




Gamma Aminobutyric Acid Increases Absorption of Glycine-Bound Iron in Mice with Iron Deficiency Anemia

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Abstract

Iron deficiency is a leading cause of anemia. Amino acids are known to promote the absorption of both soluble and insoluble iron. The bioavailability of organic iron is higher than that of inorganic iron. Therefore, the aim of this study was to evaluate the iron absorption of glycine-bound iron (an organic iron) and a combination of glycine-bound iron and gamma aminobutyric acid (GABA) in mice with iron deficiency anemia (IDA). Mice were fed an iron-deficient diet for 3 weeks, followed by oral administration of GABA, inorganic iron, glycine-bound iron, or GABA plus glycine-bound iron for 5 weeks. Ferritin storage in the spleen was measured by immunohistochemistry (IHC). Iron deposition in the liver and spleen tissues was analyzed using atomic absorption spectrometry. Expression levels of iron absorption-related genes were measured by quantitative real-time polymerase chain reaction (qPCR). Iron absorption was enhanced in the glycine-bound iron-treated group compared with the inorganic iron-treated group. Hemoglobin, serum Fe, ferritin, and liver iron levels did not increase in mice treated with GABA alone. However, mice administered GABA in combination with glycine-bound iron showed higher iron absorption than those administered organic iron alone. Our results indicate that glycine-bound iron in combination with GABA might exert a synergistic effect on iron absorption and bioavailability, suggesting that the addition of GABA to existing iron supplements might increase their effectiveness for treating IDA.

Keywords GABA · Organic iron · Inorganic iron · Iron deficiency anemia · Iron absorption

Introduction

Iron is a trace nutrient essential for almost all living organisms. In humans, iron deficiency may reduce or inhibit cellular function [1] and is a leading cause of anemia, especially in developing countries [2]. The global prevalence of anemia in 2010 was reported to be 32.9% [3]. Although the proportion of iron deficiency anemia (IDA) varies according to the factors, for instance, age, gender, and the local conditions, approximately

50% of cases of anemia are considered to be due to iron deficiency (ID) [4]. Risk factors for IDA include insufficient intake, low availability, increased demand, and excessive loss of iron [5, 6]. In general, IDA patients have low serum iron levels and high total iron-binding capacities.

In mammals, the iron metabolism consists of three main processes: iron ingestion, storage, and loss [7]. In addition, the intestinal absorption rate of iron is largely affected by the form of iron ingested. Iron ingested as heme iron, i.e., such as hemoglobin or myoglobin, has a higher intestinal absorption rate than non-heme iron [8]. Non-heme iron in the diet is mainly absorbed in the duodenum in ferrous iron form [9], whereas heme iron in food is absorbed in ferric iron form. Absorption of non-heme iron is determined by the solubility of the form of dietary iron [10]. Peptides and amino acids generated during protein digestion promote iron adsorption by keeping iron in soluble form resulted in the promotion of iron transport across cell membranes [11]. Therefore, protein deficiency may lead to a decrease in iron absorption, indicating that digestion of protein and absorption of iron may be closely related.

Inorganic iron in the form of ferrous sulfate is the most commonly used iron dietary supplement. Amino acids,

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especially cysteine-reduced N-terminal cysteine peptides, may promote the absorption of both soluble and insoluble iron, e.g., iron bound to methionine has greater bioavailability than iron bound to inorganic ions such as sulfate [12, 13]. It is widely known that the bioavailability of organic iron is higher than that of inorganic iron, and in 2-day-old piglets, the bioavailability of iron combined with methionine or glycine has been reported to be greater to that of ferrous sulfate [14]. Organic forms of iron bound to various amino acids are currently being developed.

Gamma aminobutyric acid (GABA) is an ubiquitous non-protein amino acid produced by decarboxylation of glutamic acid, catalyzed by glutamate decarboxylase [15, 16]. GABA has several well-known physiological functions, including tranquilizing and diuretic effects, neurotransmission, and induction of hypotension [17, 18]. Among well-known neurotransmitters, serotonin, norepinephrine, and dopamine have been studied on the relation to ID-dependent altered behavioral and neural development [19–21], while the effect of GABA on ID-related neurochemical alterations has been less studied [22]. GABA is widely distributed among plants and is also found in humans. It is contained in rice gums, gabaron tea, brown rice, and glutamate decarboxylase-producing bacteria [23, 24]. A probiotic bacterium, *Lactobacillus rhamnosus* increases the production of GABA [25], and a high GABA-producing *L. brevis* can be used as a functional starter for manufacturing GABA-rich fermented dairy foods [26].

In this study, we investigated the bioavailability of an organic iron, glycine-bound iron, in IDA mouse models. In addition, the synergistic effects of co-administering glycine-bound iron and GABA were assessed using hematological indices and tissue analysis.

Materials and Methods

Animals

Three-week-old male C57BL/6 mice were purchased from Koatech (Pyungtaek, Korea). Mice were acclimated for 1 week under a 12-h light: 12-h dark cycle in a room with constant temperature (20 ± 2 °C) and humidity ($50 \pm 5\%$). After acclimation, mice were randomly divided into the following eight groups ($n = 8/\text{group}$): negative control group (NC), positive IDA control group (PC), 0.5 mg/100 g body weight (BW) GABA-treated group (G0.5), 1 mg/100 g BW GABA-treated group (G1), 2 mg/100 g BW GABA-treated group (G2), inorganic iron (0.4 mg/100 g BW iron sulfate)-treated group (IO), organic iron (0.4 mg/100 g BW glycine-bound iron)-treated group (O), and 2 mg/100 g BW GABA + organic iron (0.4 mg/100 g BW glycine-bound iron)-treated group (G2 + O). NC mice were maintained on a standard iron diet (45 ppm iron) for 8 weeks. All groups except the NC group

were maintained on an iron-deficient diet (4.5 ppm iron) for 8 weeks [27]. For the last 5 weeks of the experiment, GABA and/or iron was orally administered daily to all groups except the NC and PC groups. GABA used in this study was produced using a *Lactobacillus brevis* strain in a glutamic acid medium (Milae Bioresources Co., Ltd., Seoul, Korea). Organic iron (B-traxim Fe, Milae Bioresources Co., Ltd.) and inorganic iron (Sigma-Aldrich, St. Louis, MO, USA) were used. The compositions of the AIN-93G rodent diet and the iron deficiency diet (Deyts, Bethlehem, PA, USA) are shown in Table 1. Mice were housed and used in strict accordance with the guidelines established by the Korea University Institutional Animal Care and Use Committee (KU-IACUC No. 2017–13), and all experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, 1996). Feed intake and body weight were measured once a week. The feed efficiency ratio (FER) was calculated by the following formula: FER (%) = [body weight gain (g/day)/feed intake (g/day)] \times 100. On the last day of the experiment, all animals were anesthetized with isoflurane (2%) and sacrificed to obtain blood and tissues. Euthanasia for the animals was performed by CO₂ inhalation (10–30% chamber volume/min) according to AVMA Guidelines for the Euthanasia of Animals (2013).

