Original article

Differential Expression of MicroRNAs (9-3p, 106b-5p, 15a-5p and 30a-5p) as Predictive Biomarkers at Seizure Onset and Post Seizure in Sera of Strox-Intoxicated Patients

BY

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ABSTRACT

Background: Strox is the street name of the novel synthetic cannabinoids widely abused in the last few years in Egypt causing many toxic effects including seizures. **The aim of the work:** The study evaluated the potential seizurogenic effect of strox toxicity and the possible use of microRNA (miR) expressions as predictive biomarkers for diagnosis and follow up. **Methodology:** A cross- sectional prospective observational study was carried out on 60 patients presented to ER of Zagazig University Hospitals following strox intake during the period from March 2018 to May 2020. Epidemiological data, route of consumption & frequency of drug abuse during the last 12 months were recorded. All patients were subjected to clinical examination, Electroencephalogram (EEG), measurement of serum miR-9-3p, miR-106b-5p and miR-15a-5P, and miR 30a-5P expressions using real time-polymerase chain reaction (qRT-PCR) at time of presentation, after 72hrs and 28 days of presentation. **Results:** The median age of patients included were 19-35, most of them were male (96.6%). Of the included cases, 46.6% presented with seizures which was mainly in the form of generalized tonic clonic convulsions (43%). EEG revealed abnormal changes in only 35% of patient who developed seizures at time of presentation and in 9% of those who developed seizures after 72 hours with no EEG changes were recorded after 28 days of follow up. Assessment of genes expressions revealed upregulation of miR-9-3P, miR-106b-5p and miR- 30 a-5P and downregulation of miR-15 a-5P at time of presentation in patients with seizures with both miR9-3P, and miR 30 a-5P persisted upregulated during follow up periods, while miR106b-5p and miR 15 a-5P returned to normal. **Conclusion:** miR9-3p and miR 30 a-5p can be used as predictive biomarkers in diagnosis and follow up of strox induced seizures.

Keywords: Strox, microRNA. Seizures, EEG

I. INTRODUCTION

Use of novel psychoactive substances containing synthetic cannabinoids (SCs) has been increased significantly during the last few years. These substances are more potent and can cause more physical and psychological health hazards than those associated with natural cannabis (Cohen and Weinstein, 2018). They are sold under different names as spice, Scooby Snax or Black Mamba (Cooper, 2016) and are marketed as herbal fragrances, meditation potpourris, bath additives, air refreshers or tropical car perfumes and labelled (not for human consumption or for aromatherapy only) and declared to be purely herbal, containing plant ingredients and considered inert (Dresen et al., 2010 & Zuba et al., 2011 and Fattore and Fratta, 2011).

In 2018, The Egyptian Ministry of Health listed 11 of Synthetic cannabinoids in schedule (1) of prohibited drugs Law No. (182) for year 1960 and warned against dangers of these drugs with legal measures were undertaken to control their distribution (France 24: The Observer, 2018)

Over the past few years, use of these synthetic cannabinoids (SCs) under a street name called " Strox" had been increased significantly in Egypt. Chromatographic analysis of strox revealed other ingredients such as xylene, methylene dioxy methamphetamine, ketamine and trihexyphenidyl. These components can vary considerably from package to another even within the same products (El-Masry & Abdelkader, 2021 & Tawfik, 2021). Strox abusers consume it via smoking using a pipe or a cigarette paper, in electronic cigarette and sometimes orally as herbal tea (Bilici, 2014) and it can be sold through the Internet from both national and international sources (Zawilska & Wojcieszak, 2014). Abusers of drugs containing synthetic cannabinoids present to ER with neurological, cardiovascular and psychological manifestations (Seely et al., 2012, Spaderna et al., 2013 and Tournebize et al., 2017). To the moment all descriptions of acute strox toxicity is based on history and clinical observation and no sufficient data is available about its sequelae. The reported neurological manifestations associated with strox toxicity included disturbed conscious level, hallucination, agitation and seizures (El-Masry & Abdelkader, 2021)

The producers continuously change the chemical structure of the synthetic cannabinoids in a trial to escape legal prohibition (Tellioglu, 2018) which can explain the diversity in clinical presentation associated with strox toxicity (El-Masry & Abdelkader , 2021). Another challenge is that synthetic cannabinoids are not easily detected by the simple immunoassay tests or even confirmatory techniques such as gas chromatography–mass spectrophotometry (GC-MS), liquid chromatography-GC/MS, since these tests identify THC and its metabolites, which are structurally different from synthetic cannabinoids (Auwärter et al., 2009). This forces clinical toxicologists to rely on clinical picture and search for new biomarkers for diagnosis and follow up of the drug associated toxic effects (El-Masry & Abdelkader , 2021).

Seizures onset has sudden and unpredictable nature. Several methods have been introduced to predict their occurrence

and recurrence (Mormann et al., 2007). EEG plays a crucial role in diagnosis of seizures disorders since it is a suitable and inexpensive way to demonstrate abnormal excitability that underly seizures. However, it has several limitations including low sensitivity and specifity because normal EEG doesn't exclude seizurogenic activity and abnormal EEG doesn't itself indicate seizurogenic disorder since the interictal epileptiform discharge can be seen in small percentage of normal individuals and in patients with neurological disorders other than epilepsy (Smith , 2005)

MicroRNAs are known to play an important role in the post-transcriptional regulation of messenger RNA (mRNA)which in turn regulate many neurophysiological functions through control of cell structure, neurotransmitter receptors, ion channels and transporters. Alteration of MicroRNAs expressions may affect neuronal and glial functions leading to hyper excitability and epileptogenic activity in brain (O'Brien et al. 2018). Di Leva et al. (2014) stated that MicroRNAs -mediated gene expression control is critical for the cellular response to starvation, hypoxia, oxidative stress, drugs and DNA damage, thereby being implicated in human diseases.

several studies highlighted the possibility of using microRNAs as diagnostic and predictive biomarkers in drug-induced seizures since they are expressed distinctively within specific brain regions and can be easily measured extracellulary either in CSF and plasma (Raoof et al., 2017 & Rzepka-Migut et al., 2021)

The aim of this work was to evaluate the potential seizurogenic effect of strox toxicity and the possible use of MicroRNAs expressions as predictive biomarkers for diagnosis and follow up.

