Received: May 2, 2023
Accepted: May 18, 2023

Acad Med J 2023;3(2):107-116
UDC: 340.66:[616-001-056.7:575.22
https://www.doi.org/10.53582/AMJ2332107b
Original article

A NEW METHOD TO DETERMINE WOUND AGE IN HUMAN SKIN INJURIES

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Abstract

Wound age determination is a significant part of everyday practice in forensic medicine. Determining the wound age answers a large number of questions arising from a criminal-legal event. The RT-PCR technique enables qualitative and quantitative analysis of mRNA and can register minimal changes in gene expression, which makes it more sensitive and accurate compared to the immunohistochemical method.

The study included 51 wound samples of autopsy cases. In this study we conducted comparative analyses for different methods of homogenization and isolation. An analysis was carried out for the selection of a suitable reference gene that will be used for normalization of the expression levels of the investigated target gene. Two reference genes, GAPDH and 18S, were analyzed in the study.

A biphasic expression of IL-6 information RNA (mRNA IL-6) was determined in relation to the other groups, including the control group. 18S has better efficiency of amplification then GAPDH.

RNA was isolated from all examined tissues with the TriXact kit on the MagCore Plus II instrument. It can also be concluded that mechanical maceration of the tissue was more effective than the other methods of homogenization. 18S is a more stable reference gene for the analysis of skin samples. IL-6 can could be used as a marker in wound age determination.

Keywords: wound age; real-time PCR; autopsy cases; human dermal injuries; Il-6

Introduction

Wound age determination is a significant part of everyday practice in forensic medicine[1-3]. Determining the wound age answers a large number of questions arising from a criminal-legal event[4]. There are various methods for determining the age of wounds such as macroscopic analysis, histological analysis, immunohistochemical, biochemical, etc.[2,5-6]. Despite the fact that immunohistochemical methods have a significant role in determining the wound age, they are also characterized by a large number of shortcomings. With the application of immunohistochemistry, it is not possible to quantify the expression of biological mediators, and also a longer period of time is required to conduct the analyses. The interpretation of the results obtained with this method depends on the intensity of the staining and on the morphometric analysis (subjective analyses)[5,7-9]. This is the reason that in the last two decades a growing

number of studies have been conducted at the molecular level to determine the age of injuries using RT-PCR. RT-PCR is a variant of PCR used to detect RNA expression levels (gene expression)[10]. The RT-PCR technique enables qualitative and quantitative analysis of mRNA and can register minimal changes in gene expression, which makes it more sensitive and accurate compared to the immunohistochemical method[8].

Wound healing is a complex process in which different immune and biological systems are involved and interconnected[11]. The wound healing process involves complex, precise, temporally precisely regulated interactions between tissue, cells, biochemical molecules (cytokines, chemokines and growth factors)[12-15]. It is crucial to know and understand all of these interactions when conducting wound age research[2,7]. Immediately after the injury, the levels of mRNA (informational ribonucleic acid) for certain cytokines and enzymes that are released in response to the broken integrity of the skin can be detected using real-time PCR[16].

Analyzing gene expression by RT-PCR from postmortem tissue takes great care in carrying out all the steps, because a number of quality problems can arise in the workflow which can affect the accuracy and the reliability of the results and conclusions. Because of this, it is necessary to pay special attention to the design of the experiment itself in terms of sample collection, RNA extraction, as well as selection of appropriate reference genes for normalization of the expression levels of the studied genes[17].

There is a smaller number of studies that have been published where comparative analyses have been performed to evaluate the skin sampling process, sample storage and the homogenization step. These three steps are critical procedures in skin processing in molecular biology for gene expression analysis. At this point, there are two strategies for blocking the activity of RNases either by rapid freezing, immediately after taking the sample, or by using solutions that maintain the stability of the RNA molecule[18-21]. Homogenization of the skin is usually achieved by fine chopping with a scalpel, with a mechanical homogenizer or with a specialized so-called hammer used to crush frozen tissue. During the homogenization process, commercially prepared solutions are used in order to cause degradation of cell membranes and inhibition of RNases[22-26]. In recent years several methods of RNA isolation from tissue have been published, and the difference in methods refers to the use of different reagents in the entire isolation procedure[27]. However, in general, there are two basic ways to extract RNA from a sample, mechanical extraction and automated extraction. There are several methods for determining the quality of isolated RNA from a sample. In our study, we determined the quality of isolated RNA by RT-PCR through the expression of reference (housekeeping) genes.

