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Bioimaging of copper deposition in Wilson's diseases mouse liver by laser ablation inductively coupled plasma mass spectrometry imaging (LA-ICP-MSI)

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ABSTRACT

Unbalance of metals play an important role in the development of liver diseases. Wilson's disease for example is an autosomal recessive disorder in which the liver does not release copper into bile and resulting metal buildup leads to organ damage and liver failure. Laser ablation inductively coupled plasma mass spectrometry imaging (LA-ICP-MSI) is an established analytical technique for the determination of trace metals in biological tissue. This work demonstrates a new application of LA-ICP-MSI of trace metal imaging (Mn, Fe, Cu, and Zn) to study Wilson's disease in mouse liver tissue (vs. control sections). The quantification of tissue trace metals was performed using in-house produced tissue standards from murine brain with well-defined element concentrations. The results show that the average concentrations of Mn in control (0.7 $\mu g\,g^{-1}$) and Wilson's disease liver samples (0.6 $\mu g\,g^{-1}$) were not different. In contrast, Fe, Cu, and Zn in Wilson's disease liver samples (80 $\mu g\,g^{-1}$ for Fe, 143 $\mu g\,g^{-1}$ for Cu, and 32 $\mu g\,g^{-1}$ for Zn) were found significantly higher than in control tissue samples (41 $\mu g\,g^{-1}$ for Fe, 4 $\mu g\,g^{-1}$ for Cu, and 18 $\mu g\,g^{-1}$ for Zn).

1. Introduction

Wilson's disease is an inherited autosomal recessive genetic disorder that results in severe copper accumulations in tissue resulting in liver disease and variable neurological disturbances [1]. On the molecular level, a large number of different mutations within the *ATP7B* gene encoding for a copper-transporting ATPase with six putative metal-binding regions that is primarily expressed in liver account for the observed phenotypic alterations in patients with Wilson's disease [2–5]. The global incidence of this disease is approximately 1:30,000 and all ethnic groups are affected [6].

Copper is a redox active essential trace element that is incorporated into a variety of diverse proteins and metalloenzymes that are essential for maintenance of diverse biological functions including growth control and development, red blood cell formation, metabolism of cholesterol and glucose, and immune regulation. In the normal state, copper absorbance and homeostasis is tightly regulated by a complex network of different mechanisms that affect absorbance, transport, distribution, storage and excretion [6]. Both, the lack and excess in copper can lead to tissue injury and disease. In humans, the histopathology of copper-induced toxicity is extremely variable ranging from asymptomatic to chronic liver disease and severe neurological or psychiatric disorders due to toxicity in the brain [1,7]. Novel studies in *ATP7b*$^{-/-}$ mice suggest that elevated concentrations of copper induce oxidative stress along with severe dysfunction of mitochondrial energy production and further alterations in sterol metabolism resulting in molecular impairments that are causative involved in the formation of liver disease and formation of neurological abnormalities [8].

Presently, diagnostic procedures to exclude or confirm Wilson disease include the occurrence of copper depositions in the cornea causing the Kayser–Fleischer rings, the measurement of several biochemical parameters (e.g. urine copper, serum Ceruloplasmin), genetic testing, analysis of plasma clearance and liver uptake of intravenously administered radiocopper, D-penicillamine challenge tests, ultrasound scan of the liver, and magnetic resonance imaging of the brain [1,6].

Abbreviations: ATP7B, gene encoding the ATPase-dependent Cu^{2+}-transporting beta polypeptide; LA-ICP-MSI, laser ablation inductively coupled plasma mass spectrometry imaging; WD, Wilson disease.

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When Wilson disease is diagnosed at early stages, the prognosis for patients with this overload disease is excellent. However, most of the present strategies and screening tests cannot definitely rule out Wilson disease and many patients may not possess the characteristic findings when their clinical disease is relatively mild [9]. Therefore, the earlier detection of the disease and the identification of slight cooper accumulates is still challenging. Novel technologies in copper imaging including near-infrared fluorescent sensors [10] and quantitative positron emission tomography [11] for identification of abnormal copper metabolism are presently experimentally investigated.

We have previously established a novel quantitative imaging technique that is based on laser ablation inductively coupled plasma mass spectrometry imaging (LA-ICP-MS) of brain tissue [12–14]. These protocols also allow to measure trace metal distribution in cryo-sections with a thickness of 30 μm in healthy and diseased liver tissues [15].

In the present study, we performed elemental imaging for copper in liver tissue of normal and *Atp7b*-deficient by LA-ICP-MS with a spatial resolution at 60 μm. We found that this novel technology is suitable to detect hepatic copper deposits and to determine at once iron and zinc distribution that are both extensively altered in liver tissue of *Atp7b* deficient mice.

