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Iron, copper, zinc and bromine mapping in cirrhotic liver slices from patients with hemochromatosis studied by microscopic synchrotron radiation X-ray fluorescence analysis in continuous scanning mode[☆]

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Abstract

Iron (Fe) and copper (Cu) are essential metals in physiological cell metabolism. While Fe is easy to determine biochemically in histological slices, Cu and zinc (Zn) distribution is frequently critical in confirming the presence of an overload in disturbed Fe/Cu metabolism. To analyze Fe, Cu and Zn in a near histological resolution, energy dispersive microscopic synchrotron radiation X-ray fluorescence was applied.

In normal liver tissue, after fixation and imbedding in paraffin, mean Fe, Cu and Zn concentrations were 152 ± 54 , 20.1 ± 4.3 and $88.919.5 \mu\text{g/g}$ sample weight, respectively. No substantial, characteristic differences in their distribution were found in the two-dimensional scans. In slices from patients with hemochromatosis mean Fe, Cu and Zn concentrations were 1102 ± 539 , 35.9 ± 14.6 and $27.2 \pm 6.7 \mu\text{g/g}$ sample weight, respectively. Additionally, a significant decrease in phosphorus and sulphur concentrations existed. An increased Cu around cirrhotic regenerations nodules is mostly associated with a lymphocytic infiltration in this region. Analyzing concentrations of Fe in different regions of the samples show a clear negative dependency between Fe and Cu, Cu and Zn, but a positive one between Fe and Zn.

Conclusion: With a focal beam size of $15 \mu\text{m}$ in diameter a resolution of the elemental distribution was achieved which is widely comparable with stained histological slices ($20\times$ light microscope). The analysis of simultaneous determined elements reveals metabolic differences between Fe, Cu and Zn in liver tissue from patients with hemochromatosis.

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1. Introduction

Iron (Fe), copper (Cu) and zinc (Zn) are essential metals in physiological cell metabolism. Their importance for the survival, replication and differentiation is well established [1,2]. Induced effects of these trace elements are dependent on one another [3–5]. Iron in excess is toxic, particularly in man, where hemochromatosis (iron overload characterized by

hyperabsorption of dietary iron) is one of the most frequent genetic disorders [6], leading in the end-stage to liver cirrhosis. On the other hand, zinc supplementation has shown to prevent acute hepatitis by reducing copper absorption and interfering with iron [7]. Diagnosis of hemochromatosis can be made by biochemical parameters, genetically and/or histologically by liver biopsy [8]. However, while iron is relatively easy to dye for the investigation of its distribution in human tissue slices, copper staining are often not sensitive enough or the chemicals used introduce impurities which may induce misleading results [9,10]. The widely applied rhodanine staining for copper detection identifies free copper, but not copper bound in copper binding proteins [11]. Orcin stains a copper binding protein corresponding to metallothionins. The analysis of homogenized

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tissue, e.g. by atomic absorption spectroscopy [12], can only provide coarse information about the local distribution of these metals in human tissues. Especially, if one is interested in quantitative two-dimensional element mapping in near histological resolution, physical methods such as scanning nuclear microprobes [13], secondary ion mass spectroscopy [14], laser microprobe mass analysis [15], energy-dispersive X-ray analysis [16] have been applied. However, they showed low precision or poor detection limits. Using laser ablation-inductively coupled plasma mass spectrometry an elemental mapping of Cu and Zn was achieved in sheep liver slices [17].

The aim of the study was to visualize and quantify the distribution of iron, copper and other elements in human liver slices by microscopic synchrotron radiation X-ray fluorescence analysis (μ -SRXRF). The microfocus setup at HASYLAB, beamline L, provides a microbeam of 15 μ m diameter. Two-dimensional elemental maps of near histological resolution were recorded and compared with histochemically prepared samples. The measurement of physiologically relevant areas of some 10 mm² in reasonable time was facilitated by the implementation of a continuous scanning mode and a new detector system. The investigation of normal liver tissue and cirrhotic hemochromatosis affected liver tissue from a transplanted patient is the first application of the (sub-) second data acquisition setup.

2. Experimental

2.1. Sample system

Liver slices from a liver transplanted patient (age: 54 years) were investigated. Transplantation had to be performed due to the insufficient liver function caused by liver cirrhosis induced by hemochromatosis. Additionally liver slices from a patient who died from cardio-vascular failure were investigated. Liver sections received from the Pathological Department, Medizinische Universität Wien, were about 10 cm² in size and imbedded in paraffin. Slices of 10 μ m thickness were fixed on trace element free Ultralene[®] foil of 4 μ m thickness for investigation.

