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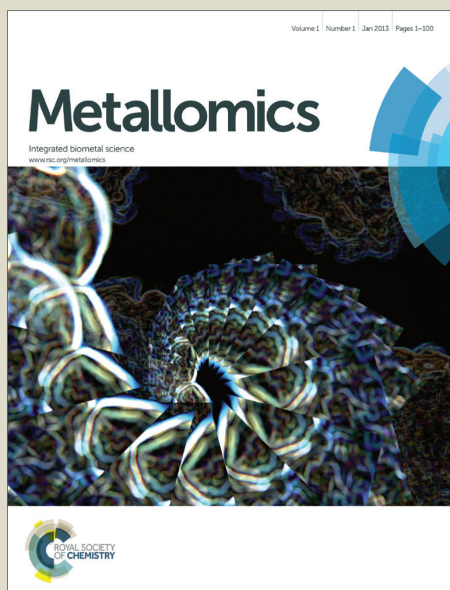

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ARTICLE TYPE

Metals and (metallo)proteins identification in vitreous humor focusing on post-mortem biochemistry

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This work describes the evaluation of metals and (metallo)proteins in vitreous humor samples and their correlations with some biological aspects in different post-mortem intervals (1-7 days) and taken into account decomposing and non-decomposition bodies. After qualitative evaluation of the samples involving 26 elements, representative metal ions (Fe, Mg and Mo) are determined by inductively coupled plasma mass spectrometry after using mini-vial decomposition system for sample preparation. A significant trend for Fe is found over time post-mortem for decomposing bodies, once that a significant increase of iron concentration is noted when comparing samples from bodies presenting 3 and 7 days post-mortem. An important clue to elucidate the metals role is the coupling of liquid chromatography with inductively coupled plasma mass spectrometry for identification of metals linked to proteins, as well as mass spectrometry for the identification of these proteins involved in the post-mortem progress.

Introduction

Biological and chemical changes have been monitored for criminal investigations and natural deaths in post-mortem interval (PMI). This approach is becoming increasingly essential to the routine activities involving forensic pathology, and considerable progress has been made over the past years.[1-3] Several body fluids can be the target for this task, such as blood, cerebro-spinal fluid, and vitreous humor. Many fundamental constituents in these targets are metal ions, proteins, and wide range of biomolecules. More than analytes, Metallomics can offer a new insight in the knowledge of biochemistry of the death, since this area has the ambition to study the entirety of components in the biological system, searching for the biological comprehension.[4-6]

Vitreous humor has been used for several decades in analysis of chemical changes and it presents some attractive advantages compared to other body fluids: (I) it has a decelerated diffusion, which is responsible for keep the integrity of constituents; (II) even in late post-mortem intervals, vitreous was hardly contaminated; and (III) it is also subjected to less bacterial degradation due to the protected environment inside the eye (Figure 1). Contrasting to the blood, vitreous humor can be considered a site well suitable for investigations of chemical and biochemical changes after death.[7,8] Vitreous humor is composed by 99% of water, with the remaining 1% made up of sugar, salts and proteins. The vitreous humor is also held together by a delicate network of fibrils, and its viscosity is due to the presence of hyaluronic acid and collagen.[9-15]

Integrating chemical and biochemical scopes, the aim of this work was therefore to present an comparative ionomic approach and protein analysis to investigate possible pattern of metals in vitreous humor, as well as to establish some correlations between decomposing and non-decomposing bodies at different post-mortem intervals (1 to 7 days). For this task, a multielemental analysis was previously carried out by ICP-MS, and Fe, Mg and Mo, they were quantified in vitreous humor. Additionally, the identification of some proteins involved in the transport of metals to inside the eyeball was carried out using LC-ICP-MS and ESI-LC-MS/MS.

