Science & Justice 59 (2019) 418–424

Contents lists available at ScienceDirect

Science & Justice

journal homepage: www.elsevier.com/locate/scijus

"The big sleep: Elucidating the sequence of events in the first hours of death to determine the postmortem interval"

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ARTICLE INFO

Keywords:
Postmortem interval (PMI)
Autophagy genes
Oxidative stress
Melatonin
Melatonin receptor 2

ABSTRACT

Recent developments on postmortem interval estimation (PMI) take an advantage of the autolysis process, pointing out to the analysis of the expression of apoptosis and autophagy genes towards this purpose. Oxidative stress plays a role in this signaling as a regulatory mechanism and/or as a consequence of cell death. Additionally, melatonin has been implicated on apoptosis and autophagy signaling, making melatonin a suitable target for PMI determination. The aim of this study was to investigate the early PMI through the analysis of the expression of autophagy genes as well as oxidative stress and melatonin receptor. Our results demonstrated a rapidly increased on the expression of autophagy genes according to the expected sequence of events, then a marked decrease in this expression, matched with the switch to the apoptosis signaling. These results revealed potential candidates to analyze the PMI in the first hours of death, helping to estimate the time-since-death.

1. Introduction

In forensic science, the accurate determination of the time-since-death is crucial for elucidating possible criminal acts and determining appropriate civil repercussions [1]. Currently, the determination of postmortem interval (PMI) is based on the different changes that a corpse suffers after death [2–6]. Current research is focused on improving this estimation based on quantifiable approaches, leading to the field called "thanatochemistry" [7]. Under this term several techniques have been proposed to determine the PMI based on chemical changes by taking into account influencing factors, such as temperature and humidity [8–15]. In addition, current trends use the "thanatomicrobiome" to pursue this purpose [16,17].

Furthermore, some reports have recently demonstrated the utility of RNA-based analyses in the body to determine the time-since-death. The majority of studies have been focused on analyzing the stability of RNA postmortem as a diagnostic tool [18–23]. Only our previous work [1] and a few recent studies [24,25] revealed the usefulness of RNA expression analysis for PMI estimation.

Decomposition begins approximately four minutes after death with a process called autolysis, inducing destructive changes in cells. Nevertheless, the nucleus remains intact until four days after death, thus allowing the application of classic methods of cellular and molecular biology for PMI estimation and the study of potential signaling pathways during this period. In fact, our study as well as recent studies [24,25,1] analyzed the expression of various genes related to apoptosis and autophagy with the time-since-death.

Autophagy (also called Macroautophagy) is an essential and multistep process that maintains cellular homeostasis via the degradation and recycling of long-lived proteins, intracellular aggregates and damaged organelles [26]. The hallmark of this process is the formation of double-membrane vesicles (autophagosomes) that non-selectively sequester cytoplasmic components, delivering them to the lysosome or vacuole for degradation. Hence, initiation, nucleation, elongation and expansion of the phagophore assembly site, formation and maturation of the autophagosomes, fusion with the lysosomes and digestion are steps involved in the autophagy process [27–29]. This is a very complex mechanism regulated by numerous factors, such as mammalian target

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https://doi.org/10.1016/j.scijus.2019.03.001
Received 14 December 2018; Received in revised form 24 February 2019; Accepted 3 March 2019

of rapamycin (mTOR), protein 53 (p53), B-cell lymphoma 2 protein (Bcl-2), ribosomal protein S6 kinase (p70S6 kinase), class III phophoinositide-3-kinase (PI3K-III), Beclin-1 and death-associated protein kinase (DAPK) [29]. At the molecular level, autophagy is controlled by AuTophaGy-related genes (ATG) and their respective Atg proteins. Among them, it is well established that autophagy depends on Atg5/Atg7, is associated with microtubule-associated protein light chain 3 (LC3) truncation and lipidation. It may originate directly from the Endoplasmic Reticulum (ER) membrane and other membrane organelles, although other independent pathways have been proposed [30].

Autophagy is regulated by numerous stresses such as nutrient starvation, hypoxia, ATP/AMP ratio, intracellular Reactive Oxygen Species (ROS) levels, bacteria and virus infection or chemical drugs [26]. In autophagy signaling, ROS seem to play a role in its regulation directly through oxidation of ATG4 [31], indirectly by AMPK [32] or through the regulation of NF-kB [33,34].