2.2. Microscopic synchrotron radiation X-ray fluorescence analysis

Microscopic synchrotron radiation X-ray fluorescence analysis (μ -SRXRF) measurements were performed at beamline L of the DORIS III storage ring at HASYLAB/DESY in Hamburg. For details concerning the beamline and standard experimental setup, see Refs. [18,19]. The white beam of the bending magnet was monochromatized by a double multilayer monochromator (NiC). The energy of the incoming beam was set to 17.5 keV for all measurements. The monochromatic X-ray beam was focused by a polycapillary half-lens to a cross section of 15 μ m providing a flux of 10¹¹ photons/s [18,20]. Liver slice samples were mounted on a XYZ-sample stage and scanned relative to the X-ray micro-beam. In the present study a constant sample time of 1.25 s per point was used. The increment in horizontal direction was 25 μ m and 15 μ m in vertical direction. Investigated areas and the position of the measurement were controlled by a long-distance light microscope connected with a CCD camera for controlling areas of interest in an incident- or transmitted light mode. Fluorescence photons and scattered radiation are detected in an energy-dispersive semiconductor detector. Previous studies on tissue slice samples [21] revealed that the sensitivity of the spectrometer and time of measurement is limited not by the flux of the incoming beam, but by the maximum processable countrate of the X-ray detector and electronics and the overhead time of the motor control system between successive points of the scan. Therefore, the standard setup was modified by the use of a detector collimator designed to reduce air scattering, the replacement of the Si(Li) detector by a silicon drift detector and the implementation of the continuous scanning mode for the reduction of overhead time.

2.2.1. Detector collimator

The collimator has a conical inner shape. Its inner diameter opens from 1 mm at the tip to 6 mm close to the detector window. The distance of the collimator tip to the detector crystal is 25 mm. A detector crystal of 50 mm² area is completely illuminated at a distance of 3 mm from the sample to the

Fig. 1. Single spectrum of normal liver tissue fitted using the AXIL software. Observed elements are P, S, Ar, Ca, Fe, Cu, Zn, Se, Br, Sr. Acquisition time 1.25 s.

Table 1

Mean values of count rate (cts/s) and concentration (ppm) of elements in normal liver tissue of 10 μm thickness after fixation and imbedding in paraffin

Elem	P	S	Ca	Fe	Cu	Zn	Se	Br	Comp
Cts/s	54.3	308	3177	1475	304	1868	14.3	15.1	7500
ppm	1604	2482	2287	152	20.1	88.9	0.6	0.55	–

collimator tip, but sees only 2 mm air path excited by the incoming beam. The front part facing the detector is made of solid molybdenum of 99.95% purity (minimum wall thickness of 4 mm). The collimator shields the detector crystal also efficiently against fluorescence radiation emitted by the housing of the polycapillary and facilitates contamination-free detection.

2.2.2. Silicon drift detector

A silicon multi-cathode X-ray Spectrometer VORTEX-EX (Radiant Detector Technologies) has been employed in combination with a CANBERRA 2060 digital pulse processor. The system provides an excellent energy resolution of 150 eV (5.8 keV) for all count rates up to 80 000 cts/s. Shifts in peak

position with count rate or other types of instabilities have not been observed [22]. The large active area of 50 mm² and the detector snout length of 100 mm enable a solid angle of detection similar to that of a standard Si(Li) detector.

2.2.3. Continuous scanning mode

In the standard scanning mode for the measurement of XRF maps motor movement spectrum acquisition, readout and transfer are executed sequentially, which results in an overhead time of ~ 2 s per measurement point at our experiment. Fast scans of (sub-) second time per measurement point are made feasible with the implementation of the continuous scanning mode [23]. The motor of the inner loop is moving continuously with speed v while spectra are collected in the multi channel analyzer (MCA) for a predefined sample time Δt . During readout of the data and writing the complete MCA spectrum on hard disk the spectrometer is “blind” for a constant time δt . Time $\Delta t + \delta t$ and speed v determine the pixel size Δy . However, for typical values of 1.25 s sample time and 25 μm pixel size the coverage $\Delta t / (\Delta t + \delta t)$ is 98% and can be neglected. After

Fig. 2. a–d: Normal liver tissue (20 $\times$ light microscope) with Hematoxilin–Eosin (HE) staining in comparison with (b) iron (Fe), (c) copper (Cu), (d) zinc (Zn) and (e) bromine (Br) distribution determined by μ -SRXRF. Sample time/pixel 1.25 s, area 4000 $\times$ 3750 μm^2 , 25 $\times$ 15 μm^2 step size. The color bar at the right indicates the element concentration in ppm.

completion of a line, the motor moves back with maximum speed, the other motor is moved by one step and the next line is measured. The motor positions, MCA file names, counters for ionization chamber signal, DSP dead time and DORIS current and predefined regions of interest (ROI) of the MCA for every pixel are compiled in a single ASCII file, which is updated after every pixel. This file is also used for the display of the ROI maps during the measurement.

