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ABSTRACT

Hepcidin, secreted by hepatocytes, controls iron metabolism by limiting iron egress in plasma. Hepcidin is up-regulated during inflammation through the activation of the signal transducer and activator of transcription 3 (STAT3) transduction pathway, which decreases iron bioavailability and may explain the anemia of chronic inflammatory disease. *In vitro*, it has been shown that curcumin can decrease hepcidin synthesis by decreasing STAT3 activity. We conducted a proof-of-concept study to assess the effect of curcuma on hepcidin synthesis in human. This was a placebo-controlled, randomized, double-blind, cross-over, two-period study performed in 18 healthy male volunteers. Subjects received a single oral dose of 6g curcuma containing 2% of curcumin or placebo. Serum hepcidin, and iron parameters were assessed repeatedly until 48h after dosing. When compared with a placebo curcuma decreased hepcidin levels significantly at 6h (-19%, p=0.004), 8h (-17%, p=0.009), and 12h (-17%, p=0.007), and tended to decrease hepcidin at 24h (-15%, p=0.076). Curcuma also significantly increased serum ferritin levels at 6h and 8h (+7% for both times, p=0.018, p=0.030, respectively) and had no effects on serum iron, transferrin and transferrin saturation. This pilot study showed that curcuma decreases serum hepcidin levels in human and supports the idea that curcuma could be useful in treating hepcidin over-production during inflammatory processes. Confirmatory studies in patients with chronic inflammation are now required to determine the optimal dose and therapeutic scheme of curcuma.

Key words: Hepcidin, Curcuma, iron metabolism, healthy volunteers

Abbreviations:

signal transducer and activator of transcription 3 (STAT 3); interleukin-6 (IL-6)

INTRODUCTION

Hepcidin, a circulatory peptide mainly expressed in the liver [1], is a central regulator of iron metabolism [2, 3]. It lowers serum iron concentrations by negatively regulating intestinal iron absorption and macrophage iron release. Hepcidin acts by inducing the internalization and degradation of the cellular iron exporter ferroportin. As a result, iron export from intestinal cells, hepatocytes and macrophages is blocked and iron bioavailability in plasma is diminished [4, 5]. Hepcidin expression is down-regulated during iron deficiency and hypoxia and is up-regulated during iron-overload and inflammation. During chronic inflammation the phosphorylation of the signal transducer and activator of transcription 3 (STAT 3) is induced by plasma interleukin-6 (IL-6) [6, 7]. Phosphorylated STAT3 interacts with a responsive element located in the hepcidin promoter and induces hepcidin expression and secretion [8-10] which limits iron release to the plasma. Iron is therefore sequestrated in cells and is not available for hematopoiesis which explains the anemia of chronic inflammatory disease [11, 12]. This anemia has a major impact on quality of life [13], and unfortunately no treatment exists apart from treating the etiology of inflammation. The control of hepcidin overexpression during inflammation could prevent the induction of anemia [3, 14] but, to date, no specific anti-hepcidin therapy is available for use in humans [15].

Our study group previously showed that curcumin, a STAT3 inhibitor, decreased hepcidin mRNA expression *in vitro* [16]. This observation suggests that curcumin could be a potential treatment to reduce hepcidin over-production in anemia of inflammation. Curcumin (diferuloylmethane) is a component of curcuma, derived from the rhizome of the plant *Curcuma longa* [17]. This yellow pigment is largely used as *a* spice, also called turmeric, and food-coloring agent. It belongs to the polyphenol family and possesses diverse anti-inflammatory and anti-cancer properties [18]. Although curcuma is widely consumed around the world, no data is available on the impact of curcuma on iron metabolism in humans. We thus conducted this proof-of-concept study to assess the effect of curcuma on hepcidin synthesis in healthy volunteers.

METHODS

Subjects

As hepcidin and iron parameters vary during the menstrual cycle [19, 20], only healthy male volunteers were included. They were medication-free, non-smokers, and they did not have excessive alcohol consumption (ie more than 30 g of alcohol per day). All subjects had no abnormality identified at clinical examination, and normal routine biological tests, including serum iron parameters (iron, transferrin, transferrin saturation, and ferritin). All subjects gave written informed consent before study participation. The study (EudraCT 2011-001925-26; ClinicalTrial.gov NCT01489592) was approved by the Ethics Committee of Rennes University Hospital.