Melatonin is an endogenous indolamine, which is produced by the pineal gland with a threshold at night [35–39]. It is highly conserved throughout evolution. Its lipo- and hydrosoluble nature, allows it to cross all biological membranes, including hematoencephalic barrier [40–42]. Its synthesis has been widely observed in retina, digestive tract and other organs [43]. Melatonin was initially studied as a key regulator of circadian rhythms and sleep [44] and a modulator of the reproductive behavior in animals with seasonal reproduction [45,46]. Later on different studies demonstrated a broad spectrum of melatonin functions including antioxidant [47–50], immunomodulatory [51,52], antitumor activities [53,54], with both oncostatic [55–57] and cytotoxic effects [58–61]. Besides, recent studies pointed out the role of melatonin on the regulation of autophagy [62,63], and this effect seems to be mediated by melatonin receptors [63].

Thus, our previous data on the relationship of apoptosis signaling with the PMI [1] together with recent works showing the implication of autophagy genes towards this estimation [24,25] prompted us to investigate the early postmortem interval, between 2 and 8 h, through the analysis of the mRNA expression of autophagy genes, and to elucidate the possible role of oxidative stress and melatonin receptor in this signaling.

2. Materials and methods

2.1. Animal protocol

Adult male Wistar rats (n = 4; 3 months old) were housed individually under standard lighting (light/dark periods of 12 h), temperature (22–24 °C), and relative humidity (55–65%) conditions. All rats were housed and handled daily under laboratory conditions for at least 15 days before the study. Water and food (Harlan 2014) were available ad libitum. All procedures were conducted in accordance with the guidelines set forth in the Care and Use of Animals. The experiment was developed in the same conditions of light, temperature, and relative humidity described above. Rats were placed in supine position in the bench and killed under anesthesia with intraperitoneal injection of ketamine (100 mg/kg of body weight)/xylazine (10 mg/kg of body weight) and left at room temperature between 0 and 8 h postmortem. Immediately after death, as a time 0 or control, 20 mg of gastrocnemius muscle were biopsied from each rat. This tissue is mainly used in rigor mortis studies [64,1], and it is easy to access and take several samples from the body. From 2 until 8 h after death, 20 mg of this muscle were collected bi-hourly.

2.2. RNA isolation and quantification

Ten milligrams of each time-sample were homogenized and total RNA extracted using TRIzol (Thermo Fisher Scientific, Wilmington, DE, USA), according to the manufacturer's protocol.

RNA quantification and quality were assessed using NanoDrop 2000c (Thermo Fisher Scientific, Wilmington, DE, USA). The RNA levels were measured as a concentration (ng/μl).

Table 1
Primers used for RT-PCR.

Primers name	Sequence 5′ 3′
GAPDH-Fw	CCATGGAGAAGGCTGGGG
GAPDH-Rv	CAAAGTTGTCATGGATGACC
MT2-Fw	ATGTTCGCAGTGTTTGTGGTTT
MT2-Rv	ACTGCAAGGCCAATACAGTTGA
LC3-Fw	ACCCTCCCTGCATGCAGCTGTCC
LC3-Rv	ACCAGGGACATGACGACGTACACAACC
ATG7-Fw	GCTCCTCACTTTTTGCCAACA
ATG7-Rv	GGAGCCACCACATCATTGC
ATG12- Fw	CACCACTGCACCTGCCTCATTTTTTAACTC
ATG12- Rv	ATGGCACACATGGCTGAGGACTACTCTG
BECN1-Fw	GGTAGCTTTTCTGGACTGTGTGCAGCAG
BECN1-Rv	GTCTTCAATCTTGCCTTTCTCCACGTCC

2.3. cDNA synthesis

RNA was subjected to reverse transcription using Superscript II RT-PCR System (Invitrogen Life Technologies), according to the manufacturer's protocol.

