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Thanatotranscriptome Study on Particular Hepatic Genes and Their Correlation With Postmortem Interval in the Presence or Absence of Postmortem Heat Stress

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ABSTRACT

The present work designed to estimate postmortem expression of mRNA of certain hepatic genes in rats in the presence or absence of postmortem heat stress using qRT-PCR technique in correlation to postmortem interval (PMI). Rats were sacrificed and divided into, control group (PMI=0 h), room temperature and heat stressed (41ºC) groups. In control group, liver samples were collected immediately after death, while in the second and third groups, samples were collected at 1, 3 and 6 hours postmortem. The results showed significant reductions in pro-inflammatory gene transcripts (TNFα and IL-1β) at all-time points compared to control group with an inhibitory effect of postmortem heat stress on expression of TNFα at 1 and 6 h after death. Whereas postmortem expression of genes encode apoptosis was significantly increased at 3 and 6 h postmortem compared to control group. Postmortem heat stress reduced the hepatic expression of Bcl-2 and Caspase-3 at 3 and 6 h of PMI and increased the expression of Caspase-3 at 1 h after death compared to room temperature group. Meanwhile, postmortem hepatic mRNA expression of c-fos was significantly induced after death at 3 and 6 h PMI. Postmortem heat stress significantly reduced c-fos expression at 3 and 6 h after death. Thanatotranscriptome of genes encode inflammation, apoptosis as well as c-fos varied in their correlations to PMI. Additionally, the results showed disparities in the effect of postmortem heat stress according to the studied gene and time.

1. INTRODUCTION

Molecular investigations particularly gene expression researches are well-established components of ante-mortem studies either for clarifying basic normal physiological mechanisms or discovering therapeutic targets in pathophysiological disorders. Several studies have proved that there is a wide range of gene transcripts that are susceptible to degradation shortly after death by the action of RNases (Phang et al. 1994; Trotter et al. 2002; Catts 2005). However, certain studies indicate that isolation of intact RNA is possible even several days after death (Inoue et al. 2002; Catts 2005; Heinrich et al. 2007). The articles that have elucidated postmortem gene expression "thanatotranscriptome" are rather few. Thanatotranscriptome was previously applied to give hints related to possible cause of death (Ikematsu et al. 2005; Zhu et al. 2008). Moreover, the postmortem apoptotic gene expression patterns were determined in hepatic tissues collected from dead bodies with postmortem intervals (PMIs) between 6 and 48 hours (Javan et al. 2015). The results revealed that mRNA expression of pro-apoptotic genes as Caspases was up-regulated whereas the mRNA expression of the anti-apoptotic genes like Bcl-2 was down-regulated. A postmortem transcriptome provides awareness about gene activity after death with potential applications in medico-legal investigations (Javan et al. 2015). Hence, the notion of existence of genes that still alive within

the internal organs of the dead body arose (Pozhitkov et al. 2017).

In a recent study, postmortem mRNA transcript for several genes was identified (Pozhitkov et al. 2017). Functionally, these genes are related to stress, apoptosis, inflammation, immunity, transport, epigenetic regulation, development and cancer. The profile of these genes was assessed, in a part of the study, through postmortem time in mouse liver, mRNA transcript levels were decreased with postmortem time (Pozhitkov et al. 2017). It was suggested that thanatotranscriptome can be used in forensic science for precise estimation of PMI.

Besides traditional approaches that help in determination of PMI as cooling (Muggenthaler et al. 2012) and rigor mortis (Dalal et al. 2006), biomarkers like proteins and enzymes have been used for PMI evaluation (Sabucedo and Furton 2003; Kikuchi et al 2010). Most of thanatotranscriptomic studies provided the principle that some genes are still active after death using different body tissues either in human or in animals (Franz et al. 2005; Javan et al. 2015; Pozhitkov et al 2017), however very few of these studies consider factors like heat (thermal) stress in their analysis (Kikuchi et al. 2010; Ma et al. 2015), the issue that reflects, in some cases, the actual circumstances and the native environment during some crimes and may affect the mRNA expressions and subsequent PMIs. In the present study, the thanato-transcripts of genes related to inflammation, apoptosis and stress were analyzed after death in the liver of rats using qRT-PCR technique either at room temperature or with postmortem heat stress in relation to time passed since death. Liver was chosen because it had slower DNA degradation suggesting its suitability for forensic studies (Heinrich et al. 2007; Ebuehi et al 2015).

