Research Article

MicroRNAs molecular biomarkers for determination of drowning death in relation to postmortem interval in different organs of albino rats

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Abstract

Background: Forensic professionals have a crucial responsibility when estimating the postmortem interval (PMI), particularly in criminal situations. Drowning is a deadly asphyxiation incident brought by extended submersion in a liquid. Diagnosing death toll from drown remains the most complex challenge for forensic pathologists. This work aimed to determine drowning deaths in relation to PMI using histopathological changes and the expression of microRNA-23b-3p and microRNA-381-3p in brain and lung tissues. **Methods:** In this study, thirty adult albino rat models were used. Three groups were created out of them (0, 12, 24 hrs. after death), each of which contains 10 rats. Brain and lung tissues from each rat were taken out and divided into two pieces. The first was prepared for histopathological analysis, and the second portion was prepared for the analysis of microRNA expression by RT-PCR. **Results:** The brain tissues showed pyknotic nuclei of the neurons with mild vacuolation at the time of death, which progressed with an increased postmortem interval to marked vacuolation. While lung tissues showed overdistention of the alveoli, which became marked with an increased postmortem interval with marked dissolution of interstitial cells at 24 hrs postmortem. MicroRNA-23b-3p and microRNA-381-3p levels showed a significant difference in brain and lung tissues at different postmortem intervals. **Conclusion:** MicroRNA-23b-3p and MicroRNA-381-3p expression could be helpful as new biomarkers for the determination of drowning death in relation to time since death.

Keywords: Drowning, MicroRNA-23b-3p, MicroRNA-381-3p

Introduction

The most common leading cause of unusual deaths worldwide is drowning. This particular type of asphyxia death is caused by aspirating fluid into the airways. Since the majority of clinical findings associated with drowning are not diagnostic, diagnosing drowning deaths in forensic science is a challenging task. Numerous causes can lead to drowning death, such as secondary injuries, laryngeal spasms, hypothermia, asphyxiation, vagal inhibition, and irregularities in electrolytes and osmotic balance **(1).**

Among the most difficult challenges in forensic medical science is estimating the exact moment of a person's death. Since estimating the post-mortem interval can be an incredibly challenging task, all professionals who interact with corpses have struggled to estimate the post-

mortem period since beginning of time **(2).**

Morphological alterations such as temperature decay, cadaveric rigidity (rigor mortis), and body color alterations (livor mortis) start to show at early PMI. Determining these morphological alterations is useful in calculating the PMI. However, several variables, including the environment and the cause of the death, may affect how frequently these outward morphological alterations occur. So, other techniques, like the degradation of RNA, have been researched for PMI estimation. Among the RNA species, microRNAs are short, non-coding RNAs that typically have 22 nucleic bases in length that can control gene expressions involved in several pathways through post-transcriptional means **(3).**

MiRNAs among the RNA species have the potential to be useful in determining PMI at advanced stages because of their various qualities, particularly stability, as they have a tiny size and are protected by a lipid or protein matrix. MicroRNAs are less prone to degradation than mRNA. Factors like the kind of tissue, environmental factors (such as high temperatures), or bodily conditions like putrefaction might affect the stability of miRNA **(4).**

Due to the fact that water breathed can affect ion transport or water channel control, specific micro-ribonucleic acids could be utilized as trustworthy indicators for confirming drowning since some miRNAs influence the genes that code for ion channels. Additionally, because of the micro-environmental changes connected to the dying process, distinct miRNA expression patterns may be determined. So, specific microRNAs could be utilized as valid biomarkers in order to determine the type of drowning **(5).**

This study was designed to determine drowning as a cause of death in relation to postmortem interval through microRNA-23b-3p and microRNA-381-3p expression at three different postmortem intervals and histopathological changes in brain and lung tissues.

Materials and methods:

This study involved thirty adult albino rats, each weighing around 200–250 gm. They were derived from the laboratory animal growth center of Minia University in Minia, Egypt. The experimental rats were resided in clean, optimally ventilated plastic enclosures. They also had unrestricted access to tap water and an ordinary diet of pellet food that was well-balanced. They were stayed at a steady temperature and humidity level and subjected to a 12-hour day and 12-hour night cycle. The experiment was carried out in compliance with Minia University's animal use and care committee regulations (Approval No. 704:2023)

Experimental design:
Rats were classified into 3 groups (0, 12, and 24 hours after death), each of which contains 10 rats. Rats were fully conscious and drowned by submersion in the water basin till death.

