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Micro-RNA evaluation, specification, and stabilization study in mixed/non-mixed body fluids as a specific molecular marker

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ABSTRACT

Body fluids are frequently recovered by forensic investigators from crime scenes, and their identification is an important aspect of forensic case analysis. Determining the type and origin of human fluids recovered at a crime scene will give essential information for crime scene reconstruction by establishing a link between sample donors and actual criminal activity. The expression levels of microRNAs (miRNAs) such as miR-10b, miR-135b, miR-16, miR-126, miR-124-3p, and miR-372 in seminal fluids, blood, vaginal fluid stains, and their mixture, were measured using a quantitative real-time PCR technique. Using SNORD-47 as a reference gene, the target genes' identification and stability were assessed. miR-10b and miR-135b were expressed at higher levels in seminal fluids stains than in vaginal secretion stains; miR-124-3p and miR-372 were expressed at higher levels in vaginal stains than in seminal fluids stains; miR-16 and miR-126 were expressed at a higher level in bloodstains than in both seminal fluids and vaginal stains, and six miRNAs were expressed in seminal fluids/vaginal/blood mix stains. In conclusion, the results indicate that the expression levels of miR-10b and miR-135b (seminal fluids), miR-124-3p and miR-372 (vaginal secretion), and miR-16 and miR-126 (blood) were higher in these samples compared to the housekeeping gene SNORD-47.

INTRODUCTION

In various incidents, including sexual assaults, stains might be a combination of different body fluids, such as seminal fluids and vaginal material, or blood and saliva. Even though several procedures for effectively resolving DNA mixtures exist, there are presently no analytical methods for resolving the mixing of physiological fluids. RNA analysis is becoming increasingly common, with micro-RNAs (miRNA) and messenger RNA (mRNA) being used to identify body fluids [1–3].

miRNAs are small RNA structures, evolutionarily conserved non-coding RNA (about 20–25 nt) plays an essential regulative role in many cellular processes. miRNAs can downregulate gene expression by base-pairing with the 3′ untranslated regions (3′UTR) of target messenger RNAs (mRNAs) and miRNA mediated regulation is now acknowledged to represent a new instance of regulatory control over gene expression programs in many organisms [4].

The main role of miRNA is negatively regulating gene expression by silencing (blocking) the messenger RNA or degrading it, miRNAs suppress gene expression based on their complementarity to a part of one or more mRNAs, usually at a site in the 3′-UTR. The annealing of the miRNA to the target mRNA inhibits protein translation. In some cases, the formation of dsRNA through the binding of miRNA triggers the

degradation of the mRNA transcript, although, in other cases, it is thought that the miRNA complex blocks the protein translation machinery or otherwise prevents protein translation without causing the mRNA to be degraded [5].

The study of mRNA and miRNA expressions in bodily fluid, particularly in the last decade, has been a key emphasis. It can generate a DNA profile with evidential strength and provide important information on the origin of the bodily fluid. miRNAs are known to regulate gene expression inside the cells that make them, and they can also be released as extracellular molecules to regulate other cells or interact between cells [6]. miRNAs are expressed differently in various cell types, according to their expression levels, suggesting the potential application of miRNAs in identifying cell types and tissues [7]. One of the major challenges in utilizing miRNAs as body fluid identification markers are their stability inside the samples. Seminal fluids can be detected on materials or clothes in sexual assault cases, and they may also be collected from the victim's body. Vaginal secretions are one of the most important forensically relevant bodily fluids [4]. Vaginal secretions, unlike other body fluids, lack specific proteins that can be used to identify them with traditional protein-depending on methods, making their detection impossible. miRNAs have recently been explored as an alternative tool for the identification of forensic body fluids [8].

It has been suggested that miRNAs can be employed as potential markers for body fluid recognition in forensic science. For instance, previous studies reported miRNA markers that could be used to detect seminal fluids are miR-10b and miR-135b and vaginal secretions were miR-124-3p and miR-372 [4,7–10]. Meanwhile, another study showed that miR-16 and miR-451 could be used as biomarkers for bloodstain [9,10]. Therefore, the goal of this study is to see how mixed samples or stains such as seminal fluids and vaginal secretions, blood and vaginal secretions, and blood and seminal fluids affected the expression of miR-10b and miR-135b, miR-124-3p and miR-372, miR-16, and miR-126 in fresh and dried samples.