Experimental

Sample collection

The vitreous humor samples were obtained from 11 bodies attended by team of forensic medicine from the West zone (decomposing bodies) and South zone (non-decomposing bodies) of the São Paulo city, from the Medico-Legal Institute of Police

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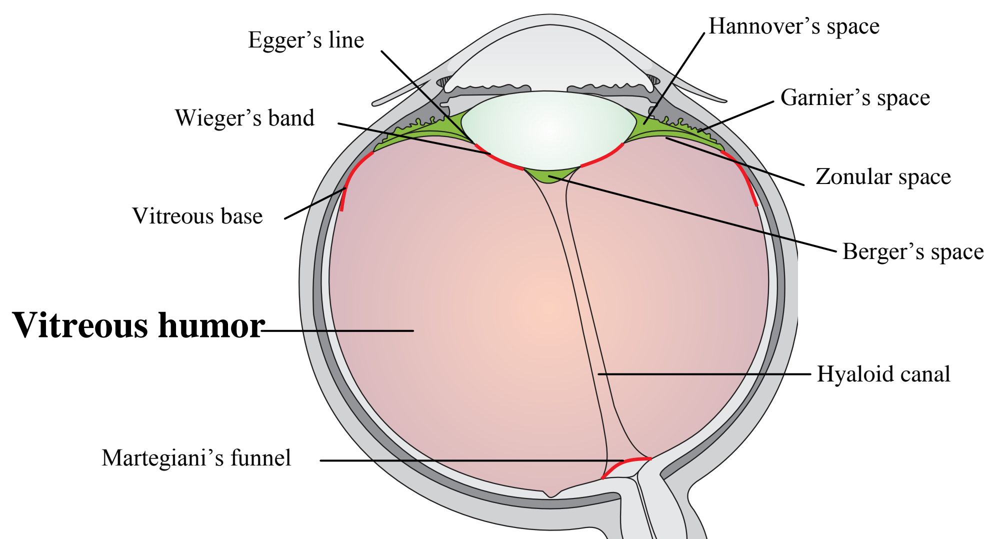

Figure 1 Vitreous Humor and adjacent spaces. Adapted from reference 16.

Table 1 Vitreous humor samples profile (pool) and post-mortem interval.

	Identification of samples	Post-mortem interval
CA	2091 – R/M/NDB	1 DAY
	2091 – L/M/NDB	
	2093 – R/F/NDB	
	2093 – L/F/NDB	
	2094 – R/F/NDB	
	2094 – L/F/NDB	
A1	2209 – R/DB	1 DAY
	2209 – L/DB	
	2210 – R/DB	
	2210 – L/DB	
A2	2202 – R/DB	2 DAYS
	2202 – L/DB	
	2206 – R/DB	
	2206 – L/DB	
A3	269/12 – L/DB	3 DAYS
	278/12 – R/DB	
	278/12 – L/DB	
A4	248/12 – R/DB	7 DAYS
	248/12 – L/DB	
	256/12 – R/DB	
	256/12 – L/DB	

R – right / L – left eye, DB - decomposing bodies, NDB - non-decomposing bodies, M – male / F – female, CA – control group, and case sample A1, A2, A3 and A4

Technical Scientific Superintendence — SPTC of São Paulo, Brazil. The case samples (vitreous humor) were collected by puncture using a trocar with 0.51 mm external diameter, trifacetaded and bevelled metal mandrel with external diameter of 0.42 mm. Ideal access to bisect the angles of the upper quadrants of the eye, with the help of eyelid retractors, thus escaping from the most vascularized tissues such as the outer corner of the eye.

All material removed was separated and identified according to the left and right eye, and also depending on the body gender. Then, the material was collected with a syringe with 5 ml capacity to set to a needle of 0.80 x25mm and packed in gray-top tubes

Table 2 Instrumental parameters used in ICP-MS and LC-ICP-MS analysis.