Histopathological study:
After specimens of brain and lung tissue had been fixed in 10% neutral buffered formalin, trimming was carried out. Washing and dehydration were performed using progressively stronger alcohols, which were then cleaned in xylene, fixed in paraffin, and divided at a 4-6 U thickness. Finally, they underwent light microscopy viewing by being stained with hematoxylin and eosin.

Genetic study:
1- MiRNA expression through qRT-PCR (Quantitative Real-time Polymerase Chain-Reaction):
By utilizing PCR real-time technology, the miRNA qRT-PCR Detection All-in-One™ Kit system 2.0 (Cat. No. QP115, Cat. No. QP116) (obtained from Gene Copoeia Company, USA) quantitatively measures microRNAs. The experimental procedure includes two major steps. The first step is single-step cDNA synthesis, which is carried out by adding the 3' terminus of miRNAs with poly-A tails using poly A polymerase and reverse transcribed the miRNA-tailed poly-A simultaneously using Sure Script™ RTase Mix with a unique primer for Oligo-dT adaptor. The second step is qPCR detection, in which the All-in-One™ qPCR Mixture including

Green SYBR® specifically is utilized to precisely identify the reverse-transcription miRNA (the PCR Primer of Universal Adaptor is used in conjunction with the microRNA qPCR Primer All-in-One™).

2- Reaction tube of real-time PCR:
2×All-in-One qPCR Mixi (10µl) was added to All-in-One™ miRNA qPCR Primers (2µl), a Universal Adaptor PCR Primer (2µl), First-strand cDNA (2µl), ROX Reference Dye IV if necessary (0.4~0.1µl) and double distilled volumes of water (3.9~3.6µl) if ROX Reference Dye was used and (4µl) if ROX Reference Dye was not used. All miRNA primers were obtained from Gene Copoeia Company, USA.

Statistical analysis:
The statistical package for social sciences, SPSS version 25 program was used to code, tabulate, and perform statistical processing of the gathered data. Descriptive statistics were carried out using the mean, the range's lowest and highest values as well as its standard deviation (SD). Quantitative analyses were conducted between times in drowning deaths using the One Way ANOVA-test, with a post hoc LSD analysis conducted in between each two intervals. The relationship between variables and cause of death was ascertained by use of Pearson's coefficient correlation and analysis of simple linear regression analysis with a P value ≤ 0.05 level of significance.

Results
Histopathological results:
Brain sections obtained from drowning rats at the time of death showed pyknotic nuclei of the neurons with mild vacuolation **(Figure 1)**. However, brain sections obtained at 12hours PM, showed moderate vacuolation in the brain paryenchma **(Figure 2).** While those obtained at 24hrs. PM, showed prominent vacuolation of the brain parenchyma and pyknotic nuclei of the neurons **(Figure 3,4).**
Lung sections obtained from drowning rats at the time of death showed overdistention of the alveoli **(Figure 5).**

However, those obtained from rats at 12hrs. PM, showed moderate overdistention of the alveoli and moderate dissolution of interstitial cells **(Figure 6).** While sections were obtained at 24hrs. PM, revealed overdistention of the alveoli and marked dissolution of interstitial cells **(Figure 7).**

Genetic results:
Table (1) showed statistically significant time-dependent declines in miR-23b-3p levels in brain and lung tissues among 3 different postmortem intervals with increased PMI in drowning deaths. There is also a statistically significant drop in levels of miR-23b-3p between each of the two time intervals **(Figure 8).** The levels of miR-381-3p revealed a statistically significant time-dependent increase in brain and lung tissues among 3 postmortem intervals with increased PMI in drowning deaths. There is also a statistically significant increase in miR-381-3p levels between each of the two time intervals **(Table 1, Figure 8)**.

The levels of miRNA 23-b.3p and miRNA 381.3p were correlated with PM intervals in brain and lung tissues. **Table (2)** showed a statistically significant strong negative correlation of miR-23-b.3p levels in brain and lung tissues with the PM intervals in drowning deaths **(Figure 9)**. **Table (2)** also showed a statistically significant strong positive correlation between miR-381.3p levels in brain and lung tissues and the PM intervals in drowning deaths **(Figure 9)**.

For miRNA 23b-3p and miRNA-381-3p levels using RT-PCR in brain and lung tissues, **Table (3)** offers a simple linear regression analysis. The accuracy of miRNA 23b-3p and miRNA-381-3p brain levels was higher for the estimated interval since death in drowning deaths ($R^2 = 0.569$, $R^2 = 0.665$, respectively). While the miRNA 23b-3p and miRNA-381-3p lung levels showed higher accuracy for the estimated period of death in drowning deaths ($R^2 = 0.709$, $R^2 = 0.581$, respectively). PMI can be calculated by PMI = constant + (sloping of unstandardized coefficient*miRNA23b-3p or miRNA-381-3p levels in brain or lung) ±SEE.