II.SUBJECTS & METHODS

This is a cross sectional observational prospective study conducted during the period from March 2018 to May 2020 on patients presented to Emergency Department, Zagazig University Hospitals with history of strox intake and developing toxic manifestations. Sixty patients met the inclusion and exclusion criteria of this study design. The inclusion criteria were history of strox intake during the last 12 months (Smith, 201). Exclusion criteria were past medical history of epilepsy, febrile convulsions, past history of seizures following drug abuse or head injury, family history of epilepsy or seizures, intake or abuse of any other addicting drugs as detected by urine drug screening, chronic neuropsychiatric, hepatic, renal and cardiovascular diseases. Patients with abnormal liver function, renal function tests or abnormal blood glucose levels were also excluded.

The study procedures were approved by the Ethical Committee for Research (Institutional Review Board), Faculty of Medicine, Zagazig University (ZU-IRP#:9021). Informed consent had been provided either by the patient or by their legal guardians in cases of disturbed conscious level. Epidemiological and clinical evaluation data, pattern of drug abuse (route, frequency) and delay time to onset of seizures in relation to strox intake were recorded. All patients were subjected at time of presentation to ER, after 72hrs and 28 days of presentation (Kretschmann et al., 2015) to (Digital Electroencephalography (EEG at

neurology department, Faculty of Medicine, Zagazig University and measurement of serum miR-9-3p, miR-106b-5p and miR-15a-5P, and miR-30a-5P expressions using real time-polymerase chain reaction (qRT-PCR) at Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Zagazig University.

II.1. METHODOLOGY

II.1.1.EEG: scalp digital EEG was performed to all subjects who presented with seizures using EB Neuro machine (Italy) in quiet room while patient was relaxed under normal standard conditions. The electrodes international 20-were placed according to 10 r as system of electrode placement. Bipola well as deferential montages were applied Hyperventilation and photic stimulation were done as provocative methods. The EEG tracing were analyzed carefully regarding; background activity, presence of epileptogenic activity: Focal, primary or focal with secondary generalized generalization

II.1.2. Measurement of miR-9-3p and miR-106b-5p and miR-15a-5P, and miR 30a-5P expression in

II.1.2.1. Blood sampling & miRNA extraction:

A 5 ml blood sample was drawn from all subjects and was collected into plain tube and serum was separated immediately and stored at -20 °C until time of analysis. MiRNA was extracted from serum by (miRNEasy isolation kit Qiagen, Germany) following to the manufacturer's instructions and elution was done using 30 µL of ribonuclease-free H_2O. The miRNA quality was quantified by A260 using UV/ spectrophotometer.

II.1.2.2. Real time-PCR analyses of miRNA -9-3p and miRNA-106b-5p, miR-15a-5P, and miR 30a-5P expressions:_A 100 ng miRNA was then used in reverse transcription by a miScript II RT Kit (Qiagen, USA) to produce cDNA. Real time-polymerase chain reaction (RT-PCR) was done using StepOne Plus™ System (Applied Biosystems Inc., USA). /Small nucleolar RNA (SNORD- 80-1) was used as the internal control gene (Gene Globe ID: MS00078686). RT-PCR was done in 20 µl as a final volume containing cDNA (5µl), pmol/ml each primer (0.5 µl for each) of miRNA-9-3p (Gene Globe ID: MS00006510, Qiagen) or miRNA-106b-5p (Gene Globe ID : MS00078707), miRNA-15a-5p (Gene Globe ID MS00003178) , miRNA-30a-5p (Gene Globe ID MS00007350)10 µl of 1X Quantitect sybr green PCR master mix (Qiagen), and 4 µL DdH2O according to the manufacturer's instructions. Amplification protocol consisted of: initial denaturation with polymerase activation at 95 °C for 15 min, then 40 cycles of denaturation 94 °C for 15 s; annealing at 55 °C for 30 s; extension at 70 °C for 30 s. Expression levels of each miRNAs were normalized to SNORD-80-1 as it is one of unbiased endogenous control genes for normalization (Kok et al., 2015). Each miRNA expression levels was normalized by calculating ΔCt value based on subtracting of its Ct value from that of SNORD-80-1, internal control. Then a relative expression of gene expression was calculated as ΔΔCt. Finally, the value of the change in miRNA expression calculated in cases in relation to controls and was analyzed

according to 2 -ΔΔCt method (Livak & Schmittgen, 2001).

II.2. STATISTICAL ANALYSIS

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Qualitative data were described using number and percent. The Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), mean, standard deviation, median and interquartile range (IQR). Significance of the obtained results was judged at the 5% level. The used tests were Chi-square test for categorical variables, to compare between different groups, Fisher's Exact or Monte Carlo tests for correction of chi-square when more than 20% of the cells have expected count less than 5 if the tables 2X2 or not 2X2 tables respectively Sensitivity test to correctly identify diseased individuals in a population "TRUE POSITIVES". The greater the sensitivity, the smaller the number of unidentified case "false negatives", Specificity test to correctly exclude individuals who are free of the disease "TRUE NEGATIVES". The greater the specificity, the fewer "false positives" will be included, Positive Predictive value (PPV to detect the probability of the disease being present, among those with positive diagnostic test results and Negative Predictive value (NPV) to detect the probability that the disease was absent, among those whose diagnostic test results were negative.

III. RESULTS

The mean age of patients presented with strox intoxication were 19-35 with 96.6% of them were male. The main route of consumption was by smoking (93.4%), with only 6.6% consumed it through E-Cigarettes (Table 1). As regard clinical presentations, out of the 60 patients included in this study, 80% had tachycardia and 52% had hypertension. Other manifestations were reported including chest pain (16%), vomiting (36%), diaphoresis(36%), dry mouth (4%), headache(4%) and blurred vision (4%). Severe manifestations in the form of cyanosia (8%) and apnea (4%) were also recorded. 20% of the patients represented with hallucinations, anxiety (12%), agitation (16%), delirium (8%), panic attacks (12%), paranoia (4%), confusion (16%), loss of consciousness (12%). Abnormal arterial blood gases were detected in 61% of the patients included in the study with 47 % and 14% revealed metabolic acidosis and respiratory acidosis respectively. Twenty eight patients (46.6%) developed seizures at time of presentation ; eight patients (28%) of them developed seizures after half an hour of strox intake while, 12 patients (42%) and eight patients (28%) developed seizures 1 hour and 2 hours after strox intake respectively. All patients were admitted and received symptomatic and supportive treatment according to critical care guidelines. A significant difference between patients who developed seizures and those who didn't develop seizures regarding frequency of strox abuse was detected(MCp<0.001*) (Table 1). Fifty percent (50%) of the patients who didn't develop seizures were first users, while only 3.1% were heavy users. on the other hand, heavy users represented 32.1 % of the patients who developed seizures with only 10.7 % of them were first users (Table 1,

