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Estimation of PMI depends on the changes in ATP and its degradation products

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ABSTRACT

Estimating the time since death, or postmortem interval (PMI), has been one of the biggest difficulties in modern forensic investigation. This study tests if the concentrations of breakdown products of adenosine triphosphate (ATP) correlate with PMI in multiple organs from rat. Brains, spleens, and kidneys of rats were harvested at different time points in carcasses maintained at 4 °C or 20 °C. High Performance Liquid Chromatography (HPLC) was used to quantify concentrations of metabolites related to ATP degradation. A *K* value (Kv = 100 × (Hx + HxR)/(ATP + ADP + AMP + IMP + HxR + Hx)) was calculated and correlated with PMI for each organ and temperature. The results indicate that the *K* value is a robust index for the estimation of PMI based on highly significant linear correlations between PMI and concentrations of ATP breakdown products. Compared with other current research methods, the changing tendency of ATP and its degradation products may be potentially a better way for the estimation of PMI in medico-legal practice.
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1. Introduction

Determining an accurate time of death, usually assessed by the postmortem interval (PMI), is a critical component of forensic science with great medico-legal significance [1]. Determining the PMI is complicated by many factors that contribute to postmortem changes [2]. Moreover, there are a multitude of methods to estimate PMI, including cerebrospinal fluid analysis [3]; testing levels of calcineurin A, MARCKS, CaMKII, and protein phosphatase 2A [2]; quantification of melatonin [4]; photometric measurement of color changes in livor mortis [5]; postmortem activity of lactate and malate dehydrogenase in human liver [6]; nerve conduction patterns [7]; morphology of sweat glands [8]; compound muscle action potential analysis [9]; immunohistochemical detection of glucagon in pancreatic α-cells [10] and insulin in pancreatic β-cells [11]; analysis of the cardiac protein troponin I [12], application of biochemical and X-ray diffraction analyses [13]; and Fourier transform infrared spectroscopy [14–17] as well as the EIS technique [18]. However, none of these methods are widely accepted by the industry experts.

Because of its central role in energy metabolism, adenosine-5′-triphosphate (ATP) may be a conserved and highly specific marker for determining the PMI across many causes of death. ATP is metabolized into the following compounds: ATP → adenosine-5′-diphosphate (ADP) → adenosine-5′-monophosphate (AMP) → inosine-5′-monophosphate (IMP) → inosine (HxR) → hypoxanthine (Hx) → xanthine (X). The *K* value takes into consideration the concentration of ATP and its metabolites to measure this pathway's speed of degradation. More specifically, the *K* value (expressed as a percentage) is calculated by dividing the sum of the HxR and Hx concentrations by the concentrations of all the compounds in the ATP catabolic pathway described above. This index is used by the food industry to evaluate the freshness of many species and correlates linearly with the storing time of fish [19–23]. This study described here tests the validity of the *K* value as a reliable measure for the proper estimation of PMI.

2. Material and methods

2.1. Animals preparations

Sprague–Dawley rats (n = 160, 200–220 g) were provided by the Animal Center of Xi'an Jiaotong University. All animal protocols used were approved by the Animal Care and Use Committee of Xi'an Jiaotong University. All rats were anesthetized, sacrificed by means of an overdose of a mixture of isoflurane and oxygen, and then stored in a thermostatically-regulated cabinet maintained at 4 °C (group 1) or 20 °C (group 2). In group 1 (4 °C), the brains,

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spleens, and kidneys of eight animals were harvested at 1, 24, 48, 72, 96, 120, 144, 168, 192, 216, or 240 h following sacrifice. In group 2 (20 °C), the brains, spleens, and kidneys of eight animals were harvested at 1, 3, 6, 9, 12, 24, 36, 48, or 72 h following sacrifice.