2. Experimental setup

2.1. LA-ICP-MS imaging of trace metals in murine liver tissue sections

A quadrupole-based inductively coupled plasma mass spectrometer (ICP-MS, XSeries 2, Thermo Scientific, Bremen, Germany) coupled to a laser ablation system (NWR 213, New Wave Research, Fremont, CA, USA) was used to study elemental distributions in tissue sections of mouse livers (30 μm thickness). Laser ablation of biological tissue was performed using a focused Nd:YAG laser beam in the scanning mode (wavelength 213 nm, repetition frequency 20 Hz, laser spot diameter 60 μm, scanning speed 60 μm s^{-1}, laser fluency 0.24 J cm^{-2}). The ablated material was transported by argon gas (as carrier gas) into the inductively coupled plasma (ICP). The ions formed in the atmospheric pressure ICP were extracted in the ultrahigh vacuum mass spectrometer via a differential pumped interface, separated in the quadrupole mass analyzer according to their mass-to-charge ratios and detected by an ion detector. No reference standard materials for quantification of metals in mouse liver were available. Therefore, matrix-matched (mouse brain) laboratory standards with well-defined element concentrations were used for the calibration of analytical data. Quantitative images of elements using prepared matrix-matched laboratory standards for calibration was described previously [14]. Briefly, five laboratory synthetic standard solutions with all elements of interest in well-defined concentrations were prepared. Five slices of the same biological tissue (each of about 0.65 g) were spiked with selected standard solutions. An additional slice was not spiked and was used for blank correction. The spiked biological tissues were properly mixed and centrifuged at 5000 rpm for 5 min. Samples were then frozen below a temperature of −50 °C and cut with a microtome into thin sections with a thickness of 30 μm and placed on glass slides. The mouse liver tissue and the brain standards were mounted in the laser ablation chamber to perform LA-ICP-MS imaging under identical experimental conditions. LA-ICP-MSI measurements of liver tissue were performed by line scanning ablation (line by line) with a focused laser beam under the optimized experimental parameters given in Table 1. The experimental parameters of LA-ICP-MSI were optimized with respect to the maximum ion intensity of $^{63}Cu^{+}$ using a matrix-matched laboratory standard. To validate the metal ion images, two isotopes of the same element were simultaneously analyzed when possible. From the continuous list of raw pixel values elemental images were reconstructed using the IMAGENA LA-ICP-MS Image Generation software created at Forschungszentrum Juelich [16]. Trace metal concentrations were calculated from ion intensities averaged throughout freely drawn regions of interest (ROIs) within ion intensity images using PMOD version 3.0 (for details see www.pmod.com).

Table 1
Optimized experimental parameters used for LA-ICP-MS imaging of mouse liver samples.

ICP mass spectrometer	ICP-QMS (Thermo XSeries II)
Rf power	1450 W
Cooling gas flow rate	16.0 L min^{-1}
Auxiliary gas flow rate	0.7 L min^{-1}
Carrier gas flow rate	1.0 L min^{-1}
Dwell time	20 ms
Extraction lens potential	3400 V
Mass resolution ($m/\Delta m$)	300
Scanning mode	Peak hopping
Analysis time per liver sample (10 mm × 10 mm)	4 h
Laser ablation system	**New wave (NRW213)**
Wavelength of Nd:YAG laser	213 nm
Laser fluence	0.24 J cm^{-2}
Repetition frequency	20 Hz
Laser spot size	60 μm
Scan speed	60 μm s^{-1}
Ablation mode	Line scan

2.2. Animals

Total livers were taken from $Atp7b^{-/-}$ mice with a genetic 129/Sv background ($n = 5$) in which an early termination codon in the mouse *Atp7b* mRNA was introduced by substitution of a portion of *Atp7b* exon 2 with a neomycin cassette oriented in the opposite transcriptional frame [17]. Wild type 129/Sv mice ($n = 5$) served as controls. All mice were housed at the University of Heidelberg, according to the guidelines of the Institutional Animal Care and Use Committees and in accordance with governmental requirements [8].

3. Results and discussions

3.1. Pathogenesis of Wilson's disease and metal imaging strategy

Wilson's disease is a classical disease that results from mutations within the *Atp7b* gene that under normal conditions acts as an ATP-dependent pump that removes cytosolic Cu^{1+} from the hepatocyte into the bile (Fig. 1A). In the present study, we measured the hepatic content of different metals by LA-ICP-MS in normal mice and in an experimental mouse model that is characterized by a homozygous disruption of the *Atp7b* gene (Fig. 1B). In the experimental workflow, liver tissue is first cryo-sected into specimen of 30 μm thickness and mounted on adhesive glass slides. These samples are then analyzed by LA-ICP-MS in which the ablation of liver tissue is done with a focused Nd:YAG laser beam. Following the ablation step, the ablated material is transported by an inert argon carrier gas into the inductively coupled plasma and formed ions are extracted in an ultrahigh vacuum mass spectrometer via a differential pumped interface, and separated in the quadrupole mass analyzer (Fig. 1C). Expression and distribution of individual elements is then calculated and plotted for each specimen (Fig. 1D). The optimized experimental parameters that are suitable to