The aim of this study was to introduce a method for determining the age of wounds by analyzing IL-6 mRNA expression. We analyzed the factors that can influence the expression levels of the studied gene, that is, we determined which was a more suitable method of sample homogenization, RNA isolation, the influence of the postmortem period, as well as the selection of a suitable reference gene.

Material and methods

The study included 51 wound samples of cases upon which autopsy was performed at the Institute of Forensic Medicine, Criminalistics and Medical Deontology, Faculty of Medicine, Ss. Cyril and Methodius University in Skopje. The analysis comprised samples from intact skin and lacerations. The following data were obtained when samples were taken: age, information on the mechanism of wounding, exact time of the wound occurrence, exact time of death, exact date and time when autopsy took place. The study included cases with post-mortem period up to 24 hours and deceased persons at the age up to 60 years.

Wound samples were taken with scalpel with sterile cut within the injury margin. Taken samples were of 1 cm to 1.5 cm length and of 0.2 to 0.3 cm width or of about 1 cm^2 area. The

sample were immediately frozen at -96.6ºC with R134a tetrafluoroethane (CF3CH2F). Frozen specimens were stored in plastic cryotubes of 2 ml volume in a freezer at -80ºC until the day of molecular biological analysis. Prior to initiation of the RNA isolation process, the weight of each examined sample was measured with an analytical scale (Sartorius, Germany).

Sample homogenization was manually done by pressing the frozen sample with a macerator in a plastic tube (BioMasher). During the manual homogenization of the examined sample, a solution of 400 μL of RB buffer and 4 μLβ-mercaptoethanol in a ratio of 100:1 was used. RB buffer is part of the MagCore TriXact kit. The other way of homogenization which was performed was homogenization by finely cutting the frozen sample using a scalpel and then further macerated in a plastic tube using a glass probe.

RNA isolation was performed automatically with the automated extractor MagCore Automated NucleicAcid Extractor Plus II (RBC Bioscience, Taiwan) using the program with 631 code aimed at RNA extraction from tissues with TriXact kit and manual isolation using Trizol method. Quantification of RNA isolated from tissue samples was made with the help of Qubit® RNA HS or BR Assay Kit of Qubit® 2.0 Fluorometer.

Reverse RNA transcription (cDNA synthesis) was performed by using High-Capacity cDNA Reverse Transcription Kit. Not more than an aliquot of 1 μg RNA should be added in the reaction. Prepared reactions were incubated in a PCR apparatus according to the following program: 10 min. at 25ºC, 120 min. at 37ºC, 5 min. at 85ºC and indefinite period at 4ºC.

The expression of the target gene, IL-6, was analyzed in this study. The relative quantity of the transcripts of interest in each of the phases of injured tissue was measured by real-time PCR by using commercial reaction mixture HOT FIREPol®EvaGreen® qPCR Mix Plus at 7500 Real-Time PCR System according to the manufacturer's instructions. Real-time PCR reactions (in triplicate) were performed for each sample for examined transcript (target) and one for the reference gene (housekeeping). GAPDH and 18s genes were analyzed as a housekeeping gene. The primers for each of the examined genes were taken from relevant literature data (Table 1).

Table 1. GAPDH, glyceraldehydes-3-phosphate dehydrogenase, Interleukin 6, 18 S Statistical analysis of results

Gene	Primer		References
GAPDH	Forward	TGCCAAATATGATGACATCAAGAA	28
	Reverse	GGAGTGGGTGTCGCTGTTG	
IL-6	Forward	CTGCAAGAGACTTCCATCCAG	29
	Reverse	AGTGGTATAGACAGGTCTGTTGG	
18S	Forward	GCAGAATCCACGCCAGTACAAG	30
	Reverse	GCTTGTTGTCCAGACCATTGGC	

The results obtained from RT-PCR analysis, that is, the C_T levels of transcripts of interest and C_T levels of housekeeping genes were calculated with the formula 2^(-ΔΔCt). The values obtained were statistically analyzed with the Pearson correlation coefficient Student T.

