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Expression of cell death genes estimates time since death in rats

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Abstract: Estimation of postmortem interval (PMI) is one of the challenges in forensic science. There are several methods estimating the time since death (TSD) involving physical and biochemical methods. Decomposition is initiated by a process called autolysis which induces detrimental alterations in the cell leading to cell death. On the basis of the process of cell death signaling, the current study investigated the early PMI (2–8h after death) using the analysis of the mRNA expression of Fas Ligand (FasL), Caspase-3 and phosphatase and tensin homologue deleted on chromosome 10(PTEN) in gastrocnemius muscles of rats by real-time PCR. The results revealed a time-dependent increase in the mRNA levels of these genes until 6 h after death. Moreover, a positive linear correlation was detected between the mRNA expression of these genes and the TSD using a regression analysis in the first 6h after death. This study supplies a quantitative tool for determining the early PMI until 6 h after death based on expression of these cell death genes. Further research is recommended to find other cell death markers and extend the time period for TSD determination.

Key Words: postmortem interval, time since death, mRNA levels, FasL, Caspase-3, PTEN.

Postmortem interval (PMI) estimation is very important task in the forensic medicine. The accurate PMI determination is necessary for including and excluding suspects relied on their whereabouts at the death time and to provide a time frame in that remains decomposed beyond identification may be connected with missing individuals [1]. The postmortem changes determining time since death (TSD) are typically classified into physical, physiological, and biochemical categories. Physiological changes including livor mortis, rigor mortis, and supravital activity are principally utilized to determine early PMI. The physical category like arthropod activity and physiological changes such as the classical decomposition signs are regularly used to estimate longer PMI [2, 3]. Most of these changes for PMI estimation are relatively still inaccurate. However, some biochemical changes of the body could be used as markers for PMI determination. Novel research lines including protein, RNA, and DNA degradation are starting to provide more precise methods for PMI estimation [4, 5].

The accurate determination of the TSD necessitates the evaluation of parameters that change regularly with time after death that including the post mortem degradation of nucleic acids. In fact, the analysis of time-dependent of nucleic acid RNA and DNA degradations drew an attention in clinical medicine and forensic science [4, 6]. For example, the potential of RNA has been investigated in postmortem assessment of the functional status of cells and organs targeting the diagnosis of etiology and mechanism of death [7]. The PMI estimation through studying the decay of RNA may be within reach since RNA degradation or loss of RNA transcripts after death appears to be rapid and dependent on death time. After death, RNA is decayed by ribonucleases actually present in the cell or arising from

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bacteria or other environmental contaminants [1].

The cadaver decomposition starts 4 min postmortem with a process called autolysis. Progressive cell destruction occurs during decomposition. Afterward, there is a release of cellular components and metabolites. To our knowledge, little literature is available on the alterations associated with the TSD within the first hours of death [6, 8]. Therefore, our study aimed to estimate the early PMI between 2 and 8 h using the expression analysis of cell death genes including Fas Ligand (FasL), Caspase-3 and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) by real-time PCR in gastrocnemius muscle of rats.

MATERIALS AND METHODS

Experimental design

Fifteen healthy adult male Sprague-Dawley rats (4 months of age) weighing 200±10 g were obtained from The Animal Research Unit, Faculty of Veterinary Medicine, Zagazig University, Egypt. The animals were acclimated to laboratory conditions for 2 weeks before starting the experiment. Rats were kept in metal cages during experimental period and maintained on 12 h light-darkness cycle with a controlled temperature (21–24 °C) and a relative humidity (50-60%) and given a standard diet and water ad libitum. The protocols were approved by the ethics of animal use in research committee (EAURC), Cairo University.

