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ORIGINAL CONTRIBUTION

Assessment of iron absorption in mice by ICP-MS measurements of ^{57}Fe levels

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Abstract
Background The study of iron metabolism is essential in nutritional sciences as iron deficiency is one of the most common nutritional deficiencies in humans and represents a serious health problem worldwide. The mouse is utilized as a unique and powerful model for the identification and characterization of genes involved in iron metabolism and for studying the pathogenesis of iron disorders. Thus, sophisticated and sensitive techniques have been developed to study iron metabolism in this animal model. In particular, iron absorption has been studied in mice by using the radioisotopes ^{55}Fe and ^{59}Fe in tied-off or dissected and everted duodenal segments. Nevertheless, several drawbacks discourage the extended use of these approaches.
Methods and results Here, we report the use of the stable isotope ^{57}Fe to measure iron absorption in mice. We show that after oral administration of ^{57}Fe-containing solutions, it is possible to measure both duodenal iron retention and duodenal iron transfer to specific organs, using inductively coupled plasma mass spectrometry (ICP-MS). As ^{57}Fe is administered orally, no surgical operation is needed before the end of the experiment, thus allowing the measurement of iron absorption under physiologic conditions. Moreover, the use of ICP-MS for ^{57}Fe detection ensures high sensitivity and provides quantitative data. Finally, the use of a stable isotope enables the measurement of both iron absorption and histologic and/or biochemical analyses in the same animal.
Conclusions The use of ^{57}Fe to measure iron absorption in mice, therefore, represents an alternative to radioisotope-based methods, providing a new tool to extend our knowledge on the mechanism of iron absorption.

Keywords Iron absorption · Mice · ICP-MS

Introduction

Iron is an essential nutrient for all forms of life as it participates in many biological processes, including oxygen transport, as well as behaving as a cofactor to several enzymes. In humans, iron deficiency represents a common health problem both in Western and developing countries. The main causes of iron deficiency are poor absorption of iron by the body, inadequate daily intake of iron, pregnancy, growth spurts or blood loss due to trauma, infection, malaria or haematological disorders. Conversely, iron is toxic when in excess, and therefore, body iron levels are tightly regulated. As the body cannot actively excrete iron, the body iron content is regulated at the absorption step when it is taken up by duodenal villus enterocytes. Main stimuli for iron absorption are represented by changes in body iron stores and according to the requirements of the developing erythroid mass [1].

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Most of our knowledge on iron homoeostasis relies on studies carried out on mice. Several genetic models are available, including both natural and gene targeting-generated mutations of the main genes involved in iron absorption, recycling, storage and utilization [2–6]. The mouse also represents a unique model to identify novel players in iron homoeostasis [7–9]. Finally, it has been shown that the occurrence, in mice, of genetic mutations responsible for human iron disorders, for example hemochromatosis and anaemia, fully recapitulate the human diseases, thus stressing the importance of mouse models for studying the pathogenetic mechanisms of these diseases and for testing new therapies [10, 11].

Up until now, iron absorption has been measured in mice by using the tied-off duodenal technique along with the radioisotopes ^{55}Fe and ^{59}Fe [12, 13]. ^{55}Fe is a beta emitter that allows the determination of mucosal uptake after administration of an oral dose of iron or incubation of a duodenal segment in the presence of the radioisotope, whereas ^{59}Fe is a gamma and beta emitter that, besides measurement of mucosal uptake, enables the determination of iron transferred from the duodenum to the rest of the body. The main disadvantage of using ^{59}Fe is that gamma emissions have a significant penetrating and shallow external exposure hazard, thus requiring laboratories specifically equipped for handling animals treated with this isotope. Here, we report an alternative approach to assess iron uptake and distribution in mice, based on the use of the stable iron isotope ^{57}Fe, detected by inductively coupled plasma mass spectrometry (ICP-MS). ^{57}Fe is one of the four naturally occurring isotopes of iron, and accounts for 2.119% of total iron, the other isotopes being ^{54}Fe (5.845%), ^{56}Fe (91.754%), and ^{58}Fe (0.282%) [14]. Natural iron isotope variations in the blood may serve to identify differences in intestinal iron absorption between individuals and genotypes [15]. Moreover, ^{57}Fe and ^{58}Fe have already been used as tracers in nutritional studies on humans due to their low natural abundance [16–18].

