Journal of Trace Elements in Medicine and Biology 52 (2019) 48–52

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

Review

Ultraclean paired sampling for metal analysis in neurodegenerative disorders

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ARTICLE INFO

Keywords:
Cerebrospinal fluid
ICP-MS
Metals
Metallopathies
Neurodegenerative
Sampling

ABSTRACT

The causes of neurodegenerative disorders are largely unknown. Environmental factors seem to contribute to neurodegeneration in genetically susceptible individuals. Increasing evidence point towards a key role for environmental exposure in the causation of neurodegenerative disorders and specifically for metal exposure. Alterations of metalloproteins seem to be a common motif in neurodegeneration and enough evidence has now accumulated to designate these disorders as metallopathies. Paired sampling refers to the simultaneous sampling of CSF and blood and by comparing metal concentrations between CSF and blood conclusions about exposure and barrier properties can be drawn. However previous reports on metal concentrations in body fluids in neurodegenerative disorders show a wide variation in results hampering firm conclusions on the role of metals in degeneration of nerve cells. Here we suggest some steps and measures to minimise this variation, most important sampling performed in a cleanroom with filtered air and the use of acid washed perfluoroalkoxy vials. By strict adherence to ultraclean paired sampling technique conclusive results concerning the role of metals in neurodegenerative disorders can be generated.

1. Introduction

Neurodegenerative disorders include Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease and multiple sclerosis. Alzheimer's disease is the third leading cause of death in industrial countries [1]. The common denominator for these disorders is relentless degeneration of nerve cells in various parts of the nervous system. The causes of these degenerations are largely unknown.

Amyotrophic lateral sclerosis is often considered a model disorder for neurodegeneration. Anterior horn cells in the spinal cord degenerate in ALS, leading to progressive limb paresis and eventually to respiratory failure and death [2]. Increasing evidence point towards a key role for environmental exposure in ALS etiopathogenesis and specifically for metal exposure [3–5]. Metals have key functions in the brain and spinal cord but can also in situations of excess induce toxic effects on nerve cells [6]. Alterations of metalloproteins seem to be a common motif in neurodegeneration and ample evidence has accumulated to refer to neurodegenerative disorders as metallopathies [5].

In order to examine the precise role of metals in neurodegenerative disorders sampling of tissues and body fluids is essential and measurements of metal concentrations in various compartments necessary. Compartments of interest are red blood cells [7], reflected in samples of whole blood and blood plasma [8], cerebrospinal fluid (CSF) [3] and brain [9] and spinal cord tissue [10]. The barriers between blood and CSF are known as the blood-CSF barrier anatomically represented largely by the choroid plexus (CP) endothelium [11] and the blood-brain barrier constituted by the continuous nonfenestrated capillaries separating blood from brain tissue [12]. This review discusses the plasma-to-CSF trafficking of potentially toxic levels of metals and their effects on the central nervous system. Paired sampling refers to the simultaneous sampling of CSF and blood. By comparing metal concentrations between CSF and blood conclusions about barrier properties can be drawn.

In relation to the barriers between blood and CSF and blood and brain respectively, various metals show varied degrees of toxicity towards the CP endothelium concerning injury of the barrier endothelium itself, selectivity for certain metals, adherence to membranes and degree of permeability for metals.

Publications on metal concentrations in body fluids from patients with disorders of the nervous system report widely varying concentration mean values and standard deviations for a specific metal in CSF and blood in a population [13,14]. There are several explanations for these discrepancies related to general problems of sampling, metal contamination from the environment, inadequate definition of

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https://doi.org/10.1016/j.jtemb.2018.11.007
Received 30 November 2017; Received in revised form 1 November 2018; Accepted 13 November 2018
0946-672X/

reference groups and poor quality of the chemical analyses [13,15]. The greatest variation is found between the individuals themselves. Yet proper sampling technique and adequate cleanliness routines are necessary to ensure conformity of results. The variation noted precludes firm conclusion concerning the role of metals in neurodegenerative disorders and methods to reduce this variation are required. The need for ultraclean sampling and analysis has been noted for decades [16]. Sources of metal contamination of body fluid samples containing the metal studied must be eliminated as far as practically and economically possible [13,17].

