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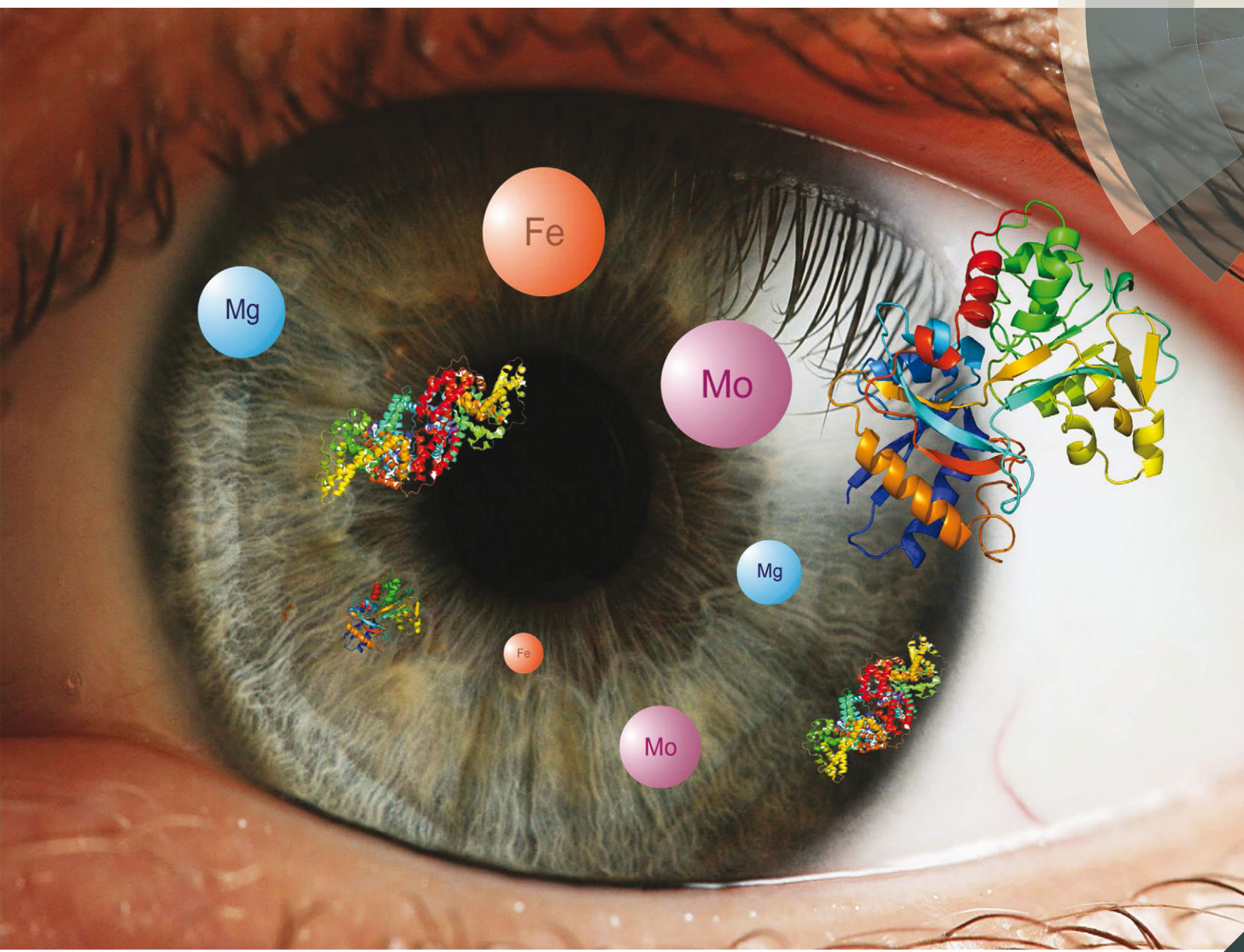

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Metals and (metallo)proteins identification in vitreous humor focusing on post-mortem biochemistry

Júlio César Santos Júnior,[a] Pedro Carlos Mollo Filho,[b] Ruggero Bernardo Felice Guidugli,[b] Marcos Nogueira Eberlin,[c] Gustavo de Souza Pessôa,[d] Elidiane Gomes da Silva,[d] Marco Aurélio Zezzi Arruda[d] and Nelci Fenalti Höehr*[a]

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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), taking into account both decomposing and non-decomposing 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 with post-mortem time for decomposing bodies because of a significant increase of iron concentration when comparing samples from bodies presenting 3 and 7 days post-mortem interval. An important clue to elucidate the role of metals 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 those proteins involved in the post-mortem interval.

