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High-resolution identification of mercury in particles in mouse kidney after acute lethal exposure

A study by Scanning Electron Microscopy coupled with X-ray Elemental Microanalysis

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Abstract

Contamination of the food chain by mercury is a major concern of Public Health of our day. Kidney and nervous system are the major targets of mercury toxicity in mammals. We show here that the detailed subcellular *in vivo* topography of microparticles of mercury in tissues can be achieved by scanning electron microscopy (SEM) coupled with X-ray elemental microanalysis (XRM). SEM-XRM offered the fine topography of mercury in the kidney of BALB/c mice that were submitted to an intraperitoneal lethal injection of mercuric chloride ($HgCl_2$). All of the renal mercury was seen inside blood vessels located in both cortex and medulla of the mouse kidney. This blood-born mercury was organised in spheroid particles of less than 50 nm in diameter (31.4±14.1 nm). They were seen attached either to aggregates of plasma proteins or to the surface of blood cells. No evidence of internalisation of mercury by blood, endothelial or kidney cells was found. The average kidney density of mercury microspheres was 1920±1320 particles per mm^2. We propose SEM-XRM as an elective approach to further investigations, at the subcellular level, on the quantitative dynamics of mercury particles in the tissues.

Introduction

In nature, mercury can be found in organic and inorganic forms, and in different oxidation states. Mercury compounds that are taken up by animals may undergo transformation between inorganic and organic forms, as part of metabolic oxidation-reduction cycles of the host. Organic mercury (e.g., methylmercury) is liposoluble and, thus, more rapidly internalised than water-soluble inorganic forms of the metal. Methylmercury readily enters the central nervous system causing neuromotor and behavioural disorders. Ingestion of inorganic salts causes gastrointestinal disease, kidney failure, and death with acute lethal doses for humans ranging from 1 to 4 g. Divalent salts of mercury (such as $HgCl_2$ used here) are more toxic than the monovalent ones. Minute amounts of mercury are enough to induce disease in kidney and nervous system, or to cause autoimmune disorders (Timbrel *et al.* 1982; Goering *et al.* 2000; Rumbeika *et al.* 2000). Recently, ingestion of food contaminated with mercury was associated with increased frequency of heart disease (Guallar *et al.* 2002; Yoshizawa *et al.* 2002).

Since the industrial disaster of the Minamata Bay of the 1950s, resulting in the contamination of the food chain with methylmercury and in severe injury and death of hundreds of citizens, environmental levels of mercury have been submitted to close scrutiny (Clarkson 2002; Watts 1997). Mercury pollution of other areas of the world has also been reported during the second half of the last century (Falnoga *et al.* 2000; Myers *et al.* 2000; Timbrel 1989). The last decade has witnessed a growing concern regarding the widespread tainting of fish and shellfish with mercury. Recent alerts by American health authorities have recommended that pregnant women should not eat tuna fish more than twice a week, in order to avoid reaching teratogenic levels of mercury in their blood (Ginsberg

& Toal 2000; Goldman & Shannon 2001; Pless & Risher 2000; FDA 1999).

Methods for high-resolution localization of microparticles of mercury at the subcellular level are needed to better understand the mechanism of toxicity of the metal. Because the kidney is a major target for toxic activity of mercury, in its different chemical presentations, we have adopted here this organ to illustrate that the *in vivo* topography of mercury particles can be achieved by scanning electron microscopy coupled with x-ray elemental microanalysis (SEM-XRM). We also show here that quantitative data on the distribution of individual microparticles of the metal can be achieved using SEM-XRM.

Materials and methods

Animals

Seven female BALB/c, 6–8 weeks old, weighing 25 g were used in this study. They were obtained from a Spanish breeder (Charles River Laboratories Spain, SA). The animals were kept under standard animal house conditions and had free access to water and balanced food. They were treated in accordance with the European Union laws on animal protection (86/609/EC).

Mercury injection

Five mice were injected with a lethal dose of mercuric chloride ($HgCl_2$, 25 mg in 500 ul of PBS) in the peritoneal cavity. This amount of mercury killed all animals within 2 to 4 min. Two mice were used as controls; they were sacrificed after no treatment and their kidneys were used to confirm that no mercury is detectable in organs of untreated mice.