MATERIALS AND METHODS

Collection and preparation of samples

Healthy volunteers provided five samples of seminal fluid, vaginal secretions, and bloodstain. Male donors donated freshly ejaculated sperm in sealed Falcon tubes, which were subsequently transferred to sterile cotton swabs. Vaginal secretions were obtained using a sterile cotton-tipped swab, and the mixture of seminal fluids and vaginal secretions were mixed with various amounts, while venipuncture was used to draw blood into EDTA vials. All samples were dried at room temperature in the dark for two weeks before being processed for RNA extraction.

Every volunteer was introduced with written informed permission. This research received ethical approval (DSM/HO-16642) for scientific research from the Ministry of Health MOH and Ministry of Higher Education and Scientific Research (MOHESR) ethics committees in Iraq.

Selection of miRNA

miR-10b and miR-135b were chosen for seminal fluids, whereas miR-124-3p and miR-372 were chosen for vaginal secretions, and miR-16 and miR-126 were selected for bloodstain [4,7–10], and all markers for mixed seminal fluids/vaginal secretions, blood

stains/vaginal secretions and bloodstains/seminal fluids. For all samples, SNORD-47 was chosen as the housekeeping gene to normalize miRNA expression levels.

RNA analysis

Total RNA was extracted using the RNAzol RT reagent (Sigma-Aldrich/USA), as directed by the manufacturer. The yield and integrity of RNA were measured.

A housekeeping gene was used in a normalization strategy to calculate the sample's RQ (Relative Quantification) [11]. SNORD-47 was previously characterized as a housekeeping transcript for miRNA normalization in the context of expression measurement in a variety of biological samples [12]. Other housekeeping genes for forensically related body fluids have been proposed and should be considered in the future [13]. A Microspectrophotometer nanodrop (Biodrop / UK) device was used to evaluate the concentration and purity of samples at 260 nm and 280 nm. Pure RNA A260/280 ratios typically range from 1.8 to 2.2. Total RNA was isolated from three body fluids samples and utilized in the MiRNA All-In-One cDNA Synthesis Kit from abm / USA for cDNA synthesis.

The Bright Green miRNA qPCR Master Mix reverse transcription and RT-qPCR experiments were performed according to the manufacturer's instructions. The expression level of the reference gene was measured in triplicates in all samples with the negative control. RT-PCR mixture was in total 20 µl including 10 µl (1x) Bright Green miRNA qPCR Master Mix, 300 nM for each primer, 3 µl cDNA product. thermocycler set up was 95℃ for 10 minutes, followed by 40 cycles of 95℃ for 10 seconds, followed by 63℃ for 15 seconds, and a 30-second extension at 72℃.

Statistical analysis

SPSS statistical program for social sciences was used to analyse the data (version 20.0 for Windows, SPSS, Chicago, IL, USA) [14].

RESULTS

Expression of miRNA

SNORD-47 was used to compare the miRNA targets that were examined. The average CT value was calculated using the triplicates of each sample. CT = CT (Target miRNA) –CT (Endogenous Control) was computed by subtracting the average CT for the endogenous control from the target miRNA of interest [11]. The expression of miRNAs target genes in the mixture of vaginal secretions and seminal fluid samples were varied according to fluids concentration which was prepared as opposite concentrations sorting as shown in Table 1.

Table 1. The average of Ct and ΔCt values for mixed seminal fluid and vaginal secretion stain.

Untreated Samples No.	(Conc. ng) S/V.S.	Ct				ΔCt				
		miR-10b Ct	miR-135b Ct	miR-124-3p Ct	miR-372 Ct	HKG SNORD-47 Ct	miR-10b ΔCt	miR-135b ΔCt	miR-124-3p ΔCt	miR-372 ΔCt
M1	100/0	20.205	21.435	NR	NR	19.28	0.92	2.15	NR	NR
M2	80/20	20.88	22.22	27.48	25.07	19.88	0.99	2.33	7.59	5.19
M3	60/40	21.54	23.28	24.64	23.98	19.61	1.93	3.66	5.02	4.37
M4	40/60	21.48	23.285	24.22	23.34	19.45	2.025	3.83	4.76	3.89
M5	20/80	22.77	23.28	24.1	23.22	19.45	3.325	3.83	4.65	3.77
M6	0/100	NR	NR	24.17	22.89	19.58	NR	NR	4.58	3.31

S: seminal fluids, V.S.: vaginal secretion, HKG: housekeeping gene, NR: nonreadable, Conc.: concentration.