Figure 1). Table 2 showed that the highest incidence of seizures and its recurrence were found in heavy abusers ($^{MC}p<0.05$). In this study, 46.6% of patients with strox intoxication presented with seizures which was mainly in the form of generalized tonic clonic convulsions (43%) followed by focal (21%), myoclonic (18%), status (11%) and tonic (7%). Most of patients developed seizures revealed normal EEG with only 35% and 9% of patients showed abnormal changes at time of presentation and after 72hrs respectively while no abnormal EEG changes were recorded in patients after 28 days (Table 1, Figure. 2). Significant up regulation of miR-9-3P and miR-30 a-5P genes expression were detected in patients with seizures at time of presentation ($p_1<0.05$) and during follow up durations (p_2, $p_3<0.05$) when compared with those without seizures. Persistent upregulation in the expression levels of these two miRNAs were detected when compared with control group (p control <0.05) with no significant changes after 72hrs and 28 days were found when compared with their levels in patients with seizures at time of presentation (p_4, $p_5>0.05$ respectively) (Tables 3&4). Also, there was no significant change between patients with seizures at 72hrs and those at 28 days of follow up regarding expression levels of miR-9-3P and miR-30 a-5P ($p_6>0.05$). Significant upregulation in the expression levels of miR-106b-5p in patients with seizures at time of presentation and after 72hrs when compared with patients without seizures ($p_1,p_2<0.05$) was found, while there was no significant difference between the expression level of this gene in patients who developed seizures after 28 days when compared with either control group (p control>0.05) or patients without seizures ($p_3>0.05$) (Tables 3&4) . Also, significant changes in the expression levels of miR-106b-5p were found in patients after 28 days when compared with patients at time of presentation($p_5<0.05$) and after72hrs ($p_6<0.05$). Regarding the expression levels of miR-15 a-5P, there were significant downregulations in patients with seizures at time of presentation and after 72hrs when compared with patients without seizures($p_1,p_2<0.05$) while, there was no significant difference when the expression level of this gene after 28 days was compared with its expression in patients without seizures ($p_3>0.05$) (Tables 3&4) . The expression levels of miR-15 a-5P returned to normal in patients after 28 days with significant difference was detected when compared with those at time of presentation($p_5<0.05$) and in patients after72hrs ($p_6<0.05$) (Tables 3&4. Figure. 3). Sensitivity of miR9-3P, miR 106b-5p, miR 15 a-5P and miR 30 a-5P were 86.67%, 81.36%, 81.36% and 67.65 respectively, specificity of miR9-3P, miR 106b-5p, miR 15 a-5P and miR 30 a-5P were 77.78%, 72.60%, 72.60% and 87.50% respectively, PPV of miR9-3P, miR 106b-5p, miR 15 a-5P and miR 30 a-5P were 76.47%, 70.59%, 70.59% and 85.19% respectively, NPV of miR9-3P, miR 106b-5p, miR 15 a-5P and miR 30 a-5P were 87.50%, 82.81%, 82.81% and 71.79% respectively and accuracy of miR9-3P, miR 106b-5p, miR 15 a-5P and miR 30 a-5P were 81.82%, 76.52%, 76.52% and 77.27% respectively (Table 5). Comparison between expression levels of MicroRNAs between patients developed seizures at time of presentation and during

follow up periods showed that miR9-3p and miR 30 a-5p had the highest accuracy when compared with miR106b-5p and miR 15 a-5P (Table 6)

Table 1: Patient demographics and seizure characteristics at time of presentation and during follow up duration

	Patients without seizures N=32	Patients with seizures at time of presentation N= 28	Patients with seizures at 72hrs N=22	Patients with seizures at 28 days N=18	Test of sig.	p value
Demographics						
-Median age (range)	25 (19-35)	22 (19-35)	22(19-35)	26(22-35)	-	-
-Gender (Male/female)						
Male	31	27	21	18	χ^2= **1.132**	^{MC}p= 1.000
Female	1	1	1	0		
Pattern of drug abuse						
-Route of abuse N (%)						
- Smoking	30 (93.7)	26 (92.8)	20 (90.9)	17 (94.4)	χ^2=	^{MC}p=
- E-cigarette	2 (6.3)	2 (7.2)	2 (9.1)	1(5.5)	0.552	1.000
-Frequency of abuse during past 12months						
-Single use (first time)	16 (50.0%)	3 (10.7%)	1 (4.5%)	1 (5.0%)		
-1-2 days/month	7 (21.9%)	3 (10.7%)	1 (4.5%)	1 (5.0%)	χ^2=	^{MC}p
-1-2 days /week	6 (18.8%)	5 (17.9%)	3 (13.6%)	1 (5.0%)	42.414*	<0.001*
-3-4 days/week	2 (6.3%)	8 (28.6%)	8 (36.4%)	7 (%)		
-5-6 days/week	1 (3.1%)	9 (32.1%)	9 (40.9%)	8 (%)		
Sig. bet. grps.	$^{MC}p_1$<0.001*, $^{MC}p_2$<0.001*, $^{MC}p_3$<0.001*, $^{MC}p_4$=0.836, $^{MC}p_5$=0.709, $^{MC}p_6$=0.943					
Seizure type n=						
Generalized tonic-clonic	–	12 (43%)	6 (27%)	1 (5%)	χ^2=7.579*	0.023*
Sig. bet. grps.	p_1=0.254, p_2=0.006*, $^{FE}p_3$=0.105					
Myoclonic		5 (18%)	4 (18%)	1 (5%)	χ^2=1.606	^{MC}p=0.427
Tonic		2 (7%)	1 (5%)	1 (5%)	χ^2=0.406	^{MC}p=1.000
Focal		6 (21%)	9 (41%)	14 (80%)	χ^2=14.264*	0.001*
Sig. bet. grps.	p_1=0.136, p_2<0.001*, p_3=0.019*					
Status		3 (11%)	2 (9%)	1 (5%)	χ^2=0.438	^{MC}p=1.000
Frequency of recurrence						
-Once	–		20 (91%)	6 (34%)	χ^2=	<0.001*
-More than once			2(9%)	12 (66%)	14.426*	
(EEG)	–					
-Normal n=		18 (65%)	20 (91%)	18 (100%)	10.326*	^{MC}p=
-Abnormal n=		10 (35%)	2 (9%)	0		0.003*
Sig. bet. grps.	p_1=0.029*, p_2<0.001*, $^{FE}p_3$=0.492					
·Generalized spike wave discharge		6	2	–	1.006	^{MC}p= 1.000
·Slow wave discharge		2	0			
·Diffuse discharge		2	0			