Size Exclusion chromatography conditions	
Column	Superdex 200 HR 10/30
Column dimension	10 x 300 mm, 13µm average particle size
Mobile phase	100 mmol L^{-1} ammonium acetate buffer, pH 7.2
Elution	Isocratic
Flow rate	0.5 mL min^{-1}
Injection volume	60 µL
ICP-MS conditions	
Radiofrequency power (W)	1240
Nebulizer gas flow (L min^{-1})	1.1
Auxiliary gas flow (L min^{-1})	0.9
Sweeps	5
Replicates	20
Dwell time (ms)	50
Acquisition Mode	Peak hopping
Detector Mode	Dual
Methane gas flow (mL min^{-1})	0.9
RP*q* (V)	0.6
Monitored isotopes	^{56}Fe; ^{98}Mo and ^{24}Mg

containing 2% (v/v) of sodium fluoride and stored at –20°C, in order to avoid proteolysis. This study was approved by the Research Ethics Committee of the School of Medical Sciences at the University of Campinas – CEP/FCM (Ethics Protocol Approval number *1270/2010*) as well as by Scientific Committee of the Institute of Forensic Medicine of Technical Police Scientific Superintendence – DTD-IML (Ethics Protocols Approval numbers *09/11*, *687/2012* and *736/2012*).

The vitreous humor samples analyzed (both controls and cases) were composed by the addition of several other similar samples pools) and were classified into control group (*CA*: non-decomposing bodies) and four case samples, all consisting in decomposing bodies: *A1* (1 days post-mortem interval), *A2* (2 days post-mortem interval), *A3* (3 days post-mortem interval) and *A4* (7 days post-mortem interval) collected between June and September of 2012 (Table 1).

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Table 3 Parameters of the analytical curves

	Fe	Mo	Mg
Dynamic range ($\mu g\ L^{-1}$)	1 – 150	0.1 – 15	1 – 150
Slope ($L\ \mu g^{-1}$)	2682.3	1153.1	506.8
r	0.9999	0.9987	0.9998
LOD ($ng\ L^{-1}$)	200	1	30
LOQ ($ng\ L^{-1}$)	800	5	100

Ionomic approach

Sample preparation and metal determinations

For decomposition of vitreous humor samples, a mini-vials system was used.[17] A volume of 200 µL of samples was added to 200 µL of HNO_3 sub-distilled and 100 µL of H_2O_2. A closed-vessel DGT100 Plus microwave oven (Provecto Analítica, Jundiaí, Brazil) was used for sample decomposition, and the following program was applied: (1) 60 s at 300 W, (2) 60 s at 500 W, (3) 60 s at 800 W and (4) 60 s at 500 W. After decomposition, samples were made up to 10 mL with deionized water, thus comprehending a 50 times dilution factor of the samples. Fe, Mg and Mo concentrations were determined by ICP-MS (Elan DRC-e, PerkinElmer, Norwalk, CT, USA), using the conditions shown in Table 2. Methane was employed as collision gas in dynamic reaction cell (DRC) to avoid polyatomic interferences.

Metallo and metal-binding proteins identification

LC-ICP-MS procedure

After establishing those target metals, the hyphenated technique – LC-ICP-MS was used for exploratory analysis of metals and proteins. Then, vitreous humor samples were diluted in mobile phase, in ratio equal to 1:5. Then, a centrifugation step was carried out for 5 min at 5000 g using a Bio-Spin-R ultracentrifuge (BioAgency, São Paulo, Brazil). After filtration, samples were ready to analysis.

LC-ICP-MS analyses were performed inside a clean room class 1000. The chromatographic ones were carried out on a PerkinElmer Series 200 liquid chromatograph (PerkinElmer, USA) equipped with a quaternary pump, degasser, autosampler, column oven, and diode array detector. SEC was performed on a Superdex 200 HR 10/30 (GE Healthcare Bio-Science AB, Uppsala, Sweden) column. The calibration was performed using the following protein standards: equine ferritin (440 KDa); bovine immunoglobulin A (300 kDa); bovine serum albumin (66 kDa); carbonic anhydrase (29 kDa) and lactalbumin (14.4 kDa).

Tryptic digestion and protein identification

After defining the protein fraction through the LC-ICP-MS hyphenated technique, the identification of the (metallo)proteins were carried out by ESI-MS/MS. The effluent of the SEC column from the LC was collected for 2 injections, in order to guarantee the protein mass for protein identification. This strategy was made using a Gilson Inc. fraction collector (Middletown, USA) at time intervals that represented the elution of metal peaks previously identified using ICP-MS. Instrumental and chromatographic parameters are listed in Table 2.

