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Electronic Supplementary Information for "Quantitative distribution of Zn, Fe and Cu in the human lens and study of the Zn-metallothionein redox system in cultured lens epithelial cells by elemental MS"

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1. Experimental Procedures

1.1 Instrumentation

ORS-ICP-MS, SF-ICP-MS and LA-ICP-MS operating conditions, acquisition parameters, and optimized chromatographic conditions are shown in Table S1.

1.2 Total multielemental determination in lenses and capsules by Isotope Dilution-ICP-MS

Mineralized lenses and capsules were analyzed by ID-ICP-MS. A known amount of ^{68}Zn, ^{54}Fe, ^{65}Cu and ^{74}Se enriched stable isotopes solution was mixed with the digested samples and introduced to the plasma by conventional nebulization. The following isotopes were monitored by ICP-MS 7500 ce: ^{64,66,68}Zn, ^{54,56,57}Fe, ^{63,65}Cu and ^{74,77,78}Se. A flow of 4 mL·min^{-1} of hydrogen was used to pressurize the octapole chamber for elemental determinations. Finally, the use of mathematical calculations based on ID analysis allowed the accurate quantitative determination of the elements in the capsule and lens samples.

1.3 Quantitative speciation of Zn, Fe and Cu in lenses and capsules by SEC-ID-ICP-MS

Separation of the water-soluble protein fraction of lens and capsules was carried out by Size exclusion column (SEC-Superdex 200 10/300 GL, Amersham Biosciences, UK), with theoretical fractionation range from 10 to 600 kDa of molecular mass. SEC column was previously calibrated using a mixture of known molecular size protein standards (i.e., thyroglobulin, IgG, ovalbumin, α-lactalbumin and vitamin B12) and UV-Vis detection, obtaining the following equation: time (min)=-3.558 Ln[MM(KDa)]+36.552. Fifty microliters (1.5-2.0 mg of lens-protein and 0.4-0.8 mg of capsule-protein) of water-soluble protein extract were injected in the column and fractionated following the conditions shown in Table S1, as previously reported.[1] Quantification of Zn, Fe and Cu-binding proteins was carried out by on-line post-column ID analysis. To this end, a standardized solution of known concentration of a mixture of the enriched stable isotopes ^{68}Zn, ^{54}Fe and ^{65}Cu, was added online after the chromatographic separation. The continuous measurement of the corresponding isotope ratios ^{66}Zn/^{67}Zn, ^{56}Fe/^{54}Fe and ^{63}Cu/^{65}Cu in the ICP-MS allowed us to obtain the "mass flow chromatogram" after applying the isotope dilution equation.[2] Integration of the area under each chromatographic peak using Origin 8 software (Microcal Software Inc., Northampton, MA, USA) provided the absolute mass of Zn, Fe and Cu bound to proteins.

1.4 Distribution of zinc and copper in human lens by LA-ICP-MS

The LA-ICP-MS system was daily tuned by continuous ablation of a reference glass sample (NIST SRM 612) for maximum intensity of ^{59}Co$^+$, ^{139}La$^+$, ^{232}Th$^+$ and ^{238}U$^+$, and low elemental fractionation by optimizing the ^{238}U$^+$/^{232}Th$^+$ ratio to 1. Bio-imaging analysis on lens sections were performed by line scan ablation using a 25-µm spot diameter and 10-µm distances between adjacent lines, in a selected area of 6.5 mm^2 corresponding to the anterior capsule region (see Table S1 for operating conditions). Two dimensional-images of Zn- and Cu-elemental distribution were created using ImageJ-Fiji software.[3]

1.5 MT distribution in human lens by immunohistochemistry

Eyes from *post mortem* human donors (cadavers) were formalin fixed and paraffin embedded (FFPE) following conventional protocols. Sections (5 µm thick) from FFPE blocks were de-paraffinized and stained with a mouse monoclonal antibody to

MT1/2 (ab12228, Abcam, UK). Tissue sections were incubated overnight at 4°C with the primary antibody (dilution 1:100), rinsed in PBS, and further incubated with the secondary antisera (1:200 fold–diluted, rhodamine conjugated goat anti-mouse IgG) for 60 min at 4°C. After washing in PBS and mounting in a solution of glycerol medium, the images were captured with a Leica DM6000 microscope equipped with epifluorescence, a DFC310 Fx Leica camera and the Advanced Fluorescence AF6000 software (Leica Microsystems CMS GMBH, Germany).