χ^2: Chi square test, MC: Monte Carlo, FE: Fisher Exact, p: p value for comparing between the studied groups, p_1: p value for comparing between Patients without seizures and Patients with seizures at time of presentation, p_2: p value for comparing between Patients without seizures and Patients with seizures at 72hrs, p_3: p value for comparing between Patients without seizures and Patients with seizures at 28 days, p_4: p value for comparing between Patients with seizures at time of presentation and Patients with seizures at 72hrs, p_5: p value for comparing between Patients with seizures at time of presentation and Patients with seizures at 28 days, p_6: p value for comparing between Patients with seizures at 72hrs and Patients with seizures at 28 days, *: Statistically significant at $p \leq 0.05$

Table 2: Relation between Frequency of abuse during past 12months and seizures at time of presentation and during follow up

	Patients without seizures n=32	Patients with seizures at time of presentation n = 28	Patients with seizures at 72hrs (n=22)	Patients with seizures at 28 days (n=18)	χ^2	P
Single use (1st time)	16 (50.0%)	3 (10.7%)	1 (4.5%)	1 (5.6%)	21.258*	$^{MC}p<0.001^*$
Sig. bet. grps.	$p_1=0.001^*, p_2<0.001^*, p_3=0.001^*, ^{FE}p_4=0.621, ^{FE}p_5=1.000, ^{FE}p_6=1.000$					
1-2 days/month	7 (21.9%)	3(10.7%)	1(4.5%)	1(5.6%)	4.022	^{MC}p 0.258
1-2 days /week	6(18.8%)	5(17.9%)	3(13.6%)	1(5.6%)	1.753	^{MC}p 0.634
3-4 days/week	2(6.3%)	8(28.6%)	8(36.4%)	7(38.9%)	9.557*	0.023*
Sig. bet. grps.	$^{FE}p_1=0.035^*, ^{FE}p_2=0.010^*, ^{FE}p_3=0.007^*, p_4=0.558, p_5=0.466, p_6=0.870$					
5-6 days/week	1(3.1%)	9(32.1%)	9(40.9%)	8(44.4%)	14.569*	0.002*
Sig. bet. grps.	$^{FE}p_1=0.004^*, ^{FE}p_2=0.001^*, ^{FE}p_3=0.001^*, p_4=0.522, p_5=0.399, p_6=0.822$					

χ^2: Chi square test, MC: Monte Carlo , p: p value for comparing between the different categories, p_1: p value for comparing between Patients without seizures and Patients with seizures at time of presentation, p_2: p value for comparing between Patients without seizures and Patients with seizures at 72hrs, p_3: p value for comparing between Patients without seizures and Patients with seizures at 28 days, p_4: p value for comparing between Patients with seizures at time of presentation and Patients with seizures at 72hrs, p_5: p value for comparing between Patients with seizures at time of presentation and Patients with seizures at 28 days, p_6: p value for comparing between Patients with seizures at 72hrs and Patients with seizures at 28 days, *: Statistically significant at $p \leq 0.05$

Table 3: MicroRNAs expression profile at time of presentation and during follow up durations

	Control patients N= 32	Patients without seizures N=32	Patients with seizures at time of presentation N=28	Patients with seizures at 72hrs N=22	Patients with seizures at 28 days N=18	χ^2	P
miR9-3P (up regulation)	3 (9.4%)	5 (15.6%)	23 (82.1%)	18 (81.8%)	11 (61.1%)	57.0*	<0.001*
$p_{Control}$		$^{FE}p=0.708$	<0.001*	<0.001*	<0.001*		
Sig. bet. grps.	$p_1<0.001^*, p_2<0.001^*, p_3=0.001^*, ^{FE}p_4=1.000, ^{FE}p_5=0.170, ^{FE}p_6=0.173$						
miR106b-5p (up regulation)	4 (12.5%)	7 (21.9%)	25 (89.3%)	17 (77.3%)	5 (27.8%)	54.371*	<0.001*
pControl		0.320	<0.001*	<0.001*	$^{FE}p=0.253$		
Sig. bet. grps.	$p_1<0.001^*, p_2<0.001^*, ^{FE}p_3=0.735, ^{FE}p_4=0.277, p_5<0.001^*, p_6=0.002^*$						
miR 15 a-5P (down regulation)	3 (9.4%)	5 (15.6%)	21 (75.0%)	16 (72.7%)	3 (16.7%)	49.672*	<0.001*
pControl		$^{FE}p=0.708$	<0.001*	<0.001*	$^{FE}p=0.654$		
Sig. bet. grps.	$p_1<0.001^*, p_2<0.001^*, ^{FE}p_3=1.000, p_4=0.856, p_5<0.001^*, p_6<0.001^*$						
miR 30 a-5P (up regulation)	3 (9.4%)	5 (15.6%)	26 (92.9%)	15 (68.2%)	17 (94.4%)	73.953	<0.001*
$p_{Control}$		$^{FE}p=0.708$	<0.001*	<0.001*	<0.001*		
Sig. bet. grps.	$p_1<0.001^*, p_2<0.001^*, p_3<0.001^*, ^{FE}p_4=0.032^*, ^{FE}p_5=1.000, ^{FE}p_6=0.054$						

χ^2: Chi square test, FE: Fisher Exact, p: p value for comparing between the studied groups, $p_{control}$: p value for comparing between Control and each other groups, p_1: p value for comparing between Patients without seizures and Patients with seizures at time of presentation, p_2: p value for comparing between Patients without seizures and Patients with seizures at 72hrs, p_3: p value for comparing between Patients without seizures and Patients with seizures at 28 days, p_4: p value for comparing between Patients with seizures at time of presentation and Patients with seizures at 72hrs, p_5: p value for comparing between Patients with seizures at time of presentation and Patients with seizures at 28 days, p_6: p value for comparing between Patients with seizures at 72hrs and Patients with seizures at 28 days, *: Statistically significant at $p \leq 0.05$

Table 4: Expression levels of different miRna at time of presentation and follow up duration