Treatment

The study was designed as a placebo-controlled, randomized, double-blind, cross-over, two-period study. Randomization was centralized and performed by the pharmacy of Rennes university hospital. Subjects received, in random order, a single oral administration of 6 g of curcuma (Laboratoires Cooper, Melun, France). The preparation was a mixture of curcuminoids (curcumin, bisdemethoxy curcumin and demethoxy curcumin) extracted from the rhizomes of Curcuma longa L (turmeric). The powder was inserted into pills by the pharmacy of Rennes University Hospital. The appearance of pills was strictly identical for curcuma and placebo. The dose of 6 g (corresponding to 120 mg of curcumin) was chosen because it appeared as the maximal dose that the volunteers could ingest (12 pills of 500 mg) without risk of vomiting, and because it was close to the maximum daily intake recommended by the FDA (180 mg curcumin). Moreover, previous studies showed that pharmacodynamic effects were observed with doses of curcumin starting at 20 mg [21]. Except for the treatment given, the two study periods were strictly identical. Subjects were admitted at the clinical investigation unit of the Inserm 1414 Clinical Investigation Centre of Rennes University Hospital at 8:30 am after an overnight fast. Treatment (12 pills of 500 mg each of curcuma or placebo) was administered at T0 (between 9:00 and 9:30 am for all subjects), and a standardized breakfast (orange juice, tea or coffee, bread, butter and jam) was immediately given.

Blood samples were taken before treatment administration (baseline) and 0.5, 1, 2, 3, 4, 6, 8, 12, 24, and 48 hours after. Subjects received standardized meals between 3h and 4h (pasta, chicken, yogurt, applesauce, bread, butter) and at 10h (ham, mashed potatoes, apple sauce). Each participant had to consume exactly the same meal, in the same quantities and at the same times during the two treatment periods. They had to avoid curcuma consumption all along the study. They also had to avoid alcohol consumption and intense physical activity

during the week before each period. Subjects were discharged after 12h, and came back to the Clinical Investigation Unit after an overnight fast at 24h and 48h. The two periods (48h each) were separated by a wash-out interval of at least 14 days.

Biological variables

Iron assays

Baseline biochemical tests were performed on fresh blood samples collected after an overnight fast. For all other biochemical tests, blood samples were centrifuged, and sera were collected and stored at -80 °C. Serum iron concentration was measured on an autoanalyzer (AU2700 Olympus, Rungis, France) with a colorimetric method that used TPTZ[2,4,6-Tri-(2-pyridyl)-5-triazine] as a chromogen. Serum transferrin and ferritin levels were measured with an immunoturbidimetric method (AU2700 Olympus, Rungis, France). Reference interval of local laboratory values of serum iron, serum transferrin saturation and serum ferritin were 12.5-25 µmol/L, 20-45% and 15-300 µg/L, respectively.

Serum hepcidin assay

Serum hepcidin was measured by an immune-enzymatic assay (EIA Bachem, Bubendorf, Switzerland) without preliminary extraction. The limit of detection was 0.1 nmol/L, and the normal reference range was 4-30 nmol/L [22].

Serum glucose

Due to a potential effect of glycaemia on serum hepcidin levels [23], serum glucose was measured at each time-point by standard biochemical technique.

Statistical analysis

This study planned to include 18 subjects in order to show a decrease of at least 30% of serum hepcidin level (primary outcome) with curcuma as compared with placebo with an effect size of 0.9, in a two-sided paired test with 95% power. Statistical analyses were performed with SAS statistical software, V9.3 (SAS Institute, Cary, NC, USA). In the text and tables, results are expressed as the median [fist quartile-third quartile], and in the figures they are presented as box plots. Baseline values were compared between curcuma and placebo treatments with a 3-way (subject, period, treatment) non-parametric analysis of variance. Thereafter, changes in biological variables over time (during 48h) were analyzed with a 3-way (subject, time, treatment) non-parametric repeated-measures analysis of variance (Friedman test), adjusted for baseline values. For this analysis, a time effect reflects changes in mean values during the period of observation; a treatment effect reflects a difference between placebo and curcuma; and a time × treatment interaction reflects that the evolution of the variable over time is different between the two treatment groups. In case of significant treatment effect or time × treatment interaction, time-point comparisons were performed using the least square means procedure. In time-point comparisons, reported effects are differences between adjusted rank values with placebo and curcuma. For all analyses, a p-value <0.05 was considered statistically significant.

