THE ROLE OF NASAL CYTOLOGY AND VITREOUS HUMOR DURING FORENSICS PATHOLOGIST'S INVESTIGATION TO DETERMINE THE POSTMORTEM INTERVAL

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ABSTRACT

Introduction*: The Post-Mortem Interval (PMI) estimation is very important in medico-legal practice for the related possible legal repercussions. The most commonly used method to determine the time of death is the Henßge - Madea method, but it has several limitations. Other research criteria include the study of different body fluids, such as vitreous humor (HV), with particular reference to potassium (K^+) levels; however this analyte alone was not sufficient for an adequate estimate of the PMI and therefore further new parameters were taken into consideration.*

Materials and methods*: In this paper we propose, for the first time, the simultaneous analysis of biological matrices represented by the most studied components of the vitreous humor (K^+, Na^+) and by the nasal mucosa aimed at finding a connection between the intravitreal levels of K^+ and Na^+, the degradation of the nasal ciliated cells, the presence of bacterial and/or mycotic colonies for a more accurate estimate of the PMI. The statistical processing was carried out by the "multivariate linear regression" model for the determination of the forecast value of the PMI (expressed in hours) in relation to the values assumed by the empirical variables previously selected.*

Results*: The results obtained using the equation we proposed to calculate the PMI. show a confidence interval of 94% between the values obtained and the known values.*

Conclusion*: The results demonstrate that the chosen matrices could play a leading role in improving the accuracy of the PMI estimate in support of the work of the forensic pathologist in the dating of the time of death.*

Keywords: *nasal cytology, vitreous humor, potassium, degradation cells, Post-Mortem Interval.*

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Introduction

Determining the exact time of death represents one of the biggest issues in terms of forensic pathology and forensic investigation. It often presents many difficulties and limitations for an accurate estimation, specifically caused by the interference of multiple intrinsic and extrinsic factors that occur from time to time, also corresponding with transformative phenomena, even more evident in the cases of cadavers in an advanced state of decomposition[1-3].

During the postmortem phases the cadaver undergoes several alterations, which can be distinguished in consecutive and transformative phenomena, in terms of time. The first group of transformations includes body temperature dispersion, hypostasis and the beginning of cadaveric stiffness; while the second group includes putrefaction, maceration, mummification and saponification[4]. The use of consecutive phenomena is necessary to identify the amount of time elapsed between the subject's death and the autopsy analysis (PMI expressed in hours). The observation of the above-mentioned phenomena is limited to the first 48-72 hours from death, since after the second and third day the transformative phenomena that occur are related to autolysis, loss of cellular membrane permeability and numerous other degenerative processes, which overlap

with the first set of transformations and alter them[5,6].

Based on studies found in the international literature, other than estimation techniques of PMI traditionally used, some biologic parameters have been considered providing a more accurate determination of time of death[7-9].

One of the most studied body fluids is vitreous humor (HV). This element was chosen for its chemical and physical characteristics and because it's found in a well isolated compartment, it remains stable after death, meaning its composition is subject to a slower alteration compared to blood and cerebrospinal fluid, and it's rarely subject to bacterial contamination than other biological matrices even after several hours of the actual end of vital functions[10-13].

After death, the levels of plasma potassium increase progressively and constantly, mainly during the first hours immediately after death, due to the deactivation of the sodium/potassium pump and to the fact that the cell wall becomes semipermeable, allowing potassium to migrate across the membrane reaching a state of equilibrium with the extracellular liquid. This phenomenon is best observed in the vitreous humor given that the intracellular concentration of potassium is 2 to 40 times higher than its concentration in plasma[14,15].

Another promising biological substrate is the nasal mucosa, for which a limited amount of studies has been performed.

The nasal mucosa consists of a pseudostratified columnar epithelium that includes ciliated cells, muciparous cells or Goblet cells, striated and basal cells. The ciliated cells are the most numerous and most differentiated[16].

The goal of this study is to find a correlation between the intravitreal levels of potassium (K^+) and sodium (Na^+) together with the degree of degradation of nasal ciliated cells, including an analysis of the possible presence of bacterial or mycotic colonies, and the postmortem interval; these results will provide new valid parameters useful in medico-legal practice for a more accurate estimation of time of death.