	Control patients N= 32	Patients without seizures N=32	Patients with seizures at time of presentation N=28	Patients with seizures at 72hrs N=22	Patients with seizures at 28 days N=18	H	p
miR9-3P (upregulation)							
Min. – Max.	0.30 – 1.40	0.55 – 1.60	0.50 – 4.0	0.60 – 4.30	0.40 – 4.30		
Mean ± SD.	0.68 ± 0.24	0.84 ± 0.23	1.96 ± 0.91	2.54 ± 1.15	2.37 ± 1.47	58.728*	<0.001*
Median (IQR)	0.66(0.54–0.80)	0.86(0.72–0.93)	1.90(1.45–2.21)	2.85(1.70–3.20)	2.90(0.80–3.60)		
$p_{control}$		0.052	<0.001*	<0.001*	<0.001*		
Sig. bet. grps.		p_1<0.001*,p_2<0.001*,p_3=0.002*,p_4=0.381,p_5=0.805,p_6=0.308					
miR 106b-5p (upregulation)							
Min. – Max.	0.30 – 1.70	0.48 – 1.70	0.70 – 4.0	0.40 – 4.0	0.20 – 4.30		
Mean ± SD.	0.74 ± 0.35	0.84 ± 0.26	1.92 ± 0.76	2.27 ± 1.17	1.40 ± 1.40	48.135*	<0.001*
Median (IQR)	0.70(0.54–0.80)	0.79(0.67–0.93)	1.85(1.55–2.05)	2.15(1.70–3.20)	0.70(0.50–2.50)		
$p_{control}$		0.124	<0.001*	<0.001*	0.130		
Sig. bet. grps.		p_1<0.001*,p_2<0.001*,p_3=0.835,p_4=0.971,p_5=0.001*,p_6=0.001*					
miR 15 a-5P (downregulation)							
Min. – Max.	0.30 – 1.90	0.60 – 2.10	0.30 – 1.90	0.70 – 1.90	0.40 – 2.10		
Mean ± SD.	1.41 ± 0.35	1.47 ± 0.41	0.92 ± 0.43	0.98 ± 0.39	1.36 ± 0.45	30.114*	<0.001*
Median (IQR)	1.50(1.30–1.60)	1.50(1.20–1.80)	0.80(0.60–1.05)	0.80(0.70–1.30)	1.40(1.30–1.50)		
$p_{control}$		0.688	<0.001*	0.001*	0.475		
Sig. bet. grps.		p_1<0.001*,p_2<0.001*,p_3=0.291,p_4=0.653,p_5=0.007*,p_6=0.031*					
miR 30 a-5P (upregulation)							
Min. – Max.	0.30 – 1.70	0.48 – 1.20	4.0–0.40	4.0–0.20	4.70–0.30		
Mean ± SD.	0.69 ± 0.28	0.80 ± 0.19	0.74±1.96	1.32±2.08	1.23±2.79	62.534*	<0.001*
Median (IQR)	0.66(0.54–0.80)	0.73(0.66–0.93)	–1.90(1.70 2.05)	–2.05(0.70 3.20)	–2.80(1.80 3.87)		
$p_{control}$		0.158	<0.001*	<0.001*	<0.001*		
Sig. bet. grps.		p_1<0.001*,p_2=0.001*,p_3<0.001*,p_4=0.417,p_5=0.237,p_6=0.064					

H: H for Kruskal Wallis test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Dunn's for multiple comparisons test), p: p value for comparing between the studied groups, $p_{control}$: p value for comparing between Control and each other group, p_1: p value for comparing between Patients without seizures and Patients with seizures at time of presentation, p_2: p value for comparing between Patients without seizures and Patients with seizures at 72hrs, p_3: p value for comparing between Patients without seizures and Patients with seizures at 28 days, p_4: p value for comparing between Patients with seizures at time of presentation and Patients with seizures at 72hrs, p_5: p value for comparing between Patients with seizures at time of presentation and Patients with seizures at 28 days, p_6: p value for comparing between Patients with seizures at 72hrs and Patients with seizures at 28 days, *: Statistically significant at $p \leq 0.05$

Table (5): Agreement (sensitivity, specificity and accuracy) for miRna

	Without seizures	With seizures	Sensitivity	Specificity	PPV	NPV	Accuracy
miR9-3P							
Down regulation	56	16	86.67	77.78	76.47	87.50	81.82
Up regulation	8	52					
miR 106b-5p							
Down regulation	53	21	81.03	71.62	69.12	82.81	75.76
Up regulation	11	47					
miR 15 a-5P							
Down regulation	8	40	83.33	66.67	58.82	87.50	72.73
Up regulation	56	28					
miR 30 a-5P							
Down regulation	56	10	87.88	84.85	85.29	87.50	86.36
Up regulation	8	58					

PPV: Positive predictive value, NPV: Negative predictive value

Table (6): Agreement (sensitivity, specificity and accuracy) for miRna

	Patients with seizures at time of presentation n=28	Patients with seizures N=40	Sensitivity	Specificity	PPV	NPV	Accuracy
miR9-3P							
Down regulation	15	1	75.00	93.75	97.05	53.57	79.41
Up regulation	13	39					
miR 106b-5p							
Down regulation	3	18	46.81	14.29	55.0	10.71	36.76
Up regulation	25	22					
miR 15 a-5P							
Up regulation	7	21	47.50	25.00	47.50	25.00	38.24
Down regulation	21	19					
miR 30 a-5P							
Down regulation	19	1	81.25	95.00	97.05	67.86	85.24
Up regulation	9	39					

