35	0.18
Age (years)	26.5 ± 0.950	26.5 ± 0.950	27.5 ± 0.80	-	-	0.81
% Irregular menstrual	20.50 ± 0.450	67 ± 1.801	80.5 ± 1.03	0.03**	0.087	0.02**
Hirsutism (G-F score)	5.83 ± 1.314	15.06 ± 0.980	19.13 ± 1.07	0.00**	0.021**	0.00**

[1]. **WWEb**: women with epilepsy before treatment with VPA
[2]. **WWEa**: women with epilepsy after treatment with VPA
[3]. **WWP**: women with polycystic ovarian syndrome
* Data presented as mean and Standard Error were made using ANOVA test. Tukey test was applied for discrete variables comparison of group.
** P value < 0.05

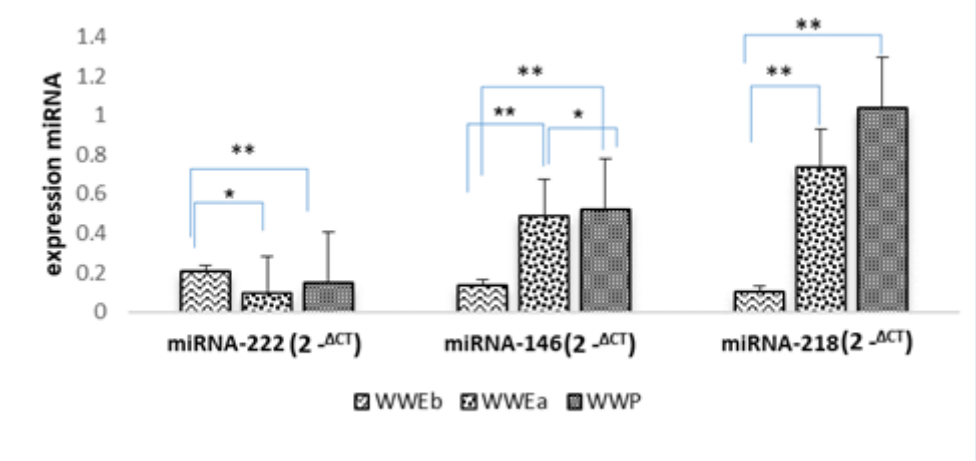

Figure 1: The expression of miRNAs in WWE patient group before and after treatment by VPA and WWP patient group. Data presented as mean and Standard Error were made using the ANOVA test. Tukey test was applied for discrete variables comparison of groups. * P value < 0.05. ** P value < 0.01. **Abbreviations**: **VPA**: Valproate; **WWEa**: women with epilepsy after treatment with VPA; **WWEb**: women with epilepsy before treatment with VPA; **WWP**: women with PCOS

