Ferric Citrate Hydrate Has Little Impact on Hyperplasia of Enterochromaffin Cells in the Rat Small Intestine Compared to Sodium Ferrous Citrate

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Ferric citrate hydrate · Sodium ferrous citrate · Tryptophan hydroxylase · Enterochromaffin cells · Anorexia

Abstract
Introduction: The most detrimental factor preventing the use of oral iron in the treatment of iron deficiency anemia is gastrointestinal side effects accompanied by nausea and vomiting. Anorexia is a known secondary effect of nausea and vomiting. The important gastrointestinal signaling molecule 5-hydroxytryptamine (5-HT) is critically involved in not only physiological function but also nausea and vomiting. The present study was designed to compare the effects of the administration of sodium ferrous citrate (SF) and ferric citrate hydrate (FC) to rats on anorexia and hyperplasia of enterochromaffin cells, which mainly synthesize and store 5-HT. Methods: Rats received either SF (3 or 30 mg/kg/day) or FC (30 mg/kg/day) orally for 4 days. Food and water intakes were measured every 24 h during the study. At 96 h after the first administration of the oral iron preparation, the duodenal and jejunal tissues were collected for analysis. Enterochromaffin cells were detected by immunohistochemical analysis. Results: Administration of 3 mg/kg SF had no effect on anorexia but led to increased hyperplasia of enterochromaffin cells in the duodenum ($p < 0.1$). Administration of 30 mg/kg SF significantly decreased food and water intakes and significantly increased hyperplasia of enterochromaffin cells in the duodenum and jejunum. Alternatively, administration of 30 mg/kg FC had no significant effect on food and water intakes or hyperplasia of enterochromaffin cells. Conclusion: The lower impact on the hyperplasia of enterochromaffin cells of FC compared to SF may contribute to the maintenance of rats' physical condition.

Introduction

Almost one-third of the population worldwide is iron deficient, which is why iron deficiency anemia (IDA) is the most common type of anemia [1]. Because excess iron in the body is toxic and there are no active mechanisms for excreting iron except through blood loss or cell turnover, intestinal iron absorption is strictly controlled in order to maintain healthy iron homeostasis [2].
fore, the first-line drugs for the treatment of IDA are oral iron preparations, most of which are ferrous preparations. This is because iron is mainly absorbed from the duodenum or jejunum as ferrous iron via the divalent metal transporter 1.

A common side effect of iron preparations is gastrointestinal (GI) disorders, including nausea, vomiting, abdominal pain, diarrhea, and constipation [3–5], which directly impair patients’ quality of life. Thus, a reduction in GI side effects is closely correlated with the success rate of treatment. In general, ferrous forms are more toxic than ferric forms because ferrous forms easily generate free radicals [6]. Indeed, ferrous forms have been reported to more strongly affect Caco-2 cell viability than ferric forms [7].

Although oral ferrous sulfate preparations are the most popular treatments for IDA worldwide [8], sodium ferrous citrate (SF), a soluble nonionic iron preparation, is widely used for IDA treatment in Japan [9]. Compared to other oral ferrous preparations, SF releases fewer iron ions and is absorbed from the duodenum and jejunum as a low-molecular-weight polymer [10, 11]. Therefore, among oral ferrous preparations, SF is assumed to have fewer GI side effects. However, it still causes GI side effects in almost 50% of patients [5]. Komatsu et al. [5] showed that ferric citrate hydrate (FC) also increases hemoglobin to levels comparable to IDA treatment with SF. They also showed that FC has fewer GI side effects than SF [5]. Interestingly, although the incidence of diarrhea is the same for SF and FC administration, the incidence of nausea and vomiting is significantly lower with FC than with SF.

Nausea and vomiting are also induced by other agents, such as anticancer drugs. The main mechanism of emesis induced in the acute phase by anticancer drugs is thought to be stimulation of the vomiting center of the medulla oblongata via stimulation of 5-hydroxytryptamine 3 (5-HT3) receptors on abdominal vagus nerve fibers by 5-HT, which is released from intestinal enterochromaffin cells following the use of anticancer drugs [12, 13]. Intestinal 5-HT is synthesized by a rate-limiting enzyme, L-tryptophan hydroxylase (TPH), and is stored mainly in enterochromaffin cells, which are sparsely located in the intestinal mucosa; nearly 90% of 5-HT content in the body is present in enterochromaffin cells [14]. Therefore, the direct effect of these drugs on the intestinal mucosa is critically involved in the development of GI side effects. Substance P also plays an important role in the elicitation of delayed emesis stimulated by anticancer drugs; it is also contained in enterochromaffin cells [15–