PPV: Positive predictive value, NPV: Negative predictive value

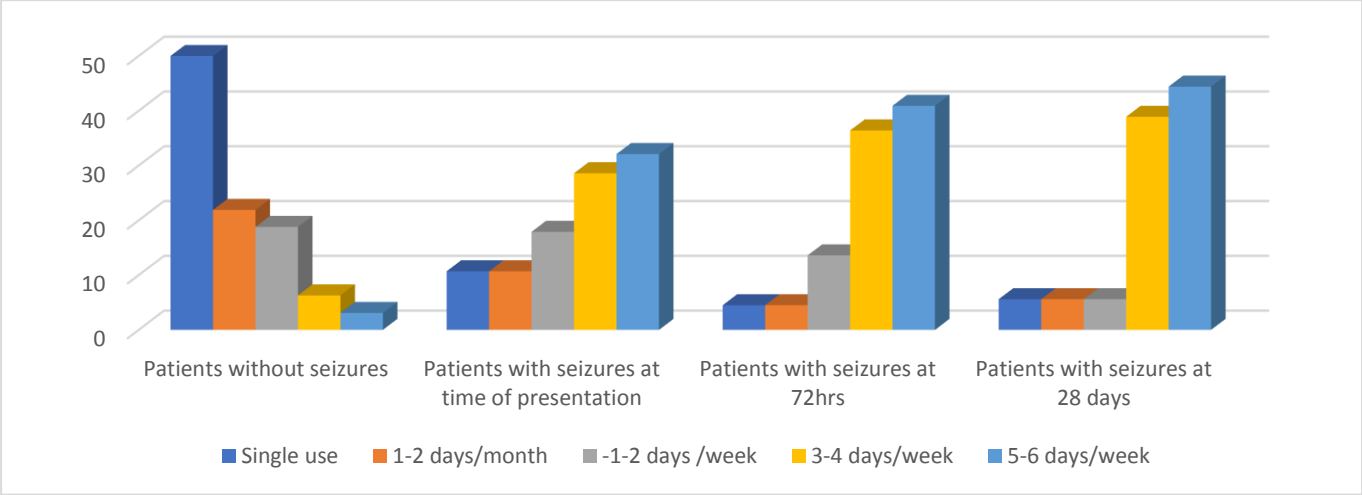

Figure (1): Relation between frequency of abuse during past 12 months and seizures at time of presentation and during follow up.

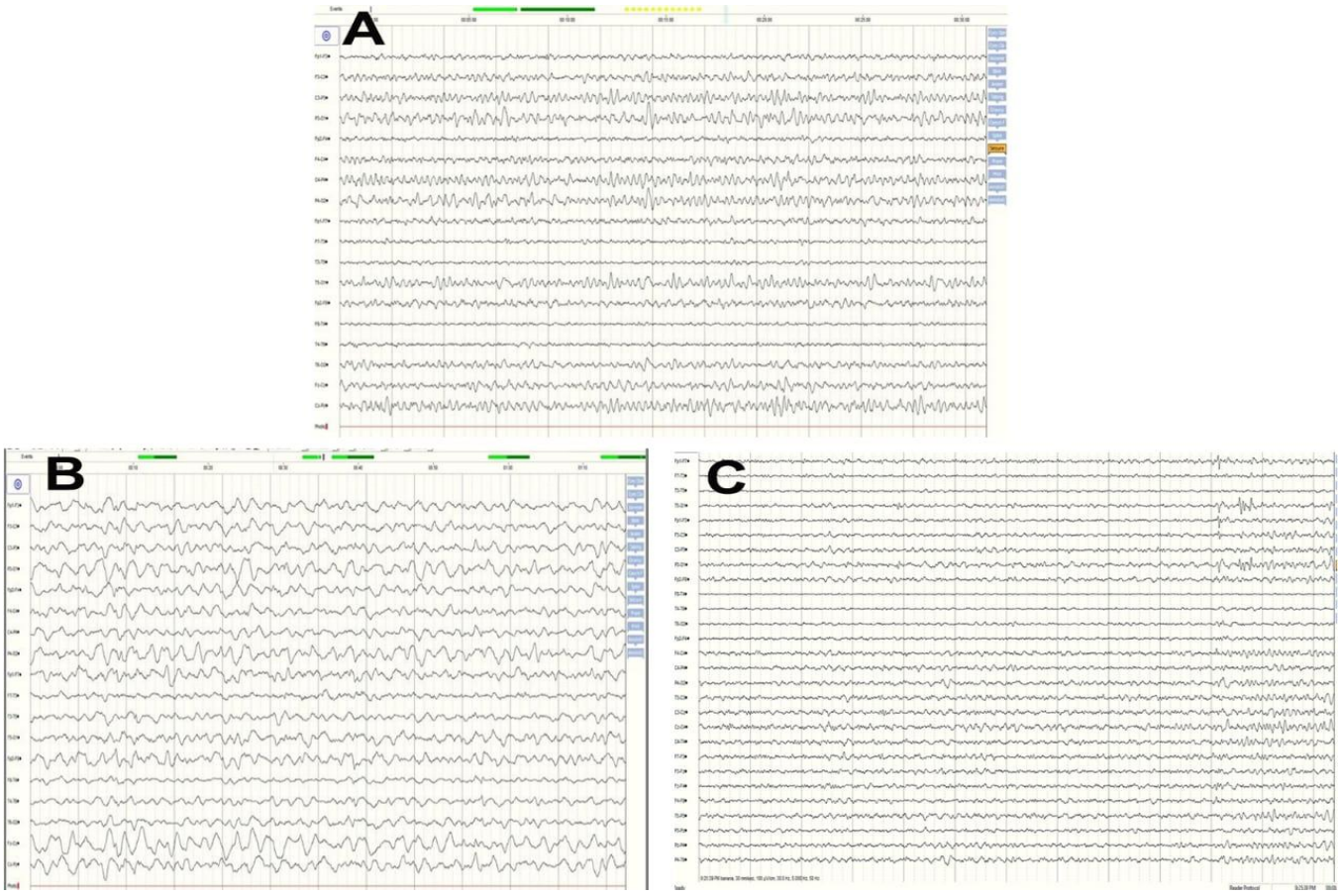

Figure (2): Electroencephalogram (EEG) (A) show normal background activity of alpha wave(8-12 HZ) with no recorded abnormal discharge, (B) shows diffuse generalized slow wave discharge of theta activity & (C) show focal spikes at the left frontal region on mild slow background.

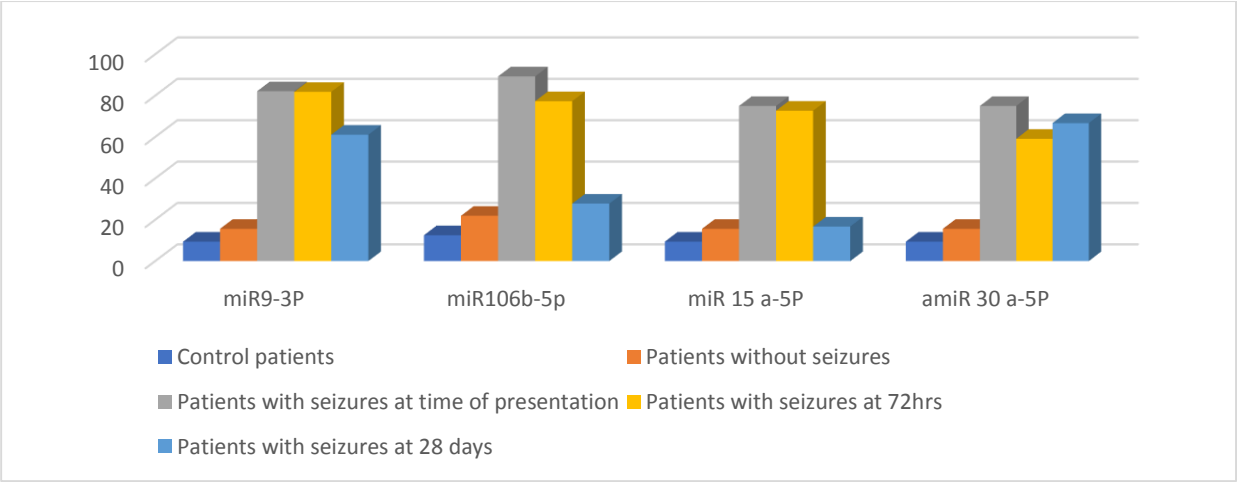

Figure (3): MicroRNAs expression profile at time of presentation and during follow up durations