Blood Analysis

Blood cell counts and serum iron concentrations were determined at the end of the trial (8 weeks). Blood samples were collected by cardiac puncture under CO₂ anesthesia. Whole

Table 1 Composition of experimental diets

Ingredients	Normal Fe diet (g/kg)	Low Fe diet (g/kg)
Casein, 30 Mesh	200	200
L-Cysteine	3	3
Corn starch	397	397
Maltodextrin	132	132
Sucrose	100	100
Cellulose	50	50
Soybean Oil	70	70
<i>t</i> -Butylhydroquinone	0.014	0.014
Mineral Mix S10022G (Low Fe gr. #81062) ^a	35	35
Choline Bitartrate	2.5	2.5
Iron content	45 ppm	4.5 ppm
Total	1000	1000

^a Low Fe gr. #81062 is a mineral mix excluding iron. The iron content of 4.5 ppm in the anemia-inducing iron deficiency feed is the sum of the iron content originally contained in other feed ingredients, except the mineral mix

blood was collected in vacutainer tubes (Becton Dickinson, Franklin, NJ, USA) coated with ethylenediaminetetraacetic acid as an anti-coagulant. White blood cell (WBC) and red blood cell (RBC) indices were measured using an automatic blood cell counter (Cell Counter Analyzer MS9-5 V-Melet, Schloesing Laboratory, Osny, France). All whole blood samples were analyzed within 5 h of collection. Blood required for serum preparation was collected in plain vacutainer tubes (Becton Dickinson), and the serum was separated by centrifugation at $2000\times g$ for 10 min. All serum samples were stored at $-80\text{ }^{\circ}\text{C}$ until analysis.

Immunohistochemistry

Spleen tissues were surgically removed, fixed in 10% formaldehyde, and embedded in paraffin, following which $5\text{-}\mu\text{m}$ sections of paraffin-embedded tissue were de-paraffinized, rehydrated, and stained using avidin-biotin complex. Sections were then treated with proteinase K for 5 min, rinsed with distilled water, and treated with hydrogen peroxide for 10 min. Next, the sections were rinsed with distilled water, blocked with serum-free protein (Dako, Carpinteria, CA, USA) for 10 min, incubated for 30 min with rabbit polyclonal IgG anti-ferritin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and further incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG antibody at a 1:5000 dilution, Santa Cruz Biotechnology) for 1 h at room temperature. Then, diaminobenzidine substrate was added, and the preparation was incubated for 5–15 min at room temperature until color fixation was sufficient. To terminate the reaction, each slide was washed with distilled water. Over 24 slides per group were examined. The slides were examined for histological changes using an optical microscope at $40\times$ magnification. Spleen sections were stained with Prussian blue and nuclear red counter stains. A brown color was considered indicative of ferritin storage in the spleen.

Quantitative Real-Time Polymerase Chain Reaction (qPCR) and Data Analysis in Mouse Liver

Total RNA was extracted using Trizol reagent (Gibco BRL, Gaithersburg, MD, USA) in accordance with the manufacturer's protocol. Approximately 10 mg of tissue was thawed and homogenized in 1 mL Trizol reagent. RNA concentration was determined with a NanoDrop spectrophotometer (DeNovix DS-11+, Wilmington, DE, USA). Then, cDNA was prepared with a cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). qPCR was performed with a RT-qPCR kit (Thermo Fisher Scientific) in a StepOne Plus Real-time PCR system (Thermo Fisher Scientific). The primer sequences are listed in Table 2. The reaction was conducted under conditions of preheating for 10 min at $95\text{ }^{\circ}\text{C}$, followed by 40 cycles of $95\text{ }^{\circ}\text{C}$ for 20 s, $58\text{ }^{\circ}\text{C}$ for 20 s, and $72\text{ }^{\circ}\text{C}$ for

20 s. qPCR data were quantified based on the number of cycles needed for amplification-generated fluorescence to reach a specific threshold of detection (C_t value). Relative gene expression was quantified based on the average C_t value of each gene for equal amounts of RNA ($1\text{ }\mu\text{g}$), and ΔC_t was calculated ($\Delta C_t = C_{t_{\text{target gene}}} - C_{t_{\text{reference gene}}}$) using C_t values of genes in the same sample. Actin was used as the internal control reference gene. The $\Delta\Delta C_t$ value was calculated using the equation $\Delta\Delta C_t = \Delta C_{t_{\text{treated}}} - \Delta C_{t_{\text{untreated}}}$. Normalized expression change was recorded as $2^{-\Delta\Delta C_t}$ (actin control was set to 1) [28].

Tissue Section Staining

After washing with distilled water, liver tissue sections were stained in Harris hematoxylin for 30 s at room temperature, washed again with distilled water for 10 min at room temperature, and incubated with eosin for 1 min at room temperature in preparation for hematoxylin and eosin staining. Next, sections were washed in running tap water for 3 min and dehydrated. The sections were mounted in Canada balsam mixed with xylene on glass slides and covered with coverslips. About 24 slides per group were examined. The sections were observed with an optical microscope at $40\times$ magnification for histological changes.

Atomic Absorption Spectrometry

Quantification of iron in the liver and spleen was performed using atomic absorption spectrometry. Each tissue (100 mg) was washed with nitric acid and water prior to sterilization and stored in decontaminated plastic tubes at $-80\text{ }^{\circ}\text{C}$ for future use. Samples were acid-digested in closed Eppendorf tubes with 1 mL of ultrapure nitric acid (1:10 dilution, w/v) in a sonicator for 30 min, transferred to an oven at $60\text{ }^{\circ}\text{C}$ for 1 h, and proportionally diluted to obtain the desired concentrations. Digested tissue (100 μL) was brought to 1 mL of total volume with 2% nitric acid and analyzed for iron. Tissue samples were evaporated until dry on a rotatory evaporator to eliminate acid. Samples diluted with distilled water to a final volume of 10 mL were analyzed to determine iron concentrations. Absorption was measured on a Perkin Elmer model 3030 spectrophotometer (Waltham, MA, USA) with background correction.

Statistical Analysis

Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA). Results are expressed as mean \pm standard deviation (SD) of three independent experiments. The results were evaluated for significant differences between groups using one-way analysis of the variance (ANOVA) followed

Table 2 Sequences of the primers

Gene	Description	Sense (5'–3')	Antisense (5'–3')
<i>DMT1</i>	Divalent metal transporter 1	GCTGAGCGAAGATA CCAGCG	TGTGCAACGGCACATACTTG
<i>Trf</i>	Transferrin	GGCATCAGACTCCA GCATCA	GCAGGCCCATAGGGATGTT
<i>TfR1</i>	Transferrin receptor 1	GTTTCTGCCAGCCCCTTATT AT	GCAAGGAAAGGATA TGCAGCA
<i>Actin</i>	Actin	TGTCACCAACTGGG ACGATA	AACACAGCCTGGAT GGCTAC

by Tukey's multiple range test. A significant difference was indicated by a *p* value less than 0.05.

Results

Changes in Body Weight

AIN-93G feed was used as the basic diet, and to induce IDA, the mineral mix normally added to the feed was replaced with a mineral mix lacking in iron to reduce the iron content to one tenth of the normal iron content. The effects of supplementation of the diet with various GABA concentrations and two types of iron on the weight of mice were monitored. Body weight gain was significantly lower in the PC group than the NC group, demonstrating the effect of iron deficiency (Table 3). There were no significant differences in body weight gain between the GABA groups and the PC group, and there were no differences between the IO, O, and G2 + O groups and the NC group. The results showed that iron deficiency reduced body weight, and GABA administration did not recover body weight in iron-deficient mice, suggesting that GABA alone may not affect body weight recovery

in iron-deficient mice. There was no significant difference in feed intake between the groups, and the FER results also showed no significant differences between the groups.