Herein a procedure is reported to assess both duodenal mucosal retention and mucosal transfer of ^{57}Fe to specific organs, following administration of an oral dose of ^{57}Fe in mice.

Materials and methods

Animals

Wild-type and Hfe-null mice [19] in the 129Sv genetic background were fed on a standard diet (4RF25 GLP, Mucedola, Settimo Milanese, Milano, Italy) containing 8 and 292 mg/kg haem-iron and inorganic iron, respectively, and received water ad lib. In the low-iron experiments, mice were given a AIN93G iron-deficient diet (Mucedola, Settimo Milanese, Milano, Italy) containing 54 mg/kg total iron for 2 weeks.

All experiments were approved by the animal ethical committee of the University of Torino (Italy).

Treatment

The stable iron isotope ^{57}Fe (^{57}Fe at 94% enrichment; Frontier Scientific Inc., Logan, Utah USA) was used as tracer. A 0.4 mol/L solution of $^{57}FeSO_4$ was prepared by overnight dissolution of 22.85 g ^{57}Fe/L in 0.4 mol/L H_2SO_4 (Sigma-Aldrich, Milano, Italy). The obtained $^{57}FeSO_4$ solution was stored at 4 °C. Before its use, 87.7 mg sucrose and 0.83 mg ascorbic acid per mg iron were added to the $^{57}FeSO_4$ solution to yield a final concentration of 20 mmol/L ^{57}Fe, 5.38 mmol/L ascorbic acid and 10% sucrose.

As a negative control, an analogous solution with no tracer was prepared.

Both the ^{57}Fe-labelled and the control solution were adjusted to pH7 by adding the required volume of 1 mol/L NaOH. To assess the in vivo tissue absorption of ^{57}Fe after oral administration of ^{57}Fe-containing solutions, overnight-fasted mice were orally treated with 20 μL of ^{57}Fe-labelled solution. Control mice received vehicle solutions. During treatment, mice received water ad lib. Tissues were collected at varying times following treatment. Control mice represented the '0' time point of the treatment.

Tissue collection

Mice were anaesthetized with Avertin (2,2,2-tribromoethanol; Sigma-Aldrich, Milano, Italy) at a dose of 2 mg/kg body weight.

Blood was collected from mice by cardiac puncture. Briefly, following a wash in heparin-containing solution, the needle of a syringe was inserted at the base of the sternum into the thoracic cavity and ventricular blood was slowly withdrawn. Samples were then centrifuged at 4,000 rpm for 6 min and supernatants were recovered, weighed and stored at −20 °C prior to proceeding with the analyses.

Duodenums, livers and kidneys were excised after transcardial perfusion of mice with 0.1 mol/L phosphate-buffered saline (PBS), washed in PBS, blotted dry, weighed and stored at −20 °C prior to analyses. Tissue perfusion with PBS was necessary to reduce background signal in ICP-MS measurements of ^{57}Fe absorption, considering the high amount of physiological iron present in blood.

Bone marrow was obtained from the hind legs of the mice. Briefly, immediately before surgery, mice were sacrificed. Tibias and femurs were dissected from surrounding muscles and tendons and flushed with PBS by inserting the needle of a syringe into the ends of the bones. The flow-through was then centrifuged at 1,200 rpm for 5 min and the pellet was weighed and stored at −20 °C prior to proceeding with sample analyses.

Tissue analyses by ICP-MS

^{57}Fe isotope and total Fe were determined using inductively coupled plasma mass spectrometry (ICP-MS) (Element-2; Thermo-Finnigan, Rodano (MI), Italy) at medium mass resolution ($M/\Delta M \sim 4{,}000$). This resolution is unable to eliminate isobaric interferences of ^{54}Cr and ^{58}Ni on ^{58}Fe and ^{54}Fe isotopes. For this reason, the isotope ^{57}Fe was selected as tracer. Sample digestion was performed with 2 mL of concentrated HNO_3 (70%) by means of microwave heating at 160 °C for 20 min (Milestone MicroSYNTH Microwave labstation equipped with an optical fibre temperature control and HPR-1000/6M six position high-pressure reactor, Bergamo, Italy). After digestion, the volume of each sample was brought to 3 mL with ultrapure water and the sample was analysed by ICP-MS. A natural abundance iron standard solution was analysed during sample runs in order to check changing in the systematic bias. The calibration curve was obtained using four iron absorption standard solutions (Sigma-Aldrich) in the range 0.2–0.05 μg/mL.