IV.DISCUSSION

The use of the new psychoactive substances including strox; the street name for synthetic cannabinoids is an alarming problem that has been emerged in the last few years in Egypt. Severe intoxications with synthetic cannabinoid compounds have been associated with incidence of seizures which is an important cause in increasing mortality rate from strox abuse (Kronstrand et al., 2013 & Funada and Takebayashi-Ohsawa, 2018). This work was carried out to study strox induced seizures and the possible use of microRNAs expressions as predictive biomarkers for diagnosis and follow up of such complication.

Sixty patients who presented to ER with strox intoxication and had history of strox abuse during the last 12 months have been enrolled in this study. Clinical evaluation, EEG, measurement of serum miR-9-3p, miR-106b-5p and miR-15a-5P, and miR 30a-5P expressions using real time-polymerase chain reaction (qRT-PCR) at time of presentation, 72hrs and 28 days after presentation were carried out in this study.

Strox intoxication showed diverse and unrelated manifestations in the form cardiovascular, neuropsychiatric manifestations, vomiting, diaphoresis, loss of consciousness and apnea. These results are in agreement with Elnazeir et al. (2020) who reported that SC intoxication is associated with a group of unconnected signs.

Data retrieved from different poison control centers worldwide showed that the most common cardiovascular manifestations associated with SC intoxication were tachycardia, hypertension, dyspnea and thoracic pain (Zawilska and Wojcieszak, 2014) with nausea and vomiting were the most common gastrointestinal symptoms (Cooper, 2016). The common Psychiatric symptoms of SC use were agitation, anxiety, auditory and visual hallucinations, delusional thinking, paranoid behavior and confusion (Sweet et al., 2018). Headache, dry mouth and diaphoresis were also reported (Gunderson et al. 2014).

The results of this work showed that 46.6% of patients with strox intoxication presented with seizures which was mainly in the form of generalized tonic clonic convulsions (43%) followed by focal (21%), myoclonic (18%), status (11%) and tonic (7%). These results are in accordance with Zawilska and (&) Wojcieszak (2014) who described different types of seizures associated with use of the synthetic cannabinoids; spice which included generalized convulsions, generalized muscle tone in extremities, generalized tonic- clonic seizures and myoclonic jerking movements. Also, status epilepticus after smoking SCs which needed emergent life support and admission to intensive care unit was reported (Babi et al. 2017). De Havenon et al., (2011) reported two cases presented after smoking spice containing synthetic cannabinoids with generalized tonic clonic seizure with neither prior neurological disease history or focal features or epileptiform discharges in EEG. Also, Elnazeir et al. (2020) presented a series of several patients with SCs intoxication in which the most neurological manifestations were generalized tonic-clonic convulsions and status epilepticus. Although, synthetic cannabinoids in strox is believed to be the main ingredients involved in occurrence of

seizures, other ingredients such as methamphetamine and ketamine which may be added to strox preparations in Egypt (El-Masry & Abdelkader , 2021), can contribute to induction of seizures (Meaden and Barnes, 2019 & Onoka, et al., 2020).

The incidence of seizures in patients enrolled in this work has occurred even in patients with single time use of strox, however it was significantly increased with increased frequency of strox consumption during the last 12 months. This was in accordance with Cooper (2016) who stated that adverse effects of SCs usage vary according to the frequency of use and may occur even with single use.

In this work, at time of presentation, 65 % of cases presented with seizures showed normal EEG, while only 35% reported abnormal EEG changes of which six patients had wave discharges-generalized spike, two patients had focal sharp slow wave discharges and the other 2 subjects showed diffuse slowing. After 72 hrs of presentation, only 9% of patients who experienced seizures showed generalized spike wave discharge while no EEG abnormalities were recorded after 28 days of presentation. This was in agreement with Elnazeir et al. (2020) who stated that most of the SCs intoxicated cases represented with seizures showed normal EEG. On the other side, Funada and Takebayashi-Ohsawa (2018) mentioned that acute intoxication with SC (AM2201) provoked abnormal, high amplitude sharp – wave activity.

Kobylarek et al. (2019) mentioned that although EEG is considered the main diagnostic modality in seizure; it is of low specificity since abnormal EEG reflects brain activity during seizure but, when it is over, activity of brain returns to normal. They stated that these abnormal findings confirm but not exclude diagnosis with new predictive and diagnostic markers of seizure are needed.

Mitchell et al., 2008, An et al., 2016 and Brindley et al. (2019) and Wang et al. (2015) stated that the brain enriched miRNAs can now be easily detected in blood and serum and they have the advantage of being stable in bio fluids, rapid, noninvasive and economical. Brindley et al. (2019) mentioned that miRNAs are convenient biomarkers to assess risk of seizure and treatment response.

No significant changes were found in this study between control group and patients who didn't develop seizures regarding genes expressions. On the other hand, significant changes were detected in genes expressions when both control group and patients who didn't develop seizures were compared with patients developed seizures at time of presentation and during follow up periods. Assessment of genes expressions revealed up regulation of miR-9-3P, miR106p-5p and miR 30 a-5P and downregulation of miR 15 a-5P at time of presentation in pts with seizures with both miR9-3P, and miR 30 a-5P persisted upregulated during follow up periods, while miR106p-5 and miR 15 a-5P returned to normal.

These results agree with that of Raoof et al. (2018) who found that miR-9-3p is the most abundant miRNA in brain and blood after seizures and it remained high for period of a time. They stated that the increase in its level during the acute stage of seizures with persisting elevated after indicating that it is a

biomarker of the underlying pathophysiology of seizures rather than ictal activity.

Also, Cava et al. (2018) mentioned that miR-106b-5p is upregulated while miR-15a-5p is downregulated in cases of seizures. They stated that miR-106b-5p is the best diagnostic miRNA marker in cases of seizures with 80.3% sensitivity and 81.2% specificity. Kretschmann et al. (2015) reported that miR-30a-5p level was upregulated in hippocampus of mouse in chronic epilepsy models while it was downregulated in acute seizure models. Moreover, Kobylarek et al. (2019) stated that overexpressed levels of miR-30a-5p in patients serum was detected in seizures.