Scanning Electron Microscopy (SEM) coupled with X-ray Elemental Micronalysis (SEM-XRM)

The kidneys were fixed in an aldehyde mixture made up of 4% para-formaldehyde, 1.25% glutaraldehyde, and 10 mM $CaCl_2$ made in 0.1 M cacodylate buffer, pH 7.0–7.2 (Silva *et al*. 1987). The samples were dehydrated in ethanol and critical point-dried in a Balzer's apparatus using carbon dioxide as the transitional fluid. The preparations were mounted on metal stubs and coated by carbon under vacuum and examined in a JEOL JSM-6301F scanning electron microscope (SEM) that was coupled to a Noran Voyager x-ray elemental microanalyser (XRM) with EDS (Energy Dispersive Spectrometry) detection system. SEM-XRM allows the *in situ* identification of mercury upon the detection of the characteristic elemental spectra of the metal (see Figures 1 and 2). SEM micrographs of the samples were derived from secondary and backscattered electron imaging modes, the latter being used to detect mercury *in situ* by x-ray microanalysis (Peão *et al*. 1993).

Results

We show here that high-resolution *in situ* localization of mercury particles can be obtained by scrutiny of tissue samples by scanning electron microscopy (SEM) coupled with x-ray elemental microanalysis (SEM-XRM). This operation involves two steps. The first one is SEM imaging of the tissue sample that is obtained by treating it with secondary electrons. Because metal inclusions are opaque to electrons, all white spots seen in the sample become potential candidates for mercury-containing bodies. The second step is aimed at the specific identification of mercury in these electron-opaque inclusions: it consists in the screening of all white spots by x-ray elemental microanalysis to determine which of them contain mercury. This is achieved with the help of backscattered electrons that, being high-energy particles, require a fast exposure of the sample, otherwise mercury will be vaporised.

SEM-XRM revealed the subcellular distribution of mercury in the kidney of BALB/c mice that were intraperitoneally injected with a lethal dose of mercuric chloride ($HgCl_2$). All mice died within 2–4 min of mercury injection. We found that mercury-containing particles were present in the kidneys of all mice injected with mercuric chloride. In control (untreated) mice, no mercury was detected in the kidney. The mercury spots were spheroid in shape and of heterogeneous size with an average diameter of 31.5 ± 14.1 nm. Aggregates of individual microparticles of mercury were detected much less frequently than isolated microspheres. All mercury bodies were observed inside blood vessels; they were bound to the surface of blood or endothelial cells or to plasma protein aggregates. No evidence of internalization of mercury by blood, endothelial or kidney cells was seen. In the glomeruli, in particular, mercury could be detected inside capillaries but not inside podocytes. Both the cortical and medullary regions of the kidney presented