Calculations

After treatment with ^{57}Fe-labelled or control solution, the amount (expressed as μg of iron per g of wet tissue) of total iron ($_{tot}$Fe) and of ^{57}Fe isotope was determined for each tissue of interest by ICP-MS. For each tissue, the calculated amount of naturally occurring ^{57}Fe was subtracted from the measured value in order to get a measure of the amount of iron retained by the tissue upon the treatment with ^{57}Fe-labelled solution. The amount of naturally occurring ^{57}Fe was calculated considering the average percentage of ^{57}Fe under basal conditions (%^{57}Fe) in wild-type mice with respect to total iron observed ($_{tot}$Fe), as shown in the example reported in Table 1.

Tissue iron measurement by colorimetric method

For tissue iron determination by colorimetric measurement, the BPS-based method was performed, using 4,7-diphenyl-1,10-phenantroline disulphonic acid (BPS) as chromogen [20]. Briefly, 0,1 g of dry tissue was incubated overnight in a mixture of trichloroacetic (10%) and hydrochloric (3 N) acids, and 20 μL of supernatant reduced with thioglycolic acid (Sigma-Aldrich, Milano, Italy) and acetic acid–acetate buffer (pH 4.5). Ferrous iron content was determined spectrophotometrically at 535 nm following the addition of BPS and incubation for 1 h at 37 °C.

Statistical analysis

Results were expressed as mean ± SEM. Statistical analyses were performed using one-way or two-way analysis of variance followed by the Bonferroni correction for multiple group comparisons. An unpaired Student's t test was used when only two groups were compared. A P value of less than 0.05 was regarded as significant.

Results

Assessment of natural abundance of ^{57}Fe in mouse tissues

The preliminary experimental work for the ^{57}Fe method in mice is summarized as follows.

Firstly, after defining the experimental set-up for tissue collection (for details see "Materials and methods"), the reliability of total iron and ^{57}Fe detection by mass spectrometry in samples recovered from wild-type mice at basal conditions was assessed. Duodenum, liver and kidney tissues were dissected and subjected to ICP-MS and spectrophotometric analyses.

A good correlation between iron levels measured by mass spectrometry and the standard colorimetric method using BPS as a chromogen (Fig. 1a) was found. As expected total liver iron in Hfe-null mice suffering from haemochromatosis resulted as being significantly higher than in wild-type animals (Fig. 1b).

Table 1 Calculation of ^{57}Fe retention in tissues

Medium %^{57}Fe under basal conditions	$_{tot}$Fe (μg/g) observed after treatment	^{57}Fe (μg/g) observed after treatment	Expected naturally occurring ^{57}Fe (μg/g)	^{57}Fe retained (^{57}Fe observed–^{57}Fe expected)
2.4%	20	5	0.48 (2.4% of 20)	5–0.48 = 4.52

An example is given considering a measurement of 20 μg/g for total iron and of 5 μg/g for ^{57}Fe. The calculated amount of naturally occurring ^{57}Fe was subtracted from the measured value in order to get a measure of the amount of iron retained by the tissue upon treatment with the ^{57}Fe-labelled solution