Table 1: One Way ANOVA statistical analysis of miRNA 23b-3p and miRNA 381-3p levels in brain and lung tissues at different PMIs in drowning death.

Organs	Parameters		PMI			P value			
			At 0 hr. N=10	At 12 hr. N=10	At 24 hr. N=10	Among 3 intervals	0 vs 12	0 vs 24	12 vs 24
Brain	miRNA 23b-3p brain	*Range* *Mean* *± SD*	(1.55-2.97) 2.00±0.4	(1.03-2.19) 1.53±0.37	(0.73-1.51) 1.09±0.22	<0.001*	0.004*	<0.001*	0.008*
	miRNA 381-3p brain	*Range* *Mean* *± SD*	(1.58-2.34) 1.92±0.24	(2.23-3.02) 2.52±0.24	(2.12-3.24) 2.89±0.37	<0.001*	<0.001*	<0.001*	0.008*
Lung	miRNA 23b-3p lung	*Range* *Mean* *± SD*	(1.21-2.06) 1.69±0.23	(1.07-1.49) 1.26±0.13	(0.58-1.42) 0.91±0.26	<0.001*	<0.001*	<0.001*	0.001*
	miRNA 381-3p lung	*Range* *Mean* *± SD*	(0.93-1.53) 1.28±0.18	(1.14-2.07) 1.68±0.3	(1.59-2.39) 1.99±0.29	<0.001*	0.002*	<0.001*	0.012*

- *One Way ANOVA test for comparison of quantitative data between the three times followed by post hoc LSD analysis between each two times.*
- **: Significant level at P value < 0.05.*
- *Data expressed by Range, Mean ±SD (standard deviation).*
- *PMI=post mortem interval.   - hr. = hour.   - N= Number.   - vs= versus.*

Table 2: *Pearson's* Correlation between PMIs and miRNA 23b-3p and miRNA 381-3p levels in brain and lung tissues in drowning death.

Organs	Parameters	PMIs	
		R	P value
Brain	miRNA 23b-3p brain	-0.754	<0.001*
	miRNA 381-3p brain	0.815	<0.001*
Lung	miRNA 23b-3p lung	-0.842	<0.001*
	miRNA 381-3p lung	0.762	<0.001*

- *Pearson's correlation.*
- *Pearson's correlation coefficient's grades are: 0: No correlation, 0.00 to 0.24: Weak, 0.25 to 0.49: Fair, 0.5 to 0.74: Moderate, 0.75 or more: Strong, 1: Perfect.*
- **: Significant level at P value < 0.05.*
- *R: Correlation Coefficient.*
- *PMI=Post Mortem Interval*

Table 3: Simple linear regression analysis of miRNA 23b-3p and miRNA381-3p levels in brain and lung tissues for prediction of PMI in drowning death.

Organs	Parameters	Unstandardized coefficient		P value	R2	SEE	Regression Equation
Brain	miRNA 23b-3p brain	35.19	-15.09	<0.001*	0.569	6.657	PMI=35.19+(-15.09* miR23b-3p level) ±6.657
	miRNA 381-3p brain	-28.17	16.452	<0.001*	0.665	5.871	PMI=-28.17+(16.452* miR381-3p level) ±5.871
Lung	miRNA 23b-3p lung	39.813	-21.672	<0.001*	0.709	5.472	PMI=39.813+(-21.672* miR23b-3p level) ±5.472
	miRNA 381-3p lung	-19.934	19.354	<0.001*	0.581	6.568	PMI= -19.934+(19.354* miR381-3p level) ±6.568