2. MATERIAL AND METHODS

2.1 Experimental animals

Twenty one Male albino rats (150-200 g) were purchased from Faculty of Medicine, Mansoura University, Mansoura, Egypt. The animals were kept on 12 h light/dark cycle with free access to food and water.

2.2 Experimental design

At the day of experiment, all rats were killed by cervical dislocation and were divided into control group (PMI = 0 h) and two main groups; room temperature group and postmortem heat stressed group. Heat stressed-rats were kept at 41ºC postmortem in thermally controlled water bath (Mete et al. 2012). Samples were collected at 0 h (PMI = 0 h) in control group and at 1, 3 and 6 h postmortem in other groups. From each rat a part from its liver was collected for analysis of gene expression. Each rat was placed in an individual plastic bag. All experimental procedures were approved by the Animal Care Committee of Mansoura University.

2.3 RNA isolation and cDNA synthesis

Tissues from liver were homogenized (100 g/1 ml) in Trizol™ reagent (Invitrogen, UK) according to manufacturer instruction (Simms et al. 1993). The concentration of RNA was detected using nanospectrophotometer (Q5000 UV-Vis spectrophotometer, San Jose, USA) to check RNA purity and concentration. An equivalent to 1mg of RNA were transferred to cDNA with High Capacity cDNA Reverse Transcription Kit® (Applied biosystem) using random hexamers in a 20 µl reaction volume that was further diluted 1:20 for further downstream analysis.

2.4 Quantification of immune gene using Real time PCR

Gene expression was assessed by quantitative RT-PCR. Primers for genes encode inflammation, apoptosis and stress (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

The application of real-time PCR for amplification and relatively quantify the specified genes in the current study was conducted on Applied bio-system step one (Thermo Fischer Scientific, UK). Real-time PCR was conducted on selected genes listed in table (1) with their primer sequences and accession number on genbank. Real-time PCR was performed using TOPreal qPCR 2x premix (enzynomics, South Korea) with the following cycling condition: Initial denaturation 95ºC for 8 minutes, then followed by 40 cycles of 95 ºC for 40 seconds, 52 ºC for 30 seconds and 72 ºC for 40 seconds, then the reaction was terminated by final elongation cycle at 72ºC for 7 minutes. The expression analysis was done using $2^{\Delta\Delta\ ct}$ method adopted by (Livak and Schmittgen 2001).

Table 1: Sequences of forward and reverse primers used for qRT-PCR Quantitation

Gene	Primer (5´-3´)	NCBI accession numbers
IL-1β	Forward: TGCCACCTTTTGACAGTGATG	NM_008361.4
	Reverse: AAGCTGGATGCTCTCATCAGG	
TNFα	Forward: ACTGAACTTCGGGGTGATCG	NM_001278601.1
	Reverse: CCACTTGGTGGTTTGTGAGTG	
Bcl-2	Forward: CCGGGAGAACAGGGTATGAT	NM_016993.1
	Reverse: CAGGTATGCACCCAGAGTGA	
Caspase-3	Forward: GAATGTCAGCTCGCAATGGTAC	NM_012922
	Reverse: AGTAGTCGCCTCTGAAGAAACTAG	
c-fos	Forward: TGGGTTTCCTTTTTGCAAGACT	NM_008036.2
	Reverse: AGCGTCCCAAGAAATGAGGG	
B-actin	Forward: CACTGTCGAGTCGCGTCC	NM_007393.5
	Reverse: CGCAGCGATATCGTCATCCA	

2.5 Statistical analysis

Normality of quantitative parameters (mRNA expression for apoptotic and inflammatory genes) was visually assessed using normal probability plots and the Kolmogorov–Smirnov test. All experimental data are expressed as mean ± standard error of mean (SEM). To determine the effect of time on postmortem mRNA expression for either room temperature or postmortem heat stress treatments, a mixed model one-way analysis of variance, with time points as repeated measurements, was used. Post hoc multiple pairwise comparisons were done according with a Duncan's multiple comparison test. Pearson's correlation was used to determine the associations between postmortem mRNA expression and PMI either at room temperature or postmortem heat stress treatments. Statistical analyses were done using SAS® (version 9.2, SAS Institute, Cary, NC, USA). For all analyses, $P < 0.05$ was defined as significant.

