

## Short Communication

# Nutritional hepatic iron overload is not prevented by parenteral hepcidin substitution therapy in mice

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### Abstract

The peptide hormone hepcidin functions as a negative regulator of intestinal Fe absorption and Fe recycling. Since its discovery as a systemic negative regulator of Fe metabolism, hepcidin has attracted enormous interest as a potential drug for the treatment and/or prevention of several forms of Fe overload. We therefore tested whether multiple doses of intraperitoneally administered synthetic renatured hepcidin can prevent hepatic Fe loading in mice concurrently fed an Fe-rich diet, and whether the same treatment affects hepatic Fe stores in mice fed a normal diet. Cohorts of male mice were fed either a normal defined diet (180 parts per million Fe) or an Fe-rich diet (the same diet supplemented with 2% carbonyl iron for 2 weeks). Concurrently, half of the animals in each diet group received 100 µg of renatured hepcidin intraperitoneally every 12 h, for the same 2-week period. The second half of the animals received PBS only. The renatured synthetic hepcidin demonstrated biological activity by significantly decreasing transferrin saturation, which lasted for up to 24 h after a single hepcidin dose. However, the 14 d intraperitoneal hepcidin therapy did not prevent hepatic Fe overload in mice fed the Fe-rich diet, nor did it affect hepatic Fe stores in mice fed the normal diet. Both hepcidin agonists and antagonists are expected to have broad therapeutic potential. The absence of an effect of biologically active hepcidin on hepatic Fe loading shows the need for thorough future studies on the hepcidin regulation of Fe absorption and tissue distribution.

**Key words:** Hepcidin: Iron overload: Iron metabolism

Since its discovery as a mammalian systemic regulator of Fe metabolism, the peptide hormone hepcidin has attracted significant interest as a potential drug<sup>(1)</sup>. Hepcidin functions as a negative regulator of intestinal Fe absorption by enterocytes and Fe recycling by macrophages and hepatocytes. According to the generally accepted hypothesis on its function, hepcidin triggers the internalisation and degradation of the Fe exporter ferroportin, which is expressed on the surface of duodenal enterocytes, macrophages and hepatocytes<sup>(2)</sup>. Ferroportin degradation thus prevents Fe release from these, thus abrogating intestinal Fe absorption and hindering Fe recycling in macrophages.

Manipulation of systemic Fe metabolism by hepcidin or, alternatively, by a hepcidin antagonist, could theoretically be used in the therapy of Fe-loading diseases and for the treatment of anaemia in chronic disease. We tested whether multiple doses of intraperitoneally administered bioactive

synthetic hepcidin can prevent hepatic Fe loading in mice fed normal and Fe-rich diets.

### Experimental methods

#### *Biological activity of renatured synthetic hepcidin*

For *in vivo* studies, we used synthetic human hepcidin of the sequence DTHFPICIFCCGCCHRSKCGMCCCKT (Clonestar Peptide Services).

Hepcidin has an unusual structure containing four disulphide bonds, the formation of which in the synthetic peptide requires a renaturation step. Renaturation was performed at low pH<sup>(3)</sup> using CLEAR-OX beads, a highly effective polymer-supported reagent for the formation of disulphide bonds<sup>(4)</sup>. Briefly, 10 mg hepcidin were solubilised in 2 ml of 30% acetonitrile–20 mM-Tris–HCl (pH 5) and incubated for 48 h with 200 mg of CLEAR-OX beads (Peptides International) at room temperature.

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Purification of the renatured peptide was performed using reverse-phase chromatography (SepPak C18 Cartridge Waters). The eluted renatured peptide was lyophilised, dissolved in 1 ml of 0.016% HCl and diluted in PBS to a final concentration of 500 µg/ml. Single doses (100 µg) were divided into aliquots and stored at  $-20^{\circ}\text{C}$ . Peptide integrity (peptide mass) and the presence of appropriate disulphide bonds were verified by matrix-assisted laser desorption ionisation-MS. These properties, as well as biological activity (measured as a decrease in plasma Fe), were found to be preserved after 2 months of storage at  $-20^{\circ}\text{C}$ . Each dose to be injected was thawed immediately before application.

To test the biological activity of the renatured hepcidin, we measured changes in transferrin saturation after intraperitoneal hepcidin administration. Cohorts of five 4-month-old male mice were injected intraperitoneally with 100 µg of renatured hepcidin in PBS or with PBS only. After 6, 12 and 24 h, blood was drawn, blood plasma was prepared and transferrin saturation was determined by a standard colorimetric method<sup>(5)</sup>.

All procedures involving animals were approved by the Animal Care Committee of the First Medical Faculty, Charles University in Prague, Czech Republic (IGA NS/10 300-3).