2.2. Samples preparation and HPLC analysis

For each subregion in each animal, 100 mg of protein was used for the High Performance Liquid Chromatography (HPLC) analysis. The tissue extract was prepared by homogenizing the sample with 1 ml chilled 0.6 M perchloric acid at 0 °C for 1 min. The homogenate was centrifuged at 4000 rpm for 10 min and the pH of the supernatant was adjusted to pH 6.5–6.8 using 1 M potassium hydroxide solution. The potassium perchlorate that precipitated after standing at 4 °C for 30 min was removed by filtration through Whatman membrane filter. The filtrate was made up to 0.4 ml and passed through 0.20 μm Whatman membrane. The samples were stored at 0 °C until analyzed. Twenty microliters aliquots of the sample extracts were injected into the a Hewlett–Packard (hp) model 1090 HPLC equipped with a metering pump, an integrator (3392A), and an injection valve with a loop capacity of 20 μl. Separation of the nucleotides was achieved on reverse-phase Bondapak C_{18} stainless steel column (3.9 mm ID·300 mm, Water Associations), equilibrated at 35 °C. A mobile phase of 0.04 M potassium di-hydrogen orthophosphate and 0.06 M di-potassium hydrogen orthophosphate dissolved in de-ionized water was used with a flow rate of 2 ml/min. The eluent was monitored at 254 nm with full scale deflection set at 0.2 absorbance units. All solutions were filtered prior to injection onto the HPLC. The peaks obtained from fish muscle extracts were identified by injecting into the chromatograph and comparing against the standard solutions. Standard curves for adenosine 5′-triphosphate (ATP) and each compound involved in its degradation pathway – adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), inosine 5′-monophosphate (IMP), inosine (HxR) and hypoxanthine (Hx) – were constructed in the 0–1 mM range. All nucleotide standards were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). *K* values were calculated according to the following concentration ratio: K value = $100 \times (Hx + HxR)/(ATP + ADP + AMP + IMP + HxR + Hx)$.

2.3. Statistical analysis

Linear regression analysis between each sample's Kv and PMI were performed in animals from both groups 1 and 2. Linear regression analysis yielded the equations ($Y = Ax + B$) with a coefficient of determination (R^2). p values of less than 0.05 were regarded as being statistically significant. All data were analyzed by SPSS software version 11.5.

3. Results

Fig. 1 shows a significant and linear correlation ($r^2 = 0.89$, $p < 0.05$) between the *K* value and postmortem interval of brains of rats maintained at 4 °C. Fig. 1 shows a significant and linear correlation ($r^2 = 0.98$, $p < 0.05$) between *K* values and postmortem intervals of brains of rats maintained at 20 °C. Fig. 2 shows a significant and linear correlation ($r^2 = 0.88$, $p < 0.05$) between *K* values and postmortem intervals of spleens of rats maintained at 4 °C. Fig. 2 shows a significant and linear correlation ($r^2 = 0.98$, $p < 0.05$) between *K* values and postmortem intervals of spleens of rats maintained at 20 °C. Fig. 3 shows a significant and linear correlation ($r^2 = 0.94$, $p < 0.05$) between *K* values and postmortem intervals of kidneys of ras maintained at 4 °C. Fig. 3 shows a significant and linear correlation ($r^2 = 0.94$, $p < 0.05$) between *K* values and postmortem intervals of kidneys of rats maintained at 20 °C.

4. Discussion

Numerous methods to estimate the PMI have been reported [2–18]. However, almost all of them cannot be used in routine forensic practice. We report here that determination of ATP and its breakdown products may be a new and useful method to estimate the PMI.

The *K* value, defined as the amount of non-phosphorylated ATP breakdown products expressed as a percentage of all the ATP breakdown products, has been used by food sciences as a measure of freshness in many species. Research suggests that IMP and AMP are responsible for sweetness, a characteristic of fresh fish muscle, while HxR and Hx is related to the loss of freshness and flavor (bitterness) in some fish species. Quantification of concentrations of ATP and its metabolites, as determined by HPLC, were used here

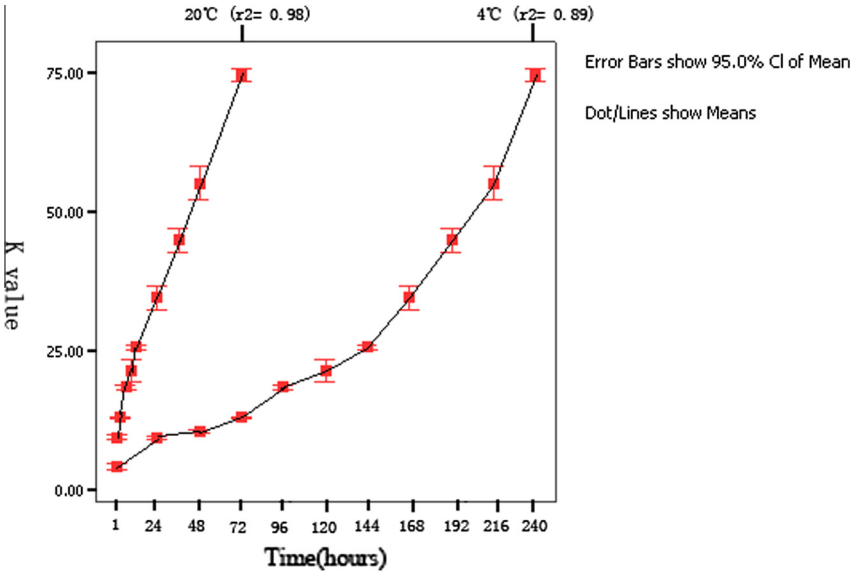

Fig. 1. Brain.

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