RESULTS

Population characteristics

The mean age and body mass index of volunteers were 22.5±3.3 years and 21.1±1.5 kg/m^2, respectively. Values of serum hemoglobin, aspartate aminotransferase, alanine aminotransferase, C reactive protein, and iron parameters (iron, transferrin, transferrin

saturation and ferritin) were within the reference ranges for all subjects. All were free of the C282Y mutation in the HFE gene, except one, who was heterozygous for the mutation.

Biological variables were not significantly different before curcuma and placebo administrations periods (Table I).

Effect of curcuma on hepcidin levels and iron parameters

Curcuma had a global significant effect on serum hepcidin levels (treatment effect, $p<0.001$, Figure 1). Time-point comparisons showed that curcuma decreased hepcidin levels significantly at 6h (-19%, p=0.004), 8h (-17%, p=0.009), and 12h (-17%, p=0.007), and tended to decrease hepcidin at 24h (-15%, p=0.076). As compared with placebo, curcuma slightly but significantly increased serum ferritin levels (treatment effect, p=0.015, Figure 2 A) at 6h and 8h (+7% for both times, p=0.018, p=0.030, respectively). Curcuma did not significantly affect serum iron (treatment effect, p=0.158, Figure 2 B), transferrin (treatment effect, p=0.229, Figure 2 C) and transferrin saturation levels (treatment effect, p=0.981, Figure 2 D).

Variations of serum glucose concentrations

Curcuma had no significant effect on serum glucose (treatment effect, p=0.159, Figure 3). Pic plasma glucose values were observed after meals at 0.5h (after breakfast), 4h (after lunch), and 12h (after diner).

Treatment tolerance

Treatment was well tolerated. No serious adverse event was reported during the study. Two volunteers reported transitory dyspepsia with bloating during curcuma treatment.

DISCUSSION

This randomized placebo-controlled study demonstrated that a single intake of 6 g of curcuma could significantly decrease serum hepcidin levels in healthy volunteers. This effect was significant after 6 hours and lasted until 24 hours after the administration of curcuma.

These results confirm in humans those obtained in our previous *in vitro study* in which we showed that curcumin reduced hepcidin mRNA transcription in a model of mouse hepatocytes co-cultured with rat liver epithelial cells [16]. In that model, mouse hepatocyte hepcidin-1 mRNA expression was associated with activation of both BMP/SMAD and STAT3 pathways. Co-cultures exposed during 6h to 20 µM of curcumin showed a 38% reduction in hepcidin-1 mRNA expression which was specifically related to the inhibition of the STAT3 pathway.

As previously reported in healthy volunteers [24, 25], we observed significant diurnal variations in serum hepcidin levels (time effect, $p<0.001$). This pattern was blunted but not suppressed by curcumin. Together with previous results of the above mentioned in vitro study, our results suggest that curcuma may limit hepcidin expression via the STAT3 pathway and that other pathways that might influence hepcidin expression are not altered by curcuma. The strict timing of biological samples for all subjects during the two periods allowed us to avoid any confusing factors due to diurnal variations of serum hepcidin.

Previous *in vitro* studies reported iron binding properties of curcumin [26, 27]. Chin et al studied the effect of a 6 month dietary supplementation with 0.2% curcumin on iron status in mice [28]. As compared with control, curcuma induced a significant reduction in iron content in the liver and in the spleen as well as a decrease of liver hepcidin expression. Jiao et al reported that curcumin could have a moderate *in vivo* iron-chelator activity [29]. In mice fed with diets containing curcumin and submitted to an iron-deficient diet, serum iron and

transferrin saturation decrease was observed (suggesting a chelator effect of curcumin). It is noteworthy that, when mice received curcumin in diets with normal iron levels, curcumin did not significantly affect serum iron parameters. In our study, all volunteers had normal iron parameters at baseline and were not submitted to iron deficient diet. Under these conditions, we did not observe any chelator effect of curcuma. On the contrary there was a slight increase of serum ferritin at 6h and 8h which could reflect an increase of iron storage secondary to the decrease of hepcidin. Finally, numerous previous clinical trials reported that curcuma was well tolerated, without significant side-effects. To our knowledge, despite the fact that iron metabolism was not specifically studied in those trials, no cases of anemia were reported [30] and curcumin is recognized as safe by FDA (http://www.fda.gov/downloads/food/ingredientspackaginglabeling/gras/noticeinventory/ucm346902.pdf).