Contamination contributes to the variation of measured concentrations found in metal studies and sources of contamination are diverse, complex and hard to eliminate. Reference values for metal concentrations exist for blood serum and whole blood (Seronorm™ materials from SERO AS, Billingstad, Norway) but not for CSF. Contamination problems apply to reference studies too [13]. Efforts have been made to standardize reference studies requirements and to eliminate or minimize variation by scrutinizing sampling conditions and providing criteria for publication in an international collaboration [13].

Method of choice for multielement trace analysis in body fluids from patients with neurodegenerative disorders is inductively coupled plasma mass spectrometry (ICP-MS). Modern ICP-MS instruments display a sensitivity down to sub-ng L^{-1} to sub-pg L^{-1} levels in aqueous solution. That means that the limiting step for sensitivity no longer is the instrument but rather various sources of contamination in the laboratory and the relation between field blanks and field samples. Recent developments in metal nanotoxicology [18,19] further emphasize the need for ultimate cleanliness in sampling and analysis. All sources of contamination leading to variation in metal concentrations measured or number of metal particles found need to be eliminated.

Sources of variation include metal contamination introduced by the investigators and by the patient, air quality in the sampling room, skin cleanliness, metal contamination from the lumbar puncture and venipuncture needles, choice of vials, protein precipitation in samples and storage conditions before analysis. Sampling should be performed in a cleanroom and analysis performed by ICP–MS in a cleanroom laboratory that meets the requirements for ultraclean trace metal analysis [15].

This article discusses in detail the challenges to be met in ultraclean sampling for metal analysis and provides firm suggestions on how to minimize variation.

2. Ultraclean sampling

2.1. Vials

Most medical studies on metal concentrations have used polypropylene (PP) tubes for sampling and storing body fluids from patients with neurodegenerative disorders prior to analysis. Vials made of PP are often considered chemically inert yet adsorption of metal to PP vial walls is a concern. Polypropylene vials are manufactured by casting hot plastic material over a specialized stainless steel amalgam mould (personal communication Dan O'Mara NNI Technical Support) and metals from the mould are found on the inner wall surfaces of the vial. Polypropylene vials seem to be less suitable as sampling vials for metal measurements for this reason and from concerns about the material itself. Polypropylene fibre has actually been used to remove metals from various geomedia [20] due to the metal binding properties of PP. Trace metals tend to adsorb to any available surface and PP may show a considerable adsorption in the range of 30–50% of metal on to vial walls [20]. This is particularly true at very low metal concentrations as present in blood and CSF samples from patients. Level of adsorption is also pH-dependent being less at acidic conditions and substantial at the neutral pH of body fluids [20,21]. Alkaline earth metals are adsorbed to PP to a lesser degree than potentially neurotoxic metals such as copper, manganese, lead and aluminium are [21]. Precise metal binding properties to PP vessel walls are insufficiently investigated and detailed studies on this topic are needed. Storage properties for metal containing body fluids in studies using PP vials may need some reconsideration before firm conclusions concerning metal concentrations in these fluids can be drawn.

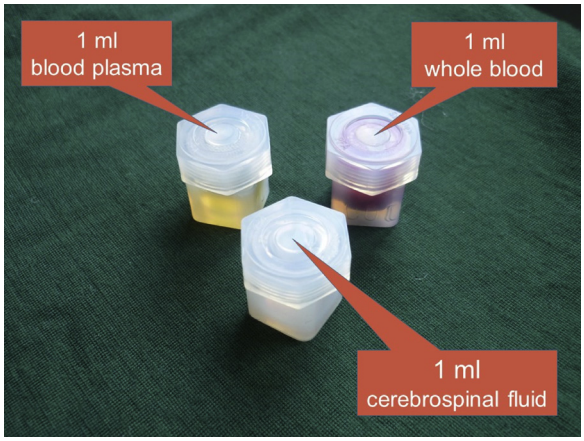

Fig. 1. Perfluoroalkoxy vials with PFA screw cap without gasket. Aliquots of 1 ml are sampled from each compartment.