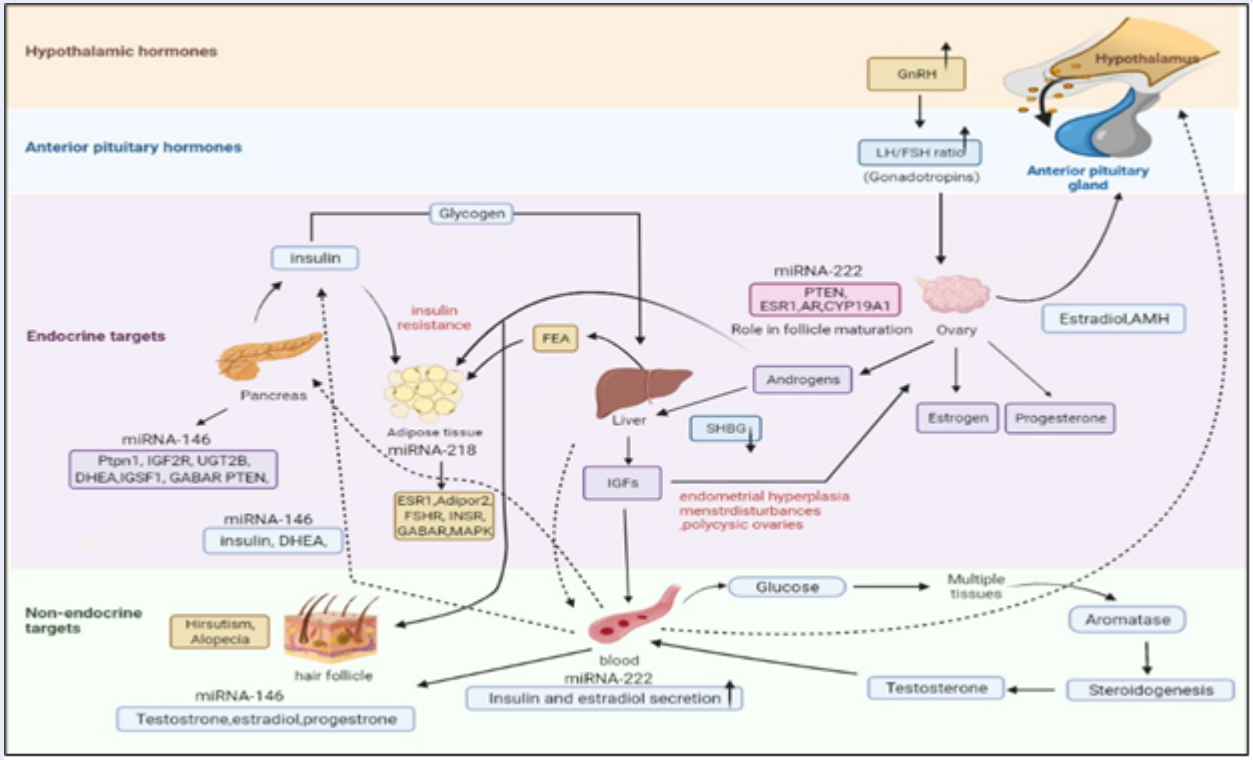

Figure 2: **The expression of miRNA-222, miRNA-146 and miRNA-218 in the hypothalamic-pituitary-gonadal axis and their cross-talk in mensterdisturbance, follicular maturation, steroidogenesis, hirsutism, insulin resistance, adipogenesis and lipid metabolism**. The elevated LH /FSH ratio that is pituitary responsiveness to GnRH secretion from GnRH neurons hyperactivity in the Hypothalamus in PCOS. Increased LH/ FSH ratio contributes to the ovarian pathology of PCOS, including endometrial hyperplasia, menstrual disturbance and increased androgen that induces hirsutism and Alopecia. Also, another feedback from gonadal steroid hormone signaling to response the GnRH neurons express estrogen receptor (ER) production is increased followed by estrogen and progesterone. In response to GnRH, neurons pulse express the AMH receptor and AMH can directly and potently stimulate GnRH neuron firing activity and increase GnRH-dependent LH secretion. Proposed mechanisms associated with insulin-stimulate androgen biosynthesis and PCOS-involved defects that are induced by PI3K and Akt. Finally, the insulin pathway results in insulin resistance which enhances androgen levels by hindering SHBG. This figure showed the associated steroid synthesis process with aromatase and other enzymes such as CYP11A1, CYP17A1, and 3β-HSD are upregulated in PCOS to cause excessive androgen synthesis and didn't explain more in this figure. **Abbreviations**: **AMH**: Anti-Müllerian Hormone; **FSH**: follicle stimulating hormone; **LH**: luteinizing hormone; **LH/FSH ratio**: luteinizing hormone/ follicle stimulating hormone ratio; **PCOS**: polycystic ovarian syndrome; **PTEN**: Phosphatase and tensin homolog deleted on chromosome 10

Table 3: Hormonal and biochemical parameters in WWE patients group before and after treatment with VPA and WWP patients group were participated in this study