MiR-10b and miR-135b were expressed at higher levels in the presence of seminal fluids and decreased with seminal fluids concentration decreasing until reach zero expression when the concentration of the seminal fluid is zeroed. while miR-124-3p and miR-372 were also expressed at higher levels in the presence of vaginal secretions stains than in seminal fluids stains as shown in in Table 1. The analysis of mixed blood/seminal fluids stains showed that miR-16 and miR-126 were expressed at higher levels in the presence of bloodstains and decreased with blood concentration decreasing until reach zero expression when the blood concentration is zeroed, the same for miR-10b and miR-135b in seminal fluids stains (Table 2). MiR-16 and miR-126 in a mixture of bloodstain and vaginal secretions were also expressed only in presence of bloodstain, and the same results for miR-124-3p and miR-372 were expressed only in presence of vaginal secretion stains (Table 3). Six miRNAs were expressed in seminal fluids/vaginal secretions, blood/seminal fluids and blood/vaginal secretion as a mixed stain but show lower expression levels in stored samples than in fresh samples (Table 1-3).

Table 2. The average of Ct and ΔCt values for mixed blood and seminal fluids stain.

Untreated Samples No.	Conc. (ng) B/S	Ct				ΔCt				
		miR-16 Ct	miR-126 Ct	miR-10b Ct	miR-135b Ct	HKG SNORD-47 Ct	miR-16 ΔCt	miR-126 ΔCt	miR-10b ΔCt	miR-135b ΔCt
M7	100/0	23.66	23.575	NR	NR	19.4	4.26	4.17	NR	NR
M8	80/20	23.8	23.64	28.5	24.2	19.01	4.79	4.63	9.49	5.19
M9	60/40	24.18	23.80	28.27	23.985	19.21	4.96	4.59	9.06	4.77
M10	40/60	25.91	25.9	24.29	23.31	19.26	6.64	6.63	5.03	4.04
M11	20/80	27.9	26.43	23.52	22.2	18.92	8.97	7.50	4.595	3.27
M12	0/100	NR	NR	23.39	22.04	19.1	NR	NR	4.29	2.94

B: bloodstain, S.: seminal fluids, HKG: housekeeping gene, NR: nonreadable, Conc.: concentration.

Table 3. The average of Ct and ΔCt values for mixed blood and vaginal secretion stain.

Untreated Samples No.	(Conc. ng) B/V.S.	Ct				ΔCt				
		miR-16 Ct	miR-126 Ct	miR-124-3p Ct	miR-372 Ct	HKG SNORD-47 Ct	miR-16 ΔCt	miR-126 ΔCt	miR-124-3p ΔCt	miR-372 ΔCt
M13	100/0	23.75	24.07	NR	NR	19.5	4.25	4.57	NR	NR
M14	80/20	23.78	24.06	24.89	24.84	19.07	4.70	4.98	5.82	5.77
M15	60/40	24.02	23.72	23.82	23.97	18.45	5.56	5.27	5.37	5.52
M16	40/60	26.18	26.39	23.30	23.39	18.27	7.91	8.12	5.03	5.12
M17	20/80	26.80	26.57	25.16	25.90	21.59	5.21	4.98	3.57	4.31
M18	0/100	NR	NR	23.735	24.24	20.09	NR	NR	3.64	4.14

B: bloodstain, V.S.: vaginal secretion, HKG: housekeeping gene, NR: nonreadable, Conc.: concentration.

Stability of miRNAs

A mixture of bodily fluid stains was incubated at room temperature for two weeks in a dark and dry environment to examine the stability of miRNAs.