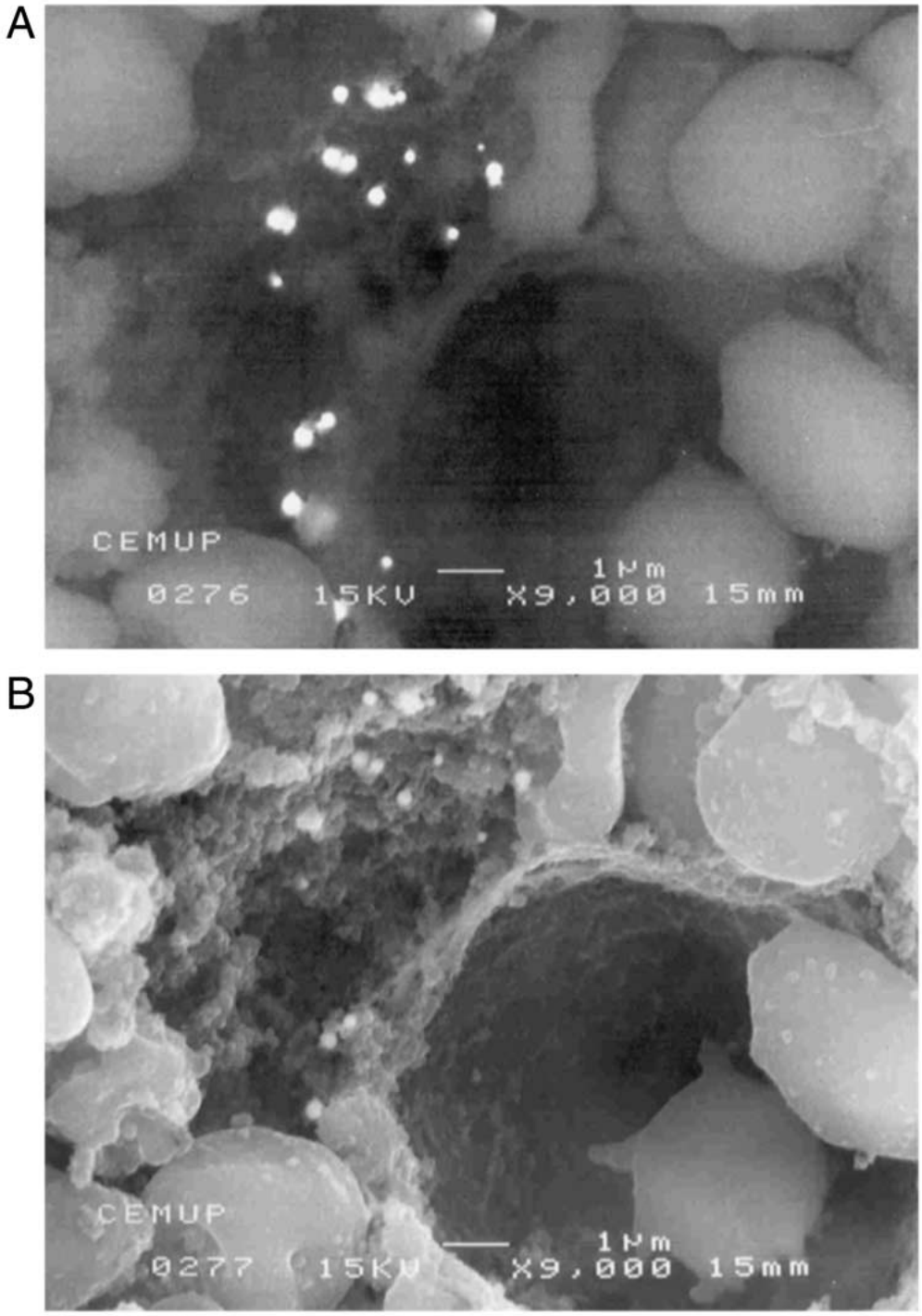

Fig. 1. Scanning electron micrographs using backscattered (Figure 1A) and secondary (Figure 1B) electrons of a kidney sample from a mercury-injected mouse. All of the white spots in figure 1A correspond to mercury. The mercury inclusions are attached either to the surface of blood cells or to plasma protein aggregates. × 9000.