After chromatographic separations, the collected fractions were submitted to a pre-concentration step, using centrifugal filter devices (10000 Da molecular weight cutoff, Microcon and Amicon Ultra; Millipore Corporation), being recovered in 150 µL of ammonium bicarbonate 100 mmol L^{-1}. First, dithiothreitol (DTT) was added to the samples for reduction and iodacetamide (IAA) was added for alkylation. Finally, trypsin was added and left for overnight digestion for at 37 ºC for 16 h.

After tryptic digestion, the peptides were analyze for LC-MS using a nanoAcquity UPLC (Waters Co., Manchester, UK) coupled to a Waters Synapt HDMS (Waters Co.) mass spectrometer equipped with nanoESI source. The sample was desalinated in a pre-column (Waters Symmetry C18; 180 µm x 20 mm) for 3 min with acetonitrile:water in 0.1% formic acid (97:3, v/v) and then transferred to a analytical column (Waters HSS T3 C18; 100 µm x 100 mm). The peptide mixture was separated using a gradient from 3–95% acetonitrile:water in 0.1% formic acid at a flow of 1 µL min^{-1}. The instrument was operated using the Data Dependent Analysis (DDA), where the equipment acquires one spectrum per second, and when multi-charged species were detected, the three most intense species were fragmented in the collision chamber (collision energy defined by m/z and precursor charge).

The mass spectra were previously processed using a Mascot Distiller (Matrix Science, London, UK) and searched against the protein databases Swiss-Prot. The search in Mascot Server (Matrix Science) was performed including oxidation of methionine as a variable modification, carbamidomethylation of cysteine as fixed modification, one missed trypsin cleavage, and a tolerance of ±0.1 Da for precursor and fragment ions.

Results and discussion

Ionomic approach

Through the use of ICP-MS, the concentration of some elements in vitreous humor was determined. Aiming to perform an exploratory analyses, a multi elemental method was used for checking the intensity profile (qualitative results based on cps – counts per second) of many metal ions (K, Sr, Zn, Cl, Ca, Br, Fe, Cu, Rb, Cr, Al, Ba, Mn, Ti, Mg, Pb, Ni, Cd, Sb, Mo, Si, V, Sn, Co, Cs and As), and searching for possible statistical correlations between metal concentration and post-mortem interval. Three metals (Fe, Mg and Mo) were noted for their biological relevance and highlighting profile in both vitreous humor samples from non-decomposing as well in decomposing bodies. In fact, these three metals were chosen by presenting the best correlations between PMI and their signal intensities. Then, quantitative analysis of these marker ions was carried out. The method showed good linear correlations for the three analytes, and detection limits in the range at ng L^{-1} (Table 3).

According to Fig. 2, Mg and Mo showed random behaviors not directly related to post-mortem intervals. In fact, Mg concentration is higher until the 2 days, decreased significantly in day 3 being slowly increased again at A4 (7 days post-mortem interval).

The literature on the subject presents with conflicting reports regarding the utility of post-mortem vitreous humor magnesium in making PMI estimations. The mean range of magnesium

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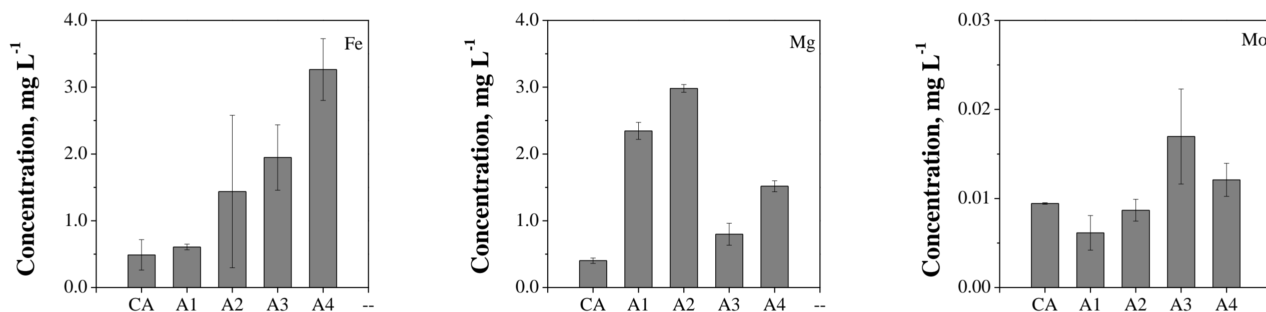

