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Investigation of the Changes in the Expression of Mir-382 and FOSBΔ Genes in the Prefrontal Area Following Alcohol Addiction in Male Wistar Rats

Elahe Shahvaroughi[1], Mostafa Ebadi[2]*

[1] Master of Molecular Genetic Biology, Islamic Azad University, Damghan Branch, Tehran, Iran.
[2]Associate Professor of Genetic Molecular Biology, Islamic Azad University, Damghan Branch, Tehran, Iran.

***Corresponding Author**

Abstract: *As different areas of the brain, including the prefrontal area, are involved in the development of addiction, the present study was conducted with the aim of investigating the changes in the expression of mir-382 and FOSBΔ genes in the prefrontal area following alcohol addiction in male Wistar rats. For this purpose, 50 male Wistar rats were prepared from Pharmacology Faculty of University of Tehran and oral alcohol at a dose of 5 mg / kg was administered to animals for 21 days .In the next step, using the guillotine device, the head of rats were separated and the considered area was removed. Having used the Real Time PCR (Qigene) method, the expression of FOSBΔ and Mir-382 genes relative to the reference gene was examined. Statistical analysis was performed using SPSS software and Independent T-test. The results showed that following the alcohol addiction for 21 days, the level of expression of Mir382 gene in the prefrontal area decreased, while the expression of the FOSBΔ gene showed an increase. The results of this study showed a reverse relationship between the expression of Mir382 and FOSBΔ genes following alcohol addiction in the prefrontal area of the brain.*

Keywords: *FOSBΔ, Mir382, Prefrontal area, Alcohol addiction, Male Wistar rats*

INTRODUCTION

Addiction is a physical-psychological disease that endangers the health of the individual, the family and the community in all aspects of life due to its progressive nature (Thombs, 2006; American Psychiatric Association, 2013). Iran is one of the most vulnerable countries in the world due to its population structure, geopolitical position and lack of strategy and comprehensive national plan to combat with supply, demand and treatment of addicts. Statistical reports suggested the increasing rate of drug addiction in Iran, so that addiction, following unemployment and inflation, is considered as the third social problem (Ritzer, 2007). One of the addictive substances is alcohol, which is a narcotic substance and is more easily accepted in the community compared to other drugs. However, its addictive power is not less than other substances (American Psychiatric Association, 2013; Ritzer, 2007). Alcohol has several side effects. Alcohol abuse causes severe metabolic and physiological effects on all systems of body. Here, the side effects of alcohol on brain and on the prefrontal area have been reviewed (American Psychiatric Association, 2013). The prefrontal

area includes non-moving anterior area of the frontal lobe (Robinson and Berridge, 1993). When the prefrontal lobe is cut off to treat certain mental illnesses, a person enters a stage called "emotional neutrality". At this stage, the person does not show any signs of happiness, sadness, hope, or disappointment. No emotion can be found in his or her words or states (Robinson and Berridge, 1993; Stricker and Psaty, 2004). Up to date, many studies have been conducted to identify the signaling pathways involved in alcohol consumption. Among them, two important signaling molecules have been identified that are involved in alcohol abuse. They include dopamine D1 (DDR1) and FOSBΔ receptors (Kreek, Nielsen and LaForge, 2004; Lishman, 1990).