- *: Significant Level at P value < 0.05.*
- *Regression equation: PMI = constant + (B * independent variable) ±SEE.*
- *The independent variable is miRNA23b-3p and miRNA381-3p levels.*
- *B: sloping of unstandardized coefficient.*
- *SEE: Standard error of estimate.*
- *PMI: postmortem interval.*
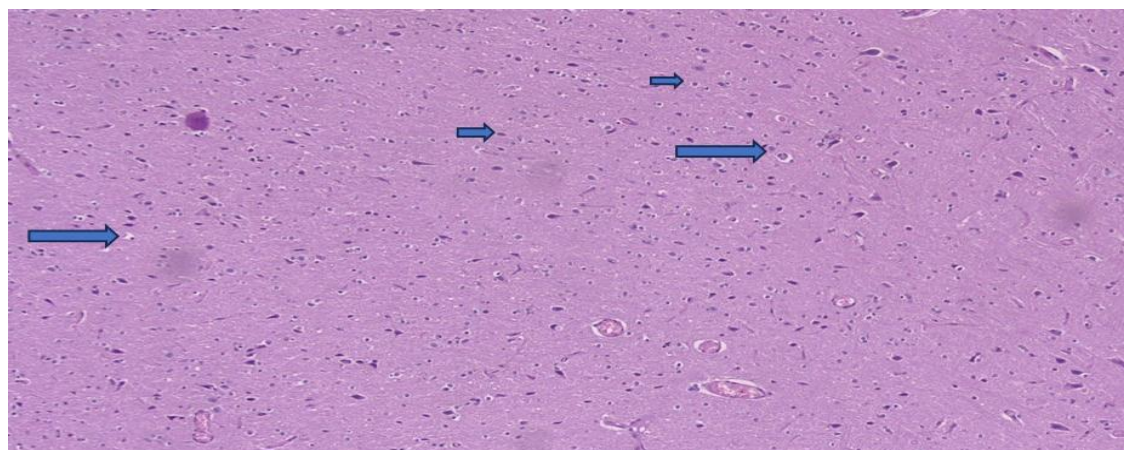- *Adjusted R^2: Effect Size.*

Figure 1: Photomicrographs of brain sections showing pyknotic nuclei of the neurons (short arrows) with mild vacuolation (long arrows) in drowning at 0 hr PM. (H&Ex40).
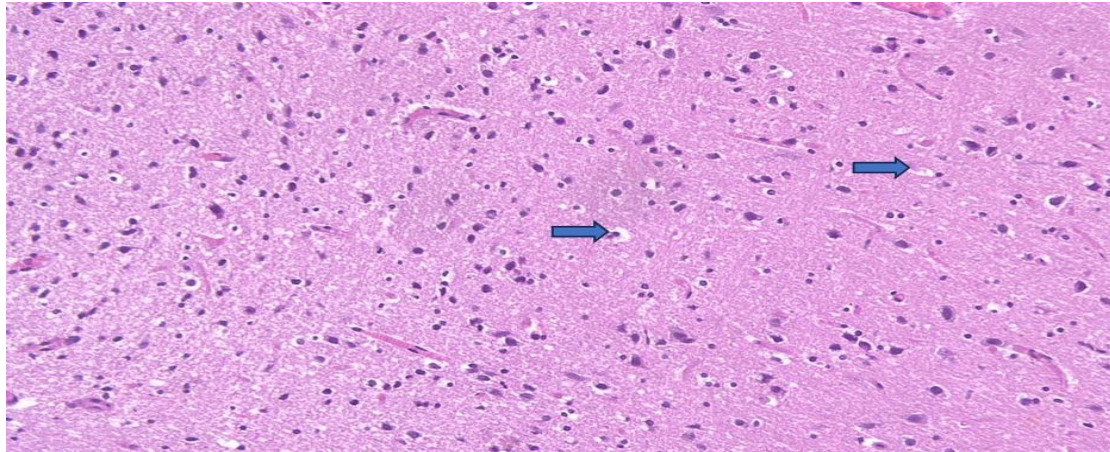
Figure 2: Photomicrographs of brain sections showing moderate vacuolation in the brain parynchema (arrows) in drowning at 12 hrs PM. (H&Ex200).
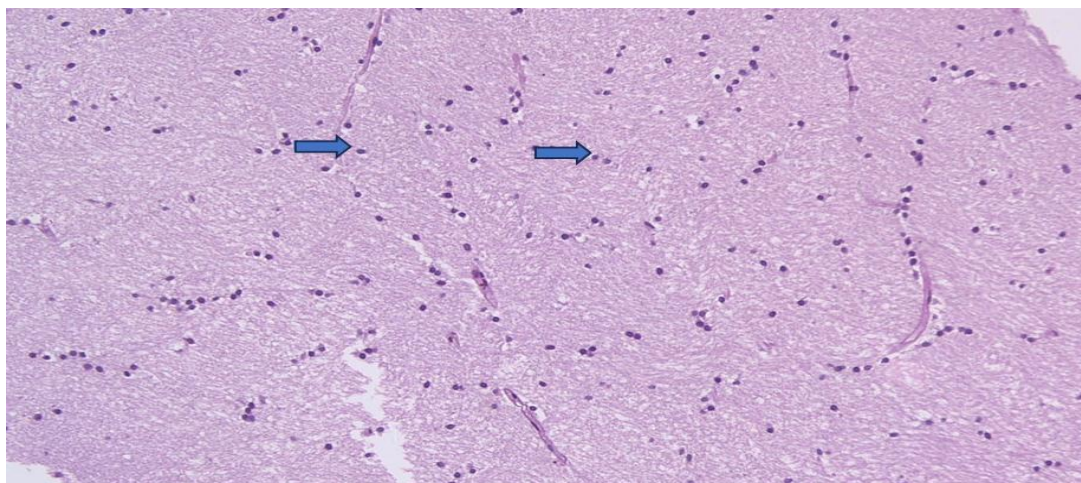