Introduction

Biological and chemical changes have been monitored for criminal investigations and natural deaths in the post-mortem interval (PMI). This approach is becoming increasingly essential to routine activities involving forensic pathology, and considerable progress has been made over the past few 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 a wide range of biomolecules. Thus, 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 maintaining the integrity of constituents; (II) even in late post-mortem intervals, the vitreous humor was hardly contaminated; and (III) it is also subjected to less bacterial degradation due to the protected environment inside the eye (Fig. 1). In contrast to blood, vitreous humor can be considered a site well suited for investigations of chemical and biochemical changes after death.[7,8] Vitreous humor is composed of 99% 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 a comparative ionomic approach and protein analysis to investigate the 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 for determination of Fe, Mg and Mo in vitreous humor. Moreover, 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.

[a] *Department of Clinical Pathology, School of Medical Sciences. State University of Campinas – UNICAMP, 181 Alexander Fleming St, 13083-881 Campinas, SP, Brazil. E-mail: nelci@fcm.unicamp.br; Tel: +55-19 3521-9455*
[b] *Team of Forensic Medicine West, Medico-Legal Institute, Police Technical Scientific Superintendence – SPTC, 307 Dr. Gastão Vidigal Avenue, 05314-000, São Paulo, SP, Brazil*
[c] *ThoMSon Mass Spectrometry Laboratory, Institute of Chemistry, University of Campinas – UNICAMP, 13085-970 Campinas, SP, Brazil*
[d] *Group of Spectrometry, Sample Preparation and Mechanization (GEPAM), Institute of Chemistry. State University of Campinas – UNICAMP, 13084-862 Campinas, SP, Brazil*

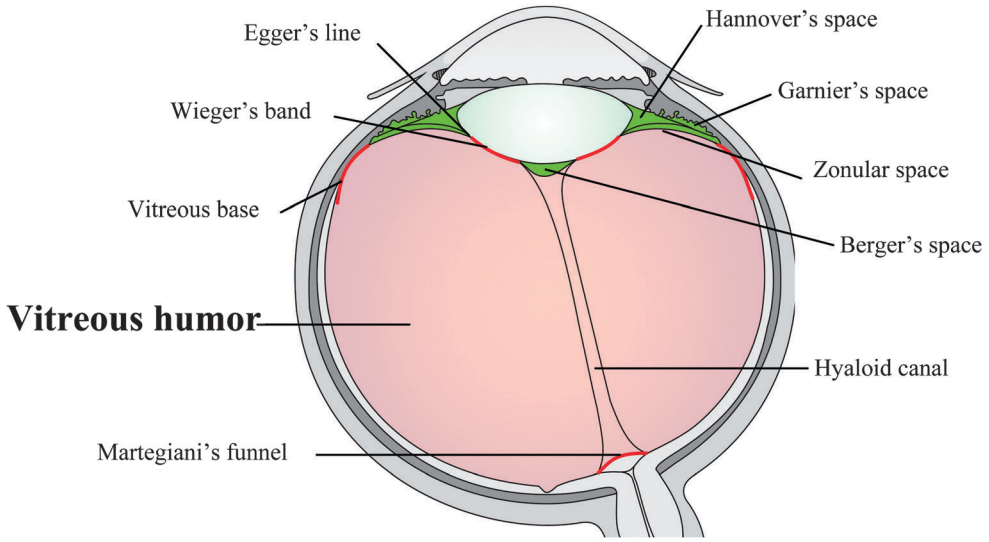

Fig. 1 Vitreous humor and adjacent spaces. Adapted from ref. 16.

Experimental

Sample collection

The vitreous humor samples were obtained from 11 bodies attended by a team of forensic scientists of the west zone (decomposing bodies) and south zone (non-decomposing bodies) of São Paulo city. The team was from the Medico-Legal Institute of Police Technical Scientific Superintendence – SPTC of São Paulo, Brazil. The case samples (vitreous humor) were collected by puncturing using a trocar with 0.51 mm external diameter, and a bevelled metal mandrel with external diameter of 0.42 mm. The angles of the upper quadrants of the eye were selected with the help of eyelid retractors thus avoided the most vascularized tissues such as the outer corner of the eye.

All material was separated and identified according to the left and right eye, and also according to the body gender. Then, the material was collected with a syringe with 5 mL capacity to set to a needle of 0.80 × 25 mm and packed in gray-top tubes containing 2% (v/v) of sodium fluoride and stored at −20 °C 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 of the addition of several other similar samples (pools) and were classified into control group (CA: non-decomposing bodies) and four case samples, all consisting of 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).

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.