## Results

The single dose of hepcidin significantly reduced transferrin saturation to approximately half the basal level at 6 h ( $P=0.002$ ) and 12 h ( $P=0.005$ ) after injection, as well as non-significantly at 24 h ( $P=0.059$ ; Fig. 1(a)). To test the potential of renatured synthetic hepcidin to modulate the levels of Fe stores in the liver, cohorts of twelve male mice of the same age were fed with either a normal defined diet (180 parts per million Fe) or an Fe-rich diet (the same diet supplemented with 2% carbonyl iron) for 14 d. Half of the animals in each diet group received intraperitoneally 100 µg of bioactive hepcidin in PBS in 12-h intervals (thirty-four doses). Control animals received injections of PBS only. At the end

of the experiment, animals were killed, livers were removed and hepatic Fe concentrations were measured by atomic absorption spectrometry.

Injected hepcidin significantly decreased the levels of available circulating Fe, as measured by transferrin saturation at 6, 12 and 24 h after injection (Fig. 1(a)). However, the thirty-four doses of hepcidin administered at 12-h intervals (for 14 d) did not prevent or decrease hepatic Fe loading in mice fed the Fe-rich diet, nor did it change basal hepatic Fe levels in animals fed a normal diet (Fig. 1(b)).

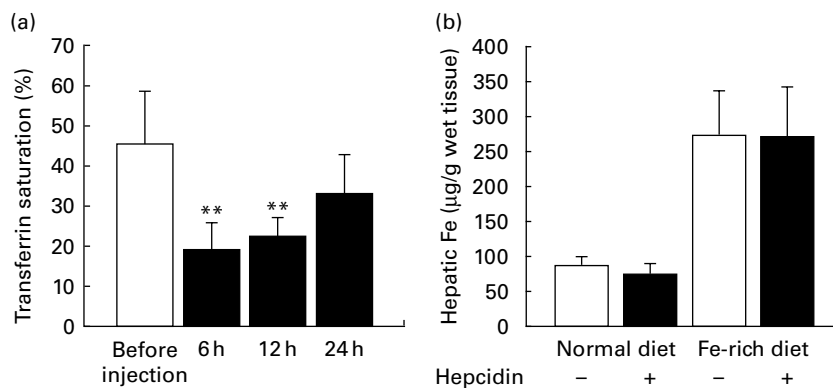
## Discussion

The absence of any measurable effect of administered hepcidin on hepatic Fe loading in animals fed an Fe-rich diet is rather counter-intuitive. However, similar results (i.e. no suppression of hepatic Fe load) have recently been obtained in a mouse model of genetic Fe overload (haemochromatosis), which were fed a standard diet (240 ppm Fe) and treated with 50 µg doses of hepcidin in 4-h intervals for 2 months<sup>(6)</sup>.

There may be several explanations for the absence of a hepcidin effect.

While hepcidin rapidly prevents Fe recycling from macrophages, Fe transport from enterocytes to the circulation is not completely blocked, as demonstrated previously<sup>(7,8)</sup>. This can result in altered tissue Fe distribution – the Fe content of macrophages and liver is 'locked' inside the cells and may even further increase as the block of Fe absorption is incomplete. The Fe content in the spleen and bone marrow remains unchanged or declines. This may even negatively affect the Fe flux to erythropoiesis, as shown in a chronic hepcidin overexpression model where anaemia is present even in Fe-overloaded animals<sup>(9)</sup>.

Alternatively, it can be speculated that in addition to the well-established direct effect of hepcidin on ferroportin, another as-yet-unknown regulatory process may be at play in systemic Fe metabolism.



**Fig. 1.** (a) Temporal effect of a single 100 µg dose of intraperitoneal hepcidin injection on transferrin saturation. Renatured synthetic hepcidin demonstrates biological activity, as documented by a long-lasting decrease in transferrin saturation ( $n$  6). □, Transferrin saturation in animals before hepcidin injection; ■, transferrin saturation in animals killed at 6, 12 and 24 h after a single dose of hepcidin. \*\* Mean value was significantly different from that before injection ( $p < 0.01$ ). (b) Effect of 2-week experimental hepcidin substitution therapy on hepatic iron levels in mice fed the normal or iron-rich diets. No effect on hepatic iron levels was observed ( $n$  6). □, Hepatic iron concentration in animals receiving PBS only. ■, Hepatic iron concentration in animals receiving injections of 100 µg hepcidin in PBS.

Thirdly, although widely accepted as an experimental nutritional Fe-overload model, the carbonyl iron diet supplementation represents neither a physiologically relevant Fe concentration nor does carbonyl iron form in animal and human food. As such, it may therefore be insensitive to hepcidin regulation.

Based on the accepted model of hepcidin function, both hepcidin agonists and antagonists can have broad therapeutic potential. However, the absence of an effect of biologically active hepcidin on hepatic Fe loading presented here demonstrates the need for future studies on the hepcidin-mediated regulation of Fe absorption and tissue Fe distribution.

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