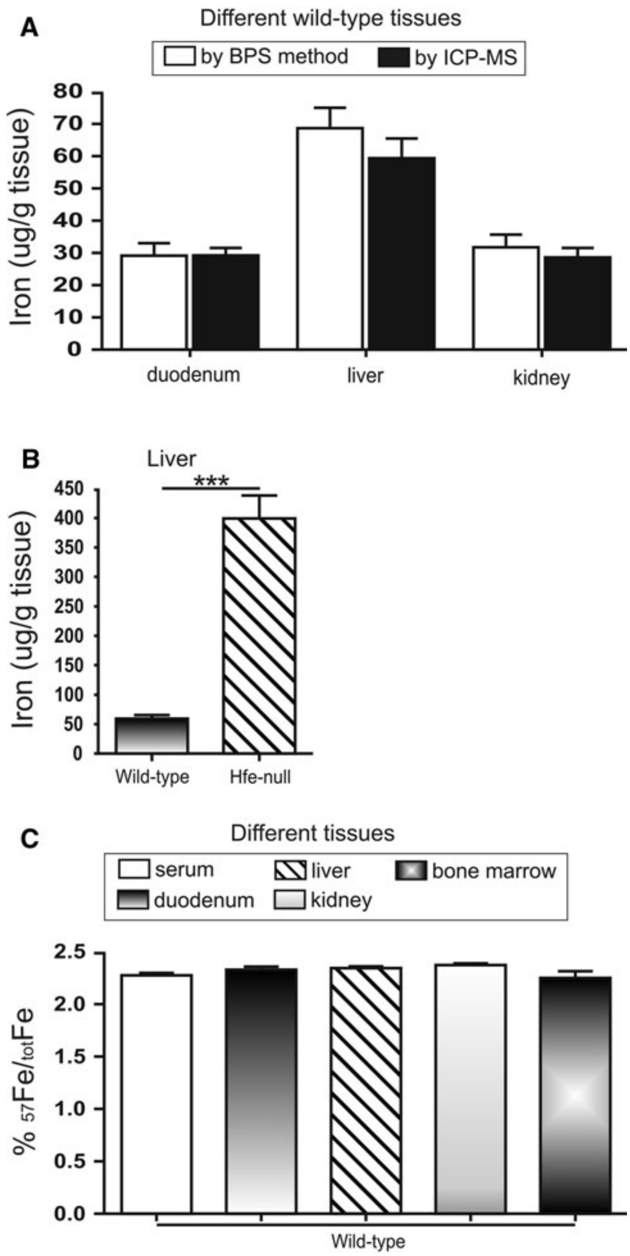

Fig. 1 Assessment of naturally occurring total iron and ^{57}Fe in mouse tissues. **a** Total tissue iron content in the duodenum, liver and kidney of 2-month-old wild-type mice, measured by the BPS-based colorimetric method or by ICP-MS. *Data* represent mean ± SEM; $n = 10$. **b** Total iron content in the liver of 2-month-old wild-type and Hfe-null mice measured by ICP-MS. Values are expressed as µg iron/g tissue. *Data* represent mean ± SEM; $n = 5$; $^{***}P < 0.001$. **c** Percentage of naturally occurring ^{57}Fe in different wild-type tissues. Values are expressed as a percentage of ^{57}Fe with respect to total iron. *Data* represent mean ± SEM; $n = 9$

In wild-type mice, the amount of ^{57}Fe with respect to total iron (%^{57}Fe/$_{tot}$Fe) was constant in the different tissues analysed, i.e. serum, duodenum, liver, kidney and bone marrow, and reached a value of about 2.3%, very close to the reported natural percentage of ^{57}Fe in the earth [14] (Fig. 1c).

Measurement of ^{57}Fe retention by duodenal mucosa after oral iron administration

The relatively low natural abundance of ^{57}Fe offers the possibility of exploiting this isotope as an easy detectable tracer upon an oral administration of solutions enriched in ^{57}Fe.

To assess the in vivo uptake of ^{57}Fe by the duodenal mucosa after oral administration of ^{57}Fe-containing solutions, 2-month-old wild-type mice were treated with 20 µL of a solution containing ^{57}Fe, and duodenum tissue was collected at different times following the treatment. A comparable volume of vehicle solution was given to control mice.

Thirty minutes after treatment, a considerably high amount of ^{57}Fe was detected in the duodenal mucosa of treated mice compared to controls, and the quantity of ^{57}Fe retained by duodenum further increased 90 min after the oral administration (Fig. 2a).

We used Hfe-null mice as positive controls, known to have enhanced duodenal iron uptake [19], and wild-type mice fed with an iron-deficient diet for 2 weeks, that display increased expression of duodenal iron transporters [21]. When ^{57}Fe was administered to Hfe-null mice, at 60 min following treatment, the amount of ^{57}Fe detected in the duodenal mucosa was already higher than in wild-type animals (Fig. 2b). The same was observed in wild-type mice fed on an iron-deficient diet (Fig. 2c).

Determination of the amount of ^{57}Fe delivered to different organs

After uptake from the duodenal lumen, iron captured by enterocytes is usually partly stored in ferritins and partly exported into the bloodstream through a process involving the iron exporter ferroportin1, located at the basal membrane of enterocytes. Once in the circulation, iron is captured by the iron carrier transferrin and transported to peripheral organs, primarily to the bone marrow [1, 22].

To test the possibility that orally administered ^{57}Fe absorbed by the duodenum and subsequently delivered to peripheral organs could be detected by mass spectrometry analyses, we treated wild-type mice as described earlier and collected the livers and bone marrow at different times following treatment. A slight increase in ^{57}Fe levels in the liver was already evident 30 min after administration of iron, and further increased up to 90 min following treatment (Fig. 3a). Furthermore, 2 days after treatment, a significant amount of tracer was detected in the bone marrow (Fig. 3b). On the other hand, the transfer of ^{57}Fe to the kidney, an organ only marginally involved in iron handling, was negligible compared to the liver and bone marrow (Fig. 3c).

