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Research Paper

α-Globin Messenger Ribonucleic Acid as a Molecular Marker for Determining the Age of Human Blood Spots in Different Temperatures

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ABSTRACT

Background: Analyzing recovered evidence, such as blood which is one of the most encountered types of biological evidence, can provide information to establish the definite time when a crime was committed. This study aims to investigate the time- and temperature-related effects on human bloodstain's α-globin messenger RNA expression and to estimate the bloodstain's age using α-globin mRNA.

Methods: A total of 22 blood samples were collected from healthy middle-aged volunteers (12 women and 10 men). After preparation, the samples were exposed to temperatures of 4°C, 24°C, and 40°C. Next, the mRNA expression of the α-globin gene was quantified by real-time RT-PCR at different time intervals of 0, 30, 90, and 150 days.

Results: The α-globin gene expression showed the highest mean values by 0 day and at 4°C and the lowest mean values by 150 days and at 40°C. Samples from male participants showed higher mean values of α-globin gene expression compared to their female counterparts. A significant negative correlation was detected between α-globin gene expression and time interval. Meanwhile, a regression equation was formulated to estimate the time interval using the α-globin gene concentration.

Conclusion: α-Globin mRNA could be a useful marker to estimate the age of human blood spots.

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1. Introduction

Body fluids are often left at physical assault crime scenes and then they are recovered as evidence. Analyzing the bodily fluids provides vital information for assessing how crimes were committed [1].

Bloodstains are among the most common biological evidence found at crime scenes, especially in violent crimes. Therefore, forensic investigators may significantly benefit from accurate age estimates of bloodstains when reconstructing the incident timeline, assessing the time of the victim's death or injury, decreasing the pool of suspects, and considering a person as a suspect [2].

In the past, the age of the bloodstain was primarily dependent on the color change of hemoglobin derivatives. Subsequently, other techniques were reported to estimate the age of human bloodstains, such as enzyme and protein activities and the aspartic acid racemization rate contained in the blood. However, none of these methods could inform the difference between human and animal blood, and their findings were also dependent on the number of used samples [3].

Despite years of attempting to ascertain the time since deposition (TSD) of a bloodstain, forensic scientists and the legal system have yet to develop a definite and validated conclusion [4].

The stability of RNA over time has been investigated to develop a more accurate mechanism for determining the age of biological samples. For several factors, RNA is considered a strong candidate for such research. As several RNA species are abundant and RNA is relatively labile, polymorphisms between species can be used to develop species-specific tests [5].

Red blood cells are primarily composed of hemoglobin, which consists of two α- and two β-globin chains. Using high-performance liquid chromatography, Inoue et al. [6] discovered a decrease in bloodstains' α-globin protein over time by investigating the patterns of the bloodstains' protein at different time intervals. Therefore, α-globin messenger RNA (mRNA) may be a good candidate for this research.

2. Materials and Methods

Study design and setting

This is a cross-sectional, prospective study. The laboratory workup was performed at the Molecular Biology Unit of the Medical Biochemistry Department, Faculty of Medicine, Cairo University, Egypt. The study was approved by the Ethics Committee of Forensic Medicine and Clinical Toxicology Department and the Ethics Committee of the Faculty of Medicine, Cairo University, Egypt.

Study population

A total of 22 blood samples were collected from healthy middle-aged volunteers (12 women and 10 men) after obtaining their informed consent. Individuals with medical disorders, such as liver and blood diseases, were excluded.

Study measurements

The human blood samples were collected in sample tubes containing 50 µL of 200 mM Ethylenediaminetetraacetic Acid (EDTA) to prevent blood clotting. Sample preparation was done by spotting 50 µL of the blood sample onto a 5×10 cm plate of sterilized glass. The samples were maintained at three different temperatures of 4°C, 24°C, and 40°C. Meanwhile, the mRNA expression of the α-globin gene was quantified by real-time RT-PCR at three different time intervals of 0, 30, 90, and 150 days.

RNA extraction and purification

The RNA extraction and purification procedures were conducted according to instructions of the nucleic acid extraction kit (NucleoSpin®), and the RNA concentration was measured using a Beckman dual spectrophotometer at 260-280 ultraviolet invisible wavelength.