1.6 Human lens cell line and common culture conditions

A human lens epithelial cell line was established in our laboratory. Briefly, a primary culture of lens epithelial cells was prepared from lens capsules, containing lens epithelial cells attached to them, dissected from a pair of eyes of a healthy 44 years old donor (cadaver), and infected with wild type SV40 as previously described.[4] The established line, named HLEsv, maintains *in vitro* the gene expression characteristics of *in vivo* lens epithelial cells when examined by microarrays analysis (data not shown).

The optimal seeding density was determined using the CyQUANT® Cell Proliferation Assay Kit (Invitrogen). The assay used CyQUANT GR dye, which produces a large fluorescence enhancement upon binding to cellular nucleic acids. The fluorescence emission of the dye-nucleic acid complexes correlated linearly with cell number. Fluorescence was measured at the wavelength Ex/Em of 485/535 nm using VICTOR™ X5 Multilabel Plate Reader (PerkinElmer).

Cell viability assay was carried out as follows: Cells were seeded in 96-well plates at a density of 10,000 cells/well. After 48 h, the medium was changed to EX-CELL® Hybridoma medium (Sigma-Aldrich, USA), and after 24 h cells were treated with different concentrations of Zn (1, 10, 25, 75 y 100 µM) in triplicates. The viability of Zn-treated cells was measured at 24 h post assay using CyQUANT cell proliferation kit as per manufacturer's instructions. Raw data were converted to reflect cell viability after Zn treatment relative to untreated controls and presented as a percentage.

1.7 Distribution of MTs in HLEsv cells by immunohistochemistry

HLEsv cells cultured on coverslips (Menzel-Gläser; Braunschweig, Germany) covered with Poly-D-Lysine (Sigma-Aldrich, USA) were fixed with cold methanol (Merck Darmstadt, Germany) followed by washing 3 times in PBS for 5 min and permeation with 0.05% Tween 20 in PBS for 10 min. To block non-specific sites we used a solution containing 10% goat serum in PBS. The cells were incubated overnight at 4°C with a mouse monoclonal antibody to MT1/2 (ab12228, Abcam; dilution 1:100). After washing 3 times with PBS for 5 min, cells were incubated at room temperature for 1 hour with Alexa 488 anti-mouse (Invitrogen; 1:500) followed by washing 3 times in PBS for 5 min. Nuclei were counterstained using 2 µg/mL 4'6-diamidino-2-phenylindole (DAPI; Invitrogen). After washing in PBS and mounting in a solution of glycerol mounting medium (Dako, Agilent Technologies), the images were captured with a Leica TCS SP8 XLeica DM6000 confocal microscope and the Leica Application Suite X version 1.8.1 AF6000 software (Leica Microsystems CMS GMBH, Germany), at the Optical Microscopy and Image Processing Unit, Scientific-Technical Services, University of Oviedo.

1.8 MT protein extraction in HLEsv cells

HLEsv-treated cells were washed three times with PBS, trypsinized, and collected by centrifugation at 200 x*g* for 5 min. The cell pellet was resuspended in 1.0 mL of TRIS-buffer (10 mM, pH=7.4 and degassed under N_2 atmosphere), and cells were disrupted with an ultrasonic cell disintegrator (Bandelin Sonopuls HD2070, Berlin, Germany) on bath-ice at 10 kHz for 30 s, three times at 30 s intervals. The cellular homogenate was centrifuged for 20 min at 15,000 x*g* and the supernatant (cytosolic fraction) was saved and stored at -80°C for further MT protein analysis (see below). Oxygen was avoided during the storage of the cytosolic fractions (by internal atmosphere of nitrogen and Teflon insulation) to prevent oxidation of MTs through cysteinyl residues.