The purpose of this test is to investigate the potential of miRNAs to be used as markers for certain bodily fluids and to examine the durability of miRNAs in samples over time. The specificity of targeted miRNAs is important, since being used to detect the most unique and specific miRNA to the targeted body fluids. However, not all miRNA has the same quantity in body fluids. Besides, a variety of events such as different diseases or metabolic pathways affects the number of miRNAs in fluids [15].

Figure 1A show the gene expression of miRNAs with fresh stains that have not been stored. The results of this research demonstrate that the expression levels of miR-10b and miR-135b (seminal fluids), miR-124-3p, and miR-372 (vaginal secretion) were

higher in these samples compared to the gene expression levels of mixed seminal fluids/vaginal secretions stain that was kept in the dark at room temperature for two weeks (treated sample) which show lower fold expression (Figure 1B).

Real-time PCR shows magnificent results of microRNA genes of vaginal secretion (miR-124-3p and miR-372) in samples M1 and Mt1 (Figure 1A and B), while all vaginal secretions showed no Ct values in contrast to microRNA genes of seminal fluids (miR-10b and miR135b) in sample M6 and Mt6, that make miR-10b and miR135b are a very suitable marker for seminal fluids also for miR-124-3p and miR-372 are very suitable for vaginal secretions.

For treated mixed sample seminal fluid/vaginal secretion which was stored in dark at room temperature for two weeks, the results are shown in Figure 1B, the sample Mt2 where the concentration 20ng of vaginal secretion RNA concentration, the expression fold was very low for both miR-372 and miR-124-3p and that may be due to the majority of microRNA in vaginal secretion are extracellularly making it more affected by environmental conditions during the time comparing to microRNA of seminal fluids which were found as extra and intracellular (inside strong disulfide structure of semen) which were more protected to withstand environmental conditions [16].

A recent study on a variety of samples including seminal fluids, saliva, blood, and menstrual blood indicated that miR-10b and miR-135b were presented in higher levels in seminal fluids, although they were stored for a year in the dark at room temperature [17].

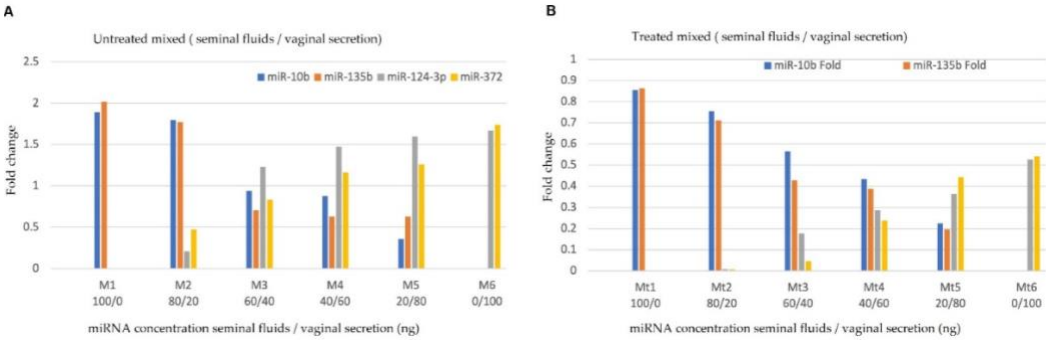

Figure 1. Gene expression values of mixed sample (seminal fluid/vaginal secretion). A) Untreated fresh sample. B) Treated sample (two weeks at room temperature and dark).

Stability of miRNAs expression in mixed fluids

Analysis of miRNAs in mixed blood/seminal fluids fresh samples showed that miR-16 and miR-126 expression levels were higher in bloodstain while the expression levels of miR-10b and miR-135b were higher in seminal fluids stain (Figure 2A), further analysis showed that miRNAs are detectable as a unique miRNA gene to bloodstain as well as the miR-10b and miR-135b to seminal fluids even after storage in dark at room temperature (Figure 2B), it has been found that the expression levels of miR-451, miR-150 and miR-126 were unchanged and very stable even after two weeks of storage in dark at room temperature (gene expression still about 1.5. for miR-150 and miR-451, while miR-126 expression decreased from 1.7 in fresh to 1.5 in treated samples), meanwhile, the expression levels of miR-16 was lower but still detectable (1.3) in fresh samples and 0.68 in treated samples) [18].