DRD1 is a subgroup of dopamine receptors and is the most abundant dopamine receptor in the central nervous system. Studies have indicated that the expression or activation of DRD1 increased significantly in the central nervous system of animals after alcohol consumption (Kreek, Nielsen and LaForge, 2004; Lishman, 1990; Madden et al., 1995). FOSB is a version of the FOS family that is complex two-component proteins. The FOS family creates heterodimer proteins with jun family proteins to form an active AP1 form. Having binded to (TGAC / GTCA) the binding site, AP1 strengthens certain methods for the regulation of their transcription. The modified isoforms of FOSBΔ (molecular mass kd35-37) accumulate in the certain parts of the brain in the face of repeated exposure to drugs, while other members of the FOS family show a tolerance to this condition. This type of FOSBΔ accumulations has been seen in cocaine, morphine, amphetamine, alcohol, nicotine (Franken, Muris and Georgieva, 2006). FOSBΔ is resulted from chronic use of antidepressants. In transgenic rats, it has been shown that over-expression of FOSBΔ in the nucleus led to increased sensitivity to the effects of cocaine, morphine, and more response of Accumbens and posterior corpus striatum to normal rewarding behaviors such as running and eating. FOSBΔ density increases the responsiveness to rewarding and drug behaviors. In terms of stability in the brain, the effects of FOSBΔ may last for more than two months in the brain. The chronic stress-induced addiction process can also be increased by FOSBΔ (Kreek, Nielsen and LaForge, 2004; Franken, Muris and Georgieva, 2006; Kachadourian, Eiden and Leonard, 2009). Evidence also suggests that miRNAs play a major role in the neuropsychiatric adaptation of drug addiction and changes in the expression of miRNA and mRNA within the mesolimbic system can play a major role in the development of drug addiction (Le Bon et al., 2004; Duffy, Iyer and Surwillo, 1989). Although many studies have been conducted on alcohol addiction, it is not yet clear if miRNAs and FOSBΔ are involved in alcohol addiction or not. Hence, the aim of this study was to evaluate the relationship between a group of miRNAs (mir-382) and FOSBΔ in the prefrontal area of the Wistar rats following alcohol addiction.

Methodology

Animals studied

In this study, a total of 50 male rats weighing 200-250 g were collected from the Faculty of Pharmacology of University of Tehran. Mature rats were kept in metal shelves under healthy conditions at room temperature with free access to water and food in a light / darkness period of 12 hours (7 am as onset of light). The rats received alcohol (5 mg / kg) for 21 days. Then, the guillotine device was used to separate head of the rats, then, the split skull and the studied area were removed.

Primer design

The primer format of mir382 and ΔFosB DNAs was ordered to Metabion Company of Germany and the primer format of Mir382 and ΔFosB RNAs was ordered to Qiagen Company of USA. To examine the proliferation of Mir382 to know whether it is synthesized with non-specific enzymes of Qiagen Company or not, a primer set was designed to proliferate the area with a length of 70bp and ordered for Metabion Company for synthesis.

Guidelines

- **MiRNA extraction by Roche Company kit**

Before using the kit, 12ml pure alcohol was added to Wash Buffer.

1. 100 mg of the brain tissue has been transferred to microtube1.5 and it was squeezed well so that no small or big piece remained. Then, 200µl of Lysis buffer was added to it.
2. We vertexed it for 10 minutes continuously.
3. For DNA deposition, we added 20µl of bivalent salts, such as sodium acetate or potassium acetate (We used sodium acetate according to our kit)
4. To remove lipid and protein, we added 180 µl of chloroform and reversed the microtube 25 times.
5. We kept the microtube for 3 minutes in a centrifuge with 12000 rpm
6. After the centrifugation, our solution was a 3-phase solution, including organic bottom phase, Buffy coat middle phase, and aqueous upper phase, we isolated the upper phase with sampler and poured into microtube.
7. We added 35% volume of 100% cold alcohol to the solution (as the volume of the solution is about 180 µl, we add 85 µl of alcohol) and then reversed the microtube 25 times and then spinned it for 2-3 seconds.
8. We transferred the contents to a filtered microtube available in the kit and re-centrifuged it with the same rpm.
9. We outed the filter and added 200 µl of 70% alcohol to the contents of the microtube and pipetaged it and transfered the contents again to the new column and re-centrifuged it.
10. At this step, we held the column and outed the microtube and poured 200µl Wash Buffer into the middle of the column and incubated for 1 minute at room temperature.
11. We centrifuged it with 12000 rpm for 2 minutes and outed the bottom solution and held the filter
12. We transfered the filter to microtube 1.5.
13. At this stage, we added 50 µl of Eloution Buffer to the column and incubated it for 3 minutes at room temperature, and then, we re-centrifuged it for 3 minutes with the same rpm. The remaining solution was MiRNA.
14. We kept at -70°C freezer