2.4. Real-time PCR

Quantitative analysis of mRNA levels for the autophagy genes (LC3, Beclin-1, ATG7 and ATG12), Melatonin Receptor 2 (MT2) as well as the housekeeper gene, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were assessed by real-time PCR in a Step-One Plus Real-Time PCR System (Applied Biosystems), using Power SYBR Green PCR Maxter Mix (Thermo Fisher Scientific, Wilmington, DE, USA) and specific oligonucleotides (Table 1). Thermocycling conditions were as follows: denaturation for 15 s at 95 °C and annealing/extension for 1 min at 60 °C, during 40 cycles. One additional step, a melting curve, was added to distinguish specific from non-specific products and primer dimers. The melting curve was constructed by increasing the temperature from 60 to 95 °C with a temperature transition rate of 0.2 °C/min. Each sample was run in triplicate, and blank controls (no template) were also included. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.5. Oxidative stress

Ten milligrams of each time-sample were homogenized in PBS. Then cell extracts were obtained by adding the homogenized samples into cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) and centrifugation 10 min 14,000 × *g* in a cold centrifuge. Supernatants were recovered and used for oxidative stress analysis in a fluorometric assay using OxiSelect In Vitro ROS/RNS Assay Kit (Green Fluorescence) (Cell Biolabs, Inc. San Diego, CA, USA), according to manufacturer's protocol. Oxidative Stress (ROS and RNS) was analyzed in a Synergy HT (BioTek, Winooski, VT, USA) and measured as Relative Fluorescent Units (RFU).

2.6. Statistical analysis

Plots of mRNA levels and Oxidative stress were performed using Sigma Plot software (Systat Software, San Jose, CA, USA). Statistical analyses were performed using SPSS 15 (SPSS Inc. Version 15.0 for Windows. Chicago, USA). The hypothesis of normality was evaluated using the Shapiro-Wilk test and homogeneity variance by the Levene test. When these two hypotheses failed, statistical analysis was performed using the non-parametric U-Mann Whitney test at 95% level of confidence.

Table 2
Quantification of RNAs extracted at different time points. RNA concentration was measured in ng/μl. TSD Time-since-death.

TSD (hours)	Subjects			
	1	2	3	4
0	700,7	483,75	860,04	784,66
2	1369,31	982,14	1141,82	1456,68
4	887,09	x	1571,39	905,3
6	934,04	1111,85	1282,39	1047,25
8	1472,15	800,9	1622,21	1324,47

3. Results

3.1. Recovery of total RNA

A total of 20 muscle samples were obtained in an 8-h period (4 rats × 5 time-points). RNA quantification showed a certain degree of variability between time periods and also between subjects, although the concentrations in general rounded from approximately 700.7 ng/μl to 1622.21 ng/μl. The lowest concentration was found on Rat 2 and the sample collected at time 0 h (483.75 ng/μl). From the same subject it was not possible to obtain enough RNA at time 4 h (Table 2). Nevertheless, the cDNA synthesis was performed using the same amount of starting RNA (2 μg) from all the samples.

3.2. Analysis of mRNA levels of autophagy genes and Melatonin Receptor 2 by real-time RT-PCR

After the reverse transcription, real-time PCR was performed to analyze the expression levels of the autophagy genes LC3, Beclin-1, ATG7 and ATG12, and Melatonin Receptor 2 (MT2). In addition, we used the reference gene (GAPDH) to normalize possible variations during RNA isolation, retro-transcription, or qPCR efficiencies, as the expression levels of this reference gene remain consistent under experimental conditions or different tissues.

The mRNA expression data for autophagy genes were in good agreement with the sequence of cellular events leading to autophagy (Fig. 1). LC3 expression, implicated in the first phase of autophagy, was found to rapidly and significantly increase with a maximum peak at 2 h that decreased thereafter. The other autophagy-related genes, Beclin-1, implicated at early and later stages, showed a time-dependent increase until 4 h after death. Similarly, ATG7 and ATG12 also implicated at later stages in autophagy signaling significantly increased with a maximum peak at 4 h postmortem.

In contrast, MT2 expression levels markedly changed along time since death. There was a expression peaking at 4 h, followed by a sudden decrease and a second peak at 8 h.(Fig. 2a).

3.3. Oxidative stress production

The production of free radicals (Reactive Oxygen Species, ROS and Reactive Nitrogen Species, RNS) measured as RFU showed a time-dependent increase at 4 h, then a modest decrease at 6 h and finally a further increase at 8 h, which can be correlated with the summit of autolysis process (Fig. 2b).