For ultraclean sampling acid washed perfluoroalkoxy (PFA) vials (Fig. 1) are suggested. Perfluoroalkoxy vials withstand the high temperatures and concentrated acids necessary for thorough cleaning. Acid wash with ultrapure nitric acid plus hydrogen peroxide followed by several rinsing steps with ultrapure water [15] is mandatory before using vials for sampling of body fluids. Perfluoroalkoxy vials are also suitable for digestion directly in the sampling vial thereby reducing number of steps in the analytical workup and limiting contamination [15]. It is recommended that no preservatives or additives are used in sample preparation and storage preceding ICP-MS [22].

2.2. Investigators

A specialist in neurology trained in contamination free sampling techniques should perform the lumbar puncture. The greatest source of contamination in a cleanroom are the people in the room, and a person moving slowly in the room will release some 5 million particles larger than 0.5 µm per minute [23]. To ensure freedom from metal contamination from assisting nurses (Fig. 2) or from the specialist several precautions are necessary. All personnel in the cleanroom should be non-smoking without dental amalgam fillings and instructed not to wear nail polish, make-up, skin cream or metal jewellery nor to use hair dye. For the helping nurse mouth protection, eye protection, a clean hood and for the assisting nurse and the specialist in addition to this also full surgical protective clothing (Fig. 2) is suggested.

2.3. Skin

The patient himself as a source of metal contamination should not be neglected. The patient is instructed to perform a thorough skin wash and shower in the morning and to arrive at the hospital in old used washed clothes [24]. At the sampling table the skin of the lumbar area and the antecubital fossa are cleaned first with uncoloured ultrapure ethanol and then with 1% ultrapure nitric acid [25]. Clean surgical draping is used to prevent contamination from unwashed skin regions (Fig. 3).

2.4. Ambient air

Hospital ventilation systems are typically a source of metal particle

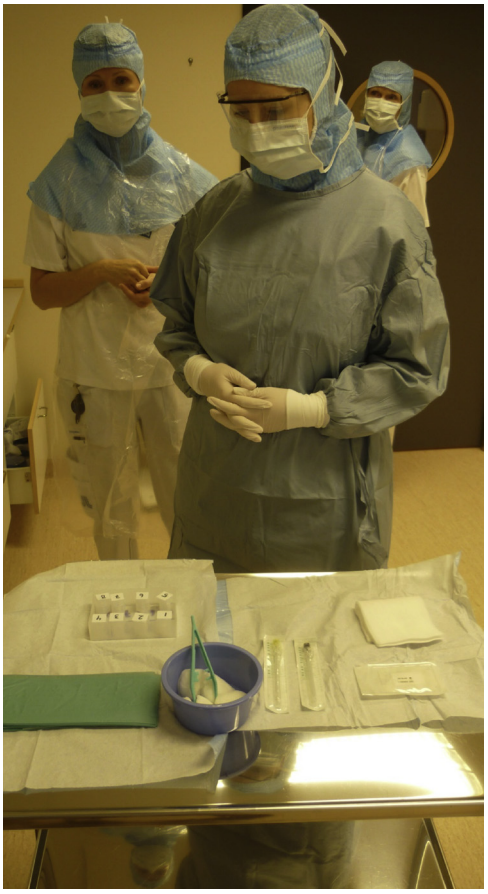

Fig. 2. Preparing for ultraclean lumbar puncture for trace element studies. Assisting nurse wears full surgical protective clothing and helping nurses wear hood and mouth protection.