Sample collection

Rats were anesthetized using diethyl ether, sacrificed by cervical dislocation and left at room temperature between 0 and 8 h after death. Then, the animals were divided into five groups (three/ each group). First group; 30 mg of gastrocnemius muscle were biopsied from each rat immediately after death, as a control or a time 0 while 2nd, 3rd, 4th and 5th groups were dissected to obtain gastrocnemius muscle at 2, 4, 6, 8 hours postmortem respectively. Muscle specimens were immediately frozen in liquid nitrogen then stored at -80°C for gene expression analysis. This tissue was selected for the study because it is easy to access and is mainly applied in rigor mortis studies [9].

RNA extraction and reverse transcription

Total RNA was extracted from 30 mg of gastrocnemius muscle using trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. The purity of total RNA was measured using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA. A total RNA (0.5µg) was reversely transcribed into cDNA using QIAGEN Long Range 2 Step RT-PCR Kit, following the manufacturer's protocol. One µl of total cDNA was mixed with 12.5 µl of 2x SYBR® Green PCR mix with ROX from BioRad, 5.5 µl of autoclaved water, and 0.5 µl (10 pmol/ µl) of each forward and reverse primers for the measured genes. The expression was normalized by an internal housekeeping control (β-actin gene).

Real-time PCR

Expressions of FasL, Caspase-3 and PTEN were performed using real time PCR. The primer sequences were listed in Table 1. PCR reactions were carried out in a Rotor-Gene Q cycler (Qiagen, Heidelberg, Germany). The real-time PCR reaction program included a 94 °C enzyme activation step for 2 min followed by 40 cycles of 95 °C denaturation for 15 sec, 60 °C annealing for 30 sec and 72 °C extension for 30 sec. The detection of a fluorescent product was performed at the end of the 72 °C extension period. The amplification data were collected by the sequence detector and analyzed with sequence detection software. For each assay, a standard curve was constructed using increasing amounts of cDNA. In all cases, the slope of the curves indicated adequate PCR conditions (slopes of 3.3-3.6). The RNA concentration in each sample was determined from the threshold cycle (Ct) values and calculated with the sequence detection software supplied by the manufacturer. The quantitative fold changes in mRNA expression were estimated relative to ß-actin mRNA levels in each corresponding group and calculated using the 2-DD CT method.

Statistical analysis

Plots of mRNA levels were carried out using Microsoft Excel 2010. Regression analyzes were performed using IBM SPSS Statistics computer software (version 22).

Table 1. Oligonucleotide primer sequences of FasL, Caspase-3, PTEN and β -actin genes.

Genes	Primer Sequences (5′ → 3′)	Size (bp)	Reference
FasL	F: CACCAACCACAGCCTTAGAGTATCA R: ACTCCAGAGATCAAAGCAGTTCCA	171	[10]
Caspase-3	F: GCAGCAGCCTCAAATTGTTGACTA R: TGCTCCGGCTCAAACCATC	144	[10]
PTEN	F: GGA AAG GAC GGACTG GTG TA R: TGC CAC TGG TCTGTA ATC CA	101	[11]
β-actin	F: TCACTATCGGCAATGTGCGG R: GCTCAGGAGGAGCAATGATG	260	[12]

RESULTS

Expression of cell death genes

FasL showed a time-dependent increase in mRNA levels, beginning 2 h after death. Conversely, mRNA levels suddenly reduced 8 h after death. The same pattern in mRNA levels of Caspase-3 and PTEN genes was found; there was a time-dependent elevation until 6 h after death and a marked decline at 8 h after death (Fig. 1).

Correlation between early TSD and mRNA levels of FasL, Caspase-3 and PTEN genes

The results of mRNA levels correlated with the early PMI. However, due to the sudden decrease at 8 h after death, the correlations were done only between 0 and 6 h after death (Fig. 2).

mRNA levels of FasL showed a strong positive linear correlation with TSD (r = 0.97; p = 0.03; with the regression formula TSD = 0.03 mRNA levels + 0.34). Similarly, a high positive linear correlation was observed in mRNA levels of Caspase-3 (r = 0.99; p = 0.01; with the regression formula TSD = 0.06 mRNA levels - 0.10) and PTEN(r = 0.98; p = 0.02; with the regression formula TSD = 0.06 mRNA levels + 0.13).