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

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 × 300 mm, 13 μm 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

and (4) 60 s at 500 W. After decomposition, samples were diluted to 10 mL with deionized water, thus producing 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 the collision gas in dynamic reaction cell (DRC) to avoid polyatomic interferences.

Metallo and metal-binding proteins identification

LC-ICP-MS procedure. After establishing the target metals, the combined technique, *i.e.* LC-ICP-MS, was used for exploratory analysis of metals and proteins. Then, vitreous humor samples were diluted in the 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 for analysis.

LC-ICP-MS analyses were performed inside a clean room of class 1000. The chromatographic analyses were carried out on a PerkinElmer Series 200 liquid chromatographer (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 using the LC-ICP-MS technique, the identification of the (metallo)proteins was carried out by ESI-MS/MS. The effluent of the SEC column from the LC was collected for two injections, in order to guarantee the protein mass for protein identification. This strategy was conducted using a Gilson Inc. fraction collector (Middletown, USA) at time intervals that represented the elution of metal peaks previously identified using ICP-MS. Note that the instrumental and chromatographic parameters are listed in Table 2.

After chromatographic separation, the collected fractions were submitted to a pre-concentration step using centrifugal filter devices (10 000 Da molecular weight cutoff, Microcon and Amicon Ultra; Millipore Corporation) and recovered in 150 μL of ammonium bicarbonate 100 mmol L^{-1}. First, dithiothreitol (DTT) was added to the samples for reduction and iodoacetamide (IAA) was added for alkylation. Finally, trypsin was added and left for overnight digestion at 37 °C 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 a nano-ESI source. The sample was desalinated in a pre-column (Waters Symmetry C18; 180 μm × 20 mm) for 3 min with acetonitrile : water in 0.1% formic acid (97 : 3, v/v), and then transferred to an analytical column (Waters HSS T3 C18; 100 μm × 100 mm). The peptide mixture was separated using a gradient of 3–95% acetonitrile : water in 0.1% formic acid at a flow rate of 1 μL min^{-1}. The instrument was operated using data dependent analysis (DDA), in which the equipment acquires one spectrum per second, and when multicharged 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 database 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 to identify 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 as decomposing bodies. In fact, these three metals were chosen as they present 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

Table 3 Parameters of the analytical curves

	Fe	Mo	Mg
Dynamic range (μg L^{-1})	1–150	0.1–15	1–150
Slope (L μg^{-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

concentration was high for the initial 2 days, and then decreased significantly in day 3 and slowly increased again at A4 (7 days post-mortem interval).

The literature presents conflicting reports regarding the utility of post-mortem vitreous humor magnesium in making PMI estimations. The mean range of magnesium concentration observed in the earlier studies varied between 0.24 and 27.9 mg L^{-1}, which is consistent with values obtained in 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 the PMI predictor.[18,19] However, those reports were based on data in particular groups only, which refers to death such as from asphyxia and from phenobarbital intoxication. In the present study, useful correlation between PMI and vitreous magnesium could not be observed in samples. The absence of diagnostic sub-groups is because of the decision of the ethics committee to protect 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 Mo, we explored whether a Mo cofactor would also be 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, which 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 ($r^2 = 0.198$); thus, it was not considered to be 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 of post-mortem interval (Fig. 2). McGahan and Fleisher[24] also reported that iron concentration rises until the 7th day. However, our work reports a linear correlation for iron for the 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. The large increase in the iron concentration found through our results in vitreous humors may have been induced by a breakdown of this barrier, which is 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 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 the variance, for example: clinical status (immune system, stunted clinical diseases and genetic factors), nutritional status, gender and age.

Metallo- and metal-binding proteins identification

In order to further investigate the behaviour of metals and their possible sources, vitreous humor samples were then inserted into a chromatographic system, and peak profiles using DAD and ICP-MS were evaluated and correlated with 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 whether metals with contrasting profiles in an ionomic approach could be bound to protein. Only Mo did not present a 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: elution volume = −8.72 [log PM] + 36.49.