Variables	WWEb[1] (n = 120) (mean ± std. Error)*	WWEa[2] (n = 120) (mean ± std. Error)*	WWP[3] (n = 120) (mean ± Std. Error)*	*P* value** (between groups)		
				1-2	2-3	1-3
Free Testosterone (pg/mL)	8.373 ± 1.390	16.02 ± 0. 864	19.51 ± 1.00	0.01**	0.038**	0.005**
DHEA-S (μg/dL)	268.80 ± 8.18	413.15 ± 2.78	432.37 ± 4.019	0.003**	0.043**	0.00**
Estradiol (pg/mL)	308.00 ± 26.710	430.42 ± 19.047	528.19 ± 13.55	0.004**	0.01**	0.00**
Hydroxy progesterone (ng/dL)	356.53 ± 4.557	394.36 ± 8.922	451.40 ± 5.349	0.018**	0.06	0.005**
Prolactin (ng/mL)	17.466 ± 0.709	39.966 ± 1.899	50.633 ± 1.324	0.010**	0.056	0.006**
SHBG (nmol/L)	51.05 ± 1.707	24.03 ± 1.47	18.86 ± 0.814	0.005**	0.06	0.001**
Fasting Insulin (μIu/mL)	11.32 ± 3.390	19.283 ±1.864	19.70 ± 1.442	0.04**	0.877	0.012**
Fasting glucose (mmol/dL)	4.353 ± 1.703	5.373 ± 1.150	6.02 ± 1. 903	0.03**	0.042**	0.01**
HOMA-IR	2.21 ± 0.631	4.63 ± 0.532	5.31 ± 0. 72	0.005**	0.023**	0.004**
AMH (ng/mL)	2.86 ± 1.721	7.50 ± 3.59	7.91 ± 4.609	0.005**	0.809	0.003**
FSH (mIu/mL)	8.55 ± 3.971	9.05 ± 2.911	11.24 ± 2.719	0.52	0.00**	0.001**
LH (mIu/mL)	10.86 ±2.479	25.59 ± 1.322	33.89 ± 2.934	0.00**	0.00**	0.00**
LH/FSH ratio	1.26 ± 0.832	3.00 ± 0.914	3.09 ± 0.742	0.002**	0.899	0.001**

[1]. WWEb: women with epilepsy before treatment with VPA
[2]. WWEa: women with epilepsy after treatment with VPA
[3]. WWP: women with polycystic ovarian syndrome
* Data presented as mean and Standard Error were made using ANOVA test. Tukey test was applied for discrete variables comparison of group
** P value < 0.05

in the WWEb group were significantly observed ($P \leq 0.05$), but not to the extent that it reached the WWP group level. In addition to the mentioned hormones, fasting insulin and glucose levels were also assessed. Our results showed increased fasting insulin and glucose levels after treatment ($P \leq 0.05$). Finally, during the treatment, the mean HOMA-IR values were significantly increased from 2.21 to 4.63 in women treated with VPA (**Table 3**). Thus, insulin resistance (HOMA-IR > 2.5) developed during therapy among the VPA-treated patients. For the women who took VPA, those who had proximate insulin resistance levels before starting therapy became obviously insulin resistant during therapy. In addition, serum levels of AMH and LH and the LH/FSH ratio increased significantly after treatment (**Table 3**).

Linear regression analysis was performed to determine the role of demographic information and hormonal and biochemical parameters on the miRNA expression in the WWEa, WWEb, and WWP groups. In the linear regression analysis, the hirsutism and AMH levels were correlated with the miRNA-222 expression level (b = 0.01; P = 0.000) (b = 0.0209; P = 0.049) (**Table 1S** in the Appendix). Additionally, significant correlations were observed between parameters, such as hydroxprogesterone (b = 0.273; P= 0.02), fasting G (b = 0.453; P = 0.00), AMH (b = -0.282; P = 0.014), LH/FSH ratio (b = −0.217; P = 0.049), free testosterone (b = 0.207; P = 0.05), and estradiol (b = 0.628; P = 0.05), LH (b = 0.094; P = 0.05) with the miRNA-218 expression level (**Table 1S** in the Appendix). Moreover, significant correlations