Figure 3: Photomicrographs of brain sections showing pyknotic nuclei of the neurons (arrows) in drowning at 24 hrs PM. (H&Ex100).
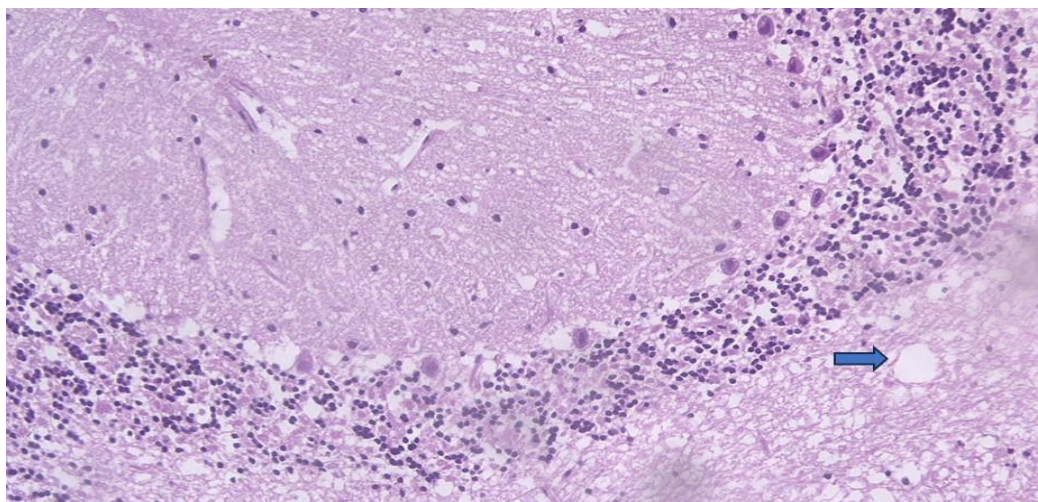

Figure 4: Photomicrographs of brain sections showing marked vacuolation of brain parenchyma (arrows) in drowning at 24 hrs PM. (H&Ex200).
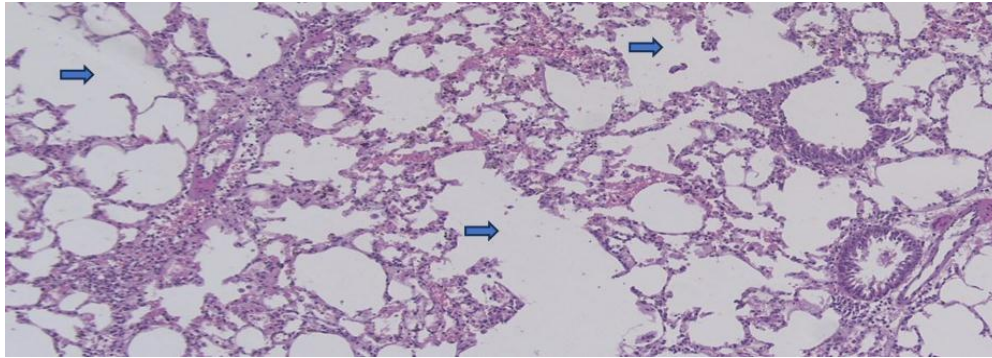
Figure 5: Photomicrographs of lung sections showing overdistention of the alveoli (arrows) in drowning at 0 hr PM. (H&Ex40).
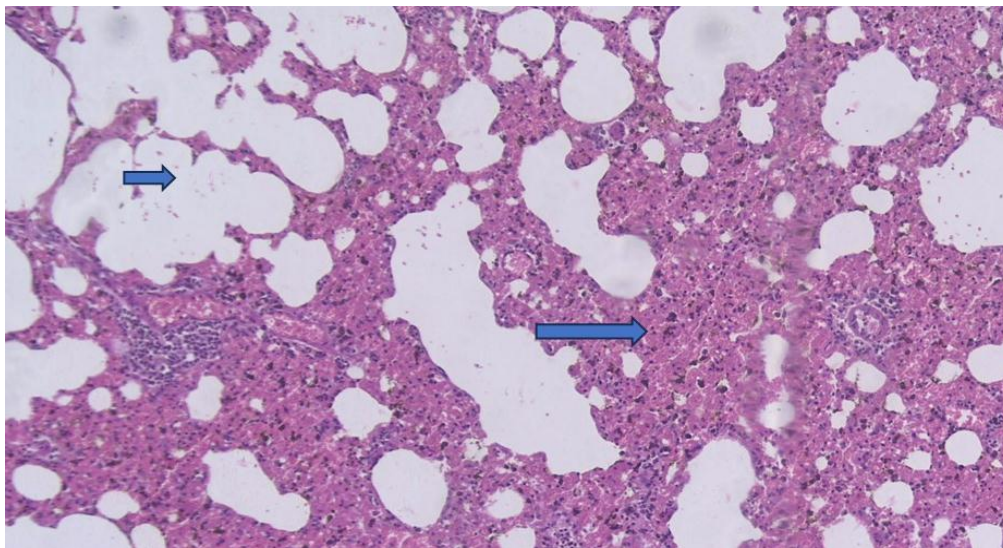