**Fig. 1.** Effects of SF on food and water intakes. After the first administration of either 3 mg/kg SF (a, c) or 30 mg/kg SF (b, d), food (a, b), and water (c, d) intakes were measured every 24 h up to 96 h. Each column represents the mean ± SE (a, c, n = 8; b, d, n = 6). **p < 0.01 versus control; ††† p < 0.001, †† p < 0.01, † p < 0.05 versus 0 h.
We previously reported that administration of anticancer drugs such as cisplatin, methotrexate, and cyclophosphamide to rats causes hyperplasia of enterochromaffin cells [17–22]. Therefore, as with the administration of anticancer drugs, oral iron preparations, especially oral ferrous preparations, may lead to hyperplasia of enterochromaffin cells as one of the mechanisms that causes nausea and vomiting. In the present study, we aimed to clarify whether the difference in the incidence of anorexia, which is caused as a secondary effect of nausea and vomiting between SF and FC, is related to a difference in the effect of SF and FC on the hyperplasia of enterochromaffin cells.

**Materials and Methods**

**Drugs and Reagents**

SF was purchased from Mitsubishi Chemical Co. (Tokyo, Japan). FC was a generous gift from Japan Tobacco Inc. (Tokyo, Japan). Other reagents used in this study were of special grade, purchased from local suppliers, unless otherwise described.

**Animals**

Nine-week-old male Wistar rats weighing 180–200 g were purchased from Japan SLC, Inc. (Shizuoka, Japan). All animals were housed under constant conditions at a room temperature of 22 ± 2°C and humidity of 50 ± 10% with a regular 12-h light (8:00–20:00)-dark (20:00–8:00) cycle and free access to water and food. All animal experiments were conducted in accordance with the
Guidelines for the Care and Use of Laboratory Animals by the Animal Research Committee of the Health Sciences University of Hokkaido.

**Drug Treatment and Measurement of Food/Water Intake**

SF and FC were suspended in distilled water (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) before administration. SF and FC were almost completely dissolved by stirring for more than 1 h. Stirring was continued just prior to administration to maintain the suspension. For 4 consecutive days, 3 or 30 mg/kg/day for SF and 30 for FC mg/kg/day as iron or vehicle alone was administered orally. According to “Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers” released by the U.S. Food & Drug Administration, the administration of 30 mg/kg/day of each of SF and FC to rats in the present study is almost equivalent to the therapeutic dose in humans based on body surface area.

To measure food and water intake, the rats were placed in individual cages and allowed access to food during the 6-day adaptation period before drug administration. Food and water intakes and body weight were measured every 24 h until the end of the experiment.

At 96 h after the first administration of either drug, the rats were euthanized by exsanguination under light anesthesia using isoflurane. The duodenum and jejunum were dissected in approximately 2-cm-long segments and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer and embedded in paraffin for immunohistochemical analysis of 4-micron-thick sections.

**Fig. 3.** Effects of 30 mg/kg SF on TPH and substance P expression in rat duodenal and jejunal tissue. Duodenal (a, e) and jejunal (b, f) tissues were dissected and fixed with 4% paraformaldehyde for immunohistochemical examination with either anti-TPH antibody (a, b) or anti-substance P antibody (e, f). Scale bar, 100 μm. Magnified view of the region indicated by the dotted square in each photograph. c, d, g, and h Show the numbers of either anti-TPH or anti-substance P antibody-positive cells expressing either TPH or substance P in the mucosa (arrows in a, b, e, and f, respectively). Each column represents the mean ± SE (n = 12). ***p < 0.001, *p < 0.05 versus control.

| Image | Description |
|-------|-------------|
| a     | Control     |
| b     | SF (30)    |
| c     | Anti-TPH antibody positive cells |
| d     | Anti-TPH antibody positive cells |
| e     | Control     |
| f     | SF (30)    |
| g     | Anti-substance P antibody positive cells |
| h     | Anti-substance P antibody positive cells |
Immunohistochemical Analysis

After deparaffinization, the specimens were incubated at room temperature for 30 min with either an anti-TPH antibody (Merck Millipore, Burlington, MA, USA) or an anti-substance P monoclonal antibody (Abcam, Cambridge, MA, USA) diluted in Tris-buffered saline. The anti-TPH antibody and anti-substance P antibody-positive cells located in the epithelial mucosa of the villus and crypt were counted under a light microscope. Cells located within the width of 20 villi were counted in two different fields of view, and a mean count was determined for each specimen. Results are presented as the number of anti-TPH antibody- or anti-substance P antibody-positive cells per villus [17, 18].

Statistical Analysis

Statistical analysis of the results was performed using either two-way analysis of variance (ANOVA) with a post hoc Tukey’s test for multiple comparisons or the Mann-Whitney U test. p values <0.05 were considered significant.