Table 4: miRNA expressions in WWE patients' group before and after treatment with VPA and WWP patients' group were participated in this study. (WWEb, WWEa, and WWP (control)) (mean ± SE)

miRNA Expression	WWEb[1] (n = 120) (mean ± std. Error)	WWEa[2] (n = 120) (mean ± std. Error)	WWP[3] (n = 120) (mean ± Std. Error)	*P* value (between groups)		
				1-2	2-3	1-3
miRNA-222 $(2^{-\Delta CT})$	0.206 ± 0.74	0.099 ± 0.89	0.15 ± 0.71	0.013**	0.072	0.00***
miRNA-146 $(2^{-\Delta CT})$	0.133 ± 0.685	0.49 ± 0.718	0.52 ± 0.885	0.001***	0.02**	0.00***
miRNA-218 $(2^{-\Delta CT})$	0.10 ± 0.936	0.74 ± 0.831	1.04 ± 0.903	0.001***	0.503	0.00***

[1]. WWEb: women with epilepsy before treatment with VPA
[2]. WWEa: women with epilepsy after treatment with VPA, and WWP
[3]. WWP: women with polycystic ovarian syndrome
*Data presented as mean and Standard Error were made using ANOVA test. Tukey test was applied for discrete variables comparison ofgroup.
** P value < 0.05
*** P value < 0.01

were shown between variables of DHEA-S (b = 0.51; P = 0.00), estradiol (b = 0.345; P = 0.00), SHBG (b = −0.004; P = 0.01), FSH (b = 0.187; P = 0.017), prolactin (b = 0.362; P = 0.002), LH (b = 0.359; P = 0.003), AMH (b = −0.201; P = 0.05) and the miRNA-146 expression level (**Table 2S** in the Appendix). Additionally, significant correlations were observed between some hormones (associated with PCOS and VPA treatment) and the miRNA expressions. A significant difference was found among PCOS phenotypes in terms of the expression of the three miRNAs and AMH level.

This study examined the expression of three miRNAs related to PCOS with the aim of early diagnosis of this syndrome and its complications during treatment with VPA. The mean expressions of miRNA-146 and miRNA-218 were significantly higher after treatment. Despite the significant increase in these two miRNAs in the WWEa group compared with the WWEb group ($P \leq 0.001$), the increase in miRNA-218 is not comparable to its level in WWP (P = 0.503). In contrast, miRNA-222 expression reduced significantly after treatment with VPA (P = 0.013) (**Table 4**) (**Figure 1**).

DISCUSSION

There are two types of anti-epileptic drugs: drugs that induce the liver enzymes, such as phenytoin, carbamazepine, and phenobarbital, and those that do not induce the liver enzymes, such as VPA[22]. There is mounting evidence that VPA can disrupt ovarian function and androgen production, presumably due to numerous effects on the hypothalamic–pituitary–ovarian axis, based on the emergence of PCOS-like symptoms in patients who use this medication. Accordingly, it has been shown that VPA alters numerous signaling pathways in the hypothalamic–pituitary–ovarian axis. Some of the most significant signaling cascades are involved in the functionality of this drug and its consequent symptoms. It has been indicated that VPA can increase GABA (an inhibitory neurotransmitter) signaling in GnRH neurons. Several studies demonstrated that this pathway might be responsible for activating the GnRH/LH system and correlate with downstream consequences in PCOS patients. Studies have reported that the GABA level is considerably higher in the cerebrospinal fluid of PCOS patients than in healthy women.

Clinical data have revealed that VPA treatment is directly linked to the development of PCOS symptoms in women. According to the results of previous studies and this study, an increase in the pulse frequency of LH leads to increased LH/FSH ratios in epileptic patients treated with VPA and in women with PCOS[23]. These pathological phenomena affect the ovarian pathogenesis of PCOS, including hyperplasia of theca cells, increased concentration of androgens, the altered response of LH to the stimulation of GnRH, and elevated serum prolactin levels.