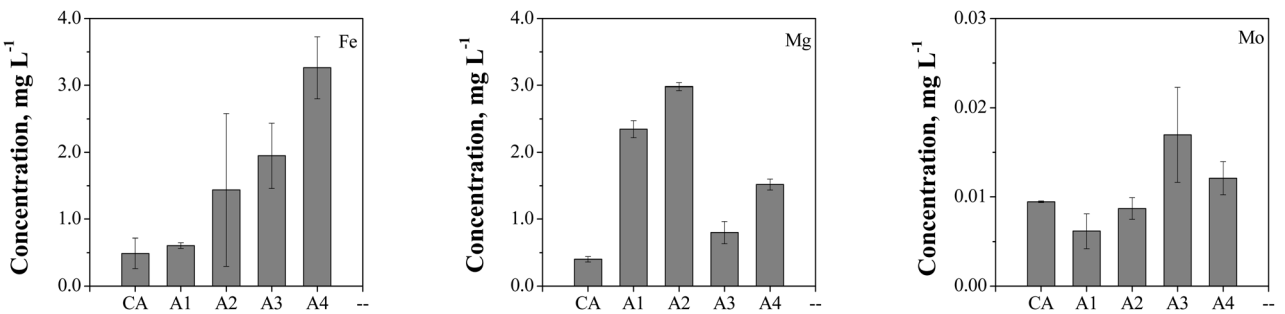

Fig. 2 Concentration of Fe, Mg and Mo at mg L^{-1} in vitreous humor samples from control group (CA) and from 1 to 7 post-mortem interval (A1–A4). For more details, see text.

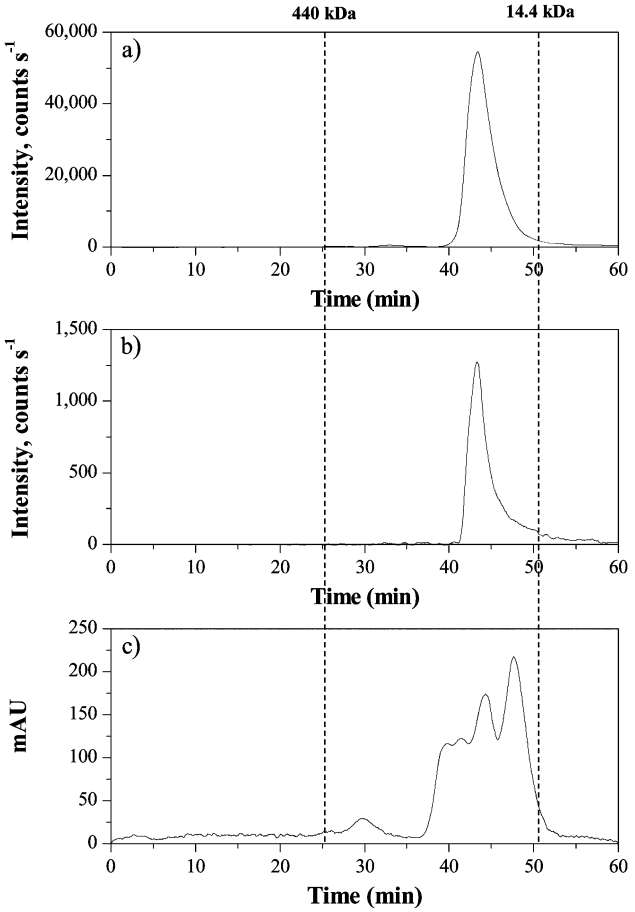

Fig. 3 SEC-ICP-MS chromatograms for Fe (a), Mg (b) and UV (280 nm) chromatograms (c) in vitreous humor samples on Superdex 200 column for A1 samples.

By 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 76.99–20.56 kDa for all the analyses sample groups, generating the same chromatographic profile. Thus, it is reasonable to assume that these compounds eluted are proteins associated with 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 fractions.

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

Serum albumin is the main protein constituent of plasma, and it 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 the blood.[28] The redox state of human serum albumin was investigated as a possible systemic redox marker in different patients. 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 tissue.[30]

As has been noted previously, the identities of only a few proteins were found; however, it is necessary to stress that vitreous humor is composed of 99% water, whereas the other 1% is composed of sugar, mineral salts and proteins. Thus, it is possible that a small amount of protein as well as the identity of protein is found. This point highlights metalloprotein and metal-binding protein identification in several human humors and organs, and the importance of the metallomic in understanding of the biological processes.

Conclusion

Three metal ions (Fe, Mg and Mo) were identified 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 us to conclude that serotransferrin is involved in binding Fe, as it is the protein that is related to transferrin receptors. The presence of serum albumin is an

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

indicator of a passive exudation, and, together with iron levels and serotransferrin identification, could suggest blood–ocular barrier failure.

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

Finally, the adopted strategy proved to be useful in verifying some aspects of the post-mortem biochemical changes. Note that Fe was found and quantified in the vitreous humor, as well as it was observed to be bound to serotransferrin, indicating that its transport to the eyeball is probably due to the disruption of the blood–ocular barrier. Thus, although there seems to be a possible explanation of the mechanism regarding Fe, some questions about Mg and Mo, and others metals still need to be answered. In this way, the strategies applied in this work and others presented in the literature are also useful in areas such as forensic pathology, thereby enlarging the applicability of the metallomic approaches in such areas.

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