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The cholesterol levels in median nerve and post-mortem interval evaluation

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ABSTRACT

Cholesterol levels in the median nerve were studied at various post-mortem intervals (PMIs). Single median nerve samples were collected from the wrists of 36 subjects during forensic autopsies of subjects with known circumstances and times of death. Although the absolute values varied, increments in cholesterol concentration were recorded. Subsequently, 16 subjects who did not suffer of any neurological and/or metabolic diseases with known times and circumstances of death were enrolled. For each enrolled subject, two samples were collected from the wrist at an interval of approximately two hours (t_1 and t_2). The obtained results revealed a gradual increase in cholesterol level with increasing time since death. The cholesterol concentration data obtained for each subject at t_1 and t_2 were correlated with the time since death, a linear interpolation was applied, and the PMI was back-calculated. Similar trends were obtained for the samples collected at similar PMIs; thus, three groups were considered: PMI $<$ 48 h, 48 $<$ PMI $<$ 78 h, and PMI $>$ 78 h. Good correlation coefficients were obtained, especially for the first group (R^2 = 0.9362) for which the PMI could be calculated with an error that ranged from −4 to 5.9 h.

Although it requires further confirmation via analyses of larger numbers of samples, the method proposed here can currently be applied to PMI determinations.

1. Introduction

It is generally accepted that the total blood cholesterol (CHOL) concentrations slightly vary after death [1–4]; however, this parameter is not considered to be sufficiently reliable to be useful in post-mortem interval (PMI) evaluations. Castellano-Arroyo et al. studied the cholesterol levels in human bone tissues and obtained good results [4–6]. These authors studied different lipid fractions extracted from human bone samples, and their thin layer chromatography-based analysis evidenced a decrease in the cholesterol levels in bone tissues sampled post-mortem. Subsequently, the same authors [7] also accounted for protein concentrations and triglyceride levels, re-processed the data, and proposed the following equation for post-mortem interval evaluations: $y = 52.2032 - 7.8213x_1 + 0.6355x_2 - 3.4930x_2$, where x_1 represents the protein concentration, x_2 represents the triglyceride concentration, and x_3 represents the cholesterol level. Based on this equation, the PMI can be estimated with a standard error of 6.47 years.

Further studies performed by Barni Comparini et al. [8] on the cadaveric adipose tissue of 300 corpses evidenced a decrease in oleic acid and an increase in palmitic acid that began in the early hours after from death, and suggested that the latter is potentially useful in thanatochronological evaluations.

Cholesterol (CHOL) levels in the peripheral nerve tissue have been investigated over the last 40 years in both normal and pathological subjects [9–11], and these investigations have provided evidence of slight differences that depend on both age and specific pathologies. Specifically, Pollet et al. reported increases in cholesterol and other myelin components with age and suggested a distinction between patients older and younger than 16 years [10]. Turpin et al. published a study of two patients with degenerative hypertrophic neuropathy that evidenced a noteworthy decrease in lipid content that correlated with the low

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myelin contents in the nervous fibres of such patients [11]. While cholesterol and other lipid components have been studied in biopsy samples (*in vivo*) and in the post-mortem nerve tissue of patients with neurodegenerative diseases, the composition of the peripheral nerve tissues of healthy subjects has not been studied in post-mortem samples. The only data available are the levels in sciatic and femoral nerves that were dissected out as soon as possible after death and reported by Johnson et al. [9]. Starting from these results, the present paper sought to investigate the post-mortem variations in cholesterol levels in the peripheral nerve tissue because after death, myelin and cell wall degradation can lead to increments in the cholesterol levels due to a process similar to the degeneration that occurs *in vivo* after nerve crush.

2. Materials and methods

2.1. Study design

Among all of the cases for which the court requested forensic autopsies, the subjects who died following roadside accidents, fatal trauma, cardiac arrest, or suicide (by hanging or defenestration) were selected so that the exact times and circumstances of the deaths were known. Moreover, the bodies were transferred to the morgue within few hours (30–90 min) after death and maintained at 2 °C until autopsy.

In the first part of the study, single samples of the median nerves were collected from the subjects' wrists during forensic autopsies that were performed at different intervals after death (within the range of 20–256 h). Thirty-six male and female Caucasian subjects (23–53 years old) without clinically evident neurological diseases and with normal physiques in terms of weight and heights relative to the Italian standards were enrolled. All samples were dissected at the beginning of the autopsy, and the differences in the time intervals depended on the different time at which the court requested the autopsies. Subsequently, 16 subjects (25–53 years old, male and female Caucasians with heights and weights that were in line with the Italian standards) without clinical evidence of neurological diseases who did not suffer from any metabolic diseases were enrolled for the study. During the forensic autopsies, two median nerve samples were collected with an intervening interval of approximately two hours. Details of the 16 enrolled subjects and the sampling times are reported in Table 1.