Reverse Transcription and PCR

The qRT-PCR assays were performed using ViPrime One Step RT-qPCR 2 × SyGreen Mix (HRox, cat. no QR8602-100, Malaysia) on a StepOne Real-Time PCR Applied Biosystems detection system using the version 3.1 software (StepOne™, USA). We used 5 µL of the total RNA and 1 µL of the gene-specific primer. The thermal cycler conditions were as follows: 1 cycle of reverse transcription for 10 min at 55°C, initial activation for 2 min at 95°C, and 40 cycles of denaturation at 95°C for 5 s, followed by annealing and extension at 60°C for 1 min. β-Actin was selected as the reference gene as it is one of the most common housekeeping genes used to normalize gene expression levels, and its transcription

Table 1. Description of α-globin gene expression at different studied time intervals

Temperature	α-Globin Gene	Time (in Days)				P
		0	30	90	150	
4°C	Mean±SD	6.14±2.40	4.82±1.24	3.528±0.57[ab]	2.57±0.58[ab]	<0.001
	Median	4.96	4.41	3.29	2.58	
	Minimum	3.45	3.12	2.70	1.24	
	Maximum	10.50	8.25	4.66	3.70	
24°C	Mean±SD	4.62±1.25	3.76±0.61	2.41±0.64[ab]	1.66±0.45[ab]	<0.001
	Median	4.31	3.85	2.38	1.69	
	Minimum	3.15	2.40	1.11	1.10	
	Maximum	7.40	4.50	3.50	2.30	
40°C	Mean±SD	2.37±0.83	1.96±0.59	1.43±0.61[ab]	0.71± 0.34 [abc]	<0.001
	Median	2.32	2.12	1.35	0.68	
	Minimum	1.10	0.96	0.59	0.21	
	Maximum	3.85	3.21	2.52	1.40	

[a] Statistically significant compared to the corresponding value in 0 day (P<0.05).

[b] Statistically significant compared to the corresponding value in 30 days (P<0.05).

[c] Statistically significant compared to the corresponding value in 90 days (P<0.05).

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level remains relatively constant in response to experimental manipulation in most tissues.

The primer sequences for the used genes

α-Globin gene (target gene):

Forward primer: 50-NED-ACG CTG GCG AGT ATG GT

Reverse primer: 50-CCC TTA ACC TGG GCA GAG

β-Actin (reference gene):

Forward primer: 5′-TGTTGTCCCTGTATGCCTCT-3′

Reverse primer: 5′-TAATGTCACGCACGATTTCC-3′

Calculation of Relative Quantification (RQ) (relative expression)

Data expression in Cycle threshold (Ct) was performed according to the RT-PCR run. The Ct values of α-globin (target gene) and β-actin (reference gene) were included on the PCR datasheet, and the α-globin gene expression was measured and compared with β-actin gene expression as follows:

$$\Delta Ct\ sample = Ct\ assessed\ gene - Ct\ reference\ gene$$

$$\Delta\Delta Ct = \Delta Ct\ sample - \Delta Ct\ control\ gene$$

Finally, the RQ was calculated using the following equation:

$$RQ=2^{-(\Delta\Delta Ct)}$$

Statistical analysis

The Data were coded and entered into the SPSS software, version 26. The quantitative data were summarized using mean, standard deviation, median, and minimum and maximum. Nonparametric Kruskal–Wallis and Mann–Whitney tests were used to compare the quantitative variables [7]. The Spearman correlation coefficient was used to determine the correlations between the quantitative variables [8]. The prediction of time using the α-globin gene was done using linear regression analysis [9]. P<0.05 were considered statistically significant.

Table 2. Description of α-globin gene expression at different studied temperatures

Time (Day)	α-Globin Gene	Temperature			P
		4°C	24°C	40°C	
0 day	Mean±SD	6.14±2.40	4.62±1.25	2.37±0.83[ab]	<0.001
	Median	4.96	4.31	2.32	
	Minimum	3.45	3.15	1.10	
	Maximum	10.50	7.40	3.85	
30 days	Mean±SD	4.82±1.24	3.76±0.61	1.96±0.59[ab]	<0.001
	Median	4.41	3.85	2.12	
	Minimum	3.12	2.40	0.96	
	Maximum	8.25	4.50	3.21	
90 days	Mean±SD	3.52±0.57	2.41±0.64[a]	1.43±0.61[ab]	<0.001
	Median	3.29	2.38	1.35	
	Minimum	2.70	1.11	0.59	
	Maximum	4.66	3.50	2.52	
150 days	Mean±SD	2.57±0.58	1.66±045[a]	0.71±0.34[ab]	<0.001
	Median	2.58	1.69	0.68	
	Minimum	1.24	1.10	0.21	
	Maximum	3.70	2.30	1.40	

[a] Statistically significant compared to the corresponding value at 4°C (P<0.05).