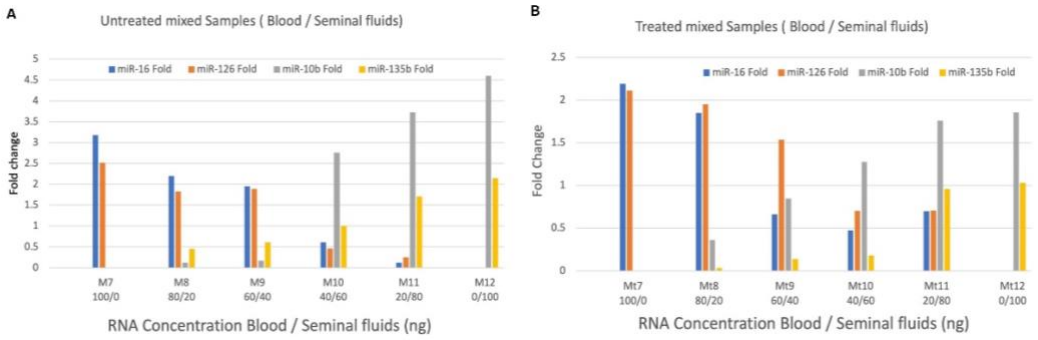

Figure 2. Gene expression values of mixed sample (blood/seminal fluid). A) Untreated fresh sample. B) Treated sample (two weeks at room temperature and dark).

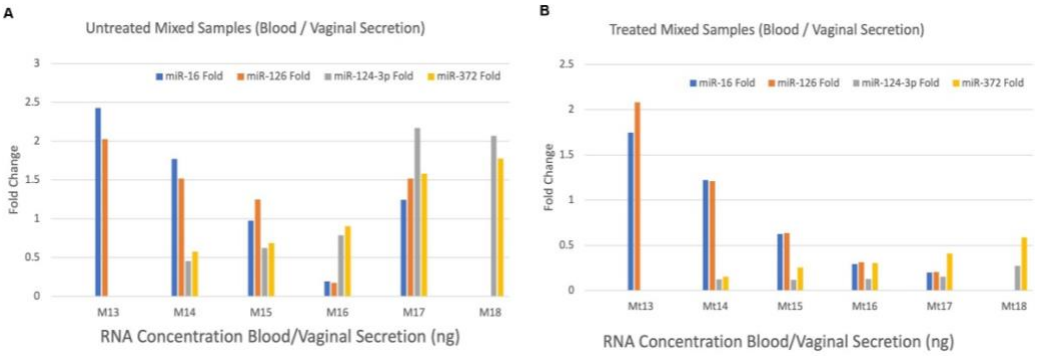

Figure 3. Gene expression values of mixed sample (blood/vaginal secretion). A) Untreated fresh sample. B) Treated sample (two weeks at room temperature and dark).

Blood/vaginal secretion stain mixture at its fresh untreated state showed that miR-16 and miR-126 (bloodstain) and miR-124-3p and miR-372 (vaginal secretion stain) were expressed higher (Figure 3A), the treated mixed samples which were stored in dark at room temperature also showed stable expression (Figure 3B), while, miR-16 and miR-126 genes were still detectable and uniquely not expressed in the absence of bloodstain, while miR-124-3p and miR-372 were also detected as unique miRNA gene to vaginal secretion stain but with lower expression level in comparison to miRNAs gene of vaginal secretion in blood/seminal fluids mixture.

Another study indicated the absence of miR-124-3p, miR-214 and miR-891in menstrual blood after storing for 1 month at room temperature under light [19].

At a crime scene, the ability to distinguish between various kinds of bodily fluids is also critical. A prior investigation using Raman spectroscopy failed to distinguish between cat, dog, and human blood samples [20]. Dry traces of bodily fluids such as blood, seminal fluids, saliva, vaginal fluid, and perspiration were analyzed using Raman microspectroscopy, which uses non-actinic (non-destructive) near-infrared light [21]. According to studies of miRNA stability in various settings, miRNAs tend to degrade in samples exposed to strong chemicals and environmental conditions [22]. Zubakov and his colleagues found that the stability of miRNAs in old samples did not seem to be deteriorated when samples were stored at room temperature for one year [23].