DISCUSSION

In this study, we used the analysis of the expression of three genes (FasL, PTEN and Caspase-3) implicated in cell death for estimating the early PMI.

The present results depicted a time-dependent increase in mRNA levels of FasL, PTEN and Caspase-3 beginning 2 h after death until 6 h with a marked decrease at 8 h after death. This sudden decrease in the mRNA levels may be owing to degradation of RNA as a result of the development of the autolysis process [8]. In our study, we found a strong positive linear correlation between mRNA levels of FasL, PTEN and Caspase-3 with TSD. These results confirmed our initial hypothesis, since Caspase-3, FasL and PTEN are implicated in cell death signaling pathways. These findings were consistent with a previous study [8].

Caspase-3 is the major cysteine protease sharing

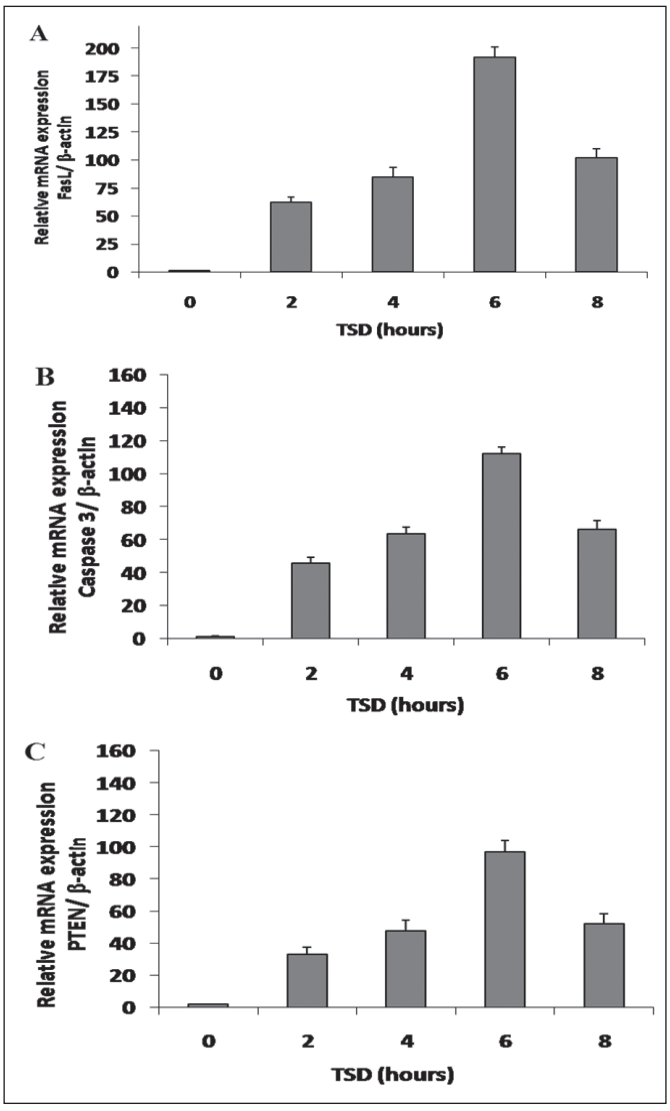

Figure 1. mRNA levels of FasL (A), Caspase-3 (B)and PTEN (C) in gastrocnemius muscles of rats from 0 to 8 h after death.