3. RESULTS

3.1 Postmortem mRNA expression of genes encode inflammation

TNF-α: A significant reduction was observed in postmortem transcript of TNF-α at 1 h (0.2549 ± 0.0362), 3 h (0.2890 ± 0.0257) and 6 h (0.2099 ± 0.0619) PMI compared to control (1.0 ± 0.1956) in room temperature group. Likewise, in postmortem heat-stressed group, mRNA postmortem expression of TNF-α was decreased at 1 h (0.0914 ± 0.0090), 3 h (0.3559 ± 0.0844) and 6 h (0.0357 ± 0.0219) PMI compared to control. The postmortem expression of TNF-α was induced by heat stress at 3 h postmortem compared to room temperature group (Fig. 1a).

IL-1β: Strong reductions were observed in postmortem expression of IL-1β at 1 h (0.0346 ± 0.0042), 3 h (0.0463 ± 0.0037) and 6 h (0.0455 ± 0.0127) PMI compared to control (1.0 ± 0.0221) in room temperature group. In postmortem heat stress group, the postmortem expression of IL-1β was greatly reduced than control at 1 h (0.0259 ± 0.0067), 3 h (0.0599 ± 0.0072) and 6 h (0.0663 ± 0.0205) PMI (Fig. 1b).

3.2 Postmortem mRNA expression of genes encode apoptosis

Bcl-2: At PMI of 1 h, a significant reduction was observed in postmortem mRNA expression of Bcl-2 either in room temperature group (0.2546 ± 0.0361) or in postmortem heat–stressed group (0.1015 ± 0.0231) compared to control (1.0 ± 0.0719). At 3 h, an increase was detected in room temperature group (6.4424 ± 0.7125) and in heat-stressed group (2.2383 ± 0.4763) compared to control. At 6 h postmortem, further increase was observed in room temperature group (10.7520 ± 0.3655), whereas in postmortem heat-stressed group, the thanato-transcript of Bcl-2 was significantly reduced (0.1824 ± 0.0171) compared to control group (Fig. 2a).

Caspase-3: In room temperature group, thanato-transcript of Caspase 3 was reduced at 1 h PMI (0.2923 ± 0.0678) and was strongly induced at 3 h (5.0696 ± 0.3937) and 6 h (4.8094 ± 0.2164) postmortem compared to control (1.00 ± 0.1546). Postmortem heat stress increased postmortem mRNA expression of Caspase 3 at 1 h (4.6582 ± 0.8594), 3 h (2.7853 ± 0.3320) and 6 h (4.2075 ± 0.3264) PMI compared to control (Fig. 2b).

3.3 Postmortem mRNA expression of c-fos

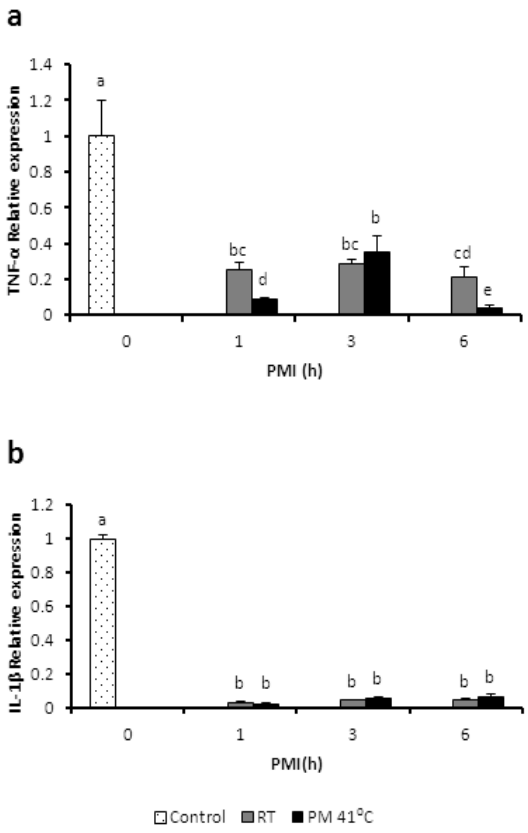

Figure 1: Hepatic postmortem mRNA expression of TNFα (a) and IL-1β (b) of the three groups; control, room temperature and postmortem heat stress groups (n = 3/group). Results are presented as means ± SEM. Means with different superscripts are significantly different ($P < 0.05$).