As shown in Figure 4A the expression levels of vaginal secretions miRNA in mixed samples (vaginal secretion/seminal fluids) which miR-124-3p and miR-372 decreased significantly between fresh and treated samples as following significance: miR-124-3p: 0.009 and miR-372: 0.008 at P-value ≤ 0.05. This means all miRNA markers of vaginal secretion in mixed samples were affected similarly by storing and the decreases were about 0.3-0.5 for vaginal secretion miRNA genes showing that miR-372 and miR-124-3p were not stable for storing, hence, it is not suitable to be chosen as a marker for vaginal secretion fluids.

miR-10b and miR-135b in seminal fluids show no significant changes as follow: miR-10b: 0.089, miR135b: 0.090 at (P≤ 0.05), which increase the chance of miR-10b and miR-135b as a specific biomarker to seminal fluids stains.

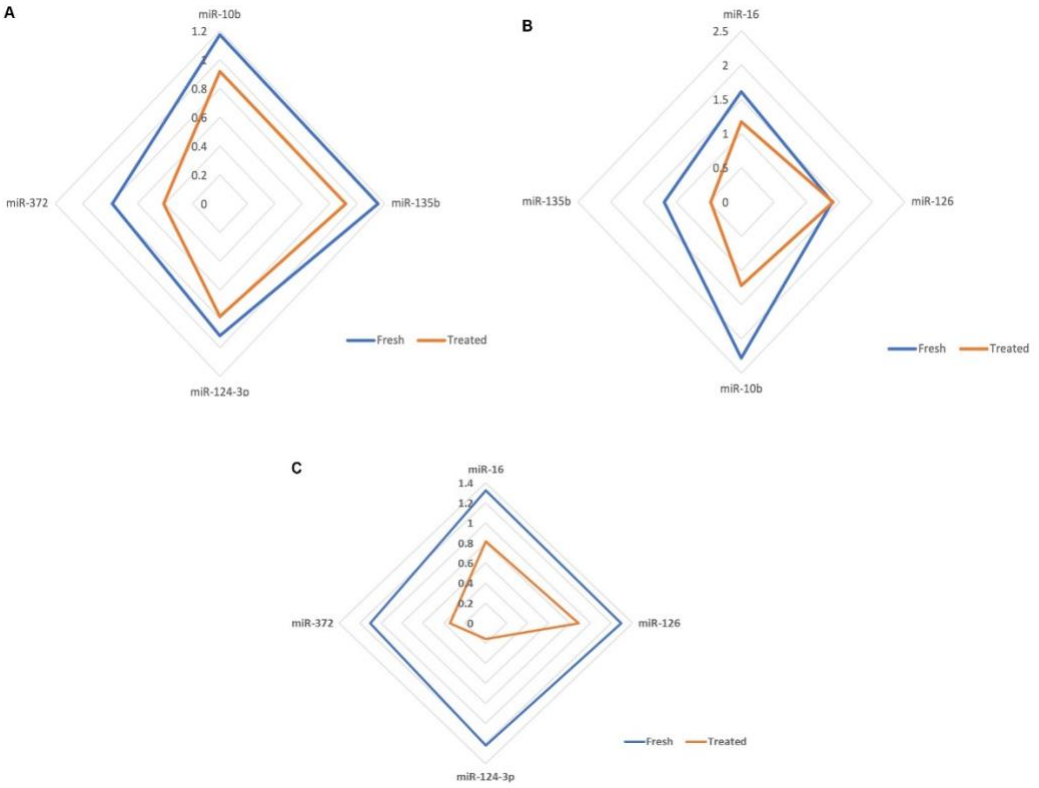

Figure 4. miRNAs gene expression fold differed significancy between fresh and treated mixed samples, A) Seminal fluids and vaginal secretions (Sig. of miR-10b: 0.089, miR135b: 0.090, miR-124-3p: 0.009 and miR-372: 0.008, P value ≤ 0.05). B) Blood and seminal fluids (Sig. of miR-16:0.526, miR-126:0.954, miR-10b: 0.323, miR135b: 0.110, P value ≤ 0.05). C) Blood and vaginal secretion (Sig. of miR-16:0.82, miR-126:0.954, miR-124-3p: 0.21, miR-372: 0.198, P value ≤ 0.05).