On the other hand, Sun et al. (2016) mentioned that the expression levels of miR-106b & miR-15a-5P and miR-30a-5p are upregulated in cases of seizures and their levels are higher during fits when compared with post seizures levels.

The alteration in the expression levels of specific miRNAs in brain tissue and subsequently in biofluids have been linked to seizure-induced neuronal death or neuroprotection through regulation of neuronal microstructure, cell death, inflammation, synaptic plasticity and apoptosis and can be used as biomarkers to detect patients at risk of epilepsy and recurring seizures (Henshall, 2013& Ma, 2018). Upregulation of miR-106b-5p in the early phase of fits suggesting its potential role in the induction of neuronal cell cycle block and neuronal apoptosis (Bot et al., 2013 & Friedman et al., 2013). Also, Teplyuk et al., 2015 stated that decreased expression levels of miR-15a-5p indicates cell cycle block with possibility of inhibition of neurogenesis together with increase of neuronal apoptosis and subsequent neuronal loss (Li et al., 2020).

Nudelman et al., 2010 stated that miRNAs are key regulators of protein production during and following seizures suggesting that miRNAs may affect neuronal excitability and remodeling responses leading to seizures. Brents et al. (2011) suggested that seizures induced by the synthetic cannabinoid compounds (JWH) are due to imbalance between inhibitory and excitory neuronal system leading to Affection of neuronal excitability and permanent neuronal damage (Akyuz et al., 2021).

V.CONCLUSION

Strox-induced seizure was associated with overexpression of miR-9-3p, miR-106p-5P, miR30a-5p at the acute stage of intoxication with miR-9-3p and miR30a-5p remained elevated in most patients during follow up. On the other hand, cases developed seizures exhibited downregulation of miR-15a-5P which returned to normal during follow up so, miR9-3p and miR 30 a-5p can be used as predictive biomarkers in diagnosis and follow up of strox induced seizures.

VI.RECOMMENDATIONS

-Attention should be paid for strox drug, since it is associated with serious manifestations including seizures

-Raising awareness about the serious toxic effects of strox and and its possible long term neurological sequalae

-Follow up is with a neurologist necessary especially for patients who developed seizures at time of presentation

Limitation:

-Diagnosis of strox intoxication was based on history of exposure either by patients' relatives or self-reports since there is no confirmatory laboratory diagnosis for SCs use.

-Out of the 28 patients who developed seizures at time of presentation, 10 patients (35 %) did not complete the follow up assessment and were excluded from the study.

-Data about seizures type, frequency, recurrence during follow up was collected according to patient's self-report

-Evaluation of EEG and gene expressions were carried out at the end of follow up period due to obstacles regarding presentation of the patients shortly after seizures recurrence, so these measurements were done at time of presentation, 72 hours after presentation for assessment of seizures onset and at the end of follow up duration (28 days) for prediction of seizures recurrence

Conflict of interest

The authors declare that they have no conflict of interest.

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الملخص العربي

التعبير الجينى التفاضلى للاحماض النووية الريبية الصغيرة (30a-5p 15a-5p, 106b-5p, 9-3p) كمؤشرات حيوية تنبؤية عند بداية التشنجات و بعدها فى أمصال حالات التسمم بالاستروكس,

يارا محمد مدحت الفخراني[1] و امل الشال[2] و ناهد شحته[3] و سماح عادل النجدى[1]
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قسم الكيمياء الحيوية الطبية -كلية الطب البشرى- جامعة الزقازيق[2] و قسم امراض المخ والاعصاب– كلية الطب البشرى- جامعة الزقازيق [3]

الإستروكس هو الاسم المتعارف عليه للقنب الصناعي الجديد الذي أسيء استخدامه على نطاق واسع في السنوات القليلة الماضية في مصر مما تسبب في في العديد من الآثار السامة بما في ذلك نوبات الصرع. الهدف من الدراسة هو تقييم التأثير التشنجي المحتمل لسمية الستروكس واستخدام تعبيرات الاحماض النووية الريبية الصغيرة(miR) كمؤشرات حيوية تنبؤية للتشخيص والمتابعة. المنهجية: أجريت دراسة رصدية مقطعية مستعرضة على 60 مريضاً تم استقبالهم بقسم الطوارئ بمستشفيات جامعة الزقازيق بعد تناولهم مادة الستروكس خلال الفترة من مارس 2018 إلى مايو 2020. تم تسجيل بيانات المرضي وطرق الاستهلاك ومعدل تعاطي هذا المخدر خلال الاثني عشر شهر السابقة . خضع جميع المرضي للفحص السريرى ومخطط كهربية الدماغ وقياس التعبيرات ل miR-9-3p و miR-106b-5p و miR 30a-5P و-miR-15a 5Pفي مصل الدم باستخدام تفاعل سلسلة البوليميراز في الوقت الحقيقي وقت دخول الطوارئ وبعد 72 ساعة وبعد 28 يوم. النتائج: كان متوسط عمر المرضى بالدراسة 19-35 ، معظمهم من الذكور (96.6٪). من بين الحالات المشمولة في الدراسة عانى 46.6٪ وقت دخول المستشفي من نوبات تشنجية كانت بشكل رئيسي في شكل تشنجات ارتجاجية معممة (43٪). كشف مخطط كهربية الدماغ عن تغيرات غير طبيعية في 35٪ فقط من المرضى الذين أصيبوا بتشنجات وقت دخول المستشفي، وفي 9٪ من أولئك الذين أصيبوا بنوبات صرع بعد 72 ساعة مع عدم تسجيل اي تغييرات في مخطط كهربية الدماغ بعد 28 يومًا من المتابعة. تقييم تعبيرات ال MicroRNAs كشف عن زيادة في التعبير عن miR-9-3Pو miR-106b-5p و-miR- 30 a 5Pونقص في التعبير عن miR 15 a-5P حيث ظل miR-9-3P و miR 30 a-5p مرتفعين اثناء فترة المتابعة بينما عاد miR106b-5pو miR 15 a-5P الي المستوى الطبيعي اثناء فترات المتابعة. الخلاصة: يمكن استخدام ظل miR-9-3P و miR 30 a-5p كمؤشرات حيوية تنبؤية في تشخيص ومتابعة اللتشنجات التي يسببها الإستروكس.