Some studies have shown that women with PCOS require higher levels of exogenous progesterone and estradiol to diminish the high and frequent pulsed release of LH[24]. The impact of anti-epileptic drugs on the concentrations of reproductive hormones could

indicate an association between the occurrence of reproductive disorders and the consumption of these medications. These correlations in epileptic patients are primarily based on several studies showing that alterations in SHBG are linked with sexual dysfunction[25]. Evidence shows that VPA effectively contributes to the metabolic pathways of estrogens and androgens. The considerable increase in the biochemical factors mentioned above following VPA treatment in this study also confirmed previous investigations.

The impact of VPA on the serum concentrations of sex hormones is complicated, and some studies have indicated increased estrogen and testosterone in patients who received VPA[26]. The development of hyperandrogenism and PCOS following the use of VPA has been extensively addressed in the literature. Several studies have demonstrated that VPA affects the hepatic cytochrome P450 enzyme system (another metabolizing system of VPA). This might result in elevated levels of biologically active and free forms of testosterone due to aromatase activity[27]. Gustavsen *et al.* showed that the treatment of human adrenal carcinoma cells, as a cell-line model of steroidogenesis, with VPA led to the downregulation of genes in response to some miRNAs regulating the early steps of steroidogenesis[28].

In this study, we found a marked reduction in the serum levels of SHBG in the WWEa group. According to previous studies and our results, VPA increases the risk of developing PCOS in women[29,30]. Heterogeneous conditions exist for diagnostic criteria of PCOS, including chronic anovulation or menstrual disorders, ovarian cyst morphology, hyperandrogenism, and hirsutism. In addition, PCOS has been associated with co-morbidities, such as type 2 diabetes, dyslipidemia, hyperinsulinemia, insulin resistance, obesity, infertility, and galactorrhea[31]. A study on Finnish women demonstrated that approximately 60% of individuals who received VPA exhibited biochemical and clinical changes related to PCOS[32]. One common side effect of PCOS is weight gain that could result from mitochondrial inhibition in response to oxidative metabolites[33]. In our study, no significant difference was detected in the weights of patients before and after VPA treatment. One randomized prospective study on women treated with lamotrigine and VPA reported serum concentrations of free and total testosterone following 6–12 months of treatment[30]. Our findings showed a significant increase in the serum levels of free testosterone in women after VPA treatment. Studies have assessed the correlation between PCOS-like symptoms and insulin resistance and obesity among patients who received VPA. VPA can alter some intracellular signaling cascades, such as mitogen-activated protein kinases (MAPK), serine/threonine protein kinase GDP (Akt), and protein kinase C (PKC)[34]. DeGrave and colleagues analyzed the effect of different doses of VPA on the activities of steroidogenic enzymes in thecal cell cultures isolated after hysterectomy from individuals with normal ovaries and PCOS patients. VPA (up to 500 μM) increased the production of DHEA, androstenedione, and progesterone in ovarian theca cells, thus increasing the expression levels of CYP17 and CYP11 α genes[35]. However, multiple investigations have found that individuals who received VPA exhibited hyperleptinemia and resistance to elevated leptin levels, mainly in those who are obese. A novel discovery concerning the hidden pharmacological effects of VPA was that it suppresses the amount of leptin mRNA in adipocytes in a dose-dependent manner, considerably reducing hormone production[36]. In this study, the serum concentrations of HOMA-IR and insulin were remarkably higher in epileptic patients after treatment with VPA than in those who did not receive VPA. Nisha and colleagues confirmed that individuals who received VPA had significantly higher insulin and HOMA-IR values than newly diagnosed patients. However, BMI did not change between the treated and untreated individuals[37].

