H82 The Big Sleep: Elucidating the Early Sequence of Molecular Events in the First Hours of Death to Determine the Postmortem Interval

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After attending this presentation, attendees will better understand the early sequence of molecular changes triggered immediately after death at the cellular level, ultimately providing a quantitative tool for determination of early postmortem interval.

This presentation will impact the forensic science community by demonstrating the added value of understanding cell death regulatory pathways and the crosstalk between autophagy and apoptosis for the estimation of postmortem interval.

Determination of postmortem interval is analyzed using multidisciplinary approaches. Among them, thanatochemistry methodologies seem to be useful and accurate in respect to classical methods. In this line, some works studied the stability of RNA expression using housekeeping genes, although only a few reports have to date investigated the thanatotranscriptome, thereby analyzing gene expression levels in internal organs of cadavers.

Decomposition begins approximately four minutes after death with a process called autolysis, inducing destructive changes in tissues and cells involving cell death; however, the nucleus remains without alterations until four days after death, thus making feasible the application of molecular and cellular methodologies for time-since-death estimation. In fact, recent trends point to the analysis of the expression of autophagy proteins (thanatophagy) toward this purpose.

Autophagy is often observed in dying cells, trying to mitigate a given stress. If the stress persists, cells can respond by activating processes of apoptosis. Even though autophagy and apoptosis represent distinct cellular processes, the protein networks that control their regulation and execution can be highly interconnected.

Based on these premises, the goal of this research was to study early postmortem interval (between two and eight hours) by analyzing: (1) messenger RNA (mRNA) and protein expression levels of both autophagy and apoptotic genes; and, (2) oxidative stress production and the expression of melatonin receptor, as a regulatory gene implicated in this process.

Four adult male Wistar rats were euthanized at the same time with intra-peritoneal injections of xylazine. The rat bodies were placed in the laboratory at room temperature and 20mg of gastrocnemius muscle were biopsied from each rat at different time points (zero, two, four, six, and eight hours) after death. Each sample was divided in two halves: one for oxidative stress and protein expression analysis and the second half for RNA extraction.

After processing on a standard lysis buffer, supernatants from the first half were divided in two for oxidative stress and protein expression analyses. Proteins were quantified by fluorometric assay, with concentrations ranging between 0.41 mg/ml and 23.18mg/ml. Standard Western blot protocol was used to study protein expression, followed by densitometric quantification.

Oxidative stress production was measured by fluorometric assay and the results expressed as Relative Fluorescent Units (RFUs).

RNA was extracted using Trizol protocol, and quantified by spectrometry, ranging concentrations between 483.5ng/µl and 1,622.1ng/µl. The next step was complementary DNA (cDNA) synthesis by reverse transcription, followed by quantitative Polymerase Chain Reaction (qPCR) to analyze the mRNA expression levels of autophagy (LC3, Beclin-1, ATG7 and ATG12) and apoptotic regulatory genes (FasL and PTEN) as well as the melatonin receptor (MT2). The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, and mRNA levels were normalized to Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) levels.

The mRNA expression data for autophagy genes were in good agreement with the sequence of cellular events leading to autophagy. Thus, LC3 expression, implicated in the first phase of autophagy, was found to rapidly increase with a maximum peak at two hours. The other autophagy-related genes, implicated at later stages, showed a time-dependent increase until four hours after death. At this point, it seems that apoptotic signaling was triggered since FasL and PTEN mRNA levels as well as Cytochrome C protein expression consistently increased until six hours after death. In contrast, oxidative stress similar to autophagy expression pattern was induced until four hours, then it decreased at six hours, and finally it further increased at eight hours, which can be correlated with the summit of autolysis process. Furthermore, as expected, induced MT2 expression parallels free radicals production at four and eight hours. Applying a multivariate regression analysis, a strong positive correlation was found between the expression levels of autophagy and apoptotic genes and the time since death.

These findings provide a proof-of-principle for a novel quantitative method to estimate early postmortem interval based on the crosstalk between autophagy and apoptosis. Future research may be directed to search for additional markers extending time-since-death estimates.

Postmortem Interval, Autophagy, Apoptosis

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