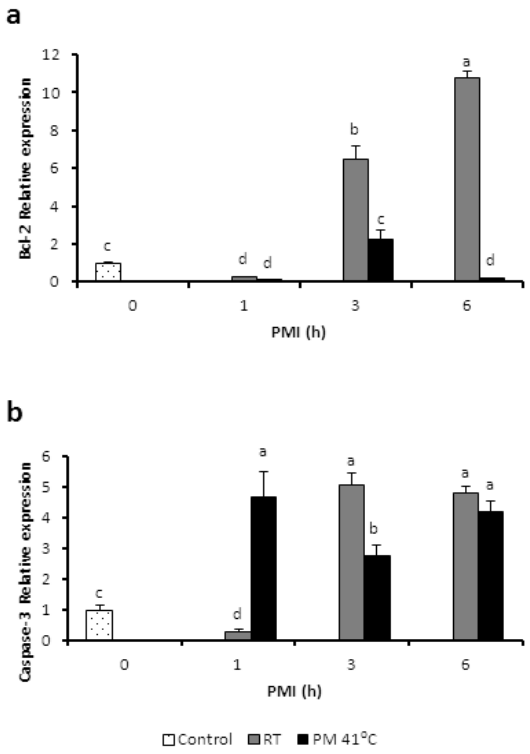

Figure 2: Hepatic postmortem mRNA expression of Bcl-2 (a) Caspase 3 (b) of the three groups; control, room temperature and postmortem heat stress groups (n = 3/group). Results are presented as means ± SEM. Means with different superscripts are significantly different ($P < 0.05$).

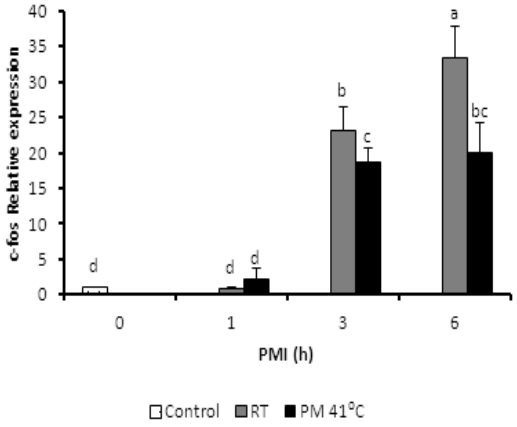

Figure 3: Hepatic postmortem mRNA expression of c-fos of the three groups; control, room temperature and postmortem heat stress groups (n = 3/group). Results are presented as means ± SEM. Means with different superscripts are significantly different ($P < 0.05$).

Table 2: Correlation between postmortem mRNA expressions and PMI in liver of rats

Genes	Room temperature		Postmortem heat stress (41ºC)	
	Correlation	P value	Correlation	P value
TNFα	-0.063	0.675	0.318	0.232
IL-1β	0.225	0.124	0.818	< 0.0001***
Bcl-2	0.373	0.009*	0.574	0.024*
Caspase-3	0.263	0.071	-0.156	0.586
c-fos	0.556	< 0.0001***	0.589	0.019*

ostmortem mRNA expression of c-fos was reduced at 1 h PMI (0.8220 ± 0.1494) and increased at 3 (23.1829 ± 3.3418) and 6 h (33.3032 ± 4.6362) in room temperature group compared to control (1.00 ± 0.0740). Postmortem heat stress increased the expression of c-fos in a time-dependent manner (2.2433 ± 1.4022, 18.5874 ± 2.0478 and 19.8994 ± 4.3167) at 1, 3 and 6 h PMI, respectively compared to control. Postmortem heat stress reduced c-fos expression significantly at 3 and 6 h postmortem compared to room temperature group (Fig. 3).

3.4 Correlation between postmortem mRNA expression and PMI

Positive correlations were found between hepatic postmortem expressions of IL-1β, Bcl-2 and c-fos and PMI in postmortem heat stress group. In room temperature group, positive correlations were observed between PMI and mRNA expressions of Bcl-2 and c-fos (Table 2).