Figure 4B shows that the gene`s expression of mixed miRNA genes which were miR-10b, miR-135b for seminal fluids and miR-16, miR-126 for blood samples were showed no significant change at $P \leq 0.05$ in treated samples with the following significance: miR-10b: 0.323 and miR135b: 0.110) comparing to fresh samples, the same results shown for miR-16 and miR-126 for bloodstain were also showed no significant change as follow: miR-16: 0.526 and miR-126: 0.954. This means all miRNA markers in mixed samples (blood/seminal fluids) were stable after storing due to no significant change between fresh and treated mixed samples, except miR-126 in blood samples which shows higher stability with almost no change in its levels in treated samples, suggesting that miR-126 is the most stable one after storing and that increases the chance of being selected as a marker for blood samples. Figure 4C show the gene's expression fold of mixed (blood / vaginal secretion) miRNA genes which were miR-124-3p and miR-372 for vaginal secretion were significantly decreased at ($P \leq 0.05$) in treated samples (miR-124-3p: 0.045 and miR-372: 0.032) in contrast to fresh samples, while miR-16 and miR-126 for bloodstain were showed no significant changes (miR-16: 0.323, miR-126: 0.405) between fresh and treated samples showing that all miRNA markers in mixed samples were affected by storing except the miR-126 and miR-16 genes in blood samples, implying its higher stability, thus, miR-126 and miR-16 are the most stable miRNAs after storing which increases their chance to be.

DISCUSSIONS

The main idea of this study was to evaluate the miRNA as a specific molecular marker for three fluids (blood, seminal fluids, vaginal secretions, and their mix) fresh and stored. The results briefly show that miR-16 and miR-126 are strongly stable biomarkers for bloodstain even after storage for two weeks when they were found as a mixed sample (as a simulation of the crime scene) in both states, fresh and stored mixed samples, while miR-124-3p and miR-372 show good response as a specific biomarker for vaginal secretion but with less stability after storing comparing to blood and seminal fluids miRNAs.

While miRNA-16 and miRNA-126 show very stable and specific markers for bloodstains in fresh and treated mixed samples. miRNAs stability was important to make this study complete since the detection of old or deteriorated forensic samples is a challenge due to the degradation of samples in forensic crime scenes as a result of exposure to all unfavorable conditions like humidity, temperature, sunlight, etc. [24]. Because of their small length of 18-25 nucleotides, degradation has a lower influence on miRNAs (miRNA) than it does on mRNA. Since 2009, a group of researchers has been assessing the feasibility and usefulness of forensic miRNA and miRNA expression analysis using quantitative PCR (qPCR) in forensic situations [15,24]. A recent study on a variety of samples including seminal fluids, saliva, blood and menstrual blood indicated that miR-10b and miR-135b were presented in higher levels in seminal fluids, in spite of the fact that they were stored for a year in the dark at room temperature [17]. It has been found that the expression levels of miR-451, miR-150 and miR-126 were unchanged and very stable even after two weeks of storage in dark at room temperature (gene expression still about 1.5. for miR-150 and miR- 451, while miR-126 expression decreased from 1.7 in fresh to 1.5 in treated samples), meanwhile, the expression levels of miR-16 was lower but still detectable (1.3) in fresh samples and 0.68 in treated samples) [18]. Another study indicated the absence of miR-124-3p, miR-214 and miR-891in menstrual blood after storing for 1 month at room temperature under light [19]. Besides, it has been evidenced that miR-124-3p and miR-372 were

decreased significantly after storage for two weeks and sensitivity was also weak compared to blood miRNA which was miR-451 and miR-16 [25].

The long-term preservation of miRNA molecules in dry circumstances might be due to a variety of factors. According to Fordyce and colleagues, drying a sample lowers the activity of RNases, which protects the nucleic acid against destruction. Another explanation for RNA stability might be their capacity to withstand hydrolytic depurination due to stronger N-glycosidic bonds and depyrimidination processes, as well as their capacity to create secondary and tertiary structures that limit phosphodiester bond hydrolysis [26].