Figure 6: Photomicrographs of lung sections showing moderate overdistention of the alveoli (short arrows) and moderate dissolution of interstitial cells (long arrows) in drowning at 12 hrs PM. (H&Ex100).
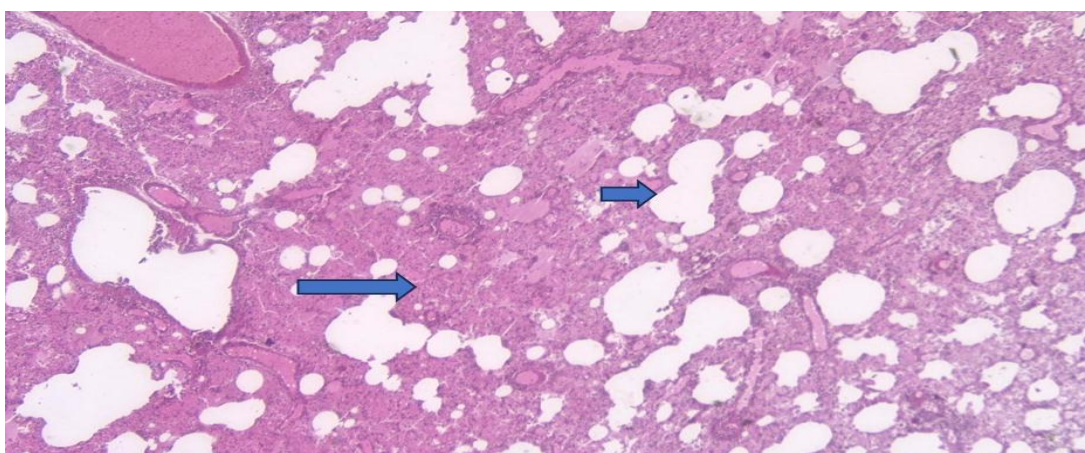