Serum and Tissues Iron Contents

To investigate iron homeostasis, the iron contents in serum and tissues were measured. The serum iron content in the NC group was 344.0 ± 41.4 $\mu\text{g/dL}$, whereas that in the PC group was approximately 59.0% lower than that in the NC group (Fig. 1A). This result indicates that the iron-deficient diet may induce anemia. The serum iron contents in the GABA groups (G0.5, G1, and G2) were not significantly elevated compared with the PC group. Although there was no statistical significance, the serum iron content of the O group increased by 17.0% compared with that of the IO group. The highest serum iron content was observed in the G2 + O group, which showed a 90.0% increase compared with the PC group. The serum iron content of G2 + O group also significantly increased compared with the O group. These findings demonstrate that although GABA alone may not significantly elevate serum iron levels, it may, in combination with organic iron, exert a synergistic effect to elevate serum iron.

Table 3 Effect of GABA and iron on body weight gain, feed intake, and FER in the experimental mice (*n* = 8/group)

Group	Body weight (g)		Body weight gain (g/day)	Feed intake (g/day)	FER (%)
	Initial	Final			
NC	18.3 \pm 0.4	29.5 \pm 3.6 [#]	0.19 \pm 0.05 [#]	1.5	12.49 \pm 3.81
PC	18.4 \pm 0.6	25.6 \pm 3.2 [*]	0.12 \pm 0.04 [*]	0.8	14.98 \pm 3.89
G0.5	18.2 \pm 1.2	25.5 \pm 3.6 [*]	0.12 \pm 0.04 [*]	0.8	15.21 \pm 4.04
G1	18.6 \pm 0.4	26.3 \pm 1.8 [*]	0.13 \pm 0.02 [*]	0.9	14.17 \pm 3.75
G2	18.1 \pm 0.8	26.9 \pm 4.9 [*]	0.15 \pm 0.07 ^{*,#}	1.1	13.29 \pm 4.31
IO	18.3 \pm 0.4	29.2 \pm 1.7 [*]	0.18 \pm 0.02 [#]	1.3	13.97 \pm 1.65
O	18.2 \pm 0.4	29.7 \pm 4.5 [#]	0.19 \pm 0.07 [#]	1.3	14.69 \pm 4.32
G2 + O	18.4 \pm 0.5	30.9 \pm 2.4 [#]	0.21 \pm 0.03 [#]	1.6	13.00 \pm 2.57

Data are expressed as mean \pm SD; NC, negative control (diet iron content of 45 ppm); PC, iron deficiency anemia (diet iron content of 4.5 ppm); G0.5, gamma aminobutyric acid (GABA) 0.5 mg/100 g body weight (BW)/day; G1, GABA 1 mg/100 g BW/day; G2, GABA 2 mg/100 g BW/day; IO, inorganic iron 0.4 mg/100 g BW/day; O, organic iron 0.4 mg/100 g BW/day; and G2 + O, GABA 2 mg/100 g BW/day + organic iron 0.4 mg/100 g BW/day. * *P* < 0.05, compared with the negative control; # *P* < 0.05, compared with the positive control

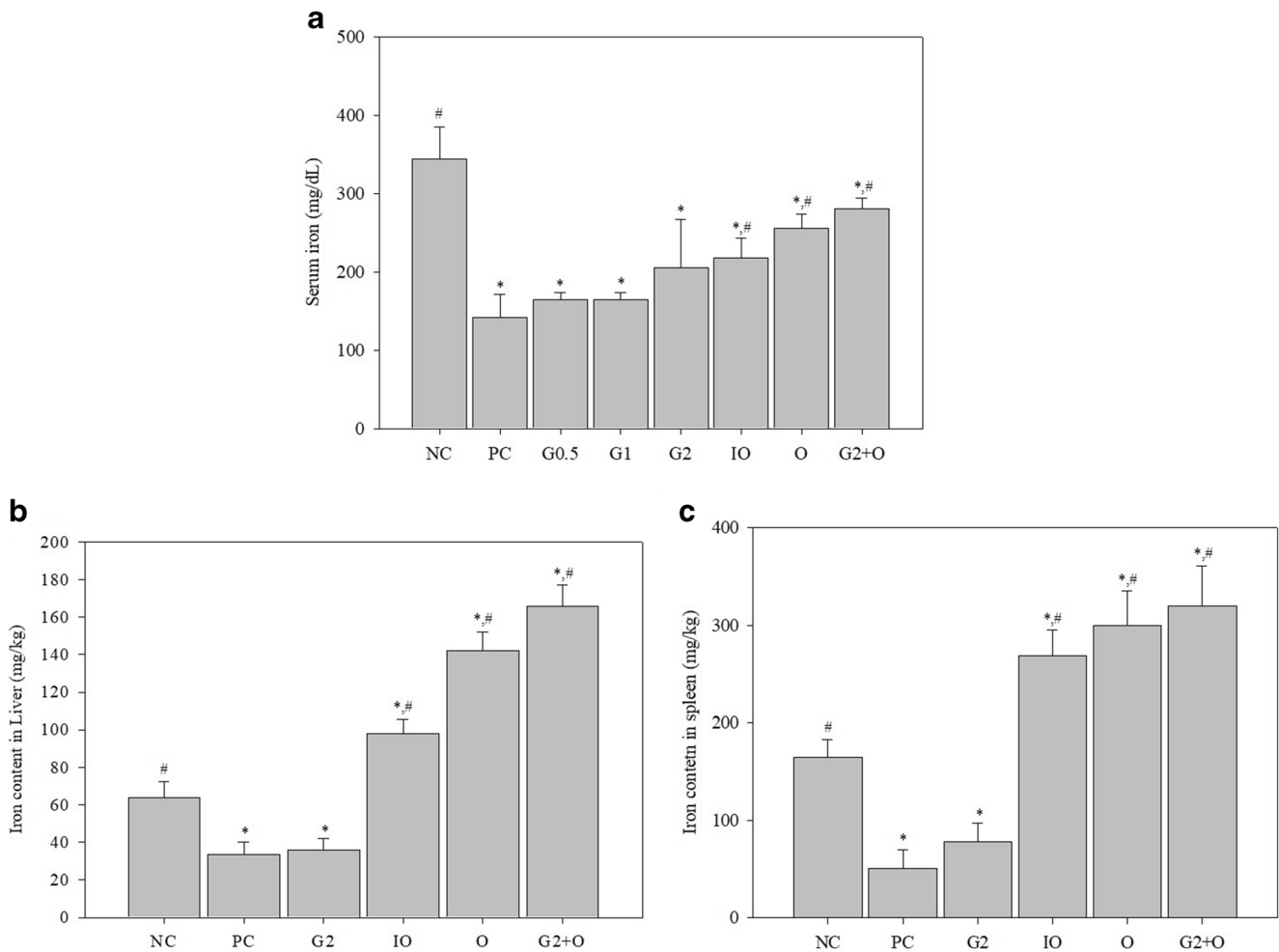


Fig. 1 Serum iron and iron contents in the liver and spleen of mice ($n = 8$) fed various concentrations of gamma aminobutyric acid (GABA) and two types of iron for 8 weeks. Data are expressed as mean \pm SD of triplicate in each group. **(A)** Serum iron in mice, **(B)** iron content in liver tissue, and **(C)** iron content in spleen tissue. NC, negative control (diet iron content of 45 ppm); PC, iron deficiency anemia (diet iron content of 4.5 ppm);