4. Discussion

The herein presented findings fit well with our previous work on apoptosis signaling and recent studies related to autophagy genes and PMI [1,24]. Thus, we found that the expression of autophagy genes rapidly increased two hours after death. Particularly, the early occurrence of LC3 called “marker of autophagy” [65] which showed its maximum expression 2 h after death, while decreasing thereafter. In contrast, other late-stage autophagy genes showed a maximum increase at 4 h and then their expression decreased. These results are in agreement with the data reported by Javan et al. [24]. Even though they started to study the expression of autophagy genes at 6 h, a similar tendency was observed with time-dependent expression changes of Beclin-1 involved on the initiation steps of autophagy [30], and also ATG7 levels later increasing at 58 h. Of note, these authors found a time-dependent increase of LC3 from 16 h, which was attributed mainly to suppression of autophagic degradation thereby causing accumulation of LC3 protein rather than increased autophagy. However, this could be a consequence of the autophagy process itself and/or a switch between the signaling pathways from autophagy to apoptosis, since a crosstalk between autophagy and apoptosis has been described [66].

Initiation of autophagy is sustained by activation of ULK1 (Unc-51-like Kinase) and ULK2 complexes, which activate the complex of Beclin-1 with Class III phosphatidylinositol 3-kinase (PI3K)/vacuolar protein sorting 34 (class III PI3 kinase/Vps34), inducing the nucleation of the phagophore [67]. After that ATG7 conjugates phosphatidylethanolamine to the LC3-I, forming LC3-II, this protein binds to ATG12-ATG5-ATG16 complex, facilitating the conjugation of autophagosome. The maturation is promoted by LC3, Beclin-1 and the lysosomal membrane proteins LAMP-1 and LAMP-2, the GTP-binding protein RAB7, the ATPase SKD1, the cell skeleton, the pH of lysosomes and possibly presenilin 1 (PS1) [30]. Thus, our results seem to perfectly match with the sequence of events in the autophagy process starting from an early and rapid and significant increase in the expression of autophagy genes LC3 and particularly Beclin-1 involved on nucleation and maturation of autophagy signaling, and according to our previous findings this will be followed by a switch from autophagy signaling to apoptosis [1]. In fact, previous studies confirmed this crosstalk between autophagy and apoptosis based on the regulation of ATG7, ATG12 and Beclin-1 among others [68–70,66]. Accordingly, we detected that the expression of all these genes consistently and significantly increased at 4 h after death and completely decreased thereafter. It is worth mentioning that autophagy can be triggered in cells as an attempt to mitigate specific perturbations of homeostasis or a given stress. However if the stress persists and autophagy is no longer able to support a survival mechanism, cells may respond by activating the process of apoptosis [66]. Our previous work revealed how genes implicated in apoptosis, such as PTEN and FasL, showed a time-dependent increase after death with a maximum peak at 6 h postmortem [1]. Concomitantly, this precisely coincided with a decrease in the expression levels of various key autophagy genes, thus clearly reflecting that the signaling switch from autophagy to apoptosis occurs at this postmortem time.

As described in our previous study [1] the course of decomposition is similar to the process induced when an organ suffers ischemic or anoxic alterations. In fact, several studies proved the activation of autophagy signaling under these conditions [71,72] as well as the role of oxidative stress as a regulatory factor [73,31,74]. We considered that the herein described data on free radical species are in good agreement with our previous findings related to the decrease of PTEN and FasL expression at 8 h. Thus, the early increase of free radicals in this study could be interpreted as a positive regulator of autophagy signaling by oxidation of ATG4 leading to an increased formation of LC3-asssociated autophagosomes [31] or through regulation of NF-κB, leading to the induction of Beclin-1 expression [33,34]. In contrast, the later increase in free radicals observed at 8 h could be due to the apoptosis process itself, leading to the inactivation of PTEN through oxidation [75], which in turns cannot activate FasL, ending to the autolysis-necrosis process. Also, based on the phases of the decomposition process, it is possible that this later increase in free radicals could be due to bacteria metabolism.

Interestingly, the expression of Melatonin 2 Receptor (MT2) parallels free radicals production. This can be explained by the versatile functions of melatonin and its implication in several intracellular processes. Although few works have studied the role of melatonin in