Table 1
Details of enrolled subjects and circumstances of death. All subjects were Caucasian.

Subject	Subject		Circumstances of death	PMI	
	Age	Sex		t_1 (h)	t_2 (h)
1	35	M	Car accident	20	22
2	33	M	Cardiac arrest	21	23
3	44	M	Sudden death	35.0	37.4
4	40	M	Car accident	35.5	38.0
5	29	M	Motorcycle accident	37.0	39.0
6	35	M	Car accident	36.0	38.0
7	44	W	Cardiac arrest	48.1	50.3
8	40	W	Sudden death	49.0	51.0
9	48	M	Cardiac arrest	55	57
10	45	M	Car accident	53	55
11	67	M	Car accident	73.2	75.2
12	55	M	Motorcycle accident	75.0	77.0
13	34	W	Suicide by hanging	122.3	123.3
14	30	W	Suicide by defenestration	120.0	122.0
15	26	W	Suicide by defenestration	138	139
16	33	W	Car accident	135	136

2.2. Reagents and apparatus

Cholesterol and α-cholestane were purchased from Sigma–Aldrich (Saint Luis, MO, US). The BSTFA derivatizing agent was from Acros (Morris Plains, NJ, USA). HPLC grade-solvents were from Carlo Erba (Milan, Italy). The GC/EI-MS analyses were performed using a TraceGC 2000 series gas chromatograph connected to a PolarisQ ion trap mass spectrometer both of which were from ThermoFisher (San José, CA, USA). The gas chromatographic separations were performed using a Rxi®-5MS (30 m × 0.25 mm × 0.25 μm), capillary column (Restek, Bellefonte, PA, USA). The samples were processed and analysed using the Xcalibur software (2.0.7 version) from ThermoFisher.

2.3. Sample collection and GC/MS analysis

For each sample collection, fragments of approximately 1 cm were collected in test tubes, and the samples were maintained at −20 °C until the analysis. Cholesterol quantification was performed on aliquots of approximately 50 mg of the collected nerves to which α-cholestane standard solution was added and used as an internal standard. The samples were incubated with 1 M KOH at 65 °C for 1 h and then kept at room temperature. Two millilitres of bidistilled water was added prior to purification by means of a liquid/liquid extraction with n-hexane. The organic fractions were dried under a nitrogen stream and dissolved in 500 μL of ethyl acetate. Ten microliter aliquots were used for the gas chromatographic/mass spectrometric (GC/MS) analysisafter derivatization with BSTFA (reaction at 70 °C for 20 min). Specifically, the GC/MS *full scan* mass spectrum was recorded in the range of 50–550m/z, and the following ions were extracted: CHOL, m/z (301.1; 352.2; 368.1; and 386.0); and α-cholestane, m/z (217.1; 357.1; and 372.0). CHOL quantification of the median nerves was performed via a calibration curve over the range of 9.25–150 ng/μL. Samples exceeding the calibration curve range were diluted and reanalysed. This method was characterized by a Limit of Detection (LOD) of 0.6 ng/μL calculated with a signal-to-noise (S/N) ratio of 3/1. The Lower Limit of Quantification (LLOQ) was 1.1 ng/μL (S/N = 5/1). The results from the analysis of the quality control samples that were prepared at CHOL concentrations of 100.0, 50.0 and 15.0 ng/μL were used to evaluate the accuracy and the precision (in terms of the CV%) of the analytical method, and values ranging from −4.3% to −2.5% and from 6.0% to 7.2%, respectively, were obtained.

2.4. Statistical analysis

The statistical description of the data was performed with SPSS software version 15.0 for Windows (SPSS ITALIA s.r.l., Bologna, Italy). To eliminate the possible inter-individual variations in cholesterol increments due to differences in the collection times, the cholesterol concentrations were corrected with respect to dt ($t_2 - t_1 = \mathrm{d}t$) by creating the variable CHOL_dt (calculated as $[\mathrm{CHOL}]_t/\mathrm{d}t$). Next, two variables were considered, i.e., CHOL_dt and Time (considered equal to 1 and 2 for t_1 and t_2, respectively). The CHOL_dt was divided into two sub-distributions according to the variable Time. Shapiro-Wilk tests were used to verify the normalities of the CHOL_dt sub-distributions, and t-tests were applied.

3. Results

In the first part of the study, 36 median nerve samples from the wrists of subjects aged between 25 and 53 years who did not suffer from any neurological diseases were collected and analysed. The obtained results evidenced the presence of intra-individual variability in cholesterol levels with concentrations ranging from