G0.5, GABA 0.5 mg/100 g body weight (BW)/day; G1, GABA 1 mg/100 g BW/day; G2 GA,BA 2 mg/100 g BW/day; IO, inorganic iron 0.4 mg/100 g BW/day; O, organic iron 0.4 mg/100 g BW/day; and G2 + O, GABA 2 mg/100 g BW/day + organic iron 0.4 mg/100 g BW/day. * $P < 0.05$, compared with the negative control; # $P < 0.05$, compared with the positive control

Iron deposition in the liver and spleen tissues was analyzed using atomic absorption spectrometry. The liver iron content of the NC group was 63.9 ± 8.5 mg/kg, while that of the PC group was reduced to 47.9% (33.3 ± 6.8 mg/kg) compared with the NC (Fig. 1B). The iron content of G2 group decreased 43.5% (36.1 ± 6.1 mg/kg) compared with the NC, which was not significantly different from that of the PC group. Surprisingly, the liver iron contents of IO, O, and G2 + O groups increased to 153.5, 222.2, and 259.5%, respectively, compared with the NC group.

The changes of iron content showed a very similar trend in spleen and liver tissues (Fig. 1C). The spleen iron content of the NC group was 164.5 ± 18.7 mg/kg, whereas that of the PC group was reduced to 69.4% (50.4 ± 19.5 mg/kg) compared with the NC group. The iron content of the G2 group decreased to 52.3% (78.4 ± 18.4 mg/kg) compared with the

NC group showed an increasing trend compared with the PC group, but this increase was not significant. The spleen iron content significantly increased to 163.2, 182.2, and 194.8% in the IO, O, and G2 + O groups, respectively, compared with the NC group.

Effect of Co-treatment of GABA and Iron on RBC Indices in Mice with IDA

To investigate the effect of co-treatment of GABA and iron on RBC indices in iron-deficient mice, RBC indices were analyzed in mouse whole blood. The number of RBCs in the NC group, fed a normal diet, was $10.8 \pm 0.7 \times 10^6/\text{mm}^3$, whereas that in the PC group, fed an iron-deficient diet, was only $7.3 \pm 0.6 \times 10^6/\text{mm}^3$, indicating that IDA was induced (Table 4). There was no significant increase in RBCs in the GABA-only groups,

Table 4 Red blood cell index counts in mice fed various GABA concentrations and two types of iron for 8 weeks ($n = 8/\text{group}$)

Group	Red blood cells ($\times 10^6/\text{mm}^3$)	Hemoglobin (g/dL)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dL)	RDW (%)
NC	10.8 \pm 0.7 [#]	16.0 \pm 0.6 [#]	55.1 \pm 0.6 [#]	55.1 \pm 1.4 [#]	15.6 \pm 0.9 [#]	30.1 \pm 0.7 [#]	13.4 \pm 0.3 [#]
PC	7.3 \pm 0.6 [*]	11.7 \pm 0.7 [*]	45.6 \pm 0.8 [*]	42.3 \pm 0.9 [*]	7.8 \pm 0.4 [*]	22.8 \pm 0.6 [*]	16.9 \pm 1.4 [*]
G0.5	7.6 \pm 0.5 [*]	12.6 \pm 1.0 [*]	50.4 \pm 1.4 [*]	48.7 \pm 1.5 [*]	8.8 \pm 0.4 [*]	25.3 \pm 0.5 [*]	16.5 \pm 0.9 [*]
G1	8.4 \pm 0.7 [*]	13.0 \pm 0.5 [*]	51.4 \pm 2.7 [*]	47.8 \pm 1.0 [*]	8.9 \pm 0.3 [*]	25.2 \pm 0.3 [*]	15.1 \pm 1.1 ^{*,#}
G2	8.7 \pm 0.4 [*]	13.2 \pm 0.4 [*]	51.5 \pm 0.9 [*]	49.7 \pm 0.8 [*]	10.2 \pm 0.4 [*]	25.1 \pm 0.7 [*]	14.6 \pm 1.4 ^{*,#}
IO	9.7 \pm 0.4 [*]	13.5 \pm 0.6 [*]	57.2 \pm 0.4 [#]	54.1 \pm 0.4 [#]	14.1 \pm 0.6 [#]	27.4 \pm 0.6 [*]	14.3 \pm 0.1 ^{*,#}
O	9.9 \pm 0.6 [*]	14.5 \pm 0.6 [*]	58.0 \pm 1.4 [#]	55.3 \pm 0.9 [#]	14.5 \pm 0.4 [#]	27.4 \pm 0.4 [*]	13.8 \pm 0.4 [#]
G2 + O	10.7 \pm 0.6 [#]	15.8 \pm 0.6 [#]	60.2 \pm 1.8 [#]	56.0 \pm 0.9 [#]	14.9 \pm 0.4 [#]	27.6 \pm 0.7 [*]	13.6 \pm 0.7 [#]

Data are means \pm SD of triplicate in each group; NC, negative control (diet iron content of 45 ppm); PC, iron deficiency anemia (diet iron content of 4.5 ppm); G0.5, GABA 0.5 mg/100 g body weight (BW)/day; G1, GABA 1 mg/100 g BW/day; G2, GABA 2 mg/100 g BW/day; IO, inorganic iron 0.4 mg/100 g BW/day; O, organic iron 0.4 mg/100 g BW/day; G2 + O, GABA 2 mg/100 g BW/day + organic iron 0.4 mg/100 g BW/day; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; and RDW, red blood cell distribution width. ^{*} $P < 0.05$, compared with the negative control; [#] $P < 0.05$, compared with the positive control

whereas RBCs significantly increased in the IO and O groups. Especially in the G2 + O group, the number of RBCs was recovered to the level of the NC group. Hemoglobin analysis showed trends similar to those seen for the RBC counts. In addition, hematocrit results were similar to those for the RBC counts, except for the hematocrit levels of the iron supplementation groups, which were similar to that of the NC group. The results of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) analyses were similar to those for RBC counts and hemoglobin. The RBC distribution width (RDW) reflects the evenness of the volume and size of red blood cells, with larger values indicating more uneven distributions. RDW values showed that the PC and G0.5 groups had an uneven distribution of red blood cell volumes. Red blood cell indices of the G2 group were elevated compared with the PC group, which seems that GABA has independent effects on red cell parameters. The results of RBC analysis confirmed the induction of IDA in the iron-deficient diet-fed PC group. Moreover, RBC values as well as hemoglobin, hematocrit, MCV, MCH, and MCHC values were similar in the NC and G2 + O groups. These results suggest that GABA and organic iron may exert a synergistic effect resulting in the recovery of RBC indices in IDA mice.

Effect of Co-treatment of GABA and Iron on WBC Counts in Mice with IDA

WBC counts, including total WBC, neutrophil, lymphocyte, monocyte, eosinophil, and basophil counts, were determined in mouse whole blood. There were some differences; nevertheless, all values were in the normal range, and there were no significant differences among groups because of the large

degree of variation in the estimates (Table 5). These results suggest that iron deficiency does not affect WBC counts.