Results

Consecutive administration of 3 mg/kg SF had no significant effects on food or water intake (Fig. 1a, c). Consecutive administration of 30 mg/kg SF significantly decreased food intake at 48 h, which lasted up to 96 h (Fig. 1b), and transiently and significantly decreased water intake at 48 h (Fig. 1d). Although there was no difference in body weight between control and the administration of 3 mg/kg SF at 96 h, it tended to be decreased by the administration of 30 mg/kg SF (p = 0.078) (Table 1).

No morphological changes or injury in the duodenum and jejunum were observed by SF and FC administration with hematoxylin and eosin staining (data not shown). Consecutive administration of 3 mg/kg SF had no significant effect on the numbers of anti-TPH antibody-positive cells (Fig. 2a–d) or anti-substance P antibody-positive cells (Fig. 2e–h) in either duodenal (Fig. 2a, c, e, g) or jejunal (Fig. 2b, d, f, h) tissue but tended to increase the numbers of both anti-TPH antibody-positive cells (p = 0.083) and anti-substance P antibody-positive cells (p = 0.080) only in duodenal tissue (Fig. 2a, c, e, g). Consecutive administration of 30 mg/kg SF significantly increased the numbers of both anti-TPH antibody-positive cells and anti-substance P antibody-positive cells in duodenal and jejunal tissues (Fig. 3).

Consecutive administration of 30 mg/kg FC had no significant effects (p > 0.1) on food or water intake (Fig. 4). There was no difference in body weight between control and the administration of 30 mg/kg FC at 96 h (Table 1). In addition, consecutive administration of 30 mg/kg FC had no significant effects on the numbers of anti-TPH antibody-positive cells or anti-substance P antibody-positive cells in duodenal and jejunal tissues (Fig. 5).

Discussion

In the present study, we demonstrated that administration of 30 mg/kg of SF to rats caused hyperplasia of enterochromaffin cells in the duodenum and jejunum, in association with anorexia. The anorexia caused by 30 mg/kg SF may have contributed to the weight loss trend at.

Table 1. Effects of SF and FC administration on body weight changes in the rat at 96 h

| Group          | n  | Body weight (with 0 h at 100%), % |
|----------------|----|---------------------------------|
| Control        | 12 | 106.68±0.58                     |
| 3 mg/kg SF     | 12 | 106.62±0.33                     |
| Control        | 12 | 102.19±0.89                     |
| 30 mg/kg SF    | 12 | 99.77±0.92                      |
| Control        | 13 | 104.82±1.10                     |
| 30 mg/kg FC    | 13 | 103.18±1.00                     |

Fig. 4. Effects of 30 mg/kg FC on food and water intake. After the first administration of 30 mg/kg SF, food (a) and water (b) intake were measured every 24 h up to 96 h. Each column represents the mean ± SE (n = 9).
96 h. On the other hand, the same dose of FC had no effect on hyperplasia or anorexia. Our results with anorexia are consistent with a report showing that the incidence of nausea and vomiting is significantly lower with FC administration than with SF administration in humans [5]. Pica (the ingestion of kaolin or other nonnutritive substances) in rats is considered a behavior that is analogous to emesis induced by anticancer drugs [18, 19, 22, 23]. Although we have tried to clarify the effects of SF and FC on pica, no clear pica was observed (data not shown). This is probably because the vomiting caused by iron preparations is weaker than that caused by anticancer drugs. Indeed, the pattern of anorexia with 30 mg/kg of SF that appeared 48 h after its first administration was similar to but much weaker than the pattern of anorexia seen when rats were given methotrexate, which is classified as a mild and moderate emetogenic drug among anticancer drugs [18].

At 3 mg/kg SF, there was a trend toward an increased number of enterochromaffin cells only in the duodenum. Further studies are required to determine whether the duodenum is more prone to hyperplasia with ferrous iron than the jejunum. Because 30 mg/kg FC had no effect on the hyperplasia of enterochromaffin cells, the hyperplasia induced by iron may depend on the amount of ferrous iron that directly reaches the cell membrane.