Figure 7: Photomicrographs of lung sections showing overdistention of the alveoli (short arrows) and marked dissolution of interstitial cells (long arrows) in drowning at 24 hrs PM. (H&Ex100).
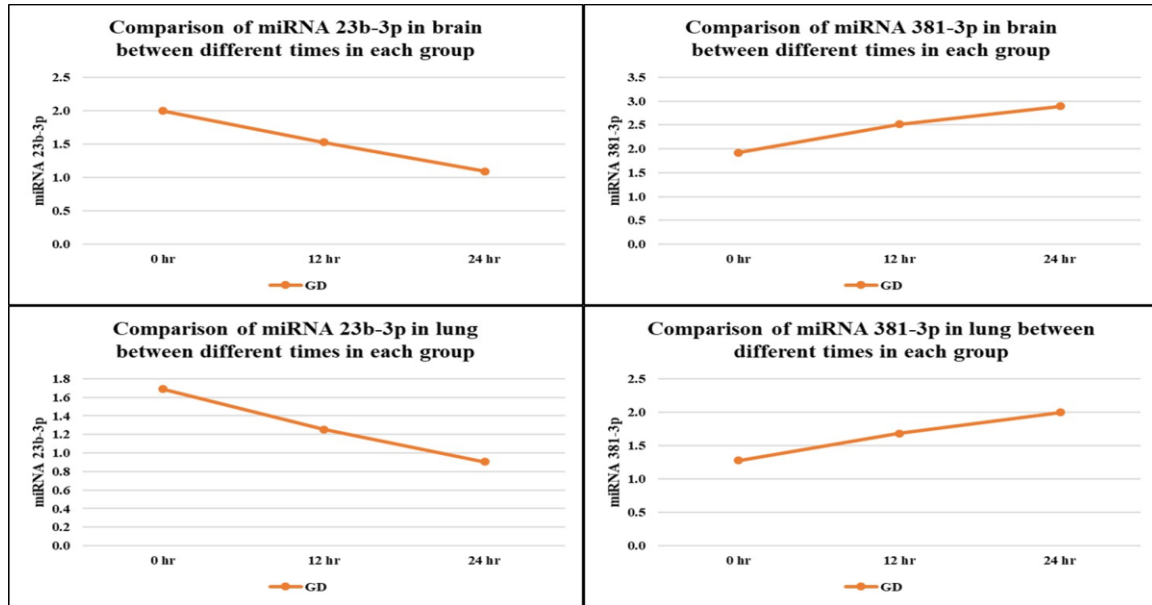
Figure 8: The relation between miRNA 23b-3p, miRNA 381-3p levels in brain and lung tissues and the different PMI in drowning death group.
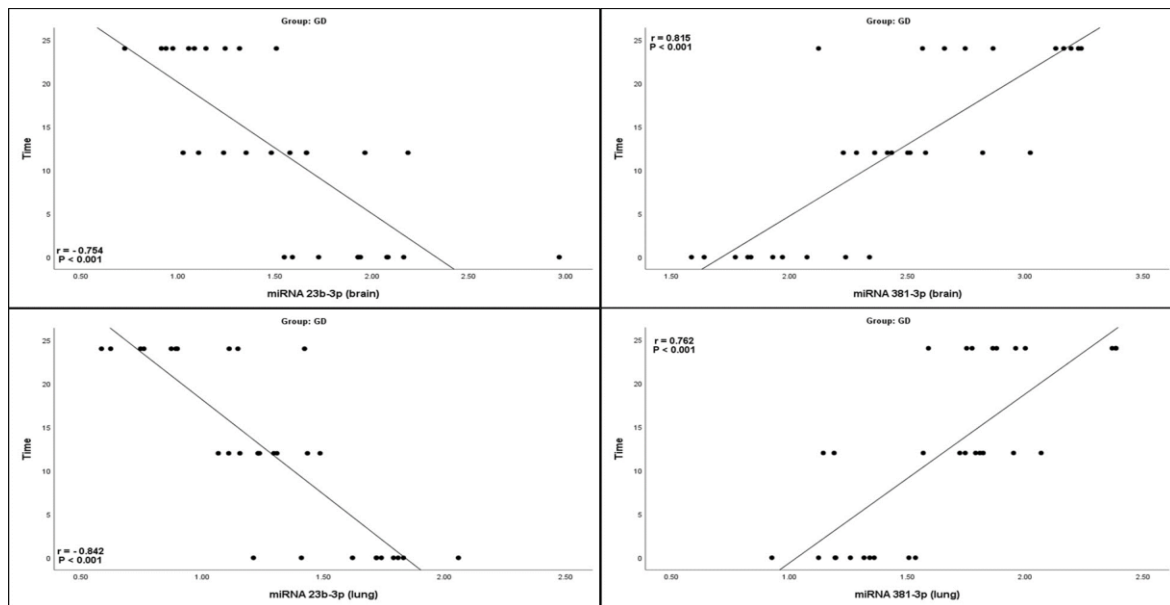

Figure 9: Correlation between miRNA 23b-3p, miRNA 381-3p levels and PMIs in brain and lung tissues in drowning death group (strong negative correlation for miRNA 23b-3p and strong positive correlation for miRNA 381-3p).

Discussion
Discussion

PMI estimation is a focus of inquiry in the forensic discipline. Even for experienced pathologists, estimating the PMI remains difficult, even though many studies on this topic have been undertaken for more than a century. Its importance is due to its civil and criminal consequences (**6**).

Throughout time, a wide variety of RNA species, including ribosomal RNA (r-

RNA), messenger RNA (m-RNA), and microRNA (mi-RNA), have been investigated for the calculation of PMI. The real-time quantitative PCR is presently the preferred technique because of its great specificity (**7**).

One of the most intricate pathophysiological conditions in forensic medicine is drowning. There is currently no reliable diagnostic test available to identify drowning-related deaths. This is

most likely because suffocation and drowning deaths are closely related **(8).**

Certain miRNAs influence ion channel-encoding genes, and distinct miRNA expression patterns may be determined by changes in the microenvironment linked to the dying process. Furthermore, certain miRNAs may serve as trustworthy biomarkers to identify the diagnosis of drowning **(9).**

The current study revealed pyknotic nuclei of the neurons with mild vacuolation at the time of death in brain tissues. While moderate vacuolation was present in the brain parenchyma at 12 hours postmortem. The brain parenchyma showed marked vacuolation and more neuronal pyknosis that increased with time until 24 hours postmortem.