Histological Analysis of Spleen and Liver of Mice with IDA

Ferritin is an iron-containing protein, which, along with hemoglobin, is a major reservoir of iron in the body [29]. Formalin-fixed and paraffin-embedded spleen tissues were prepared and subjected to immunohistochemical analysis using ferritin antibody. Ferritin storage in the spleen was indicated by the presence of a brown color (Fig. 2). The results of the immunohistochemical analysis showed a similar trend with iron contents quantified using atomic absorption spectrometry in the spleen (Fig. 1C). Brown staining indicated the presence of ferritin, where the highest frequencies of ferritin spots were observed in G2 + O group. Ferritin staining also showed that the O group had a wider ferritin spot distribution than the IO group. We also used hematoxylin and eosin staining to evaluate the toxicity of the various treatments used in this study. No clinical changes were observed in the liver and spleen as a result of any treatment (Fig. 3).

Expression Levels of Iron Absorption-Related Genes

Divalent metal transporter 1 (DMT1), transferrin (Trf), and transferrin receptor 1 (TrfR1) are expressed in the liver. The expression levels of *DMT1*, *Trf*, and *TrfR1* mRNA were measured using qPCR. *DMT1* expression significantly increased in the PC, G0.5, G1, and G2 groups compared with the NC group (Fig. 4A). However, there was no significant difference between the NC group and the iron-administered groups IO, O, and G2 + O. With regard to *Trf* expression, the PC, G0.5, and G1 groups showed low expression compared with the NC group (Fig. 4B). However, the expression levels of *TrfR1* in

Table 5 White blood cell differential counts in mice fed various GABA concentrations and two types of iron for 8 weeks ($n = 8/\text{group}$)

Group	White blood cells ($\times 10^3/\mu\text{L}$)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
NC	3.4 ± 1.4	18.3 ± 5.6	75.5 ± 5.8	1.3 ± 0.3	4.5 ± 1.9	0.43 ± 0.33
PC	3.5 ± 1.3	8.7 ± 3.5	78.3 ± 25.2	1.0 ± 0.4	3.7 ± 1.7	0.55 ± 0.28
G0.5	3.1 ± 1.8	13.7 ± 3.9	77.7 ± 3.2	1.6 ± 0.7	3.0 ± 2.3	0.59 ± 0.30
G1	3.3 ± 1.9	15.1 ± 6.5	78.9 ± 3.5	1.5 ± 0.6	4.7 ± 2.8	0.83 ± 0.54
G2	3.3 ± 1.6	11.7 ± 4.2	78.6 ± 4.2	1.6 ± 0.8	6.6 ± 6.0	0.75 ± 0.77
IO	3.4 ± 1.8	18.6 ± 5.1	79.1 ± 3.6	1.6 ± 0.7	4.5 ± 2.1	0.50 ± 0.27
O	3.1 ± 1.1	13.6 ± 6.3	80.5 ± 3.6	2.1 ± 1.0	4.9 ± 5.8	0.45 ± 0.21
G2 + O	2.9 ± 1.3	11.4 ± 2.8	81.8 ± 4.3	1.7 ± 1.0	8.4 ± 12.5	0.70 ± 0.6

Data are mean \pm SD of triplicate in each group; NC, negative control (diet iron content of 45 ppm); PC, iron deficiency anemia (diet iron content of 4.5 ppm); G0.5, GABA 0.5 mg/100 g BW/day; G1, GABA 1 mg/100 g BW/day; G2, GABA 2 mg/100 g BW/day; IO, inorganic iron 0.4 mg/100 g BW/day; O, organic iron 0.4 mg/100 g BW/day; and G2 + O, GABA 2 mg/100 g BW/day + Organic iron 0.4 mg/100 g BW/day

G2, IO, O, and G2 + O groups significantly increased compared with that of the PC group. The expression levels of *TfrR1* (Fig. 4C) gene showed a similar trend to that of transferrin.

Discussion

Iron plays a role in the biosynthesis of the neurotransmitters such as serotonin and dopamine in the central nervous system (CNS) and plays a crucial role in the functioning

of the different neurotransmitter systems, including the serotonin, norepinephrine, and dopamine systems [22]. ID and IDA are significantly associated with alterations of monoamine neurotransmitters and with psychiatric disorders including mood disorder, autism spectrum disorder, and attention-deficit hyperactivity disorder (ADHD) [30]. ID in the brain of infant and young children causes mental retardation, such as delayed development language and psychomotor disorder [31]. On the other hand, excess deposition of iron in the basal ganglia of the aged brain develops various neurodegenerative diseases, such as

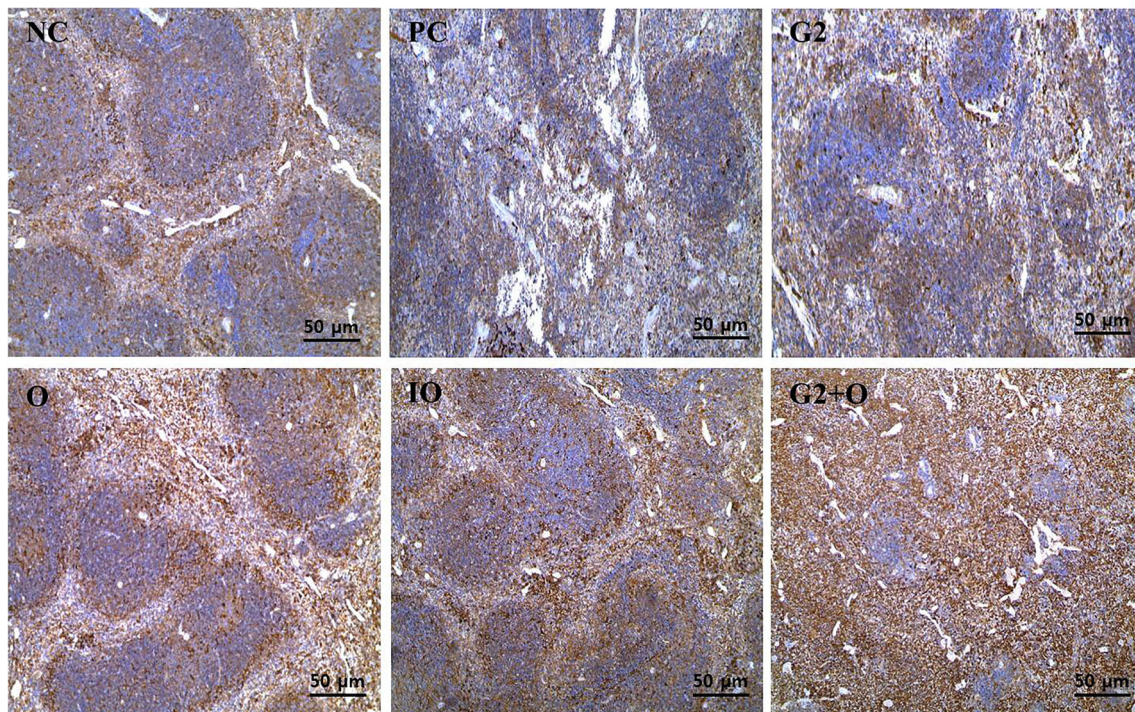


Fig. 2 Immunohistochemistry of ferritin storage in the spleen of mice ($n = 8$ per group, at least five parts were evaluated for each slide). About 24 slides per group were examined, and brown-colored spots are ferritin (magnification 40 \times). NC, negative control (diet iron content of