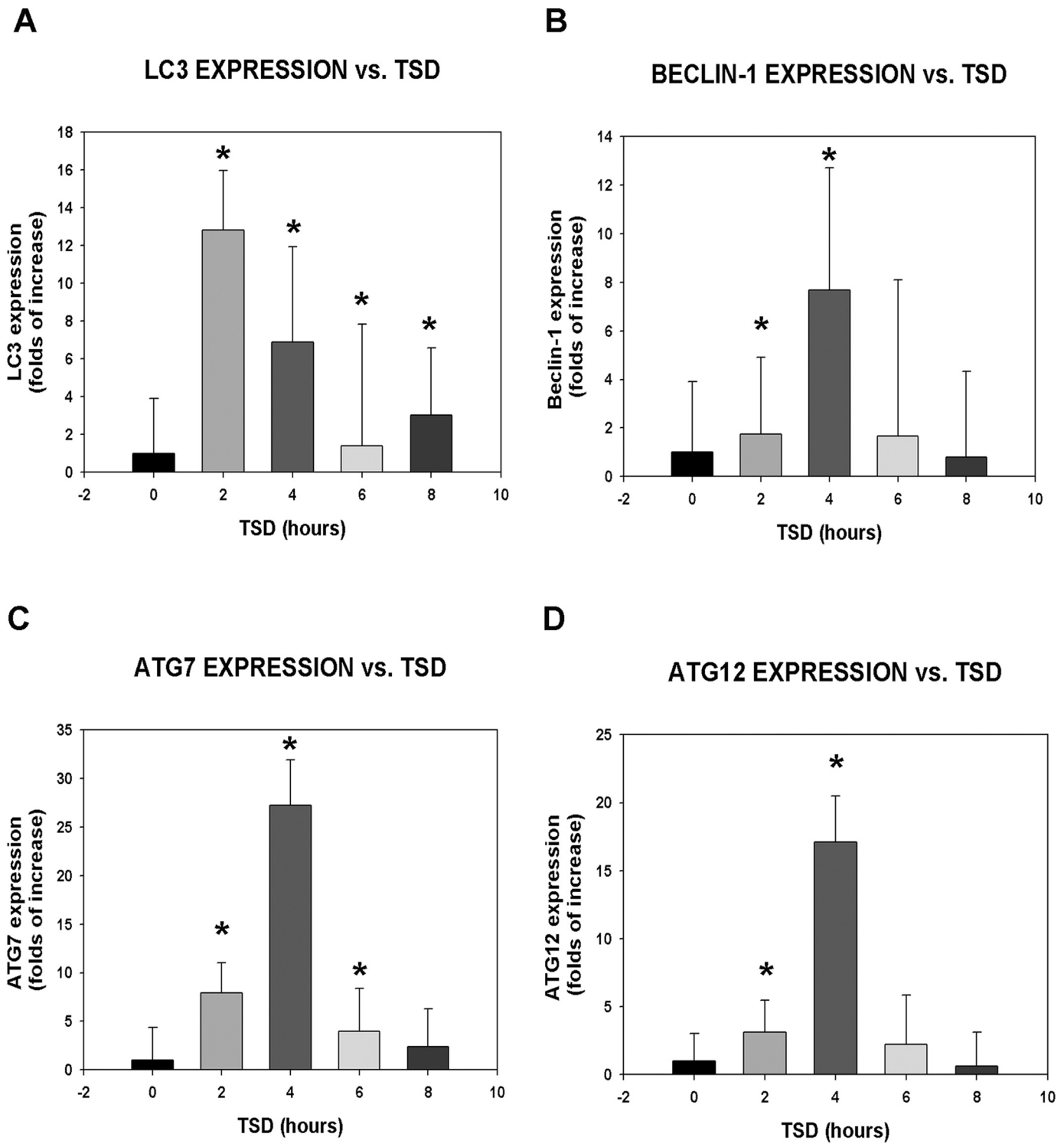

Fig. 1. mRNA levels of LC3, Beclin-1, ATG7 and ATG12 from 0 to 8 h after death. The relative mRNA levels were measured by real-time RT-PCR, and expressed as folds of increase in LC3 (a), Beclin-1 (b), ATG7 (c), ATG12 (d) levels normalized to GAPDH levels and relative to control sample (time 0) assigned value 1. * $p < 0.05$ respect to time 0 h. TSD Time-since-death.

autophagy associated to ischemia [62,76], these reports pointed out to an increase in protein levels of LC3-II and Beclin-1 upon melatonin treatment that also required ROS signaling [62]. In addition, other studies indicated that melatonin induced a partial reduction of autophagic processes [76]. Besides, autophagy suppression could be mediated by melatonin receptors [63]. In fact, recent evidence suggested that neuroprotective actions of melatonin in ischemia/reperfusion are not only the result of its free radical-scavenging properties [77,78]. Also activation of the MT1 and MT2 may be involved [79], inducing antioxidant genes, such as superoxide dismutase and catalase, through receptor-mediated transcriptional signaling [80]. Particularly, activation of MT2 receptor has been linked to melatonin protective function against neural damage that follows ischemic strokes [81,82], thereby promoting neurogenesis and cell proliferation via an MT2 receptor-dependent mechanism [82]. Therefore, our results suggest a dual-role of melatonin on this postmortem signaling. As a consequence of the