2.2.4. Quantification

The X-ray spectra were peak-fitted using the AXIL program package [24], in order to extract the net intensities of fluorescence lines. A typical peak fitted spectrum of normal liver tissue is shown in Fig. 1. The net peak intensities were normalized to the intensity of the incoming monochromatic beam intensity and 1 s sample time, and were corrected for detector system dead time. A germanium standard foil of homogeneous Ge area density of $2.6 \times 10^{-8} \text{ g/cm}^3$ was employed for external standardization [25]. An array 9×5 points was measured for 10 s before and after each 2d scan. The standard deviation of the mean Ge K- α line intensity between individual scans was 1.7% after normalization, demonstrating the stability of the experimental conditions. Element concentrations were calculated from the normalized net peak intensities using the fundamental parameter method based program package “quant” [26]. No absorption correction was necessary for the elements of interest ($Z > 25$) on thin section of organic tissue and the sample-detector geometry, sample thickness and beam energy was unchanged during the measurement series. Therefore, the same set of element specific conversion factors could be applied for all measurements.

2.2.5. Sensitivity of spectrometer for liver tissue analysis

The intensity of the micro beam provided by the combination of multilayer monochromator and polycapillary optics and the large solid angle of the detector enable a high sensitivity of the spectrometer for the elements in the range from P to Sr. Table 1 specifies the mean counts per second net peak intensities and concentrations derived from the fundamental parameter calculations obtained on normal liver tissue of $10 \mu\text{m}$ thickness. The net peak intensities are high enough to realize low-noise element maps for elements like Fe or Zn, but reasonable structures within 2D mapping can be also obtained for trace elements like Se and Br or light elements like P and S within 1 s sample time per pixel, as demonstrated in Figs. 2 and 4.

3. Results and discussion

3.1. Normal liver tissue

Fig. 2 shows the results of two-dimensional iron (Fig. 2b), copper (Fig. 2c) and zinc mapping (Fig. 2d) in a slice of not affected, normal liver tissue in comparison with a conventional, histological Hematoxylin–Eosin (HE) staining (Fig. 2a). Additionally, the distribution of bromine (Fig. 2e) is given. Bromine seems to be mainly distributed in connective tissue and vessel walls [27,28]. In normal liver tissue iron, copper and zinc

distributions revealed no greater differences in their concentration throughout the measured area, and varied only in a small range (Fig. 3a–c). However slightly elevated concentrations of Cu and Zn were found in the liver capsule (upper border of Fig. 2c, d), which consists mainly of connective tissue as indicated by the increase of bromine (Fig. 2e). Mean values of the investigated elemental concentrations are given in Tab 2. The determined elemental concentrations for Fe, Cu and Zn in normal liver tissue by using a germanium-standard for their calculation were in line with previously published data mostly

Fig. 3. a–c: Associations between (a) Fe and Cu, (b) Cu and Zn and (c) Fe and Zn in normal liver tissue determined for six different areas and total scan, each. Concentrations are given in ppm ($\mu\text{g/g}$ sample weight).

Fig. 4. a–f: Hemachromatosis (HC) with regeneration nodules. (a) Prussian Blue staining, (b) Rhodanine staining (20 $\times$, light microscope) and (c) iron (Fe) and (d) copper (Cu) elemental maps determined by μ -SRXRF. Also shown are (e) zinc (Zn) and (f) bromine (Br) elemental maps. Sample time/pixel 1.25 s, area 4000 $\times$ 3750 μm^2 , 25 $\times$ 15 μm^2 step size. The color bar at the right indicates the element concentration in ppm.

determined by using atomic absorption or X-ray fluorescence techniques [29,30]. In normal liver tissue low variations for Fe, Cu and Zn were detectable, indicating a close homeostatic balance between these elements. These results are consistent with previously published data in normal liver tissue [29–34].

3.2. Haemachromatosis

Two-dimensional scans in a liver slice from a patient with genetically confirmed iron-overload due to a hemochromatosis are shown in Fig. 4a–f in comparison with two different histochemical stainings: Fig. 4a Prussian Blue and Fig. 4b Rhodanine. The cirrhotic nodules are clearly distinguishable not only in the histological preparation but also in the element maps. As seen in Fig. 4a and Fig. 4c an excellent coherence between the histochemical preparation of Fe and the Fe-element-map was achieved, showing an in-homogenous distribution of Fe within cirrhotic nodules. By contrast, this is not the case for the copper distribution in comparison with the histochemical preparation (Fig. 4b and d). Again higher concentrations of Cu were detected for the liver capsule, but more surprising high concentrations of Cu were located around the iron-loaded regeneration nodules. Normal concentrations of Cu existed within the cirrhotic nodules. Again Br clearly demarcates the regeneration nodules, and characterizes thus