The present results are going with the study of **Zaki et al. (10)** which was conducted on twenty adult albino rat brain tissues after being drowned. At the time of death, the brains of drowned rats showed an irregular distribution of neurons with an ill-defined nucleus in the cerebrum. While at 12 hours postmortem, there was neuronal shrinkage with deep blue color staining, and they also revealed the presence of flattening in the neuronal shape with a wide distance in between with an increased time of death.

In conjunction with the study of **Elalfy et al (11)** which was conducted on 36 drowned albino rats. They discovered that the brain tissues displayed some vacuolation in the brain parenchyma and scattered neurons with pyknotic nuclei right after freshwater drowning. After 24 hours of drowning, the brain displayed increased vacuolation in the brain parenchyma, more pyknotic neuronal nuclei, and a loss of cytoplasm.

The current study revealed more overdistention of the alveoli of rats' lung tissues at the time of death, which increased progressively with the appearance of moderate to marked dissolution of interstitial cells at 12hrs and 24hours postmortem, respectively.

Elalfy et al (11) found that the rat's freshwater drowned lung displayed pulmonary alveolar overdistension at the time of death, with numerous alveoli showing atelectasis and normal interstitial

tissue. Twenty-four hours later, the lung had postmortem changes, including ruptured blood capillary walls, pyknotic nuclei, and the disintegration of interstitial cells. There were dispersed pyknotic support interstitial cells and lung cells with suppressed atelectasis with increased time of death. The current results also revealed the same results.

According to **Marella et al (12)** who studied histopathological findings observed on different cadavers recovered from the water, they demonstrated alveolar dilation in combination with alveolar wall thinning and interalveolar septal rupture. Furthermore, elastic fibers were broken down, pneumocytes were swollen and damaged, and siderocytes could be detected in the interalveolar septa. Peribronchial hemorrhage may also be detected. Histological analysis of lung tissues from drowning victims revealed reduced alveolar macrophages and a low interstitial tissue ratio.

The current study revealed that the measurements of miR-23b-3p levels in brain and lung tissues showed statistically significant time-dependent reductions by RT-PCR among 3 times with increased PMI. The levels of miR-23b-3p showed a statistically significant negative correlation with PM intervals in brain and lung tissues.

The current study's findings revealed that the measurements of miRNA 381-3p levels in brain and lung tissues showed statistically significant time-dependent increases by RT-PCR among 3 times with increased PMI. The levels of miR-381 showed a statistically significant positive correlation with PM intervals in brain and lung tissues.

The present investigation found that lung miR-23b-3p had higher accuracy for time-since-death estimation ($R2 = 0.709$) followed by brain miR-381-3p ($R2 = 0.665$) then lung miR-381-3p ($R2 = 0.581$), and finally brain miR-23b-3p ($R2 = 0.569$) in drowning deaths.

In line with the study of **Martinez-Rivera et al. (13)** who studied rat skeletal muscles at different postmortem times. He revealed upregulation of miR-381-3p levels in the 24 h-postmortem group compared to the group at time of death. Additionally, he

discovered that up to 24 hours after PMI, miR-23b-3p gene expression declined.

Yu et al., (14) who studied the brain tissues of 13 Imprinting Control Region (ICR) mice, they found that miRNA expressions were higher in freshwater drowning compared to the control group in different post-mortem intervals. This dysregulation may be due to the connection between different miRNA levels and alterations in the cellular microenvironment, particularly ion channel/transport activation.

Ma et al., (15) found that some miRNA expression remains highly stable for several days, so they can be used as endogenous control markers. They revealed that distinct miRNA levels in the brain were very stable over six days, even under high temperatures. The current results are disagree with these results.

Few kinds of literature were available on microRNA expression as a marker for the determination of drowning in relation to postmortem intervals in brain and lung specimens, which made it difficult to compare these findings. New equations were developed and applied in the current study to estimate PMI in drowning deaths.

Conclusions:

According to the current study's findings, the gene expression of miRNA 23b-3p and miRNA-381-3p was found to be a valuable biomarker in line with the histopathological alterations in the recognition of drowning as a death cause in relation to the postmortem interval from the brain and lung tissues. The equations detected in this study can help estimate the postmortem interval for drowning deaths.

Nevertheless, there aren't many studies looking into how microRNAs affect postmortem interval prediction for various causes of death. Therefore, it is advised to conduct additional research on microRNAs to summarize their functions and overview their roles.

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