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[b] Statistically significant compared to the corresponding value in group at 24°C (P<0.05).

3. Results

Comparing the α-globin gene expression within the four studied time intervals at the three different temperatures showed a significant (P<0.001) reduction in the mean values by 90 and 150 days compared to 0 and 30 days. Furthermore, at 40°C, there was a significant (P<0.001) reduction in the mean value of the α-globin gene expression by 150 days compared to 90 days (Table 1).

Concerning the α-globin gene expression at the three investigated temperatures, there was a significant (P<0.001) reduction in the mean values at 40°C compared to 4°C and 24°C at the four investigated time intervals (Table 2). By 90 and 150 days, there was a significant (P<0.001) reduction in the mean value of α-globin gene expression at 24°C compared to 4°C.

Regarding sex-related α-globin gene expression, higher mean values were detected in men than in women (Table 3). The mean values of α-globin gene expression in men were significantly higher than in women at the four studied time intervals at 4°C. At 24°C and the four investigated time intervals, the mean values of α-globin gene expression in men were higher than in women but with no statistically significant difference. Also, at 40°C, the mean expression values in men were significantly higher than those in women by 0, 30, 90 and 150 days, but with no statistically significant difference.

The analysis using the Spearman correlation coefficient revealed statistically significant negative correlations between the α-globin gene expression and time interval, with increasing time intervals being accompanied by a decrease in α-globin gene expression at the three examined temperatures (Table 4).

Table 3. Comparison of α-globin gene expression in both sexes within the studied time intervals

Temperature	Time (Day)	α-Globin Gene								P
		Female				Male				
		Mean±SD	Median	Min	Max	Mean±SD	Median	Min	Max	
4°C	0	4.51±0.67	4.48	3.45	5.68	8.09±2.27	8.90	4.40	10.50	<0.001*
	30	4.10±0.58	4.17	3.12	5.21	5.69±1.28	5.26	4.31	8.25	<0.001*
	90	3.15±0.25	3.12	2.70	3.70	3.97±0.53	4.20	3.10	4.66	0.001*
	150	2.25±0.42	2.37	1.24	2.70	2.97±0.52	2.84	2.00	3.70	0.001*
24°C	0	4.07±0.66	4.13	3.15	5.21	5.28±1.50	5.40	3.20	7.40	0.080
	30	3.60±0.68	3.79	2.40	4.45	3.95±0.47	4.12	3.13	4.50	0.140
	90	2.13±0.60	2.31	1.11	2.94	2.75±0.54	3.01	2.10	3.50	0.056
	150	1.66±0.40	1.74	1.11	2.17	1.67±0.54	1.30	1.10	2.30	0,824
40°C	0	1.90±0.69	1.78	1.10	3.60	2.93±0.62	2.98	2.14	3.85	0.002*
	30	1.64±0.57	1.55	0.96	2.83	2.33±0.35	2.21	1.95	3.21	0.003*
	90	1.33±0.45	1.20	0.59	1.93	1.55±0.78	1.50	0.66	2.52	0.656
	150	0.68±0.27	0.63	0.31	1.20	0.74±0.43	0.73	0.21	1.40	0.941

*P-value is statistically significant (P≤0.05).

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A regression equation was formulated to estimate the time interval (in days) using the parameters provided in Table 5 by subtracting the multiplication product of the α-globin gene with its coefficient from the constant coefficient as follows:

Time interval (days)=118.103–17.457×α-globin gene.

4. Discussion

Determining the age of human bloodstains is an essential tool for forensic examiners. It helps in minimizing samples submitted for DNA analysis, narrowing the investigation pool, and estimating the time the crime was committed [10].

In this study, the relationship between the α-globin mRNA expression and blood age was investigated in

Table 4. Spearman correlation Between α-globin gene concentration and time

Temperature	Statistical Parameters	Time (Day)
4°C	Correlation coefficient	-0.806-
	P	<0.001*
24°C	Correlation coefficient	-0.854-
	P	<0.001*
40°C	Correlation coefficient	-0.713-
	P	<0.001*

*P-value is statistically significant (P≤ 0.05).