Extraction of Total RNA using the Roche Kit

1. We crushed 100 g of the considered tissue thoroughly and added 500 µl of Lysis buffer to it.
2. We adjusted the Bain-**marie** at a temperature of 30 ° C and placed the samples in it for 10 minutes.
3. We added about 100 µl of chloroform to it and slowly reversed it several times.
4. We incubated it for 1 minute at room temperature.
5. We centrifuged it at 4 C° for 5 minutes in a refrigerated centrifuge with 12000 rpm.
6. We transformed the upper phase that was transparent and contained RNA to the new microtube
7. We added 250µl isopropanol to the obtained solution.
8. We reversed the microtube 50 times and incubated it for 15 minutes in a 30 C° Bain-**marie**
9. We centrifuged the solution at 12000 rpm for 15 minutes at the same temperature.
10. The upper solution was discarded (the sediment adhering to the end of the tube was colorless and not visible.)
11. We added 500µl alcohol 70% to tube and slowly tapped the tube to dissolve the RNA in it (this alcohol is in fact used to rinse isopropyl alcohol)
12. We centrifuged it at 12000 rpm at a temperature of 4 ° C for 3 minutes in a refrigerated centrifuge.
13. The upper solution was discarded and placed at room temperature for 2-3 minutes under the hood 2- so that the sediment was dried.
14. We added 50 to 100 µl of RNse free water to the tube so that the RNA sediment was dissolved completely.
15. All extracted RNAs have been treated for possible removal of DNA from the DNase enzyme.
16. We had to keep the RNA at a temperature of -70 ° C.

- **Synthesis of cDNA using the QIAgen company kit**

This kit had two buffers, named Hifeln and Hispec, in which the type of buffer was different according to the goal of test.

Hifelx ⟵ Assey

Hispec ⟵ Array

To compare the performance of two buffers, the test was designed as follows (Table 1)

Table 1: Compare the two buffer Hifelx and Hispec in the construction of cDNA

Component	1-react	2-react
5X-Buffer	2µl Hifelx	2µ Hispec
10X-Nucleics.mix	1 µl	1 µl
Rnase free water	———	———
M-RT-Mix	1 µl	1 µl
Temp (RNA)	6 µl	6 µl

Comparison of Hiflex and Hispec buffers in making cDNA

After preparing the above mixtures, we piptages and spinned each of them separately, then, we kept them at 37 ° for 1 hour and then at 95 ° for 5 minutes.

In making cDNA, we acted according to the following tables:

1-Concentration of substances in RT reaction

Table 2. Concentration of substances in making cDNA

	GAPDH	ΔFOSB	Mir-382
Temp (RNA)	5 µl	5 µl	5 µl
Primer (SP)	1 µl	1 µl	1 µl
dH2O	4 µl	4 µl	4 µl
T.V	10 µl	10 µl	10 µl
We placed them at 65 ° for 5 minutes for refolding			
Enzym(2x)	10 µl	10 µl	10 µl

RT reaction temperature program

Table 3. RT reaction temperature program

	Step1	Step2
Temperature	50 o C	70 o C
Time	60 min	10 min

- **Materials and components needed for PCR**

1) Polymerase DNA enzyme
2) Oligonucleotide primer pairs
3) Deoxynucleotide triphosphates (dNTPs)
4) Bivalent cations
5) PCR buffer
6) DNA pattern

- **Electrophoresis of PCR product**

To prepare the gel, according to the required concentration, the amount of Agarose was dissolved by heating in a suitable amount of TBE-0/5 X solution. The solution was cooled to about 60 ° C (20 mg / µl).