45 ppm); PC, iron deficiency anemia (diet iron content of 4.5 ppm); G2, GABA 2 mg/100 g BW/day; O, organic iron 0.4 mg/100 g BW/day; IO, inorganic iron 0.4 mg/100 g BW/day; and G2 + O: GABA 2 mg/100 g BW/day + organic iron 0.4 mg/100 g BW/day

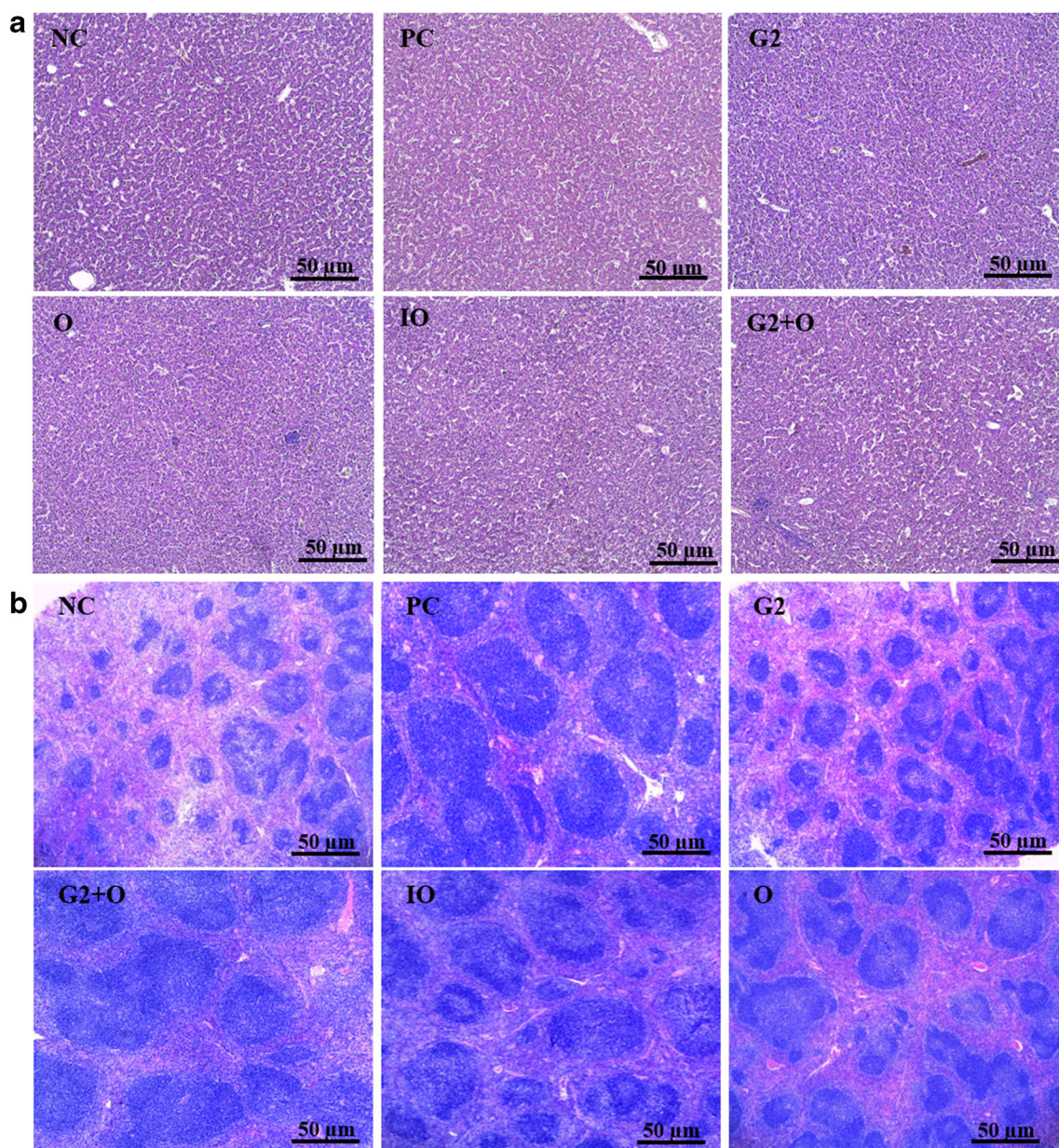


Fig. 3 Hematoxylin and eosin-stained sections from mouse liver (**A**) and spleen (**B**) ($n = 8$ per group, at least five parts were evaluated for each slide). About 24 slides per group were examined for histological changes using an optical microscope at $40\times$ magnification. NC, negative control (diet iron content of 45 ppm); PC, iron deficiency anemia (diet iron

content of 4.5 ppm); G2, GABA 2 mg/100 g BW/day; O, organic iron 0.4 mg/100 g BW/day; IO, inorganic iron 0.4 mg/100 g BW/day; and G2 + O, GABA 2 mg/100 g BW/day + organic iron 0.4 mg/100 g BW/day

Alzheimer's disease, Parkinson's disease, and Huntington disease [31, 32]. Therefore, the strict regulation of iron balance in the brain is very important to prevent and treat nervous system diseases. In addition, iron in the brain is related to the utilization of GABA, and ID in the rat brain alters the metabolism of GABA by decreasing GABA-shunt enzymes (glutamate decarboxylase and GABA transaminase) activities [33, 34].

In this study, IDA was induced in mice by the administration of an iron-deficient diet, following which the iron

absorption-enhancing effects of GABA and glycine-bound organic iron were assessed. Analysis of mouse serum iron content indicated that the iron content of the PC group was significantly lower than that of the NC group. The results demonstrated that the low iron content in the iron-deficient diet limited the absorption of iron in the intestines, resulting in insufficient levels of iron in the serum, thereby leading to the induction of IDA.

Iron homeostasis in the body is maintained through the absorption of iron in the small intestine, recycling of iron