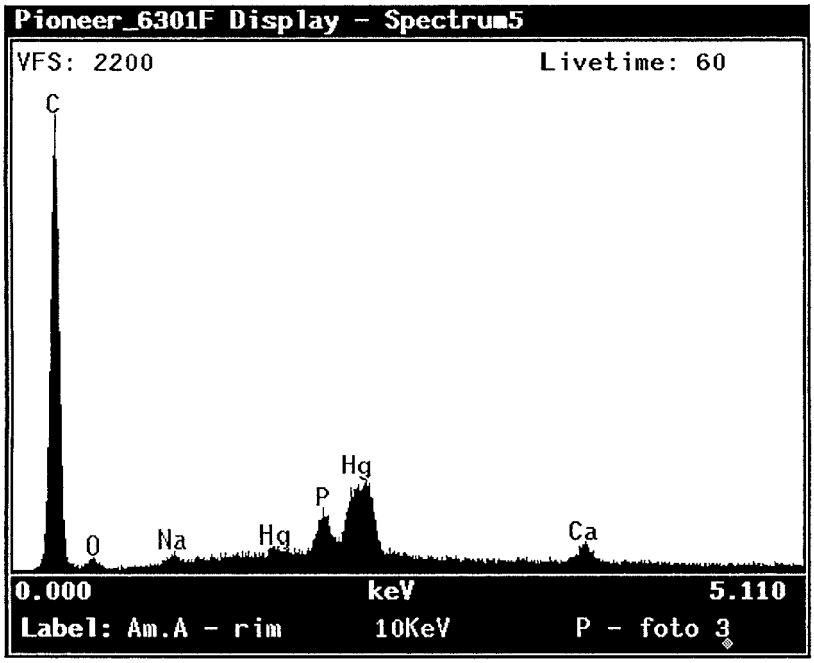

Fig. 2. X-ray elemental microanalysis spectrum of one of the electron opaque inclusions shown in the previous figure. The spectrum reveals that the inclusion contains mercury (two peaks marked as Hg), carbon (C in the figure) that is used to coat the sample for SEM viewing, and normal chemical elements of cells (Ca, Na, P and O). The two Hg peaks in the correspond different electron transitions that the Hg molecule undergoes after being bombarded by backscattered electrons; the peak on the left corresponds to the M energy level of Hg and the peak on the right represents the L energy level of Hg.

microparticles of mercury inside their blood vessels. Quantitative evaluation of mercury micropheres by SEM-XRM revealed that our samples contained a density of 1920±1320 particles of mercury per mm^2 of kidney tissue.

Discussion

This report documents a method that offers high-resolution *in situ* distribution of individual mercury particles in the kidney. Our work revealed the shape, size and topography of particles of blood-born mercury. The new data were obtained by SEM coupled with X-ray elemental microanalysis (SEM-XRM). The new structural information on the *in vivo* arrangement of microscopic of mercury can be summarised as follows: (i) mercury is carried inside blood capillaries mostly as individual spheres that have a diameter between 1–5 nm (31.5±14.1 nm); (ii) mercury is found in virtually all blood vessels of the kidney within 2–4 min of injection of the metal salt in the peritoneal cavity of mice; (iii) blood-born mercury is observed either attached to the surface of cells or to aggregates of plasma proteins.

Since we have used a high (lethal) dose of mercury, a note of caution is pertinent: the submicroscopic arrangement of Hg found in our samples may be difference if lower doses of Hg are injected. The observed spherical morphology of the microscopic particles of mercury may be explained by high surface tension of the metal (480.3 din/cm3). The attachment of mercury particles to the surface of cells or to plasma protein aggregates of kidney vessels is probably due to the chemical avidity of mercuric mercury for sulphur, in particular to sulphur present in thiol groups of proteins (Timbrel 1982, 1989). The bonding between mercuric ions and sulphydryl groups of proteins, such as albumin, has been postulated as an important factor in the pathogenesis of mercury-induced kidney disease (Zalups 2000). In our samples, we found that this binding does not lead to immediate internalisation of mercury particles since, 2–4 min after ingestion, no such mercury particle was observed inside blood, endothelial or kidney cells. In the long term, exposure to mercuric mercury is known to increase the amount of metallothionein-like proteins in the kidney (Nordberg *et al.* 1974).