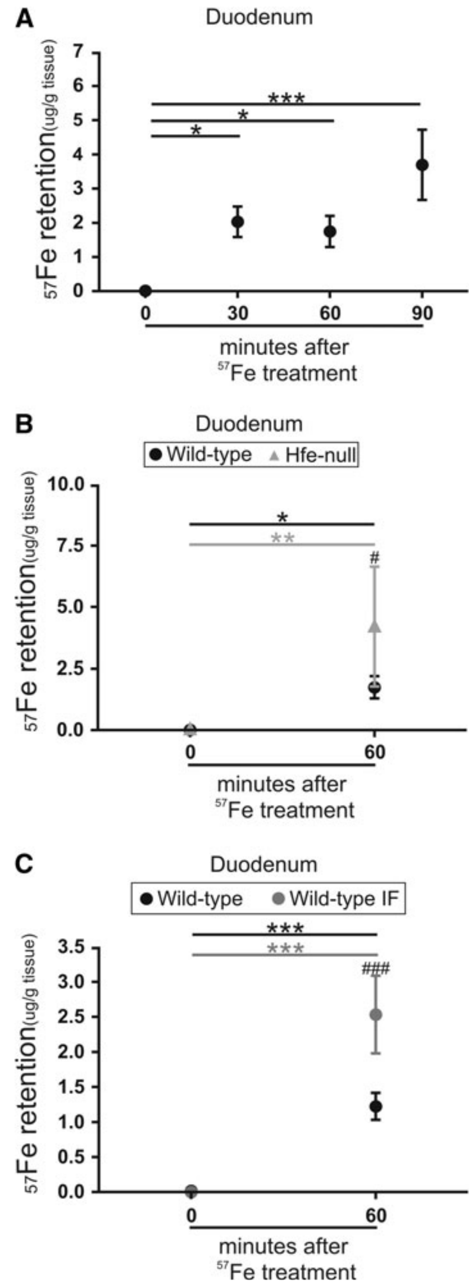

Fig. 2 Measurement of ^{57}Fe retention in the duodenal mucosa after oral treatment. **a** ^{57}Fe retention in the duodenal mucosa of wild-type mice measured by ICP-MS 30, 60 and 90 min after oral treatment with a solution containing 20 mmol/L ^{57}Fe. Control mice were treated with a vehicle solution and represented the '0' time point of the treatment in the experiment. Values are expressed as µg ^{57}Fe/g tissue. *Data* represent mean ± SEM; $n = 9$ for each experimental point; $^{*}P < 0.05$, $^{***}P < 0.001$. **b** ^{57}Fe retention in the duodenal mucosa of wild-type and Hfe-null mice measured by ICP-MS 60 min after oral treatment with a solution containing 20 mmol/L ^{57}Fe. Control wild-type and Hfe-null mice were treated with a vehicle solution and represented the '0' time point of the treatment in the experiment. Values are expressed as µg ^{57}Fe/g tissue. *Data* represent mean ± SEM; $n = 4$ for each experimental point; $^{*}P < 0.05$, $^{**}P < 0.01$ (comparing control mice with the corresponding group of ^{57}Fe-treated mice), $^{\#}P < 0.05$ (comparing the two genotypes). **c** ^{57}Fe retention in the duodenal mucosa of wild-type mice fed a standard or an iron-free (IF) diet, measured by ICP-MS 60 min after oral treatment with a solution containing 20 mmol/L ^{57}Fe. Control mice were treated with a vehicle solution and represented the '0' time point of the treatment in the experiment. Values are expressed as µg ^{57}Fe/g tissue. *Data* represent mean ± SEM; $n = 4$ for each experimental point; $^{***}P < 0.001$ (comparing control mice with the corresponding group of ^{57}Fe-treated mice), $^{\#\#\#}P < 0.001$ (comparing mice fed on the two different diets)

As expected, the transfer of ^{57}Fe to the liver of Hfe-null mice and of wild-type mice fed on an iron-deficient diet was strongly enhanced compared to wild-type animals and well detectable 60 min after oral administration of iron-containing solutions (Fig. 3d, e).