Materials and methods

The research began following approval by the Independent Ethical Committee of Fondazione Policlinico Tor Vergata, and was conducted on deceased subjects with forensic medicine implications for which it was possible to establish the exact time of death and the time of entrance to the morgue.

Six months after beginning the research project, the number of sample subjects was 45; 26 cases of traumatic death and 19 cases of non-traumatic death, which was deemed to be appropriate and satisfactory for analysis. Cases of death due to maxillofacial and suborbital trauma have been excluded because they are not compatible with the collection and the subsequent analysis.

The biological matrices including vitreous humor (VH) and nasal mucosa were collected for the purposes of this study.

Specifically, during the autopsy, vitreous humor was withdrawn from both eyeballs and biological material was immediately frozen at -80°C, while cells from the nasal mucosa were collected using a disposable sterile curette (nasal scraping) and fixed to a glass slide.

The vitreous humor was analyzed at the Clinical Biochemistry Laboratory of the Policlinico Tor Vergata, where the collected samples were gradually defrosted through agitation on vortex and centrifuge at 3000 rpm per 10 min to obtain the supernatant, reducing the interferences caused by the sample turbidity. The resulting matrix was dosed with the intravitreal levels of Na^+ and K^+ using the Abbott® Architect instrument, by indirect ion selective electrodes. The instrument was calibrated according to standard quality procedures. The quantitative determination of electrolytes was obtained by measuring the voltage of the sample compared to the voltage of the standard curve and converted into concentration with unit of measure expressed in mmol/L.

To perform the analysis of the nasal mucosa, the glass slides where stained using the May Grunwald-Giemsa method and analyzed using a 100x optical immersion microscope to observe the morphology of the ciliated cells and putrefactive changes such as ciliocytophtoria, karyorrhexis and the presence of eventual bacterial or mycotic colonies on fifty microscopic fields according to the procedure standardized by the Italian Academy of Nasal Cytology[17].

The result of the reading expresses the presence or the lack of degradation of the ciliated cells compared to the total number of cells observed, degraded and non-degraded. The presence of bacterial or mycotic colonies it was calculated by adding the areas occupied in 50 microscopic fields respectively by bacteria and spores, if present, and dividing the value obtained by the overall surface. The data are reported in the rinocytogram by using degrees from 0 to 4. The interpretative table of the results was

adapted to our study taking as reference the work by Meltzer e Jalowayski[18] (Tab. 1).

INTERPRETIVE TABLE		PERCENTAGE	DEGREE
	Semi-quantitative analysis	**Quantitative analysis**	
CILIATES	Absence	0	0
	Occasional	1-24	1
	Some	25-49	2
	Fair	50-74	3
	Numerous	75-100	4
BACTERIA	Not visible	0	0
	Occasional groups	1-24	1
	Moderate number	25-49	2
	Easily visible	50-74	3
	Cover the entire field	75-100	4
MYCOTIC SPORES	Not visible	0	0
	Occasional groups	1-24	1
	Moderate number	25-49	2
	Easily visible	50-74	3
	Cover the entire field	75-100	4

Table 1: Rinocytogram Interpretation: by Meltzer e Jalowayski (Mod.).

Statistical analysis

The analysis of the results was performed using a "Multivariate Linear Regression" model with the goal of verifying our hypothesis: determining the predictive value of PMI expressed in hours, in relation to the variables that were object of this research.

R^2 of the regression model was calculated, to assess how much our data can be compared to the theoretical pattern. Furthermore, was used the Durbin - Watson statistic to detect the presence of autocorrelation of residues in our regression analysis.

Statistical significance of the linear regression was determined using the ANOVA® method (variance analysis), that allows to compare the variability within the data groups with the variability between the groups.

Finally, the analysis of the residuals provided a normal distribution (Gauss curve or error curve), which highlights the desirable randomness of such variations.

Results

In our sample, the levels of Na in the vitreous humor showed a substantial stability of post-mortem concentrations and only in a minimum percentage of cases did they differ from the average reference values (136 - 145 mmol/L). Therefore it was not taken into consideration among the variables under study. Potassium levels, on the other hand, showed much higher values than the reference range (3,5 - 5,1 mmol/L) and highly correlated with the time elapsed since death.

The elements provided by reading the rhinocytogram show a relationship that proved to be statistically significant between the degree of degradation of the ciliated cells, the relative number of bacterial and / or mycotic colonies and the PMI values expressed in hours.