2. Results

2.1 Verification of epithelial cells attachment to the capsule

As seen in Figure S1, lens epithelial cells are intensely stained with toluidine (Panel A) and they remain attached to the capsule after its dissection (Panel B).

2.2 Responses of HLEsv cells gene expression to zinc and pro-inflammatory interleukin

The optimal Zn concentration was established between 25 and 50 µM, with a survival rate above 80% (Figure S2) using the cell proliferation assay kit.

The cellular distribution of MTs in HLEsv cells was determined by indirect immunofluorescence using MT1/2 antibodies, where the staining was detected in both the cytoplasm and nuclei by confocal microscopy (see Figure S3, panels A-C).

Table S2 shows the effects of exogenously added zinc, in the form of $^{68}ZnSO_4$, and/or pro-inflammatory interleukin (IL1α), on HLEsv cells on MTs and cytokine gene expression.

2.3 Quantification of zinc-binding proteins, including MTs, following exogenous zinc ($^{68}ZnSO_4$) and IL1α treatments in HLEsv cells

Table S3 shows the levels of natural (^{nat}Zn), exogenous (^{68}Zn) and total zinc ($^{nat}Zn+^{68}Zn$) found in all the zinc binding-proteins and biomolecules detected in HLEsv extracts, i.e., MTs proteins and proteins other than MTs, in the cytosolic fraction.

Table S4 shows the concentration of MTs and their elemental stoichiometric composition (Zn, Cu : MT ratio) in the water-soluble protein fraction of HLEsv cells containing natural (^{nat}Zn) and/or exogenous zinc (^{68}Zn), under control conditions (no treatment) and after exposure to zinc and/or IL1α.

SUPPLEMENTAL FIGURES

Figure S1. Tolouidine blue staining of 10 µm human lens sections. Panel A: Lens with capsule. Panel B: Lens without capsule.

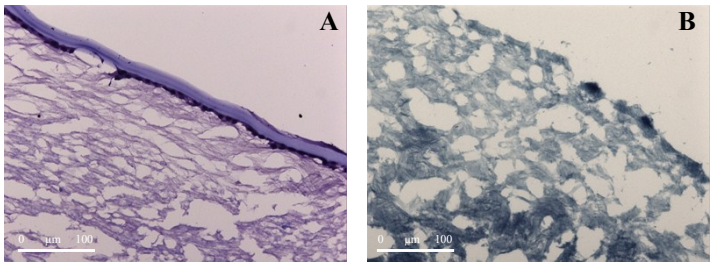


Figure S2. Percentage of relative cell viability depending upon zinc concentration (1, 10, 25, 75 and 100 µM).

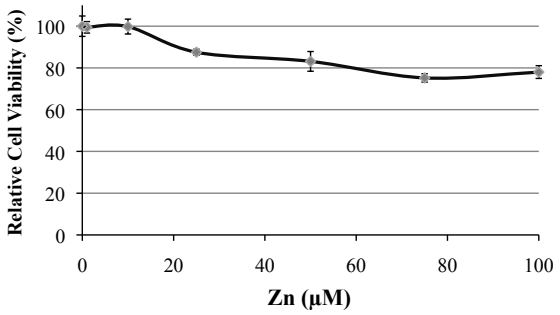
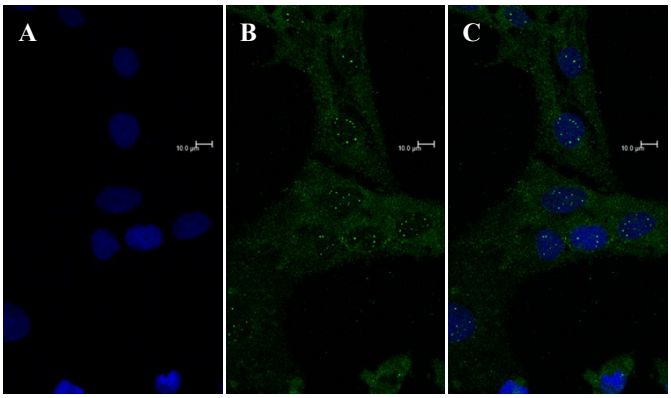