the fibrotic connective tissue between them (Fig. 4f). Interestingly, Cu distribution in HC turned out to be characteristically distributed in comparison with Fe. Higher concentrations of Cu at the border of the Fe containing regeneration nodules were found (Fig. 4d). Inspecting the corresponding histological preparation in a higher magnification (50 $\times$), this border area is characterized by a zone of lymphocyte infiltration. We therefore may suspect that the higher Cu concentration is associated with these cells. Copper metabolism is altered in inflammation. In contrast to iron levels that decline in serum in infection and inflammation, copper concentration rise. Moreover, copper-containing enzymes and even many copper complexes are capable of protecting against the organism radical oxygen species in chronic diseases [2], and may thus give a clue for the observed increased copper levels at the border of the Fe-containing regeneration nodules.

Table 2

Mean values and S.D. for determined elements in normal liver tissue (NL) and for liver tissue in hemochromatosis (HC) given in ppm ($\mu\text{g/g}$ sample weight)

ppm		P	S	Ca	Fe	Cu	Zn
NL	Mean	1604	2482	2287	152	20.1	88.9
	S.D.	511	457	394	54	4.3	19.5
HC	Mean	801	1115	1390	1102	35.9	27.2
	S.D.	307	243	261	539	14.6	6.7

Fig. 5. a–c: Associations between (a) Fe and Cu, (b) Cu and Zn and (c) Fe and Zn in hemochromatosis (HC) normal liver tissue determined for five different areas and total scan, each. Concentrations are given in ppm ($\mu\text{g/g}$ sample weight).

In Table 2 values for the concentrations are given, determined as stated above. Interestingly P, S, Ca and Zn were significantly reduced, while Cu was increased as it was the expected case for Fe. Scarce information is available about sulphur and phosphorus concentrations in HC. Quantitative hepatic phosphorus-32 magnetic resonance spectroscopy investigations in liver cirrhosis indicate that particular in decompensated liver cirrhosis metabolic disturbances in hepatic energy and phospholipids metabolism exist. A significantly decreased level of adenosine-tri-phosphate was found [39,40].

On the other hand, recent findings indicate that sulphur-containing amino acids attenuate the development of liver fibrosis [41]. In the light of these findings the decreased phosphorus and sulphur level in our HC measurements seems to be feasible. However, for comparison of absolute concentration values with other studies it should be noticed that the samples used in our study were chemically fixated and embedded in paraffin. It is known that this preparation can change the concentration of light elements significantly [42]. Potassium, for example, is almost completely eliminated.

3.3. Elemental dependencies

As suspected from Fig. 3 an inverse dependency between Fe and Cu distribution seems to exist in HC. In Fig. 5a–c this is demonstrated numerically. A high Fe concentration in liver tissue is associated with a decrease of the Cu (Fig. 5a). Moreover high Cu decreases Zn (Fig. 5b) while Fe and Zn are positively correlated (Fig. 5c). Metabolic links between Fe, Cu and Zn have been repeatedly described on the molecular basis [1]. Their interaction was found in processes such as Fe uptake in eukaryotes, ceruloplasmin ferroxidase activity [35] or metallothionein (MT) induction. MT is a cysteine-based protein that transports metals such as Cu and Zn. The recently described divalent metal transporter (DMT1) binds not only Fe but also Cu [36–38]. These biochemical dependencies should be elucidated by inspecting and analysing regions with different Fe concentrations in the scans of HC. In fact, correlations between Fe, Cu and Zn were found (Fig. 5a–c). While a negative correlation was found for Cu–Fe and Cu–Zn, a positive one existed for Fe–Zn. This seems to correspond with recent findings. Exposing Hep-G2 cells – a model of hepatic cells – to increasing extracellular Cu concentrations resulted in a decrease of Fe and Zn concentration intracellular, measuring Fe and Zn contents by total-reflection X-ray fluorescence spectrometry [38]. These data indicate that Cu overload at least in Hep-G2 cell lines reduces their Fe and Zn content presumably by downregulating DMT1 activity. Our investigated sample was a genetically caused hemochromatosis. But it seems at present that high Fe reduces Cu and increases Zn in liver tissue in HC.

4. Conclusion

In conclusion we could demonstrate that using SRXRF technique with a focal beam size diameter of 15 μm metal distributions in a near histological resolution can be achieved. The spatial resolution approximately corresponds to a 20 times magnified histologic preparation so that elemental distribution can be associated to structural differences. In determining elemental concentrations additionally metabolic links between Fe, Cu and Zn could be calculated which seem in line with biochemical investigations.

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