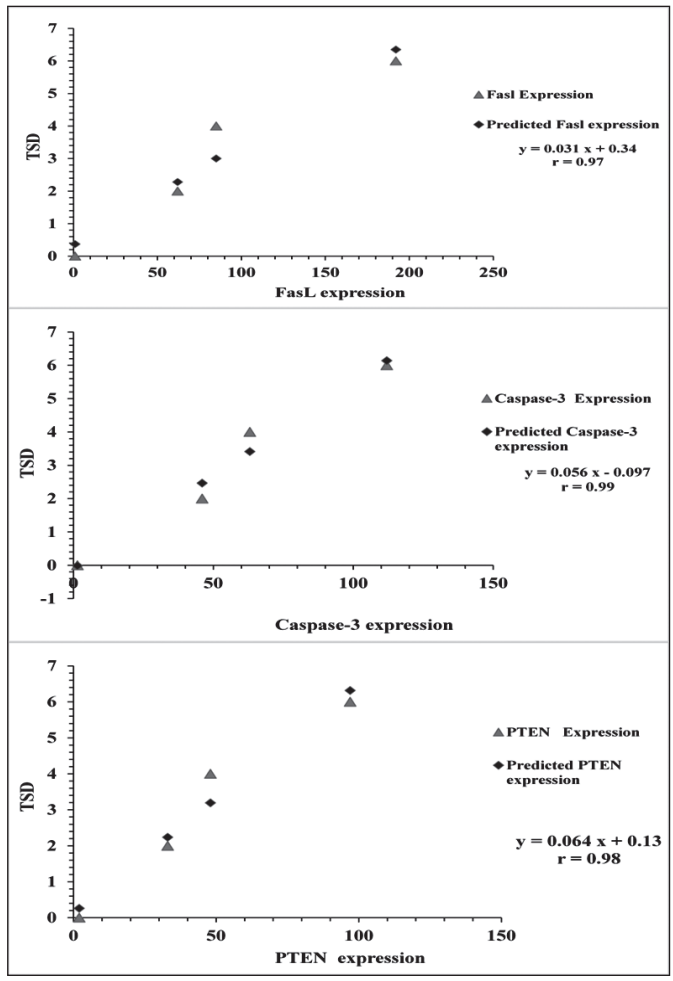

Figure 2. Correlation between expression levels of FasL (A), Caspase-3 (B), PTEN (C) and time since death (TSD). mRNA expression levels of these proteins showed a high positive linear correlation with the TSD from 2 to 6 h. the strength of the correlation is marked by "r" and the regression plot: y TSD, x mRNA expression.

in apoptosis and is the most important of the executioner caspases. It is activated by one of the initiator caspases (caspase-8, caspase-9, or caspase-10). It can activate the apoptosis pathway by cleavage of corresponding substrates in cells. Therefore, this protein is recognized as a "molecular switch" [13, 14].

FasL is a member of the tumor necrosis factor family that initiates cell death by binding to its surface receptor Fas [15]. PTEN was known as a tumor-suppressor gene located on chromosome 10. It is deleted in several types of human cancers and cell lines [16]. Moreover, PTEN plays a vital role in the regulation of cell growth and apoptosis (programmed cell death) [17]. The function of PTEN in the regulation of cell death was shown in this study by increasing its expression with the time after death. PTEN is contributed in the regulation of FasL and death signals [18]. Binding of FasL to Fas receptor initiates the formation of the death-inducing signaling complex (DISC) which in turn leads to activation of Caspase-8. Caspase-8 activates Caspase-3 which is the effector caspase to stimulate the cell death signaling. If the signal of Caspase-8 is not enough to cause cell death, the mitochondrial pathway is activated with Caspase-9 which activates Caspase-3 leading to cell death [19]. This sequence of events explains the relationship of the expression levels of FasL, PTEN and Caspase-3 in our study since the same pattern in mRNA expression levels of these genes was observed.

In conclusion, the present study provides a quantitative tool for estimating the early PMI until 6 h after death through expression analysis of cell death genes. mRNA expression levels of these proteins demonstrated a strong positive linear correlation with the TSD from 2 to 6 h after death. Further studies are warranted to apply other cell death genes for extension of both time period and precision of TSD estimation.

Conflict of interest. The authors declare that they have no conflict of interest concerning this article.

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