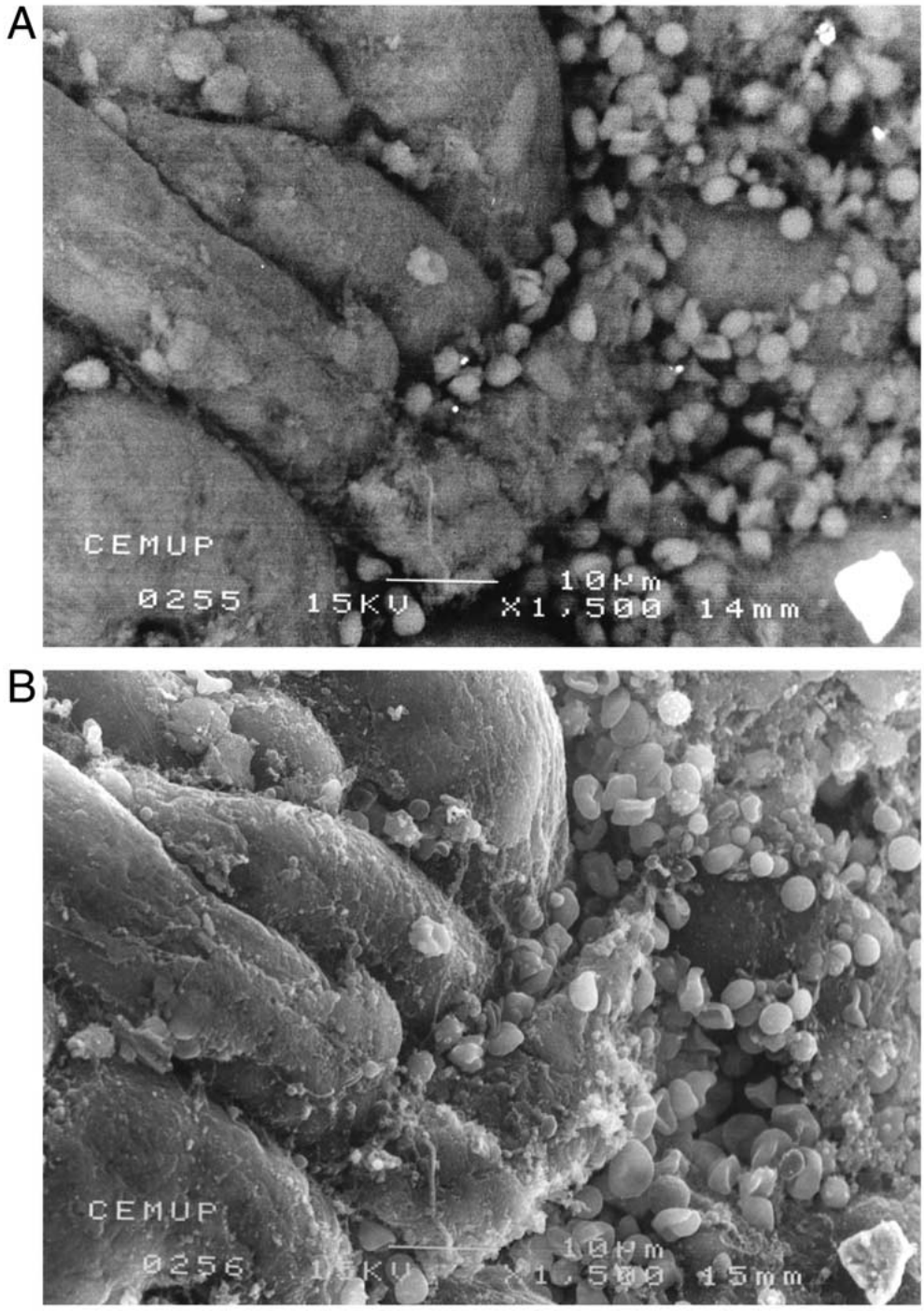

Fig. 3. Scanning electron micrographs, using backscattered (Figure 3A) and secondary (Figure 3B) electrons of a glomerular area of kidney of a mercury-injected mouse. The white spots in the centre and top right corner of figure 3A are mercury inclusions, as it was further demonstrated by *in situ* x-ray microanalysis. The mercury bodies are on the surface of blood cells of a kidney capillary. In contrast, the large white spot in the lower right corner was shown not to contain mercury (contamination of the preparation with metal debris). ×1500.