Figure S3. Cellular distribution of MT1/2 in HLEsv cells by confocal microscopy. (Panel A) DAPI staining of cell nuclei micrograph. (Panel B) Alexa 488-labeled micrograph. (Panel C) Merged image of B-C. Scale bar 100 µm



SUPPLEMENTAL TABLES

TABLE S1. ORS-ICP-MS, SF-ICP-MS and LA-ICP-MS operating conditions, data acquisition parameters and the corresponding optimized chromatographic conditions.

ORS-ICP-MS Agilent 7500ce		
	RF power/W	1500
	Plasma gas flow rate/L·min^{-1}	15
	Auxiliary gas flow rate/L·min^{-1}	1.12
	Sampling depth/mm	5.8
	Tuning solution	10 µg·L^{-1} Li, Co, Y, Ce and Tl
	Double charged species	<3%
	Oxide formation	<2%
Reaction cell parameters	H_2 gas /mL·min^{-1}	4
	Octapole bias/V	-18
	QP bias/V	-16
Data acquisition parameters: Total determination (ID analysis)	Acquisition mode	ID analysis
	Monitored isotopes	^{64,66,68}Zn, ^{63,65}Cu, ^{54,56}Fe, ^{74,78,80}Se
	Points per peak	3
	Acquisition time per point/s	4
	Replicates	5
Data acquisition parameters: Quantitative speciation (ID-post-column)	Acquisition mode	Time resolved analysis
	Monitored isotopes	^{64,66,68}Zn, ^{63,65}Cu, ^{54,56}Fe,
	Points per peak	1
	Integration time (per peak)/s	0.3
SF-ICP-MS Element 2		
Plasma parameters	RF power/W	1300
	Cool gas flow rate/L·min^{-1}	16
	Sample gas flow rate/L·min^{-1}	0.90
	Auxiliary gas flow rate/l·min^{-1}	0.87
	Tuning solution	1 µg·L^{-1} Li, In, Ce and U
	Double charged species	<3%
	Oxide formation	<10%
Data acquisition parameters (IPD-Post column)	Acquisition mode	Quantitative speciation
	Monitored isotopes	^{32,34}S, ^{64,66,67,68,70}Zn, ^{63,65}Cu
	Resolution	Medium (R=4000)
Chromatographic conditions		
SEC-HPLC	Size Exclusion Column	Superdex 200, 10/300 GL
	Mobile phase	10 mM Tris/HCl, pH=7.4
	Flow rate/ mL·min^{-1}	0.6
	Injection volume/ µL	50
AE-HPLC	Anionic Exchange Column	Mono Q HR 5/50 GL (50 × 5 mm id)
	Mobile phases	Buffer A: 10 mM Tris/HCl, pH=7.4; Buffer B: 10 mM Tris/HCl, pH=7.4, 0,25 M ammonium acetate
	Flow rate/ mL·min^{-1}	1
	Injection volume/ µL	50
	Gradient of buffer B (time in min/%B)	0/0, 1.5/2, 2/3, 5.5/4, 6/10, 10/17, 11/18, 12/99, 19/100, 23/0
Laser ablation conditions		
LA-ICP-MS	Output laser energy	80 % (4.48 mJ)
	Repetition rate	10 Hz
	Spot diameter	25 µm
	Scan speed	17 µm.s^{-1}
	Ablation mode	Single line scan
	Carrier gas (He)	1.0 L.min^{-1}
	Temperature analysis	-20 ºC
	Reference glass sample (NIST SRM 612)	^{59}Co^{+}, ^{139}La^{+}, ^{232}Th^{+} and ^{238}U
	Oxide formation	<20%