Results

Fifty-one skin wound samples were included in the study; 10 of the total number were control samples taken from intact skin at autopsy.

Based on the survival time, i.e., the period from receiving the injury and the occurrence of the death outcome, the wound samples were divided into five groups:

1. The first group included cases of immediate death and survival up to 1 hour (n=8)
2. The second group included cases with survival from 1 hour to 6 hours (n=8)

3. The third group included cases with survival from 6 hours to 24 hours (n=7)
4. The fourth group included cases with survival from 24 hours to 72 hours (n=8)
5. The fifth group included cases with survival from 72 hours to 168 hours (n=10)

A comparative analysis of two manual homogenization techniques was carried out in 10 samples from the total number of samples included in the study. The samples had a weight of 26.61 g to 179.58 g, that is, a mean weight of 100.06 g. Manual homogenization was performed using a plastic macerator and a plastic tube and homogenization by finely chopping the sample. The rest of the samples were homogenized with the first method, with a plastic macerator. After homogenization and isolation, RNA concentration in the isolate was measured using a Qubit 3.0 fluorometer. It was determined that the first method of homogenization was better, that is, a higher concentration of RNA was isolated in the isolate itself.

By applying the t-test, where we analyzed two independent variables, that is, the concentrations of RNA obtained with two different methods of homogenization. Significant differences were obtained in two studied variables (t=-1.866, p=0.04).

Two methods of RNA isolation, automatic and manual isolation, were performed in the study. Twelve samples out of the total were isolated by both methods. Student's t-test was conducted to determine if there was a difference in the two groups that we analyzed, that is, in the two groups that were isolated in two different ways. No statistically significant difference was found between the two groups, i.e., t test was 0.29 ($p > 0.05$).

In order to determine the critical parameter for the RNA concentration, the influence of the variables (postmortem period and the mass of the analyzed sample) was determined, and it was concluded that both variables had certain influence: the mass of the sample had a positive correlation (r=1), $p \leq 0.05$), while the postmortem period had a negative correlation (r=-1.59) with the RNA concentration.

The values obtained by applying Pearson's formula, which are presented in Figure 1, showed that with an increase in the postmortem period, the concentration of RNA decreased, while with an increase in the mass of the sample, the concentration of isolated RNA increased (Figure 1). Since the time of the postmortem period is an independent variable, it is necessary to unify the mass of the tissue that will be used for analysis, that is, to use a tissue with an adequate mass from which a sufficient concentration of RNA can be isolated.

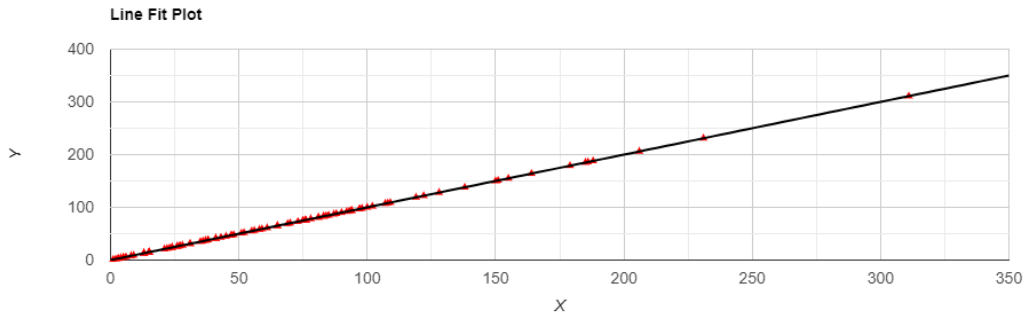

Fig. 1. Correlation between mass of the sample and concentration of isolated RNA

In the study, an analysis was made for the selection of a suitable reference gene that would be used for normalization of the expression levels of the investigated target gene. Two reference genes, GAPDH and 18S, were analyzed in the study. A parameter that we took into account to determine the choice of reference gene was determination of the amplification efficiency. We determined the efficiency in percentage by applying the following formula:

$$E= [-1+10(-1/slope)] \times 100$$

The slope of the standard curve of one of the used reference genes, 18S, was -3.285, and by applying the formula, an efficiency of 101% was obtained, while the correlation coefficient was R^2=0.994.