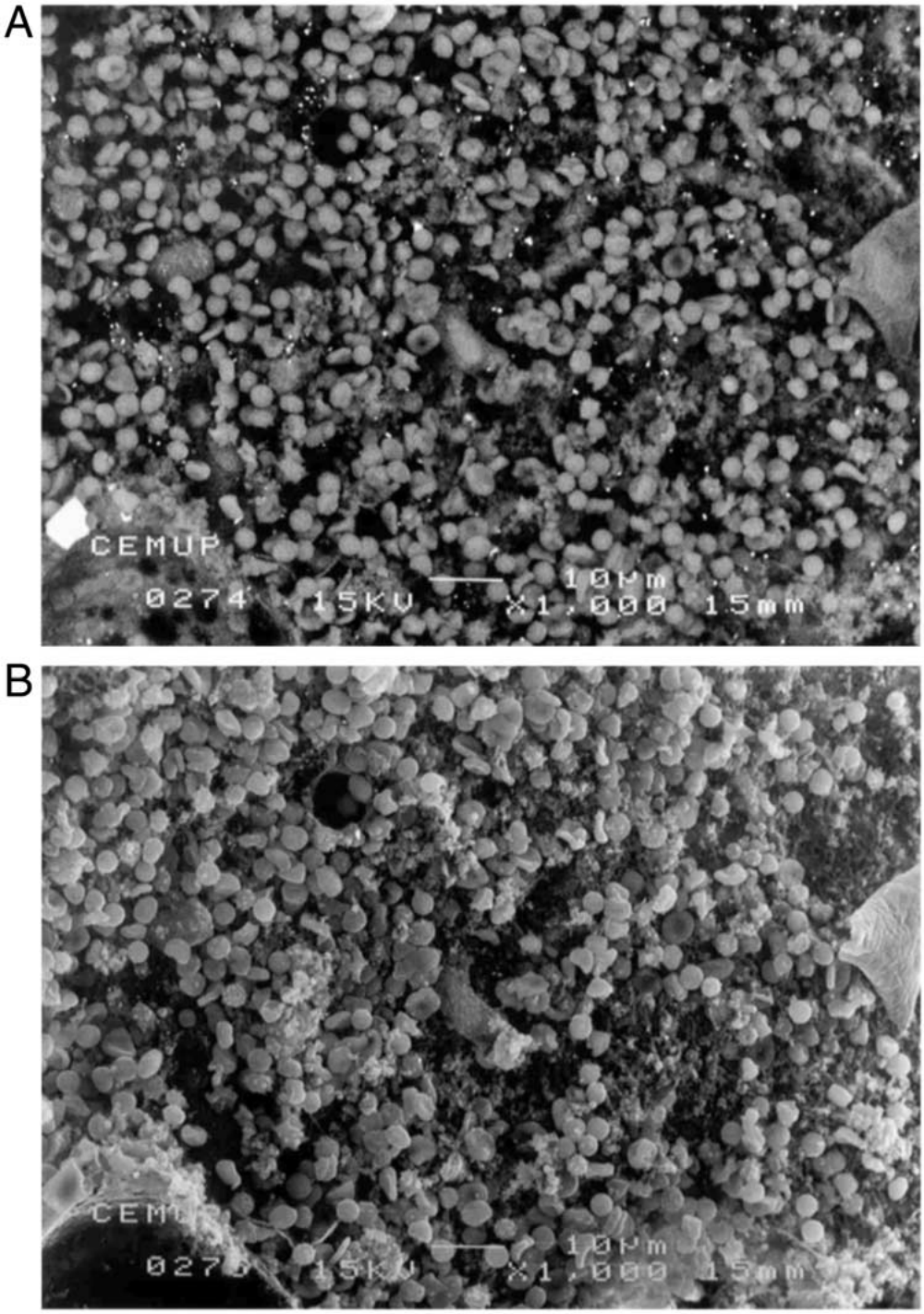

Fig. 4. Scanning electron micrographs using backscattered (Figure 4A) and secondary (Figure 4B) electrons of kidney medulla showing numerous mercury inclusions inside blood vessels. Mercury shows up as the small white spots scattered throughout Figure 4A. X-ray elemental microanalysis showed that the large white spot at the lower left corner of the figure does not contain mercury. ×1000.

Autometallography was been the major method used before to identify mercury *in situ* in tissue samples studied by transmission electron microscopy (Moller-Madsen 1994; Schionning *et al*. 1993a,b). It is a histochemical technique that makes mercury visible by turning areas of deposition of the metal into electron opaque inclusions; for that, it uses developers. Autometallography reveals the mercury that is bound to sulphide or selenic ions. We show here that SEM-XRM offers complementary information to the data obtained by autometallography, since it allows visualization of mercury by SEM. Other advantages of SEM-XRM are the detection of all mercury present on the sample, and the discrimination of mercury dots as small as 10 nm in diameter.

Most previous publications on the identification of mercury in tissues are clinicopathological investigations in humans that had suffered acute poisoning by mercury injection, namely in suicide attempts (Lupton *et al*. 1985; Garnier *et al*. 1982; Nadarajah *et al*. 1996; Netscher *et al*. 1991; Sau *et al*. 1991). These studies are focused on the pathological changes produced by the metal rather than on the fine morphology of mercury particles or on their topography in the tissues.

In conclusion, we document here how mercury particles look inside blood vessels and also that SEM-XRM can be used to obtain quantitative data on the density of mercury particles at the subcellular level. The herein report thus opens a new venue for future studies on the detailed kinetics of microscopic particles of mercury, from their entry into the host until mercury dots reach final storage in the body.

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