Our study has some limitations. First, we did not use pure curcumin because the available product pharmaceutically certified for human use was curcuma for which the curcumin content was certified. Consequently, we cannot determine if the observed effect on hepcidin was due to curcumin or to other components of curcuma. Nevertheless, this effect was significant and its intensity was of the same order of magnitude than that previously observed with other medical products such as erythropoietin [24]. Second, the relatively high dose of curcuma used in our study induced moderate effects on serum hepcidin probably due to the low oral bioavailability of curcumin. Therefore new formulations of curcuma with enhanced oral bioavailability should be tested in further studies [31]. Third, one could argue that glucose was used as placebo in our study despite recent published data that showed links between glucose, insulin metabolism and hepcidin [23, 32, 33]. At the time the protocol was written, these data were not available. Wang et al demonstrated that hepcidin could be directly up-regulated by insulin and that the effect of insulin disappeared when the STAT3

pathway was blocked [33]. These results are in line with those of Aigner et al who showed that glucose acts as a regulator of serum iron by increasing serum hepcidin concentrations [23]. However this was observed in healthy volunteers after the administration of 75g of glucose that led to an increase of glycaemia up to a mean of 150 mg/dl, which was higher that what we observed in our study. In fact, in our study the amount of glucose administered was only 6g via the placebo pills and the volunteers received strictly identical meals during the two periods. Under these conditions we did not find any significant difference regarding serum glucose levels between the two periods.

CONCLUSION

This proof-of-concept study showed that curcuma decreases serum hepcidin levels in humans and supports the idea that curcuma could be used to treat hepcidin over-production during inflammatory processes. The low cost of curcuma makes it attractive for a worldwide use. A phase 2 study is now required in patients with chronic inflammation such as inflammatory rheumatologic disease to determine the optimal dose and therapeutic scheme of curcuma before confirmatory studies.

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FIGURE LEGENDS

Figure 1. Variation in serum hepcidin over time during two treatment sequences.

Data are presented as box plots, and the lower and higher boundaries of the box indicate the 25th and 75th percentile. The line within the box marks the median, and the error bars below and above the box indicate the 10th and 90th percentiles. Results are expressed as the difference from the baseline concentration.

Figure 2. Variations in serum iron metabolism parameters with time during two treatment sequences.

Data are presented as box plots, and the lower and higher boundaries of the box indicate the 25th and 75th percentile. The line within the box marks the median, and the error bars below and above the box indicate the 10th and 90th percentiles. Results are expressed as the difference from the baseline concentration. (A) serum hepcidin, (B) serum iron, (C) serum transferrin, and (D) serum transferrin saturation.

Figure 3. Variation of serum glucose with time during two treatment sequences.

Data are presented as box plots, and the lower and higher boundaries of the box indicate the 25th and 75th percentile. The line within the box marks the median, and the error bars below and above the box indicate the 10th and 90th percentiles. Results are expressed as the difference from the baseline concentration.

Table I. Biological variables before curcuma and placebo administrations

	Curcuma sequence	**Placebo sequence**
Hepcidin (nmol/L)	5.8 (3.8-12.8)	6.2 (5.1-7.7)
Haemoglobin g/dL	14.8 (14.3-15.0)	14.9 (14.2-15.2)
Iron (µmol/L)	15.3 (13.6-18)	17.4 (14.9-22.6)
Transferrin (g/L)	2.6 (2.4-2.8)	2.6 (2.5-2.7)
Transferrin saturation (%)	24.1 (19.9-26.0)	27.8 (23.1-34.2)
Ferritin (ng/mL)	78 (67-152)	86 (72-147)
C reactive protein mg/L	0.6 (0.6-0.9)	0.6 (0.6-0.6)
Glucose (mmol/l)	4.9 (4.8-5.0)	4.9 (4.7-5.1)

Values are expressed as median (first quartile-third quartile).