Figure 2 Concentration of Fe, Mg and Mo at mg L^{-1} in vitreous humor samples from control group (CA) and from 1 – 7 post-mortem interval (A1 – A4). More details see text.

concentration observed in the earlier studies varied between 0.24 and 27.9 mg L^{-1}, which corroborates with the present study. [18,19] The linear regression correlation between vitreous magnesium and PMI was not found to be statistically significant ($r^2 = 0.312$). The results in the present work do not agree with those earlier reported using magnesium as PMI predictor.[18,19] However, those reports were based on data in particular groups only, which refers to death like asphyxia and to phenobarbital intoxications. In the present study, useful correlation between PMI and vitreous magnesium could not be observed in samples. The absence of diagnostic sub-groups is due to the decisions of the ethics committee, since this project was approved emphasizing the confidentiality and respect for the donor in question. Our results are consistent with the earlier findings that reported no significant correlation between vitreous magnesium and PMI.[20, 21]

Regarding to Mo, we explored whether Mo cofactor would be also a possible marker for PMI. As observed through the results, increasingly higher concentrations of this element were found up to 3 days, but after 7 days a decrease in Mo was noted.

Mo acts as a cofactor of xanthine oxidase that catalyzes the oxidation of xanthine to uric acid, and it contributes to the generation of reactive oxygen species.[22] Salam suggests that a significant relationship was observed between Hypoxanthine level in vitreous humor and PMI.[23] However, in our study, Mo was not found to be statistically significant ($r^2 = 0.198$), and it should not be considered a marker for PMI.

A direct correlation was found for iron (PMI = 0.0176 [Fe] + 0.425), with its concentration increasing quite linearly ($r^2 = 0.948$) from 1 to 7 days post-mortem interval (Fig. 2). McGahan and Fleisher[24] also reported that iron concentration rises until 7th day. However, our work reports a linear correlation for iron at first time in the literature. Usual levels of iron are nearly 0.05 mg L^{-1}, *i. e.*, less than 1% of the iron content of plasma, reflecting the ability of the blood–ocular barrier to prevent its entrance into the eye. A large increase in the iron concentration found through our results in vitreous humors may have been induced by a breakdown of this barrier, most likely due to protein sources, to influx of red blood cells and their subsequent hemolysis and/or to tissue necrosis.[25] These findings suggest that iron could act as a marker, as it well as has a great importance in post-mortem biochemical processes.

Controls showed great variability with respect to the metals mentioned, and this variance has its origin in the physiological state of the donor. There are several factors contributing to this, for example: clinical status (immune system, stunted clinical diseases and genetic factors), nutritional status, gender and age. This is an important finding that the vitreous with the passage of time after death is characterized by a specific elemental composition and are