Recent studies have shown a correlation between the serum concentration of AMH, which belongs to the TGF-β family, and the incidence of PCOS. Thus, they introduced this hormone as a valuable and powerful diagnostic marker in the pathogenesis of PCOS. The role of AMH during folliculogenesis is to act as a regulator, avoiding FSH overuse and maintaining homeostasis in the ovaries[38]. In the clinical assessment and intervention of PCOS, two factors, AMH levels and insulin resistance, are significant. Insulin resistance is clinically correlated with hyperandrogenism and increased AMH serum levels. Nevertheless, there is contradictory information regarding the association between high AMH levels and insulin resistance and other metabolic symptoms of PCOS[39]. Furthermore, most research has involved few participants. This prompted us to examine the blood AMH levels in the epileptic women before and after VPA treatment[40].

Biomarker identification for the early detection of PCOS-like symptoms in patients using VPA has shown considerable promise in both fundamental research and clinical applications, owing to the involvement of several signaling pathways associated with

PCOS. MiRNAs should receive greater attention as a PCOS biomarker due to their widespread occurrence and strong link with endocrine abnormalities and inflammatory disorders. Numerous studies have shown that dysregulation of miRNAs participates in the initiation, development, and progression of various illnesses, including PCOS[41]. In this study, three miRNAs, including miRNA-218, miRNA-222, and miRNA-146, were chosen based on the literature and bioinformatics analysis for the following reasons: recent research has shown that these miRNAs are increased in the oocytes and undergo substantial variations at the gene expression levels during maturation of oocytes[13,42,43]. These miRNAs target many of the genes and signaling pathways involved in hyperandrogenism, insulin resistance, and obesity, as with PCOS. By constructing the miRNA–mRNA network, we demonstrated that miRNA-218, miRNA-222, and miRNA-146 correlate with PCOS.

Similar to in PCOS, many of the genes and signaling pathways associated with insulin resistance, hyperandrogenism, and obesity are targeted by these miRNAs. By establishing an miRNA–mRNA network, we showed that miRNA-218, miRNA-222, and miRNA-146 are significantly linked with PCOS incidence. Two of the three miRNAs are of particular interest as biomarkers of PCOS. It has been reported that miRNA-222 is positively associated with insulin resistance, while miRNA-146 is inversely linked with the testosterone level[44,45]. It is now known that the insulin transport pathway is one of the most significant molecular pathways in developing PCOS, activating many detrimental pathways. The activation of PTPN1, targeted by miRNA-146, acts as an inhibitor in this pathway. Several lines of evidence indicate that miRNA-146 expression is elevated, while PTPN1 expression is considerably reduced in PCOS patients[46]. The RAS pathway could initiate insulin resistance or activation of detrimental molecular cascades, subsequently activating the MAPK pathway by phosphorylation of the RAS protein. Activating this pathway could result in the conversion of MEK to ERK, leading to increased production of androgens[47]. Of note, the fas gene, targeted by miRNA-146, can induce apoptosis in oocytes, thereby promoting folliculogenesis[48]. It has been reported that the miRNA-222 family is one of the major regulators of the expression of this protein. As shown in **Figure 2**, this miRNA is elevated in PCOS patients. It inhibits PTPN1 expression, thus stimulating the activation of the MAP kinase pathway, androgen production, and the emergence of disease complications[49].

Notably, increased expression of miRNA-218 is correlated with the inhibition of adiponectin, which is secreted by differentiated adipose tissues and is abundantly present in the blood. However, its expression is substantially diminished in those with PCOS and obesity. The MEKKs/TAK1 pathway regulates the molecular mechanism of adiponectin production in muscle. miRNA-218 inhibits the adiponectin receptor (AdipoR2), which interacts with AMP-activated protein kinase and p38 mitogen-activated protein kinase[50].

CONCLUSIONS

To identify biomarkers for early detection of PCOS-like symptoms in patients taking VPA, the expressions of miRNA-218 and miRNA-146 were evaluated. As previous evidence on PCOS-signaling pathways in valperat users has described, many genes involved in these pathways are targets of these miRNAs. According to our findings, the significant change in the expressions of these miRNAs and the results reported in previous articles on their target genes can be used for diagnostic or therapeutic purposes in patients taking VPA. Concerning the development of PCOS, serum levels of LH, FSH, and AMH are important for both early diagnosis and prognosis. Moreover, we found that serum AMH levels significantly differed among women before and after VPA treatment. However, positive correlations were observed between the other hormones that link PCOS and VPA metabolism and the expressions of the studied miRNAs.