![Fig. 5. Effects of 30 mg/kg FC on TPH and substance P expression in rat duodenal and jejunal tissue. Duodenal (a, e) and jejunal (b, f) tissues were dissected and fixed with 4% paraformaldehyde for immunohistochemical examination with either anti-TPH antibody (a, b) or anti-substance P antibody (e, f). Scale bar, 100 μm. Magnified view of the region indicated by the dotted square in each photograph. c, d, g, and h Show the numbers of either anti-TPH or anti-substance P antibody-positive cells expressing either TPH or substance P in the mucosa (arrows in a, b, e, and f, respectively). Each column represents the mean ± SE (n = 9).](image-url)
Fig. 6. Possible mechanisms underlying the nausea and vomiting induced by iron preparations. SP, substance P; 5-HT, 5-hydroxytryptamine; DMT1, divalent metal transporter 1; DcytB, duodenal cytochrome B.
The mechanisms underlying the hyperplasia of enterochromaffin cells are not fully understood, but inflammation in the intestine and/or physiological stress with histological damage in the intestine is believed to be one possible cause of the hyperplasia [20, 24, 25]. However, the hyperplasia induced by SF may not be due to either inflammation or physiological stress because there was no histological damage induced by SF and the conditions for the administration of SF and FC were the same. Another possible mechanism for the hyperplasia is the enhancement of intestinal stem cells (ISCs) by reactive oxygen species (ROS). Oxidative stress induces ISC proliferation in the Drosophila midgut [26], and ROS and 5-HT in the intestinal tissue are upregulated in ozo- treated rats [27]. Excess iron is toxic because it promotes the production of iron-catalyzed ROS, resulting in damage to cell membranes, protein, and DNA [28]. Ferrous iron is a main actor in the Fenton reaction, producing excessive free radicals that attack cell membranes and depress the stability or increase the penetrability of the membrane [29]. It has been reported that ferrous iron more strongly reduces Caco-2 cell viability than ferric iron [7]. It seems that ferrous iron mainly alters membrane stability and penetrability from outside of the cells. In fact, the release of lactate dehydrogenase from Caco-2 cells treated with ferrous iron is higher than that from cells treated with ferric iron, even though the intracellular iron concentration is almost the same [7]. The membrane damage caused by free radicals induced by ferrous may accelerate the differentiation and proliferation of epithelium cells, including enterochromaffin cells, even though SF exerted no obvious histological damage in the intestinal epithelium.

In general, the mechanisms of emesis induced by cytotoxic drugs are assumed to be as follows: (1) cytotoxic drugs evoke 5-HT release from enterochromaffin cells in the intestinal mucosa; (2) the released 5-HT stimulates 5-HT receptors on the adjacent vagal afferent nerves; (3) the depolarization of the vagal afferent nerves stimulates the vomiting center in the brainstem and eventually induces a vomiting reflex; and (4) some of the released 5-HT from the enterochromaffin cells is transmitted from the chemoreceptor trigger zone to the vomiting center via the circulation. Ferrous sulfate is reported to increase the resting level of 5-HT release from segments of the guinea pig jejunum, as iron makes its way into enterochromaffin cells and either potentiates internal calcium release or acts on the calcium-dependent release machinery to increase the basal release of 5-HT [30]. Therefore, it is speculated that consecutive administration of SF not only causes hyperplasia of enterochromaffin cells but also potentiates the release of 5-HT from each enterochromaffin cell.

Since substance P induces physiological activity via tachykinin NK$_1$ receptors, tachykinin NK$_1$ receptor antagonists are clinically used to prevent emesis. Although the main mechanism for preventing emesis involves antagonization of the receptor present in the central nervous system [31], peripheral substance P and tachykinin NK$_1$ receptors may also play a role in emesis [32, 33]. Indeed, substance P crosses the blood-brain barrier, suggesting that it can bind to NK$_1$ receptor in the vomiting center [34, 35]. Furthermore, there is crosstalk between the tachykinin NK$_1$ and 5-HT$_3$ receptor signaling pathways [32, 33, 36, 37]. Substance P potentiates the 5-HT$_3$ receptor-mediated inward current in rat trigeminal ganglion neurons [36]. 5-HT$_3$ receptor antagonists block substance P-mediated vagal afferent activation, and NK$_1$ antagonists block serotonin-induced vagal afferent activation [33]. Therefore, an elevated level of substance P induced by hyperplasia may play a role in increasing the risk of vomiting. The possible mechanisms underlying anorexia, which involves nausea and vomiting, in relation to iron preparations are presented in Figure 6. These mechanisms may be involved in the clinically observed differences in nausea and vomiting between SF and FC.

**Conclusion**

In summary, we demonstrated that administration of SF to rats caused hyperplasia of enterochromaffin cells in association with anorexia. On the other hand, FC had no effect on either hyperplasia or anorexia. These differences may be due to the amount of extraluminal ferrous iron that directly reaches the cell membrane. The lower impact of FC on the hyperplasia of enterochromaffin cells compared with SF may contribute to the maintenance of rats’ physical condition. In addition, in clinical settings where FC formulations are available, the use of FC could contribute to fewer GI side effects.

**Statement of Ethics**

Animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals by the Animal Research Committee of the Health Sciences University of Hokkaido (Protocol No. 19-094 and 21-006).
Conflict of Interest Statement

The authors declare no conflicts of interest.

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Author Contributions

Takuj Machida participated in research design, conducted all experiments, performed data analysis, and wrote the manuscript. Sachiko Hiraide administered drugs to rats, measured food and intake, and created figures. Takahiro Yamamoto and Saki Shiga performed all experiments and data analysis. Shiori Hasebe and Asuka Fujibayashi administered drugs to rats and performed immunohistochemical analysis. Kenji Iizuka participated in research design and supervised the experiments and the manuscript.

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