In the "Multivariate Linear Regression" model, four variables were considered, and their possible interactions compared to the PMI, which is our response variable (Tab. 2). In order to obtain the best definition of the postmortem interval (PMI), in this phase of the study the following interactions were analyzed:

- Degraded ciliated cells K^+
- Degraded ciliated cells - Spores
- Bacteria K^+
- Bacteria - Spores
- Degraded ciliated cells - Bacteria K^+
- Degraded ciliated cells - Bacteria - Spores
- Bacteria - Spores K^+
- Degraded ciliated cells - Bacteria - Spores - K^+

Descriptive statistics			
	Mean	Std. Deviation	N
PMI (h)	79,39400	61,368091	45
RELATIVE CILIATED CELLS	,56280	,437666	45
RELATIVE BACTERIA	,28209	,200883	45
RELATIVE SPORES	,09278	,184259	45
K^+	21,09556	10,002453	45
Na^+	124,77778	15,302835	45
CILIATES K^+ INTERACTION	14,92527	19,409478	45
CILIATES BACTERIA INTERACTION	,23087	,353548	45
CILIATES SPORES INTERACTION	,09991	,247750	45
BACTERIA K^+ INTERACTION	7,26418	7,750428	45
SPORES K^+ INTERACTION	3,02884	7,246208	45
BACTERIA - SPORES INTERACTION	,04389	,090739	45
CILIATES BACTERIA -K^+ INTERACTION	7,08889	14,097010	45
CILIATES BACTERIA - SPORES INTERACTION	,05613	,160661	45
BACTERIA SPORES-K^+ INTERACTION	1,49151	3,618645	45
CILIATES BACTERIA SPORES K^+ INTERACTION	2,11133	6,907319	45

Table 2: Descriptive statistics of the mean values and SD of the estimated variables (PMI (h)) and of the dependent variables used in the model; N is the sample number for each variable.

The R^2 of regression model is 0,916; this value indicates an almost perfect correlation between variable response (PMI) and the covariate variables remaining after the elimination of those unrelated to itself response variable (Tab. 3).

Model summary [g]					
Model	R	R-squared	Adapted R-squared	Std. Error of the estimate	Durbin-Watson d
6	,967[f]	,935	,916	17,761001	2,414

Table 3: R^2 of the regression model.

The Durbin-Watson statistic value is always between 0 and 4; the value obtained in our model is 2. With reference to the response variable, a value of 2 indicates that no autocorrelation appears; in our analysis we demonstrated the non-existence of autocorrelation of the variable PMI (h).

The regression performed is extremely correct as it is highly significant so that the correct setting of the covariate variables can be affirmed as responsible for the values assumed by the response variable. Fisher's F of 49,129 implies, in fact, a p-value <0,001 (Tab 4).

ANOVA[a]						
Model		Quadratic sum	gl	Quadratic mean	F	Sign.
6	Regression	154980,469	10	15498,047	49,129	,000[g]
	Residual	10725,407	34	315,453		
	Total	165705,875	44			

Table 4: Test ANOVA® p value < 0,001.

Table 5 allows us, through the values assumed by the predictors (regression coefficients or regressors) to make estimates on the values assumed by the response variable PMI (h). The coefficients are all highly significant. The linear relationship must be set up as follows:

$$PMI(h) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + ... + \beta_n X_n \quad (1)$$

The equation (1) parameters correspond, with exception to the first parameter (β_0: intercept), to the product of the regression coefficients (or regressors or predictors: $\beta_{1\text{-}11}$) with the assumed values of the variables measured empirically (dependent variable or covariate or concomitant: $X_{1\text{-}11}$).

The PMI (h) can therefore be estimated, case by case, using the data obtained from the reading of the rhinocytogram and from the analysis of the vitreous humor relating to the concentration of potassium.

The values obtained by applying above mentioned equation show a 94% confidence interval between the PMI calculated with the model and the known PMI detected in the cases studied. It follows that the value relating to the average deviation is very limited and equal to 6%.