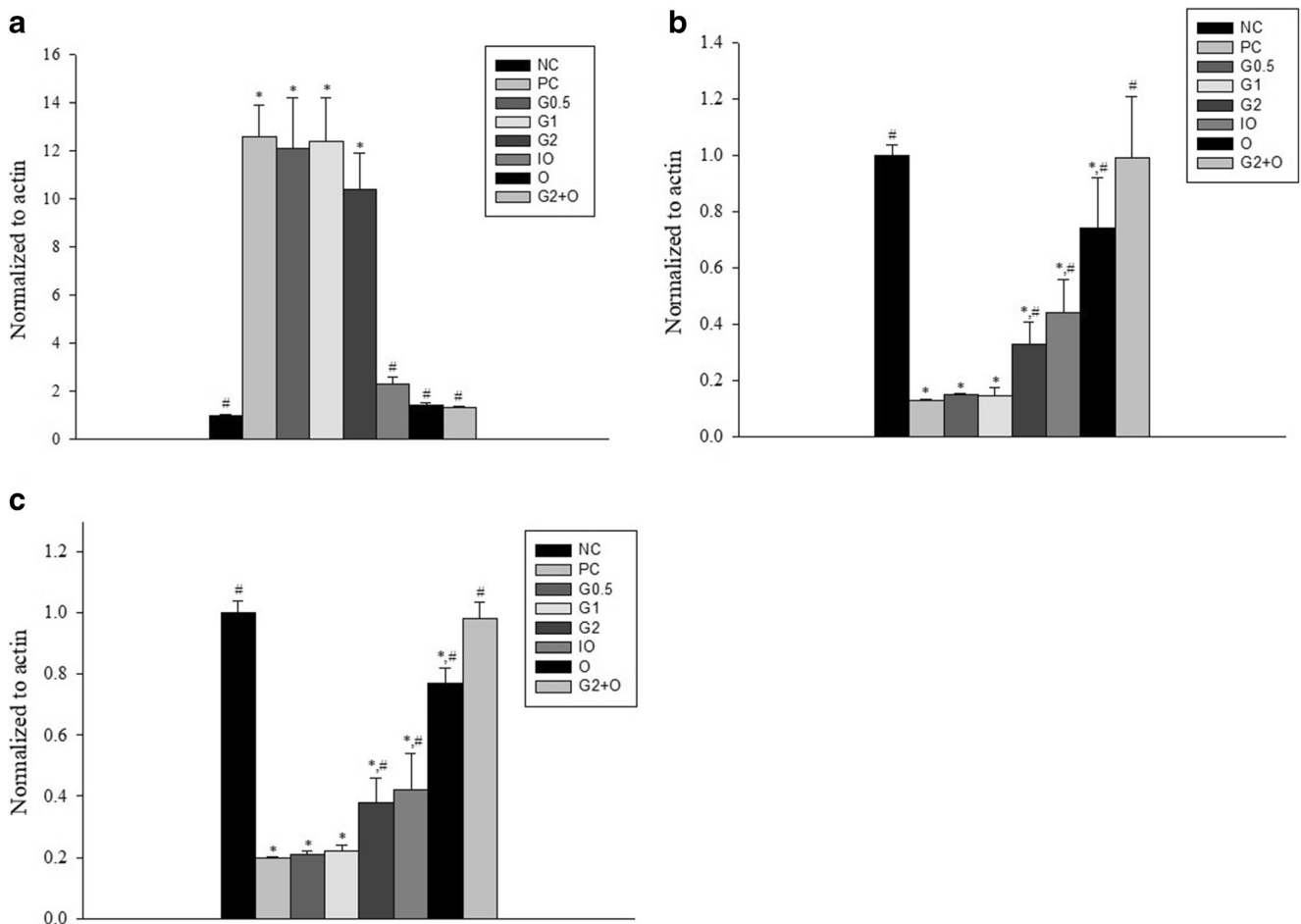


Fig. 4 Expression levels of iron absorption-related genes of mice ($n = 8$) fed various concentrations of gamma aminobutyric acid (GABA) and two types of iron for 8 weeks. **(A)** Divalent metal transporter 1 (*DMT1*), **(B)** transferrin (*TfR*), and **(C)** transferrin receptor 1 (*TfR1*). Normalized fold expression values were determined using results of triplicate in each group and are expressed as mean \pm SD. NC, negative control (diet iron content of 45 ppm); PC, iron deficiency anemia (diet iron content of

4.5 ppm); G0.5, gamma aminobutyric acid (GABA) 0.5 mg/100 g body weight (BW)/day; G1, GABA 1 mg/100 g BW/day; G2, GABA 2 mg/100 g BW/day; IO, inorganic iron 0.4 mg/100 g BW/day; O, organic iron 0.4 mg/100 g BW/day; and G2 + O, GABA 2 mg/100 g BW/day + organic iron 0.4 mg/100 g BW/day. * $P < 0.05$, compared with the negative control; # $P < 0.05$, compared with the positive control

from destroyed red blood cells, and storage and excretion of iron as ferritin or hemosiderin [35]. Abnormal serum iron levels are the first indicators of IDA [36], because iron in food is absorbed by the small intestine and enters the plasma [37]. In this study, serum iron levels in the iron-treated groups did not recover to the level of the NC group despite all the iron-treated groups having elevated tissue iron stores. Further study is needed to explain this. Nevertheless, we found that GABA showed a synergistic effect with organic iron to elevate serum iron, which means that GABA might be used as an adjuvant for absorption of organic iron.

Measurements of MCV, MCH, MCHC, RDW, serum iron, total iron-binding capacity, and ferritin, as determined by an automatic analyzer, are used as general blood tests for the diagnosis of anemia [38]. Iron is a major component of hemoglobin, which carries oxygen throughout the body. Therefore,

serum iron levels are directly related to hemoglobin levels in the blood [39]. In this study, the combination of GABA and organic iron recovered RBC indices in IDA mice, which might be derived from the increase of iron absorption by the combination treatment.

RDW is a measure of the standard RBC size and volume. Changes in the standard size of RBCs occur in certain diseases such as anemia, nutrient deficiency, and cancer [40]. A higher RDW value reflects an increase in RBC size. When anemia is diagnosed, an RDW test is performed to identify its cause, and the results are mainly used to distinguish between mixed-cause and single-cause anemia. In this study, the results indicated an increase in the RDW of the PC group compared with the other groups. This indicated that anemia was induced in the PC group where RBCs were lacking, promoting new RBC production. As a result, the proportion of less

mature RBCs increased, resulting in non-uniformity in the size of RBCs and a higher RDW value.

DMT1 mediates the absorption of the ionic forms of iron, cobalt, zinc, cadmium, and other metal ions [41]. It is a transmembrane protein that uses the proton gradient existing across the gut lumen and enterocyte cytoplasm, to symport Fe^{2+} coupled with H^+ . When metal ions such as iron are lacking, DMT1 function increases to maintain homeostasis. In this study, *DMT1* expression in the IO, O, and G2 + O groups was not different from that of the NC group but significantly increased in the PC and GABA-treated groups. This demonstrated that DMT1 significantly increased in the GABA-treated groups, as GABA is not a metal ion and did not increase the iron content, which was consistent with the results of iron contents in serum and tissues.

Most absorbed plasma iron binds to transferrin and circulates as di-ferric transferrin ($\text{Fe}[\text{III}]_2\text{-Tf}$), whereas a small proportion of iron exported into the plasma is found as non-transferrin-bound iron [42]. This explains that iron deficiency increases the expression of both transferrin and transferrin receptor 1 in this study. Interestingly, although the G2 group did not show a significantly increased iron content in the serum, liver, or spleen, expression levels of the transferrin and transferrin receptor 1 genes significantly increased in the G2 group compared with the PC group.

In this study, the results firstly demonstrate that GABA shows a synergistic effect with organic iron on iron absorption. GABA is approved as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA), and it did not show any toxicity in mice in this study. Further study is needed to explain the role of GABA in the increase of iron absorption by the combination treatment.

In conclusion, administration of GABA alone did not contribute to an increase in serum iron levels or iron absorption in mice with IDA. Glycine-bound iron, an organic iron, was more readily absorbed than inorganic iron, which means that organic iron might be more effective than inorganic iron. Finally, this study confirmed that GABA in combination with organic iron synergistically increased the iron levels in the serum, liver, and spleen of iron-deficient mice, suggesting that the addition of GABA to existing iron supplements may improve their effectiveness for treating IDA.

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Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no conflict of interests.