To prepare the buffer at needed concentration, the required value of Agarose was dissolved by heating it in appropriate value of TBE-0.5. After cooling the solution up to 60 ° C, Ethidium bromide color (20 mg / µl) was added. The buffer used in tank was the buffer used in the preparation of gel that had to cover 2-3 mm of the gel. To separate the pieces, the tank was attached to current and a 70-90 Watt flow was established. Given the presence of negative charge in DNA, the samples were placed at cathode side to migrate towards anode. The molecules were separated based on their size. Finally, gel was examined versus the UV lamp. GAPDH gene was used as the reference gene.

- **Substances needed for Real Time-PCR reaction**

1) Master Mix Syber Gree
2) Progressive primer
3) Recursive primer
4) Sample Pattern (cDNA)
5) Ultra pure water

The temperature conditions and concentration of substances in this study were as follows:
1-The concentration of substances in Real-Time reaction for GAPDH, Mir-382, and FOSBΔ genes

Table 4. Concentration of substances used in Real-Time reaction

	GAPDH	Mir-382	ΔFOSB
Syber-M-mix	10 µl	10 µl	10 µl
Forward	0.5 µl	0.5 µl	0.5 µl
Reverse	0.5 µl	0.5 µl	0.5 µl
Temp(cDNA)	3 µl	3 µl	3 µl
dH2O	6 µl	6 µl	6 µl
T.V	20 µl	20 µl	20 µl

Temperature program of Real-Time reaction

Table 5. Temperature program of Real-Time reaction

Step	Time	Temperature
activation step	15 min	95 o C
3-step cycling	15 s 30 s 30 s	94 o C 55 o C 70 o C
Cycle number	40 cycle	

Results

The effect of alcohol on changes in the expression of FOSBΔ gene

In response to the first question of whether alcohol consumption affects the expression of FOSB gene in the prefrontal area, T-test was used for the experimental and control groups. As shown in Table 6, the obtained mean was significantly higher than the constant score.

Table 6. Descriptive statistics of one-sample t-test for the change in the expression of ΔFOSB in the prefrontal area following alcohol addiction in male Wistar rats

	N	Mean	Std. Deviation	Std. Error Mean
Control1	3	1.0000	.00000a	.00000
ΔFOSB	9	1.2889	.31798	.10599

The results of the T-test showed that alcohol consumption was influential in changing the expression of ΔFOSB gene in the prefrontal area of Wistar rats and had a significant level (t (8) = 12.1, p <0.05) (Table 7).

Table 7. Results of the one-sample T test for change in the expression ΔFOSB in the prefrontal area following alcohol addiction in male Wistar rats

	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
ΔFOSB	12.160	8	.000	1.28889	1.0445	1.5333

The effect of Alcohol on the change in the expression of Mir382 gene

In response to the second question of the research, whether alcohol consumption affects Mir382 gene expression in the prefrontal area, T-test was used for experimetal and control groups. Table 8 presents the results of descriptive statistics of a one-sample t-test for changing the expression of Mir-382 in the prefrontal area following alcohol addiction in male Wistar rats.

Table 8. Descriptive statistics of one-sample t-test for change in the expression of Mir-382 in the prefrontal area following alcohol addiction in male Wistar rats

	N	Mean	Std. Deviation	Std. Error Mean
Mir382	9	0.6911	0.26592	0.08864

The results of the T-test showed that alcohol consumption was influential in expression of Mir-382 gene in the prefrontal area of male Wistar rats and had a significant level ((t (8) = 9.993, p <0.05) (Table 9).

Table 9. One-sample t-test results on the changes in the expression of Mir-382 gene in the prefrontal area following alcohol addiction in male Wistar rats

	t	Df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Mir382	9.993	11	.000	.76833	.5991	.9376

Comparison of changes in expression of ΔFOSB and Mir382 genes

In response to the third question of the study, whether changes caused by alcohol consumption are influential in the expression of FOSBΔ and Mir-382 in the prefrontal area are consistent with each other or not, independent T-test was used for comparing the two groups. The results of the T-test have been presented in Table 10.