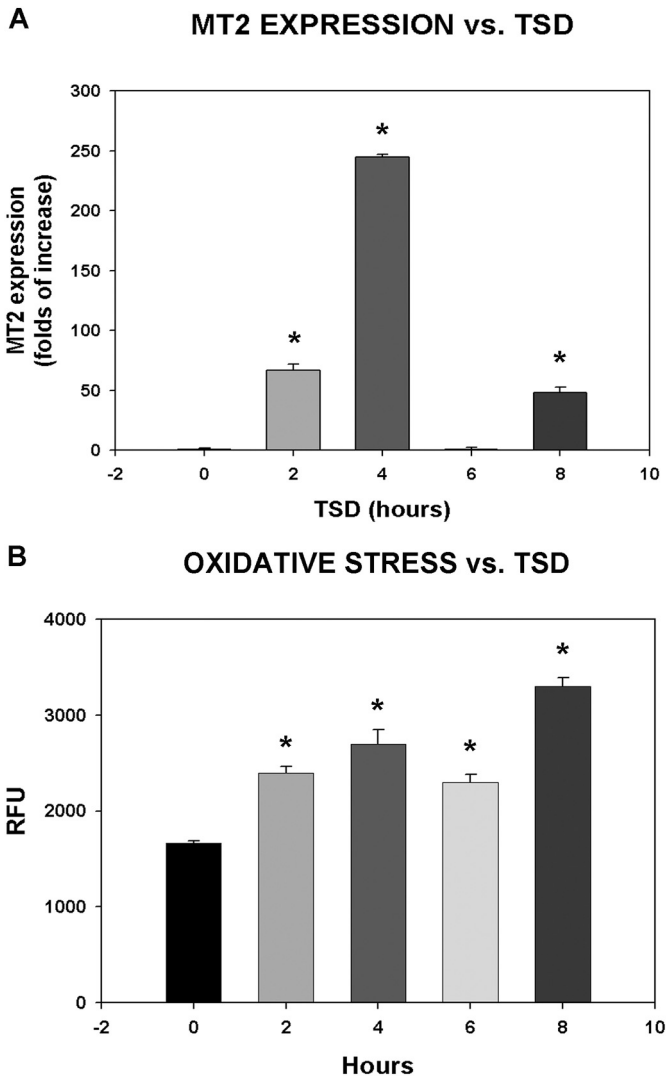

Fig. 2. A. mRNA levels of MT2 from 0 to 8 h after death, measured by real-time RT-PCR as folds of increase normalized to GAPDH levels and relative to control sample (time 0) assigned value 1. B. Oxidative stress production from 0 to 8 h after death, measured by a fluorometric assay as Relative Fluorescent Units (RFU). * $p < 0.05$ respect to time 0 h. TSD Time-since-death.

early increase of ROS production postmortem, melatonin could act as a promoter of autophagy signaling or as a protector of the cell trying to counteract the oxidative stress and/or reduce autophagy.

5. Conclusions

These findings provide a proof-of-principle of the crosstalk between autophagy and apoptosis genes within the first 6–8 h of death, as well as the implication of free radicals and melatonin in these signaling pathways. Besides, this work shows for the first time the usefulness of melatonin receptor 2 towards the estimation of postmortem interval and its application in forensic science.

The role of these genes and molecules in cell death as well as their implication in ischemia makes them potential candidates to analyze the PMI in the first few hours of death, leading to the development of a novel quantitative method to estimate the postmortem interval. Future research may be directed to search for additional markers extending time-since-death estimates.

Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

The authors acknowledge Laboratories of Analytical Biology from Smithsonian Institution for providing the space and equipment to develop this work. For their technical assistance, the authors acknowledge Dr. Saúl Álvarez-Teijeiro and Rocío Granda-Díaz from ISPA (Instituto de Investigación Sanitaria del Principado de Asturias) and Rebecca Stone Gordon from American University.

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