Table S2. Microarray analysis of the multiple metallothionein isoforms and interleukins expressed in HLEsv cells before (control) and after been exposed with either: i) $^{68}ZnSO_4$ alone; ii) IL1α alone; and iii) $^{68}ZnSO_4$ + IL1α. The relative hybridization signal obtained for each of the MT isoforms was normalized with internal controls, and the obtained arbitrary units converted into fold-change when comparing treatments (up, overexpression; down, underexpression).
*compared to control.
**compared to $^{68}ZnSO_4$ treatment

GENE	$^{68}ZnSO_4$*	IL1α*	$^{68}ZnSO_4$+IL1α*	$^{68}ZnSO_4$+IL1α**
MT1A	2.306 up	2.135 up	2.282 up	1.010 down
MT1B	1.072 up	1.007 down	1.005 up	1.066 down
MT1E	3.694 up	2.217 up	4.184 up	1.132 up
MT1F	8.323 up	1.934 up	8.387 up	1.007 up
MT1G	36.154 up	2.791 up	40.158 up	1.110 up
MT1H	44.017 up	1.334 up	56.773 up	1.289 up
MT1M	12.698 up	1.334 up	18.226 up	1.435 up
MT1X	23.058 up	1.126 up	21.682 up	1.063 down
MT2A	3.467 up	2.773 up	3.373 up	1.027 down
MT3	1.393 up	1.036 down	1.230 down	1.714 down
IL1A	1.055 up	1.427 up	1.602 up	1.517 up
IL24	1.024 up	1.176 up	1.002 down	1.027 down
IL1B	1.024 up	1.771 up	2.374 up	2.317 up
IL6	1.030 down	13.274 up	11.964 up	12.329 up
IL32	1.086 down	2.569 up	4.157 up	4.517 up
IL10	1.381 up	1.322 up	1.584 up	1.146 up
IL18	1.315 up	1.127 up	1.551 up	1.179 up
IL8	1.532 up	39.574 up	47.778 up	31.185 up

Table S3. Concentration of natural (^{nat}Zn), exogenous (^{68}Zn) and total zinc (^{nat}Zn+^{68}Zn) (in µM) found in zinc binding-proteins/molecules, i.e., MTs, other than MTs, and all zinc-binding proteins/molecules), in the water-soluble protein fraction of HLEsv cells, by IPD-HPLC-ICP-MS. HLEsv cells were either not treated (control) or exposed to $^{68}ZnSO_4$ (at 25, 50 or 100 µM) and IL1-α (100U/mL) in combination or separately for 24h.

Treatment	cells/cm^2	Zinc-binding proteins/molecules	^{nat}Zn (µM)	^{68}Zn (µM)	Total zinc (^{nat}Zn+^{68}Zn)
Control	150,500	MTs	0.81 ± 0.12	0.00 ± 0.00	0.81 ± 0.12
		Other than MTs	1.00 ± 0.24	0.00 ± 0.00	1.00 ± 0.24
		All zinc binding proteins/molecules	1.81 ± 0.36	0.00 ± 0.00	1.81 ± 0.36
25 µM $^{68}ZnSO_4$	122,000	MTs	0.25 ± 0.01	0.97 ± 0.02	1.22 ± 0.03
		Other than MTs	0.38 ± 0.05	0.29 ± 0.02	0.67 ± 0.07
		All zinc binding proteins/molecules	0.62 ± 0.05	1.28 ± 0.04	1.90 ± 0.09
25 µM $^{68}ZnSO_4$ + IL1α	68,000	MTs	0.47 ± 0.03	2.57 ± 0.06	3.04 ± 0.09
		Other than MTs	0.44 ± 0.02	0.44 ± 0.03	0.87 ± 0.05
		All zinc binding proteins/molecules	0.91 ± 0.05	3.88 ± 0.13	4.78 ± 0.18
50 µM $^{68}ZnSO_4$	118,500	MTs	0.38 ± 0.01	3.38 ± 0.11	3.76 ± 0.12
		Other than MTs	0.51 ± 0.08	0.48 ± 0.05	0.99 ± 0.13
		All zinc binding proteins/molecules	0.89 ± 0.09	4.75 ± 0.22	5.64 ± 0.31
50 µM $^{68}ZnSO_4$ + IL1α	78,000	MTs	0.44 ± 0.02	4.19 ± 0.55	4.63 ± 0.57
		Other than MTs	0.35 ± 0.18	0.49 ± 0.14	0.84 ± 0.32
		All zinc binding proteins/molecules	0.83 ± 0.26	4.68 ± 0.69	5.51 ± 0.95
100 µM $^{68}ZnSO_4$	79,500	MTs	0.94 ± 0.25	5.20 ± 0.63	6.14 ± 0.88
		Other than MTs	0.46 ± 0.12	0.22 ± 0.29	0.68± 0.41
		All zinc binding proteins/molecules	1.40 ± 0.37	5.42 ± 0.91	6.82 ± 1.28
100 µM $^{68}ZnSO_4$ + IL1α	81,500	MTs	0.85 ± 0.13	6.11 ± 0.42	6.96 ± 0.55
		Other than MTs	0.73 ± 0.13	0.41 ± 0.32	1.14 ± 0.45
		All zinc binding proteins/molecules	1.58 ± 0.26	8.04 ± 0.64	9.62 ± 0.90
IL1α	114,000	MTs	2.75 ± 0.23	0.00 ± 0.00	2.75 ± 0.23
		Other than MTs	0.90 ± 0.20	0.00 ± 0.00	0.90 ± 0.20
		All zinc binding proteins/molecules	3.66 ± 0.43	0.00 ± 0.00	3.66 ± 0.43