Table 10. Descriptive statistics of the T-test for comparing the expression of Mir-382 and ΔFOSB gene in the prefrontal area following alcohol addiction in male Wistar rats

	group	N	Mean	Std. Deviation	Std. Error Mean
results	Mir	3	.8333	.15275	.08819
	Fos	3	1.3333	.20817	.12019

The results showed that changes in the expression of Mir-382 and ΔFOSB in the prefrontal area of the male Wistar rats were not consistent with each other after alcohol consumption (Table 11).

Table 11. T-Test results for the comparison of the changes in the expression of Mir-382 and ΔFOSB in the prefrontal area following alcohol addiction in male Wistar rats

	Levene's Test for Equality of Variances		t-test for Equality of Means					
	F	Sig.	t	df	Sig. (2-	Mean	Std. Error	95% Confidence Interval of

						tailed)	Difference	Difference	the Difference	
									Lower	Upper
Results	Equal variances assumed	.500	.519	-3.354	4	.028	-.50000	.14907	-.91389	-.08611
	Equal variances not assumed			-3.354	3.670	.032	-.50000	.14907	-.92900	-.07100

Discussion and Conclusion

The results showed that following alcohol addiction for 21 days, the level of expression of Mir382 gene decreased in the prefrontal area, while the expression of the FOSBΔ gene showed an increase. The results of this study suggested a reverse relationship between the expression of Mir382 and FOSBΔ genes following alcohol addiction in the prefrontal area of the brain. In a study conducted by Jenica D. Tapocik et al. in 2013 on the effect of alcohol dependence on microRNAs in the prefrontal cortex, they concluded that 41 microRNAs and 165 microRNAs showed significant changes in the middle area of prefrontal cortex after the chronic alcohol treatment, which was consistent with the results of this study. Moreover, the results of Jngyuan Li et al. in 2012 suggested that multiple RNAs were unduly expressed after the injection of alcohol into nucleus **accumbens area of** rat .Among these microRNAs, Mir 382 decreased in alcohol-treated rats. In both nerve cultured cells in the laboratory and in the nucleus **accumbens** of rats, they found that D1 receptor dopamine was the main target of Mir382. With this target gene, Mir382 greatly modified FOSBΔ. In 2015, Dark et al. conducted studies on the effects of alcohol on various areas of the body. They concluded that alcohol in the face of cultured nerves reduced the expression of Mir9, Mir21, Mir153, and Mir335 and Mir 29b, Mir 29 a, 9, Mir133 genes decreased in neurons of Cerebellum granules isolated from alcohol-exposed rats. Alcohol reduced the expression of Mir29b gene in Cerebrum of rats. Based on the results obtained in this study, in which a reduction in mir382 in exposure to alcohol was observed, thus it can be stated that the results of this study were consistent with those of research mentioned above. Results of the research conducted by Kesmati et al. in 2008 showed that the rate of expression of FOS-C transcription factor in male rats increased after discontinuation morphine by Naloxone, while in female rats, this rate was reduced. The results obtained from this study were consistent with the results of our study. The results of our study showed that alcohol consumption was influential on the change of Mir-382 and ΔFOSB gene expression in the striatum area in male Wistar rats. Increased alcohol consumption caused a reduction in Mir-382 gene expression and an increase in αFOSB gene expression in the striatum area in male Wistar rats. In 2013, Gorini G et al. examined the effect of alcohol on cortex MiRNAs of rats and concluded that the level of MiRNA in alcohol consumption dependent rats was significantly changed compared to the non-dependent rats. Our results showed that alcohol consumption was influential in changing the expression of Mir-382 and ΔFOSB genes in the prefrontal area of male Wistar rats and an increase in alcohol consumption reduced the expression of Mir-382 genes and increased the expression of the αFOSB gene in the prefrontal area in male Wistar male rats. It was consistent with the results of the research conducted by Gorini G et al.

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