References

- Andrews NC (2000) Iron metabolism: iron deficiency and iron overload. *Annu Rev Genomics Hum Genet* 1:75–98
- Kassebaum NJ (2016) The global burden of anemia. *Hematol Oncol Clin North Am* 30:247–308
- Kassebaum NJ, Jasrasaria R, Naghavi M, Wulf SK, Johns N, Lozano R, Regan M, Weatherall D, Chou DP, Eisele TP et al (2014) A systematic analysis of global anemia burden from 1990 to 2010. *Blood* 123:615–624
- WHO (2015) The global prevalence of anaemia in 2011. World Health Organization, Geneva
- Papanikolaou G, Pantopoulos K (2005) Iron metabolism and toxicity. *Toxicol Appl Pharmacol* 202:199–211
- Ugwuja E, Akubugwo E, Ibiam U, Onyechi O (2010) Impact of maternal iron deficiency and anaemia on pregnancy and its outcomes in a Nigerian population. *Internet J Nutr Wellness* 10:1–11
- Morgan EH, Oates PS (2002) Mechanisms and regulation of intestinal iron absorption. *Blood Cells Mol Dis* 29:384–399
- Conrad ME, Umbreit JN (2000) Iron absorption and transport—an update. *Am J Hematol* 64:287–298
- Miret S, Simpson RJ, McKie AT (2003) Physiology and molecular biology of dietary iron absorption. *Annu Rev Nutr* 23:283–301
- Glahn RP, Van Campen DR (1997) Iron uptake is enhanced in Caco-2 cell monolayers by cysteine and reduced cysteinyl glycine. *J Nutr* 127:642–647
- Li Y, Jiang H, Huang G (2017) Protein hydrolysates as promoters of non-Haem Iron absorption. *Nutrients* 9:609
- Kegley E, Spears J, Flowers W, Schoenherr W (2002) Iron methionine as a source of iron for the neonatal piglet. *Nutr Res* 22:1209–1217
- Roussel G, Stevens V, Cottin S, McArdle HJ (2017) The effect of amino acid deprivation on the transfer of iron through Caco-2 cell monolayers. *J Trace Elem Med Biol* 40:82–90
- Ettle T, Schlegel P, Roth F (2008) Investigations on iron bioavailability of different sources and supply levels in piglets. *J Anim Physiol Anim Nutr* 92:35–43
- Jakobs C, Jaeken J, Gibson K (1993) Inherited disorders of GABA metabolism. *J Inher Metab Dis* 16:704–715
- Park KB, Oh SH (2007) Cloning, sequencing and expression of a novel glutamate decarboxylase gene from a newly isolated lactic acid bacterium, *Lactobacillus brevis* OPK-3. *Bioresour Technol* 98:312–319
- Komatsuzaki N, Shima J, Kawamoto S, Momose H, Kimura T (2005) Production of γ -aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods. *Food Microbiol* 22:497–504
- Wong T, Guin C, Bottiglieri T, Snead OC (2003) Gaba, γ -hydroxybutyric acid, and neurological disease. *Ann Neurol* 54:3–12
- Trenkwalder C, Winkelmann J, Inoue Y, Paulus W (2015) Restless legs syndrome-current therapies and management of augmentation. *Nat Rev Neurol* 11:434–445
- Lyu S, DeAndrade MP, Mueller S, Oksche A, Walters AS, Li Y (2019) Hyperactivity, dopaminergic abnormalities, iron deficiency and anemia in an in vivo opioid receptors knockout mouse: implications for the restless legs syndrome. *Behav Brain Res* 374:112123
- Moreno-Fernandez J, Lopez-Aliaga I, Garcia-Burgos M, Alferrez MJM, Diaz-Castro J (2019) Fermented goat milk consumption enhances brain molecular functions during Iron deficiency anemia recovery. *Nutrients* 11:E2394
- Beard J (2003) Iron deficiency alters brain development and functioning. *J Nutr* 133:1468S–1472S

23. Barrett E, Ross R, O'toole P, Fitzgerald G, Stanton C (2012) γ -Aminobutyric acid production by culturable bacteria from the human intestine. *J Appl Microbiol* 113:411–417
24. Kinnersley AM, Turano FJ (2000) Gamma aminobutyric acid (GABA) and plant responses to stress. *Crit Rev Plant Sci* 19:479–509
25. Janik R, Thomason LAM, Stanisz AM, Forsythe P, Bienenstock J, Stanisz GJ (2016) Magnetic resonance spectroscopy reveals oral *Lactobacillus* promotion of increases in brain GABA, N-acetyl aspartate and glutamate. *Neuroimage* 125:988–995
26. Wu Q, Shah NP (2017) High γ -aminobutyric acid production from lactic acid bacteria: emphasis on *Lactobacillus brevis* as a functional dairy starter. *Crit Rev Food Sci Nutr* 57:3661–3672
27. Kim YM, Lee KH, Kim DY, Kang BS, Yoon JS, Lee YB, Jeong JH, Nam SY, Yun YW, Kim JS (2012) Effect of iron-nanoparticles and ironmicroparticles on erythropoiesis and iron-storage in iron-deficiency anemic mice. *J Biomed Res* 13:119–132
28. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} method. *Methods* 25:402–408
29. Shi Z, Hu X, Yuan B, Pan X, Meyer HE, Holmboe-Ottesen G (2006) Association between serum ferritin, hemoglobin, iron intake, and diabetes in adults in Jiangsu, China. *Diabetes Care* 29:1878–1883
30. Chen MH, Su TP, Chen YS, Hsu JW, Huang KL, Chang WH, Chen TJ, Bai YM (2013) Association between psychiatric disorders and iron deficiency anemia among children and adolescents: a nationwide population-based study. *BMC Psychiatry* 13:161
31. Thirupathi A, Chang YZ (2019) Brain iron metabolism and CNS diseases. *Adv Exp Med Biol* 1173:1–19
32. Sipe JC, Lee P, Beutler E (2002) Brain iron metabolism and neurodegenerative disorders. *Dev Neurosci* 24:188–196
33. Shukla A, Agarwal KN, Shukla GS (1989) Latent iron deficiency alters gamma-aminobutyric acid and glutamate metabolism in rat brain. *Experientia* 45:343–345
34. Hill JM (1985) Iron concentration reduced in ventral pallidum, globus pallidus, and substantia nigra by GABA-transaminase inhibitor, gamma-vinyl GABA. *Brain Res* 342:18–25
35. Finch CA, Hegsted M, Kinney TD, Thomas E, Rath CE, Haskins D, Finch S, Fluharty RG (1950) Iron metabolism: the pathophysiology of iron storage. *Blood* 5:983–1008
36. Cook JD (2005) Diagnosis and management of iron-deficiency anaemia. *Best Pract Res Clin Haematol* 18:319–332
37. Conrad ME, Crosby WH, Merrill B (1963) Intestinal mucosal mechanisms controlling iron absorption. *Blood* 22:406–415
38. Haq SM (2009) Anemia analyzer: algorithm and reflex testing in clinical practice leading to efficiency and cost savings. *Stud Health Technol Inform* 143:14–16
39. Sinha N, Mishra T, Singh T, Gupta N (2012) Effect of iron deficiency anemia on hemoglobin A1c levels. *Ann Lab Med* 32:17–22
40. Evans TC, Jehle D (1991) The red blood cell distribution width. *J Emerg Med* 9:71–74
41. Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 388:482–488
42. Lane DJ, Merlot AM, Huang ML, Bae DH, Jansson PJ, Sahni S, Kalinowski DS, Richardson DR (2015) Cellular iron uptake, trafficking and metabolism: key molecules and mechanisms and their roles in disease. *Biochim Biophys Acta* 1853:1130–1144

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