Table S4. Concentration of MTs (in µM) labeled with ^{nat}Zn or ^{68}Zn, in HLEsv cells. Cells were cultured for 24h in the absence (control) or presence of (i) $^{68}ZnSO_4$ (at 25, 50 or 100 µM) alone, (ii) IL1-α (100U/mL), and (iii) a combination of $^{68}ZnSO_4$ (at 25, 50 or 100 µM) and IL1-α (100U/mL). The concentration of MTs containing natural (^{nat}Zn) and exogenous zinc (^{68}Zn) was determined by IPD-HPLC-ICP-MS. The elemental stoichiometric composition of the MTs complexes was calculated by the determination of the sulfur to metal (Zn, Cu) ratio, i.e., Zn:Cu:MT.

Treatment	Total protein (µg)	^{nat}Zn-MTs (µM)	^{68}Zn-MTs (µM)	Total MTs (µM)	Zn : Cu : MT stoichiometry
Control	462 ± 9	0.27 ± 0.04	0.00 ± 0.00	0.27 ± 0.04	3.03±0.03 : 0.23±0.03 : 1
25 µM $^{68}ZnSO_4$	384 ± 2	0.086 ± 0.002	0.351 ± 0.005	0.44 ± 0.01	2.88±0.09 : 0.10±0.02 : 1
25 µM $^{68}ZnSO_4$ + IL1α	428 ± 7	0.074 ± 0.001	0.42 ± 0.01	0.50 ± 0.01	6.30±0.30 : 0.11±0.01 : 1
50 µM $^{68}ZnSO_4$	433 ± 9	0.054 ± 0.001	0.48 ± 0.01	0.53 ± 0.01	7.07±0.04 : 0.07±0.01 : 1
50 µM $^{68}ZnSO_4$ + IL1α	479 ± 6	0.062 ± 0.003	0.59 ± 0.07	0.65 ± 0.07	7.08±0.10 : 0.08±0.01 : 1
100 µM $^{68}ZnSO_4$	380 ± 7	0.13 ± 0.03	0.752 ± 0.007	0.88 ± 0.04	7.17±0.18 : 0.17±0.02 : 1
100 µM $^{68}ZnSO_4$ + IL1α	352 ± 1	0.12 ± 0.02	0.892 ± 0.004	1.01 ± 0.02	7.11±0.16 : 0.12±0.01 : 1
IL1α	484 ± 8	0.45 ± 0.02	0.00 ± 0.00	0.45 ± 0.02	6.07±0.19 : 0.08±0.01 : 1

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