Coefficients[a]								
Model B		Non-standardized coefficients		Standardized coefficients	t	Sign. Lower limit	95,0% Confidence interval for B	
		Std. Error	Beta				Upper limit	
6	(Constant)	4,002	9,082		,441	,662	-14,456	22,459
	RELATIVE BACTERIA	270,675	68,468	,886	3,953	,000	131,531	409,819
	RELATIVE SPORES	875,210	239,502	2,628	3,654	,001	388,483	1361,938
	CILIATES- K INTERACTION	6,474	1,559	2,048	4,152	,000	3,305	9,643
	CILIATES-SPORES INTERACTION	-1578,293	312,472	-6,372	-5,051	,000	-2213,312	-943,274
	BACTERIA K INTERACTION	-10,485	3,293	-1,324	-3,184	,003	-17,177	-3,793
	BACTERIA SPORES INTERACTION	-2946,512	803,853	-4,357	-3,665	,001	-4580,137	-1312,887
	CILIATES BACTERIA K^+ INTERACTION	-5,153	2,828	-1,184	-1,822	,077	-10,901	,594
	CILIATES BACTERIA SPORES INTERACTION	2759,266	649,150	7,224	4,251	,000	1440,034	4078,498
	BACTERIA SPORES K^+ INTERACTION	87,459	17,340	5,157	5,044	,000	52,220	122,698
	CILIATES BACTERIA SPORES K^+ INTERACTION	-30,511	7,654	-3,434	-3,986	,000	-46,066	-14,956

Table 5: Regression coefficients to estimate PMI.

Finally, the residuals analysis was carried out to verify the validity of the model. The residuals correspond to the difference between the data obtained from the model (theoretical value) and the actual

Statistics of residuals (Dependent variable PMI (h))					
	Minimum	Maximum	Mean	Std. deviation	N
Predicted value	11,86126	296,23792	79,39400	59,348828	45
Residual	-32,377594	58,165581	,000000	15,612791	45
Std. predicted value	-1,138	3,654	,000	1,000	45
Std. residual	-1,823	3,275	,000	,879	45

Table 6: Reliability of the theorical model.

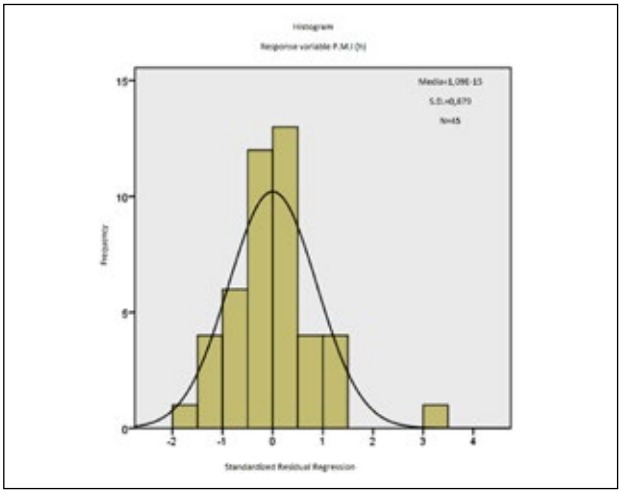

Fig. 1: The residuals analysis, carried out to verify the validity of the theoretical model, shows a normal distribution of residuals within the Gauss curve, demonstrating the good quality of our model; in fact, the more these residuals are normally distributed within the Gauss curve, the more the theoretical model reflects reality.

values of the response variable PMI (h). The more these residues are normally distributed within the Gauss curve the more the theoretical model reflects reality. The graph 1 describes the quality of our model (Tab. 6), (Fig. 1).

Discussion

Among the various mathematical methods suggested in order to determine the most accurate time of death, the most common in medico-legal practice is the Henßge Madea method. The nomogram allows for a real-time determination of thanatology data analysis by measuring rectal temperature, environmental temperature and the cadaver's weight[19]. However, this method presents many limitations due to the elevated number of conditions for which it cannot be used, such as cases of bodies found in particularly sunny locations or with artificial air-conditioning systems; suspicious cadaveric hypothermia; cases in which the location where the cadaver was discovered does not match the location of death; if circumstantial information proves a temperature variation from time of death to time of forensics pathologist's observation. Furthermore, it is not always possible, and often quite difficult, to have the three measurements available used for this specific method, which are necessary to determine PMI, with regards to weight and environmental and cadaveric temperature. These factors cause a limited applicability of this method in the forensics pathologist's routine activity[20].

Vitreous humor (VH) has been the most studied biological fluid for coroner's investigation since the 1960s[21] for its characteristics of stability, ease to collect and anatomical district when compared to blood or cerebrospinal fluid[22,24].