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Table 5. Linear regression to detect time using α-globin gene concentration

Model B		Unstandardized Coefficients		Standardized Coefficients	t	P	95.0% Confidence Interval for B	
		Std. Error	β				Lower Bound	Upper Bound
Time (in days)	Constant	118.103	5.836	-0.558-	20.237	<0.001*	106.609	129.597
	α-Globin gene	-17.457-	1.643		-10.626-	<0.001*	-20.693-	-14.221-

*P-value is statistically significant (P≤ 0.05).

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both sexes at different time intervals (0, 30, 90, and 150 days) and different temperatures (4°C, 24°C, and 40°C).

Concerning the α-globin mRNA gene expression values at the four investigated time intervals, the results of this study revealed a significant reduction in mean values by 90 and 150 days. Furthermore, the Spearman correlation between the α-globin gene expression and time interval showed a statistically significant negative correlation, suggesting that the increasing time intervals inversely affected the α-globin gene expression.

Our findings were similar to the results of Tuckprakhon et al. [10], who found an increase in Ct values with increasing bloodstain age, suggesting a decrease in the expression of α-globin mRNA in the bloodstain.

Moreover, Anderson et al. [5] and Alrowaithi et al. [11] reported that under uncontrolled room conditions, bloodstains' β-actin mRNA expression had decreased over time.

The reduction of mRNA expression over time may be because of the destructive effects of ribonucleases that exist in the cells or arise from bacteria or any other contaminants, in addition to the impact of various environmental conditions, such as temperature, UV light, pH, and humidity [12]. Regarding the effect of temperature on the α-globin mRNA gene expression, our results showed a significant reduction in the mean expression values at 40°C compared to 4°C and 24°C.

Alaeddini et al. [13] reported similar findings that revealed a high degree of damage on bloodstain samples exposed to 48°C. Another research conducted by Rebecchi et al. [14] reported that high temperatures of up to 50°C, could significantly reduce DNA stability and survival over 21 days.

In contrast, numerous studies have reported the high stability of RNA in different organs at low temperatures. For instance, Zhu et al. [15] reported a slower breakdown of β-actin mRNA extracted from mice brains at 4°C than 37°C. In addition, Harrison et al. [16] showed that postmortem cooling at 8°C, until a postmortem interval of 96 h, prevented the reduction of RNA extracted from rat brain. Furthermore, Ross et al. [17] found that rat tissue mRNA values were remarkably stable for up to 72 h when stored at 4°C.

The temperature-related findings may be attributed to the detected weak activity of ribonucleases at lower environmental temperatures as reported by Deng et al. [18], who discovered generalized stability of the ΔCt values of the brain and heart mRNA with postmortem interval at 4°C.

Regarding the effect of sex on the α-globin gene expression, our study showed a significant difference between both sexes, as the mean expression values were higher in men than women. These findings were because of sex-related differences as all other factors were similar for both sexes, such as the type, quantity, storage conditions of samples, and the ethnic origin of all subjects.

Qi et al. [19] investigated the impact of sex difference on RNA expression in bloodstains and found that sex difference had influenced the ratio of 18S rRNA: β-actin mRNA, with women having lower concentrations of bloodstain mRNA than men.

Moreover, Preece & Cairns [20] showed that sex and age might influence brain mRNA quantification. They found that women had lower quantities of mRNA than men.

However, Sakurada et al. [21] and Juusola and Ballantyne [22] reported no significant sex-related differences between the quantification of HTN3 and STATH mRNA markers in saliva.

5. Conclusion

Increasing time intervals and increasing temperatures have an inverse effect on the α- globin gene expression; nevertheless, its expression could still be detect-

ed at 40°C and after 150 days. A regression equation was formulated to estimate the time interval using the α-globin gene concentration. Therefore, α-globin mRNA could be a valuable molecular tool in the age estimation of human blood spots.

Ethical Considerations

Compliance with ethical guidelines

This study was conducted upon the approval of the Ethics Committee of the Department of Forensic Medicine & Clinical Toxicology and the Ethics Committee of the Faculty of Medicine, Cairo University, Egypt with the reference number I-191016. Informed written consent was obtained from each participant after clarifying the aim of the work.

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Authors' contributions

Conceptualization and supervision: Usama Mohammed Ibrahim Elbarrany and Sherien S Ghaleb; Methodology: Dina Sabry Abdelfattah; Investigation,Writing--original draft and Writing--review&editing: Heba Abdullah Mostafa Eid and Heba Mohamed Aboubakr; Data collection: Heba Abdullah Mostafa Eid; Data analysis: Heba Abdullah Mostafa Eid and Heba Mohamed Aboubakr.

Conflict of interest

The authors declared no conflict of interests.

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