Discussion

In this work, a novel procedure to measure iron absorption in mice using the stable ^{57}Fe isotope has been described.

Upon administration of an oral dose of a ^{57}Fe-containing solution, a considerably high amount of ^{57}Fe was detected in the duodenal mucosa of mice. Moreover, the transfer of a significant amount of ^{57}Fe to peripheral organs, such as the liver and bone marrow was assessed. As stable iron isotopes exist in nature in fixed ratios, corrections have to be made for background levels when they are used as tracers in absorption studies. For this purpose, corrections for the natural abundance of the stable isotope ^{57}Fe have been considered during the analysis of experiments. Moreover, a series of controls, such as the assessment of iron absorption in Hfe-null mice, and in animals fed on an iron-deficient diet, confirmed the reliability of the proposed method. The sensitivity of the ICP-MS technique allows detection of relatively low amounts of ^{57}Fe corresponding to an experimental detection limit of about 40 ng ^{57}Fe/g tissue.

The procedure described in the present work therefore represents a good technique to assess iron absorption in mice and, along with the classical methods, allows determination of both mucosal retention and mucosal transfer of iron, distinguishing between the two steps of iron absorption, thus offering a valuable alternative to the approaches that have been used in laboratory practices so far.

The use of orally administered ^{57}Fe to measure iron absorption in mice shows a series of advantages respect to other techniques reported in the literature [23].

Firstly, the use of a stable iron isotope instead of a radioactive one shows obvious benefits in terms of safety and ease of handling, as the use of ^{57}Fe does not require special laboratory equipment or particular skills for the operator.

Secondly, the cost of ^{57}Fe-enriched substances is considerably lower than that of radioactive iron-containing