In the last decade numerous studies of tanatochemistry have been conducted[23-26]. Tanatochemistry allows an analytical approach based on the evaluation of modifications of endogenous compounds released, produced or transformed in the body after death[27].

In vitreous humor following death many chemical compounds have been studied such as potassium, chloride, sodium, magnesium, calcium, phosphate, creatinine, lactate and urea[28,29]. The most studied compound and promising for a valuation of time of death has been potassium[30,31,12] but it alone is not enough to establish with the necessary accuracy the PMI and it should be used in association with other parameters[32].

Regarding analytical aspect, new methods and markers have been identified which are useful in the estimate of PMI; in particular, among those concerning the K^+ quantification in vitreous humor are indirect ion selective electrodes method, flame photometry and capillary electrophoresis[21].

For example, Choi et al.[33] used mass spectrometry to evaluate the degradation of skeletal muscle proteins; Sapienza et al.[34] used quantitative magnetic resonance molecular imaging (qMRMI) on animals with muscle tissue similar to that of humans.

Another biological fluid studied has been ammonium produced in the post-mortem period by the degradation of proteins.

Musile et al.[35] used a microfluidic paper to determine ammonium concentration in vitreous humor; Gotthard et al.[36], analyzed the levels of ammonium and potassium in the vitreous humor by capillary electrophoresis. Recently, Risoluti et al.[37] estimated the PMI by analyzing micro and macroelements of the vitreous humor using inductively coupled plasma optical emission spectrometry (ICP-OES). Ferreira et al.[38] studied the quantitative variation of the RNA levels that occur after death in different tissues and their gene expression to obtain an evaluation of the PMI.

The Authors of above mentioned articles have used different types of biological substrates, even simultaneously, and analytical methods alternative to that of Henßge Madea, but all these methods have not provided definitive results applicable in medico-legal practice and they are not validated for tanatochemistry; in addition, they have been conducted on a very limited number of cases[39,12].

In our study we propose for the estimate of time of death the use of two biological matrices: the vitreous humor and the nasal mucosa, which are easy to collect both during the forensic inspection and during autopsy. These materials, analyzed together for the first time, are also simple to store for later analysis and medico-legal investigation.

Potassium and its implications in forensic practice have already been widely debated; on the other hand, nasal cytology has never found application in this particular field to date; in fact the nasal mucosa has been analyzed only in an Italian study conducted on cadavers with the aim of verifying the diagnostic utility of post mortem ciliary motility as a potential tool for PMI estimation for about 12 hours after the subject's death[40].

Figure 2 illustrates what can be observed from the microscopic analysis (100x immersion magni-

fication) of a sample of nasal mucosa relating to a subject with a 122-hour PMI, where degraded ciliated cells, bacterial colonies and mycotic spores are clearly visible.

The results obtained confirm the existence of a high reliability of our model and a significant correlation between the studied variables and the time of death. In fact, all the possible interactions

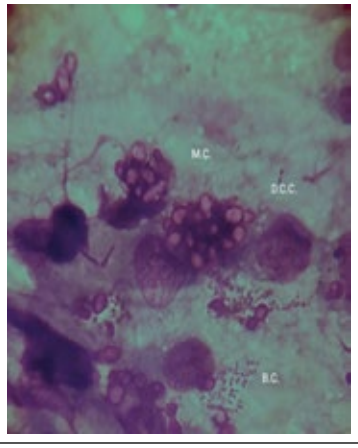

Fig. 2: Microscopic image showing the morphological aspect of the cells of the nasal mucosa at 122 hours after death; loss of cilia and karyoressis of the nucleus (D.C.C.: degradation of ciliated cells) can be observed in nasal hair cells. Generally for high PMI the probability of finding bacterial colonies (B.C.) and mycotic colonies (M.C.) increases, as clearly visible in the image presented.

analyzed between the parameters under study showed a p value <0,01.

The linear equation that we have built to derive the PMI is an excellent starting point in order to improve the accuracy of the estimate of the time of death in support of the classic medico-legal findings carried out during the course of medico-legal investigations.

Conclusions

The equation proposed in our work for the estimate of the PMI as an alternative to the common methods currently used in medico-legal practice to answer the question relating to the time of death has proved to be a valid tool to improve the accuracy in determining the time lapse between the death of a subject and autopsy assessment.

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