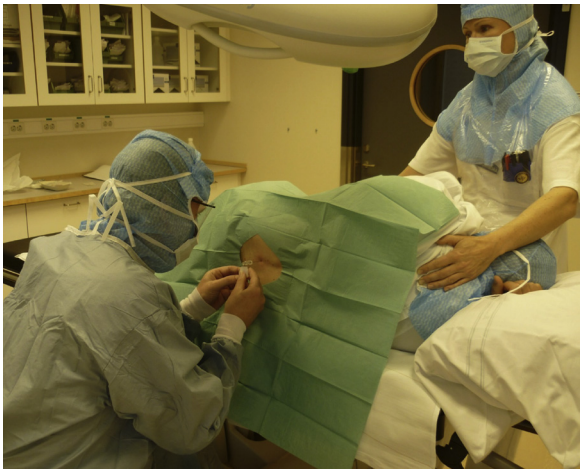

Fig. 3. Lumbar puncture performed at clean conditions in a cleanroom with filtered air. Drapings cover patient and protective gear is used by doctor and helping nurse.

contamination and air quality varies. Particles containing metals have been found in hospital air [26], specifically metals found in CSF but not in blood from patients with ALS i.e. manganese, copper and vanadium [3,4] have been noted in hospital air for manganese present at 3.5 ng/m^3 in fine particles and for Cu at 10.3 ng/m^3 and for vanadium at 5.6 ng/m^3 in hospital air [27]. Thorough air quality monitoring is necessary in ultraclean paired sampling. Incoming air to the cleanroom should pass through two high efficiency particle arresting (HEPA) filters mounted in series and the integrity of the filter system checked regularly with an aerosol photometer according to the standards ISO 14644-3. A ventilation system creating elevated pressure inside of the cleanroom and air-lock doors moving air only in the direction from the filters and outwards are necessary and cleanroom designed and constructed according to the standard ISO 14644-4.

2.5. Miscellaneous

During sampling CSF flows through the stainless steel lumbar puncture needle and some contribution of metal from the inner surface of the needle can be anticipated. The contribution for manganese and copper has been estimated to 0.00004 µg for manganese and 0.01552 µg for copper per millilitre passing through the needle [28]. Further data on contribution from the needle for other elements are needed.

Field blanks can be prepared by using a stainless steel lumbar puncture needle and flush it with ultraclean (Milli-Q ©) water on site in the cleanroom used for patient sampling. Alternatively an infusion drip bag with saline solution for intravenous infusion, mimicking body fluids, can be penetrated by the needle and samples taken from saline dripping from the needle.

Some metal contamination from shoe soles can be prevented by using dedicated cleanroom footwear and sticky mats before entering the clean area.

Surgical gloves made from natural rubber latex are potential sources of contamination as metal compounds including copper, manganese and lead are used as accelerators in the vulcanization process [29] and reside in the latex material. Other rubber additives are aluminium and antimony trioxide. Nitrile, polyvinylchloride or other plastic gloves are also potential sources of metal contamination due to metals present in the manufacturing process, most prominent for zinc and magnesium. Hospital hygiene requirements may however demand the use of gloves for lumbar puncture. If used powderless versions are preferred to avoid elemental contamination from constituents of the talcum powder sometimes used for lubrication. Thorough surgical handwash including a final step with 1% ultrapure nitric acid and rinsing with ultrapure water and no gloves is recommended for lumbar puncture in trace metal studies. Attention should be paid to handling of the needle and touching of skin avoided as zinc from keratin may contaminate the sampling. Zinc and chromium often represent great challenges in relation to contamination.