In relation to the results obtained for the slope of the standard curve for the other reference gene GAPDH, which was - 3.519, an efficiency of 92.3% was obtained. The value of the correlation coefficient was R^2=1.0.

A relative quantification of the expression of the studied gene Il-6 in relation to the expression of the reference gene 18S was performed based on Ct values. The relative quantification was determined by applying the formula 2-ΔΔCt.

A biphasic expression of IL-6 information RNA (mRNA IL-6) was determined in relation to the other groups, including the control group. The strongest expression was determined in the fifth group, that is, the group that included cases with a survival period between 72 hours and 152 hours. A higher expression was also determined in relation to the control group and in the cases with survival from 1 hour to 6 hours, that is, in the second group. No significant expression of the studied gene was found in the other groups (Table 2, Figure 2).

Table 2. Relative quantification of Il-6 with the formula $2^{-\Delta\Delta Ct}$

Groups	Δ Ct mRNA IL-6	ΔΔ Ct mRNA IL-6	2^(-ΔΔ Ct mRNA IL-6)
Control group	0.8	0.6	0.04
group 1	1.1	0.8	0.1
group 2	1.9	1.5	0.2
group 3	1.7	1.3	0.11
group 4	3.2	2.7	0.02
group 5	0.4	0.15	0.3

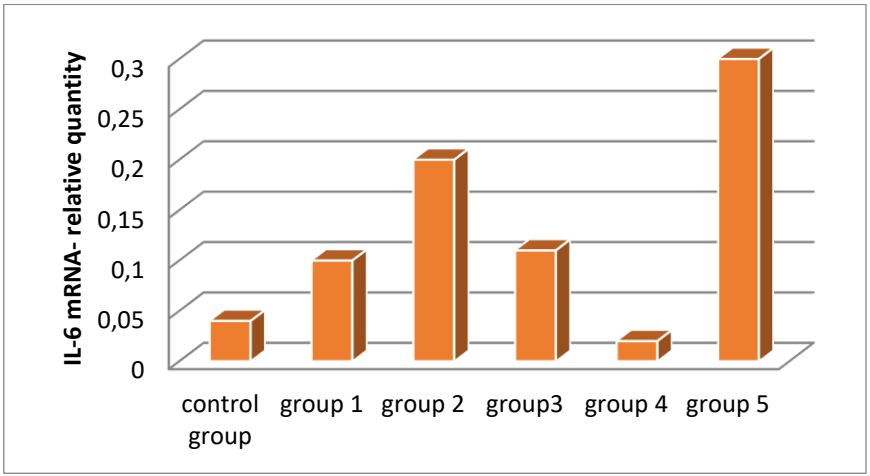

Fig. 2. Real-time PCR analyses of IL-6 mRNA expression in wounds

Discussion

Determination of the wound age continues to be a challenge in forensic medicine [31]. Different types of techniques have been used to determine the vitality and age of injuries. A large number of studies that have been conducted to determine the age of injuries have applied either conventional histological analyses or immunohistochemical analyses. However, studies say that by applying these two methods for the age of injuries, they cannot be used in practice, that is, as material evidence in court[32-35]. Apart from morphological methods, researches with biochemical methods were also carried out for the evaluation of vitality markers in wound

fluids or tissue extracts from wounds[36]. Immunological tests such as ELISA are one of the biochemical methods that have obtained quite significant results and knowledge about the wound healing process through the analysis of proinflammatory cytokines. In order to provide good and reliable material evidence, a growing number of studies are conducted on samples from living individuals as well as from samples taken during autopsy using molecular analyses. However, when taking a sample from living individuals, there may be an ethical problem. With the use of RT-PCR, it is possible to determine the age of injuries much more precisely than with other methods, and it is also possible to detect the studied mediators at the earliest stage of the wound healing process[37].