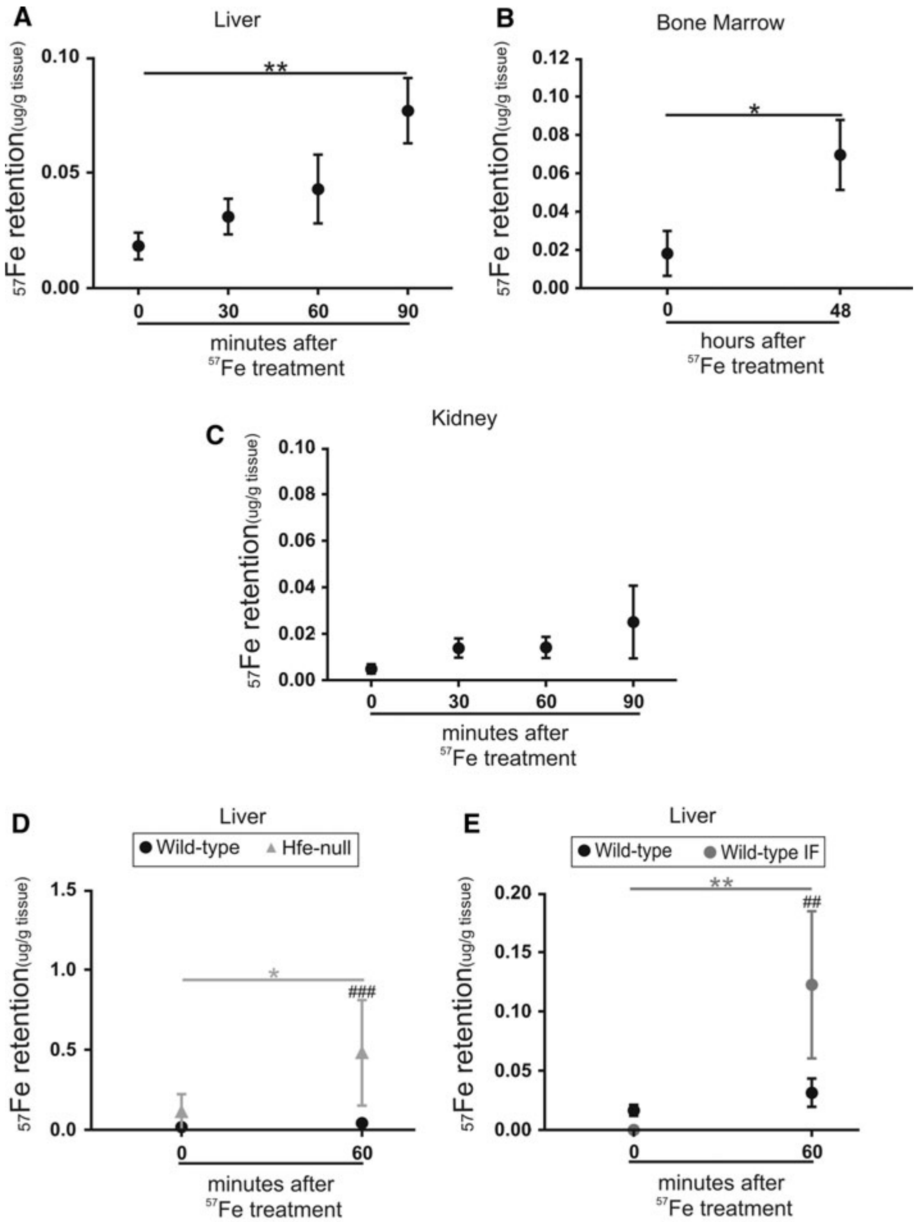

Fig. 3 Determination of the amount of ^{57}Fe delivered to different organs. (**a**, **c**) ^{57}Fe retention in the liver (**a**) or in the kidney (**c**) of wild-type mice measured by ICP-MS 30, 60 and 90 min after oral treatment with a solution containing 20 mmol/L ^{57}Fe. Control mice were treated with a vehicle solution and represented the ‘0’ time point of the treatment in the experiment. Values are expressed as µg ^{57}Fe/g tissue. *Data* represent mean ± SEM; $n = 9$ for each experimental point; $^{**}P < 0.01$. **b** ^{57}Fe retention in the bone marrow of wild-type mice measured by ICP-MS 48 h after oral treatment with a solution containing 20 mmol/L ^{57}Fe. Control mice were treated with a vehicle solution and represented the ‘0’ time point of the treatment in the experiment. Values are expressed as µg ^{57}Fe/g tissue. *Data* represent mean ± SEM; $n = 9$ for each experimental point; $^{*}P < 0.05$. **d** ^{57}Fe retention in the liver of wild-type and Hfe-null mice measured by ICP-MS 60 min after oral treatment with a solution containing 20 mmol/L ^{57}Fe. Control wild-type and Hfe-null mice were treated with vehicle solution and represented the ‘0’ time point of the treatment in the experiment. Values are expressed as µg ^{57}Fe/g tissue. *Data* represent mean ± SEM; $n = 4$ for each experimental point; $^{*}P < 0.05$ (comparing control mice with the corresponding group of ^{57}Fe-treated mice), $^{\#\#\#}P < 0.001$ (comparing the two genotypes). **e** ^{57}Fe retention in the liver of wild-type mice fed a standard or an iron-free (IF) diet measured by ICP-MS 60 min after oral treatment with a solution containing 20 mmol/L ^{57}Fe. Control mice were treated with a vehicle solution and represented the ‘0’ time point of the treatment in the experiment. Values are expressed as µg ^{57}Fe/g tissue. *Data* represent mean ± SEM; $n = 4$ for each experimental point; $^{**}P < 0.01$ (comparing control mice with the corresponding group of ^{57}Fe-treated mice), $^{\#\#}P < 0.01$ (comparing mice fed on the two different diets)

compounds, thus representing an affordable tracer for many research centres.

Moreover, the use of ICP-MS for ^{57}Fe detection ensures high sensitivity and the use of a calibration curve during measurements provides quantitative data.

Another advantage associated with the herein reported method deals with the possibility of carrying out several kinds of analyses on the tissue specimen recovered from animals treated with ^{57}Fe. Indeed, the limited handling of the mouse during the experiment and the safety of the employed tracer allow the use of excised tissues partly for ICP-MS analyses and partly for any other kind of tests, i.e. biochemical and histological analyses.

Finally, while radioactive tracers are not suitable for multi-element studies because of difficulties due to overlapping energy spectra, the absorption of different stable isotopes can be measured by ICP-MS simultaneously.

A weak point of this methodology is the impossibility to detect the amount of absorbed iron in the entire carcass, which the use of radioactive compound allows [24–26]. Of course, separated analyses on different organs of interest can be performed in order to reach a global assessment of iron distribution in the entire organism.

In conclusion, the present work proposes a sensitive, safe and reliable method to measure iron absorption in mice, thus providing an alternative technique to that reported so far and offering a new methodology to improve the study of iron absorption mechanisms.

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Conflict of interest The authors declare no conflicts of interest.

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