4. DISCUSSION

Discovering hundreds of genes with variable functions remaining awake as well as still operative after death create a new arena for scientific researchers particularly in forensic medicine (Pozhitkov et al. 2017). Thanatotranscriptome can provide a novel approach for estimation of PMI, give suggestions related to possible cause of death and deliver a new definition for death (Ikematsu et al. 2005; Zhu et al. 2008; Schober et al. 2015). It was recently revealed that in first postmortem 48 hours, some genes continue to be expressed inside cells (Pozhitkov et al. 2017). Postmortem up-regulation of mRNA transcripts proposes that the cells are still having energy reserve and resources for functioning and manufacturing new molecules for a period of time after death. Postmortem mRNA expression of myosin light chain 3 (*Myl3*), matrix metalloprotease 9 (*Mmp9*), and vascular endothelial growth factor A (*Vegfa*) genes was increased in body fluids 12 h after death (González-Herrera et al. 2013). We studied postmortem mRNA transcripts relating to inflammation, apoptosis and stress up to 6 h after death in liver of rats using qRT-PCR.

In the current work, postmortem mRNA expressions of proinflammatory genes (Il-1β and TNFα) were decreased at all-time points of PMI, with significant reduction in TNFα expression at 1 and 6 h PMI in postmortem heat stress group compared to room temperature group. Hepatic postmortem expression of IL-1β showed positive correlation with PMI in postmortem heat stress group.

In consistence, rats exposed to ante-mortem thermal stress (41ºC) showed higher expression in splenic IL-1β than in non-heated splenic-enervated rats (Ganta et al. 2004). Also, pronounced increases in IL-1β and TNFα mRNA expressions were observed in mice hippocampus exposed to heat (43ºC) (Lee et al. 2015). Thus, the thermal stress was able to affect gene expression of the proinflammatory cytokines, under investigation, even after somatic death, proving that there was still a response from the cells to heat stress.

On the other side, most of genes implicated in inflammation were up-regulated within 1 h after death in mice and continued up to 12-48 h (Pozhitkov et al. 2017). The number of genes encodes inflammation reach their peaks at 1 to 4 h postmortem, reflecting rapid and robust inflammatory event in mice (Pozhitkov et al. 2017). In zebra fish, IL-1β and TNFα mRNA expressions were up-regulated at 4 and 12 h postmortem, respectively (Pozhitkov et al. 2017). The variation in the upregulation of these inflammatory genes suggests an underlying regulatory network is involved in organismal death.

In the present work, the apoptotic genes were reduced in first hour after death and were induced at 3 and 6 h

in room temperature group. This outcome could be attributed to the mechanisms that regulate the balance between anti- and pro-apoptotic pathways are still operative after death as well as struggling of the cells to repair common signaling pathways. Likewise, the anti-apoptotic genes showed various responses in thanato-transcriptomic study applied on hepatic tissues from human cadavers (Javan et al. 2015). Postmortem expression of Bcl-2 was down regulated while XIAP was up-regulated with increasing time passed after death (6 h) compared to control. Additionally, the pro-apoptotic genes, like Caspase 3, were over expressed with increasing PMI (Javan et al. 2015). Postmortem heat stress reduced the expression at 3 and 6 h time points compared to room temperature group. Hepatic postmortem mRNA expression of Bcl-2 showed positive correlation with PMI. Likewise, induction of ante-mortem thermal stress (42ºC for 1 h) stimulated apoptosis in developing rat brain, particularly in neural regions with high proliferative activity (Khan and Brown 2002). Alternatively, adult neural cells did not show apoptosis in response to such thermal stress, but apoptosis was observed in non-neural tissues (Khan and Brown 2002).

On the other hand, pro- and anti-apoptotic gene transcripts in mice were increased after death starting at 0.5 h and continued till 48 h postmortem (Pozhitkov et al. 2017)**.** In zebra fish, taking in mind species variability, both anti-apoptosis *Jdp2* and 553 pro-apoptosis *Acer3* genes were up-regulated within 0.1 h postmortem. The upregulation of these genes was followed by the upregulation of five pro-apoptosis genes and one anti-apoptosis gene within 0.3 to 0.5 h (Pozhitkov et al. 2017). A time-dependent increase in mRNA levels of PTEN and FasL genes (both have roles in cell death signaling up to 6 h postmortem) in muscles of rats with abrupt decrease at 8 h after death. A correlation between each gene with time elapsed since death till 6 h was found (Zapico et al. 2014). The quick reduction in the mRNA levels is probably due to degradation of RNA as a result of the advancement of the autolysis process (Zapico et al. 2014).