This study introduced a protocol for extracting RNA from a skin sample for further analysis to determine the age of injuries. Two steps had been determined before RNA isolation was performed: 1. Immediately after taking the skin sample, it was frozen and stored at -80^{o} C until the isolation process; 2. Homogenization of the frozen sample. A study conducted by P.M. Lacoco *et al.* in which a comparative analysis was made in the process of taking and storing the skin sample, showed that using RNA stabilizing solutions (RNAlater) did not preserve the RNA integrity in its entirety, in contrast to the quick freezing of the sample after taking it. The same results were obtained in the study by E. Reimann *et al.* where four different storage protocols were used. In our study, by using a method of rapid freezing of the sample and homogenization of the frozen sample, RNA was isolated in a sufficient concentration for further analysis[38].

In our study, a comparative analysis was performed between two methods of tissue homogenization. One method was carried out by finely cutting the frozen sample using a scalpel and then further macerated it in a plastic tube using a glass probe. The other method was performed by directly macerating the frozen sample in a plastic tube using a plastic macerator. RB Buffer and mercaptoethanol were used in both methods. With the second method of homogenization, higher concentrations of RNA were obtained.

The integrity of the RNA molecule depends on a number of factors. After death, it is characterized by rapid degradation under the influence of various factors, primarily RNase enzymes, which are found in various types of cells in the human body. After death, the RNA molecule is degraded by the action of the RNase enzymes that are found in the cells or may originate from bacteria, or under the action of other factors from the environment. The concentration of RNases is different in different organs; for example, the liver and pancreas are rich in RNase enzymes, unlike the brain, which is why the RNA molecule is more stable in the brain. The skin as an organ is characterized by a higher concentration of RNase, but it is also exposed to external influences. There are studies on the influence of the post-mortem period on the RNA concentration in different organs, especially in the brain, but there are small number of studies on the skin[8].

In our study, ten cases with different post-mortem period were analyzed, in which in all samples a sufficient concentration of RNA was isolated for further analysis to determine the age of the injuries. With the conducted statistical analysis, it was concluded that there was influence of the length of the post-mortem period on the concentration of RNA, that is, a lower concentration of RNA was isolated in samples with the longest post-mortem period. We can conclude that the post-mortem period affects the concentration of RNA; however, can be isolate a sufficient amount of RNA with a post-mortem period of 72 hours.

Laboratory analysis of the RNA molecule compared to the DNA molecule shows that RNA is more unstable and susceptible to RNase degradation during the isolation process. In the comparative analysis conducted by Liu Chien – Ju *et al.* [39] in relation to manual extraction and automatic extraction of RNA, better results were obtained using automatic extraction and also the procedure lasted a shorter period. In our study, we applied the automatic

extraction method, whereby in all twelve samples RNA was isolated in a sufficient concentration for further analysis.

Two reference genes, 18s and GAPDH, were included in the study for normalization of the expression levels of the investigated target genes. A reaction was carried out for both reference genes. However, a better PCR efficiency of the reference gene 18S was obtained, which was 101%. Although the efficiency of the PCR reaction for GAPDH was within acceptable limits, the absence of expression was observed in a certain number of samples. Reference genes, also called housekeeping genes, are necessary for maintaining the basic cellular function and are expressed in all cells of the body in normal as well as pathophysiological conditions. GAPDH and 18S are one of the most frequently used reference genes, which is why we included them in the conducted study. In contrast to GAPDH expression, 18S was recorded in all samples. The reason for the absence of GAPDH expression may be due to the length of the post-mortem period, gender, age, and certain skin disease. IL6 is a proinflammatory cytokine that is produced by numerous cell populations involved in the injury healing process. It is found to take part in the inflammation phase of the immune response and functions as the main mediator of the acute phase reaction. In our study, increased IL-6 expression was detected in the second group with survival time from 1 to 6 hours and also in the fifth group of samples with wound age from 72-152 hours. A limitation of this study was the small number of total cases. A higher number of cases could lead to more clear interpretation of the results.

Conclusion

It can be concluded that RNA isolated from all examined tissues with the TriXact kit on the MagCore Plus II instrument is suitable for further molecular analyses. It can also be concluded that mechanical maceration of the tissue in the presence of the RB buffer and β-mercaptoethanol is more effective than the approach of crushing/mincing the tissue prior to extraction. We can conclude that 18S is a more stable reference gene for the analysis of skin samples. IL-6 can be used as a marker in wound age determination.

Conflict of interest statement. None declared.

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