ABBREVIATIONS

AdipoR2: receptor adiponectin; **AEDs**: antiepileptic drugs; **Akt/ PBK**: The serine / threonine protein kinase GDP also known as the Akt; **AMH**: Anti-Müllerian Hormone; **BMI**: body mass index; **CNS disease**: Central Nervous System disease; **DHEA-S**: dehydroepiandrosterone sulfate; **E**: estradiol; **EIAEDs**: liver enzyme inducing antiepileptic drugs; **Ers**: estrogen receptors; **FSH**: follicle stimulating hormone; **GABA**: inhibitory neurotransmitter; **HOMA-IR:** homeostasis model assessment-estimated insulin resistance; **ILAE**: International League Against Epilepsy; **Jak-STAT**: Janus Kinase and Signal Transducer and Activator of Transcription; **LH**: luteinizing hormone; **LH/FSH ratio**: luteinizing hormone/ follicle stimulating hormone ratio; **MAPK**: mitogen-activated protein kinase; **miRNA**: microRNA; **non-EIAED**: none liver enzyme inducing antiepileptic drugs; **PCK**: Protein kinase C; **PCOS**: polycystic ovarian syndrome; **PCR**: polymerase chain reaction; **PTEN**: Phosphatase and tensin homolog deleted on chromosome 10; **PTENP1**: Phosphatase

and tension homolog Pseudogene 1; **PTPN1**: Protein Tyrosine Phosphatase Non-Receptor Type 1; **qRT-PCR:** quantitative real time polymerase chain reaction; **SHBG**: sex hormone binding globulin; **VPA**: Valproate; **Wnt**: canonical pathway; **WWEa**: women with epilepsy after treatment with VPA; **WWEb**: women with epilepsy before treatment with VPA; **WWP**: women with PCOS

ACKNOWLEDGMENTS

This research is the product of a research project (code: 5374) and financial support of abortion research center, Yazd Reproductive Sciences Research Institute. I would like to thank the group of specialists and experts who took part of their precious time to share their experiences and knowledge in meetings. This group includes: Dr. Seyed Mehdi Kalantar and the Supervisor of the Genetics Laboratory of the Abortion Research Center) Dr. Fatemeh Montazeri (, also neurologists (Dr. Seyed Massoud Etemadifar, Dr. Mohsen Beshtam) from the Epilepsy Center, and (Ms. Dr. Mitra Poursirfi). I would like to Special thank Dr. Mansour Salehi and the Genetics Laboratory of Al-Zahra Hospital in Isfahan for their cooperation in sampling. I am also grateful to everyone.

AUTHOR'S CONTRIBUTIONS

All the authors have somehow played a role in the implementation of this project, including: Mahya Rajabi and Fateme Montazeri: study design, literature review, doing laboratory works including miRNA extracting, qRT-PCR, data analysis, article writing; Seyed-Mehdi Kalantar: supervision, study design, literature review

Seyed Massoud Etemadifar, Mohsen Beshtam: our coworkers as neurologists from the Epilepsy Center; Mitra Poursirfi: our coworker in the Epilepsy Center for collecting samples. All authors read and approved the final manuscript.

FUNDING

This research is extracted from a research project (code: 5374) and financial support from the Abortion Research Center, Yazd Reproductive Sciences Institute.

AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Informed consent form was achieved from all participants in this research. All procedures performed in studies involving participants were accordance with the approved standards of Yazd Reproductive Sciences Institute Ethics committee (Ethical code: IR.SSU.RSI.REC.1396.8) and the ethical standards of 1964 Helsinki declaration and its later amendments comparable standards.We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Also none of the authors has any conflict of interest to disclose.

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