3. Discussion

Paired sampling of CSF and blood by simultaneous venipuncture and lumbar puncture at ultraclean conditions as described in this article allow for conclusions about metal distribution between CSF and blood and hence conclusions about barrier properties. Metal exposure in neurodegenerative disorders seem to be varied and complex [3,30] and several exposure routes for metals reaching the nervous system such as dermal, enteric, respiratory and retrograde axonal [31,32] have to be considered. Once in the bloodstream metals behave differently in relation to barrier systems depending on metal [11] and metal species [33]. Metals with neurotoxic properties can be separated into three categories depending on their behaviour in relation to the CP: A/ General CP metal toxicants capable to destroy the CP membrane (mercury, cadmium) B/ Selective CP metal toxicants impairing specific CP regulatory pathways critical to development and function of the nervous system (manganese, lead) and C/ Sequestered CP metal toxicants that adhere to the membrane without destroying it or specifically impact regulatory pathways (iron, silver or gold) [11]. It has recently been shown that the permeability of the blood-CSF-barrier varies for selected (i.e. manganese, iron, copper and zinc) metals [14] where much higher percentages of manganese do pass the barrier systems compared to the other metals studied. Manganese has been implicated as potentially causative in ALS [34].

Endothelia of the blood-brain barrier and the blood-CSF barrier protect the nervous system against metal toxicity yet metals with known neurotoxicity such as mercury, lead or arsenic can accumulate in the CP and cause substantial damage to the CP structure [11] or alter its protective properties allowing for metal entry into the spinal cord and brain.

By comparing metal concentrations in blood and CSF information about barrier properties can be gained. There seem to be a difference in molecular size distribution between various metal species where high molecular weight species, abundant in blood for iron, copper and zinc only pass the barrier into the CSF via regulated receptor mediated pathways [11,14] in contrast to low molecular weight species abundant for e.g. manganese where less specific transporters across the barrier may be used, of importance in situations of excessive exposure to manganese as in some cases of ALS [3,10,34,35], PD [36] and AD [37].

In these neurodegenerative disorders a common denominator seems to be accumulation of neurotoxic metals in variable yet vulnerable regions of the central nervous system. In order to further characterize and quantify the impact of metal toxicity in these regions results from paired sampling in humans should be combined with recently developed advanced methods for *in vivo* quantitation of metal deposits [38,39].

Metal speciation and analysis of metal concentrations in paired samples from patients with neurodegenerative disorders will pave the way for a better understanding of the complex interplay between metal exposure, barrier properties and degeneration of nerve cells. In order to be valid these analyses have to be performed on contamination free samples drawn in an ultraclean environment and analysed in cleanroom laboratories meeting the highest standards of analytical purity [15]. For legal reasons this may not be possible in some countries where clinical standard protocols for lumbar puncture are mandatory, yet it is important to strive towards including all details of ultraclean sampling as described above

Considering the somewhat sprawling reports over several decades about the involvement of metals in neurodegenerative disorders establishing firm reference values for metal concentrations in CSF is a priority task. In these efforts ultraclean sampling is also necessary. When the principles of ultraclean paired sampling described here can be applied to populations in different parts of the world with varied exposure conditions congregated valid information can be gathered in order to understand the role of metal exposure in neurodegenerative disorders.

4. Conclusion

Evidence accumulate for a possible causative role for metals with neurotoxic properties in neurodegenerative disorders. Abnormal function of metalloproteins in these disorders further strengthen the hypothesis that metal dyshomeostasis contributes to neurodegeneration. In order to examine the precise role of metals in neurodegenerative disorders sampling of tissues and body fluids is essential, and measurements of metal concentrations in different compartments necessary. Simultaneous extraction of blood and CSF fluid can give valuable information about metal exposure and barrier properties. Variation in results of metal measurements can be significantly reduced by adherence to ultraclean paired sampling technique.

Conflict of interest

None.

Acknowledgements

Valuable and constructive suggestions from the research nurses of the cleanroom surgery facilities of kidney KBC M87 Huddinge hospital are gratefully acknowledged. Procedures described in this article were performed under ethical permit EPN Stockholm 2014/1815-31/4 with informed consent from patients and controls. Pictures are published with permission from patient and personnel. This work was supported by Ulla-Carin Lindquist ALS-Foundation and Magnus Bergvall foundation.

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