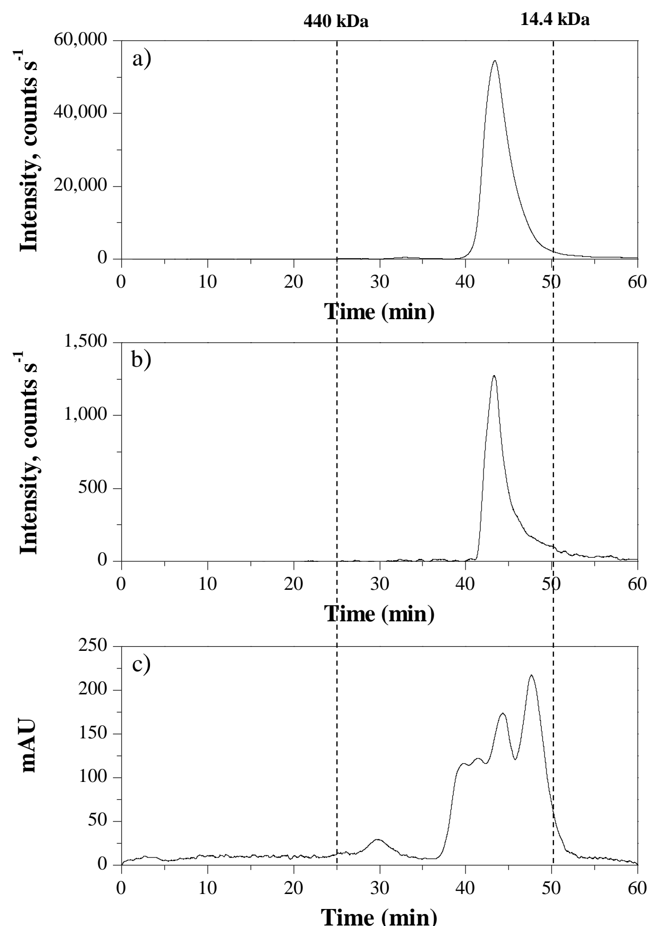

Figure 3 SEC-ICP-MS chromatograms for Fe (a), Mg (b) and UV (280 nm) chromatograms (c) in vitreous humor samples on Superdex 200 column for A1 samples.

therefore of particular functions related to decomposition processes and valuable forensic pathology by the observation highly dependent physiological roles of elements characteristic.

Metallo and metal-binding proteins identification

In order to deeper investigate on the behaviour of metals and their possible sources, vitreous humor samples were then inserted into a

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Table 4 Characterization of the identified protein species.

Sample	Protein name	Protein accession number	Mascot score	Coverage (%)	Matched peptides	Sequence of peptides
CA	Serum albumin [*Homo sapiens*]	ALBU_HUMAN	245	28	22	K.KVPQVSTPTLVEVSR.N
	Serotransferrin [*Homo sapiens*]	TRFE_HUMAN	51	9	4	R.APNHAVVTR.K
A2	Serum albumin [*Homo sapiens*]	ALBU_HUMAN	425	28	26	K.KVPQVSTPTLVEVSR.N
	Serotransferrin [*Homo sapiens*]	TRFE_HUMAN	111	17	13	K.HQTVPQNTGGK.N
A3	Serum albumin [*Homo sapiens*]	ALBU_HUMAN	244	24	18	K.AVMDDFAAFVEK.C
	Serotransferrin [*Homo sapiens*]	TRFE_HUMAN	82	13	10	R.APNHAVVTR.K
A4	Serum albumin [*Homo sapiens*]	ALBU_HUMAN	901	35	41	K.KVPQVSTPTLVEVSR.N
	Serotransferrin [*Homo sapiens*]	TRFE_HUMAN	284	13	13	R.SMGGKEDLIWELLNQAQEHFGK.D

chromatographic system, and peak profiles using DAD and ICP-MS evaluated and correlated to some proteins. The mechanism responsible for the higher levels of iron, for example, is likely to involve migration processes dependent on the distribution of mediating proteins (*i. e.* metal transporters, metallo-regulatory sensors and storage molecules).

After a simple preparation, vitreous humor samples were subjected to SEC, using an ammonium acetate 100 mmol L^{-1} buffer solution. ICP-MS detection was carried out to clarify if metals with contrasting profiles in ionomic approach could be bound to protein. Only Mo did not present peak in SEC-ICP-MS.

Compounds strictly separated on basis of their molecular weight were eluted between 25.05 and 51.36 min, which corresponds to the calibration interval ranging from 440.0 to 14.4 kDa (see Fig. 3). A significant correlation was obtained (r^2 = 0.945) and a satisfactory calibration equation was achieved, as followed: Elution volume = -8.72 [log PM] + 36.49.