Immediate-early genes (IEGs) are a group of rapidly and transiently transcribed genes in response to cellular external or internal stimuli. One of the most famous IEGs is c-fos that is rapidly activated either in neural or non-neural cells. C-fos plays a vital role in various cellular events, comprising cell survival, proliferation and differentiation, and is used as a marker for neuronal activation (Sagar et al. 1988; O'Donnell et al. 2012).

We investigated the effect of postmortem thermal stress on c-fos thanatotranscriptome. The results proved presence of c-fos in liver in non-stressed rats (room temperature group) at all-time points after death, with higher c-fos postmortem expression levels at 3 and 6 h of PMI. Postmortem heat stress reduced hepatic expression of c-fos 3 and 6 h PMI compared to room temperature group.

This study is the first record for analyzing postmortem hepatic expression of c-fos. It was demonstrated that c-fos was induced after exposure to thermal stress either in vivo or in vitro (Andrews et al. 1987; Lin et al. 1999). Rats exposed to a temperature of 37ºC for 0.5 to 3 h showed induction of c-fos in different brain regions at all-time points (Bratincsák A and Palkovits 2004). In an earlier study, c-fos expression was measured in brain and alveolar tissues of mice either after decapitation or mechanical asphyxia. In both brain and lung, mechanical asphyxia significantly induced c-fos levels (Ikematsu et al. 2008). In alveolar epithelial cells, c-fos protein showed feeble positive expression in sudden death cases while bathing (hot water bath). Moreover, c-fos was expressed in hippocampus in patients expert a cardiac disease several hours prior to death while most of individuals showed no expression of c-fos in their neuronal tissue, suggesting that drowning itself may not increase c-fos expression (Sakurada et al. 2014). Alternatively, c-Fos expression was induced by rapid hypothermia in rats' forebrain up to 3 h under conscious restraint water-immersion stress, whereas unstressed rats did not show c-fos expression in their brains reflecting non-stressed basal conditions (Zhang et al. 2009).

Thus, after somatic death, the expression of c-fos has been proved to be induced, authorizing that the expression of IEGs changed after death and that the patterns of alterations varied according to the cause of death as well as the type of organ examined (Ikematsu et al. 2008). In other words, we could state that the response of such genes (as c-fos) varied according to the type of stress. It was proved that c-fos was expressed after death in response to different stressors either before or after death, but the patterns of its expression were greatly differs with PMI.The present work evidently indicates that postmortem thermal stress is a direct inducer of c-fos (IEG) in the liver of rats compared to control. c-fos was expressed in a different pattern in relation to PMI, with positive correlation between hepatic postmortem mRNA expression of c-fos and PMI at both groups, meaning that with increasing time after death, c-fos expression

increased. Also, we proved the presence of c-fos in liver under basal conditions. Indeed, it is challenging to rely on the expression of certain IEGs to predict the cause of death rather than the time of death, as their expressions were established along with different causes of death, but the patterns by which the expression of genes were altered with time after death (PMI) is the issue.

In cases where the gene transcripts were induced, it might be the synthesis rate of mRNAs exceeded that of the degradation rate (Neymotin et al. 2014), or due to variance stability of cells types with postmortem time (Babapulle and Jayasundera 1993), in other words, cells varied in their ability to sustain the event of death. Also, this study was considered as a proof for persistence of some cell types alive for a time after death and their ability to synthesize mRNA for up to 6 h PMI. Moreover, the increase that was observed in postmortem expression of c-fos could explain the reductions in genes encode inflammation, as it was proved that c-fos has an anti-inflammatory action via suppression of NF-kB activity (Ray et al. 2006).

Put together, the postmortem expressions of genes encode inflammation, apoptosis and stress (represented in c-fos) were expressed during normal conditions as well as under stressful circumstances. Also, there was a degree of correlation between postmortem mRNA expressions of specific genes with PMI. To our knowledge, this is the first study that testified the effect of postmortem heat stress on mRNA expression of particular genes. The concept is novel and there are still many issues that are needed to be evaluated to better understand thanatotranscriptome and its applications particularly in forensic science via establishing with more precision the PMI in hours not in days, which can be useful for investigations to renovate the conditions of death.

Conflict of interest:

The Authors declare that there is no conflict of interest.

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