For instance, it has been evidenced that the presence of AU-rich motifs in the RNA sequence is linked to the rate of RNA degradation in live cells [27]. As a result, this issue should be considered when choosing RNA markers for medical purposes. Micro–RNA is particularly persistent postmortem due to its capacity to bind to proteins and subcellular compartmentalization [28].

CONCLUSION

In conclusion, this study found that miR-16 and miR-126 are strongly stable biomarkers for bloodstain even after storage for two weeks when they were found as a mixed sample (as a simulation of the crime scene) in both states, fresh and stored mixed samples, while miR-124-3p and miR-372 show good response as a specific biomarker for vaginal secretion but with less stability after storing comparing to blood and seminal fluids miRNAs. While miRNA-16 and miRNA-126 show very stable and specific markers for bloodstains in fresh and treated mixed samples. As a result, these miRNAs can be used to identify criminal body fluids samples at crime scenes when they were mixed together (Figure 5).

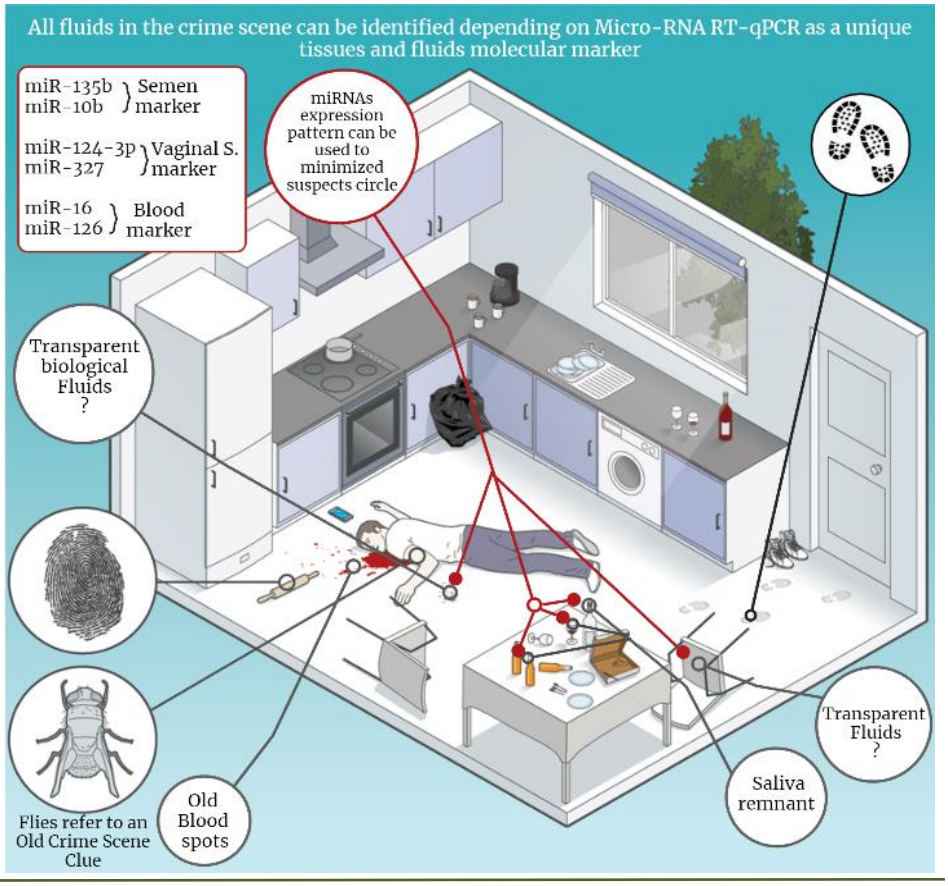

Figure 5. Summary of the study. It shows the fluid that might be found in a crime scene especially when it's found mixed with one or more another type of fluid, and the role of micro-RNA molecules to identify each type of fluid considering that the micro-RNA molecules are tissue-specific molecules. The figure is created with *BioRender.com*.

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AUTHOR CONTRIBUTIONS

Conception and design of study: Yasir Haider Al-Mawlah and Mohammed Zuhair Naji. Drafting the manuscript: Mustafa Jawad Al-Imari and Hadi Sajid Abdulabbas. Analysis and/or interpretation of data: Yasir Haider Al-Mawlah.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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