Analyzing the chromatograms, one fraction can be identified for Fe and Mg, in the SEC chromatographic separation, as observed in Fig. 3. Vitreous humor compounds were eluted within 76.99 to 20.56 kDa for all sample groups analyzed, generating the same chromatographic profile. Thus, it is reasonable to presume that these compounds eluted are proteins associated to different elements, characterizing metal-binding proteins or metalloproteins. Then, the protein fraction from SEC separation was analysed by ESI-MS-MS in order to establish the identity of possible proteins contained in such fraction.

All identified proteins were assigned to *Homo sapiens*, with molecular weights consistent with the eluted fraction (*ca.* 77 and 67 kDa for serotransferrin and serum albumin, respectively), and having good scores (Table 4). Among the proteins identified, it was found serotransferrin, which is synthesized and secreted by the lens and the retinal pigmented epithelial cells.[26] The presence of the blood–ocular barriers prevents the movement of large proteins, such as transferrin, into the eye. In normal health, the tissues residing behind such barriers make and secrete transferrin in order to capture iron for maintenance of critical physiological functions.[27] Thus, the presence of transferrin and levels of iron could suggest a disruption in blood–ocular barriers, which could be occurring by inflammatory or decomposition processes.

Serum albumin is the main protein of plasma, has a good binding capacity for water, several metals, fatty acids, hormones, bilirubin and drugs. Its main function is the regulation of the colloidal osmotic pressure of blood.[28] Redox state of human serum albumin was investigated as a possible systemic redox marker in patients with different eye. Only patients with diabetes mellitus presented the oxidized forms of serum albumin.[29] In our work, serum albumin could indicate a passive exudation as a consequence of blood-ocular barrier failure in conjunctival.[30]

As noted, few proteins identities were found, however, it is necessary to stress out that vitreous humor is composed by 99% of water, and the other 1% represents sugar, mineral salts and proteins. Then, it is plausible that a little amount of protein as well as identity of proteins is found. This point highlights metalloprotein and metal-binding protein identification in several human humors and organs, and how metallomic assumes an important function in understanding of the biological processes.

Conclusion

Three metal ions (Fe, Mg and Mo), were indentify and precisely quantified in the vitreous humor samples with different PMI for decomposing bodies and non-decomposing bodies.

The good correlation between Fe and PMI, was clearly demonstrated due to the presence of serotransferrin in vitreous humor samples, indicating the possible mechanism of Fe incorporation into the eyeball after the death of the individual. The chromatographic approach allowed to conclude that serotransferrin is involved in binding Fe, being this protein related to transferrin receptors. The presence of serum albumin is an indicator of a passive exudation, and, together with iron levels and serotransferrin identification, they could suggest a blood-ocular barrier failure.

Additionally, Mg and Mo were successfully quantified, and detection limits were in the range at ng L^{-1}. Mg was also identified in the analysed proteins fraction, also corroborating the ionomic results. However, they could not be considered as PMI marker due to the low statistical correlation between their concentrations and post-mortem interval.

Finally, the adopted strategy was proved to be useful in verifying some aspects of the post-mortem biochemical, once that Fe was found and quantified in the vitreous humor, as well as it is

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bound to serotransferrin, indicating that its transport to the eyeball is probably due to the disruption of the blood ocular barrier. If by one side the explanation of the mechanism regarding Fe is plausible, some questions about Mg and Mo, and why not others metals, are still opened. In this way, the strategies applied in this work and others presented in the literature are also useful in areas as forensic pathology, enlarging the applicability of the metallomic approaches in such areas.

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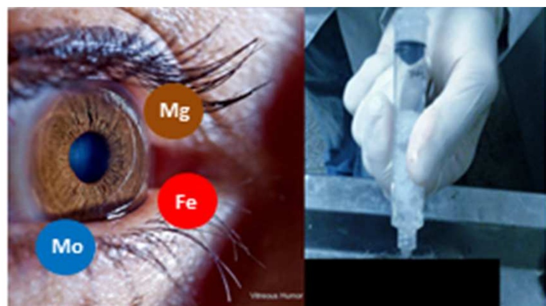

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