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THE EFFECT OF NaFeEDTA AS AN IRON FORTIFICANT ON HEMATOCRIT AND BLOOD PLATELET LEVELS OF RATS (*Rattus norvegicus* L.) SPRAGUE-DAWLEY STRAIN

PENGARUH PEMBERIAN FORTIFIKAN ZAT BESI NAFEEDTA TERHADAP KADAR HEMATOKRIT DAN PLATETET DARAH TIKUS (*Rattus norvegicus* L.) GALUR SPRAGUE DAWLEY

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Abstract

The potential of NaFeEDTA as an iron fortificant can be assessed by identifying hemoglobin and red blood cell levels and their relationship to hematocrit levels. High hematocrit values can affect blood circulation. Iron can also affect platelet levels; excessive platelet aggregation can inhibit blood flow. This study aims to identify hematocrit and blood platelet levels of rats after NaFeEDTA in soy milk (SM). The method is RAL with 25 male rats in 5 treatment groups: KK1 (without SM); KK2 (SM 10 mL/kgBW); KP1 (SM + NaFeEDTA 2.7 mg Fe/kgBW); KP2 (SM + NaFeEDTA 5.4 mg Fe/kgBW); KP3 (SM + NaFeEDTA 10.8 mg Fe/kgBW) for 14 days. Hematocrit was measured using a hematology analyzer (t_0 and t_{14}). ANOVA-LSD test ($P < 0.05$) showed hematocrit increases in KK2, KP1, KP2, and KP3 compared to KK1, highest in KP2 (6.22%). NaFeEDTA significantly reduced platelet levels, highest decrease in KP2 (3.06% compared to KK1 and 2.81% compared to KK2). Despite the decrease, platelet levels remained normal. Thus, NaFeEDTA has the potential to fortify iron absorption at an optimal dose of 5.4 mg Fe/kgBW.

Keywords: Hematocrit; Iron; NaFeEDTA; Platelet; Soy milk

Abstrak

Potensi NaFeEDTA sebagai fortifikator zat besi dapat dinilai dengan mengidentifikasi kadar hemoglobin dan sel darah merah serta hubungannya dengan kadar hematokrit. Nilai hematokrit yang tinggi dapat memengaruhi sirkulasi darah. Zat besi juga dapat memengaruhi kadar platelet. Agregasi platelet yang berlebihan dapat menghambat aliran darah. Penelitian ini bertujuan untuk mengidentifikasi kadar hematokrit dan platelet darah tikus setelah pemberian NaFeEDTA dalam susu kedelai (SM). Metode yang digunakan adalah RAL dengan 25 tikus jantan dalam 5 kelompok perlakuan: KK1 (tanpa SM); KK2 (SM 10 mL/kgBW); KP1 (SM + NaFeEDTA 2,7 mg Fe/kgBW); KP2 (SM + NaFeEDTA 5,4 mg Fe/kgBW); KP3 (SM + NaFeEDTA 10,8 mg Fe/kgBW) selama 14 hari. Hematokrit diukur menggunakan alat analisis hematologi (t_0 dan t_{14}). Uji ANOVA-LSD ($P < 0,05$) menunjukkan hematokrit meningkat pada KK2, KP1, KP2, dan KP3 dibandingkan dengan KK1, tertinggi pada KP2 (6,22%). NaFeEDTA secara signifikan menurunkan kadar platelet, dengan penurunan tertinggi pada KP2 (3,06% dibandingkan dengan KK1 dan 2,81% dibandingkan dengan KK2). Meskipun terjadi penurunan, kadar platelet tetap normal. Dengan demikian, NaFeEDTA berpotensi sebagai fortifikator penyerapan zat besi pada dosis optimal 5,4 mg Fe/kgBW.

***Kata Kunci**: Hematokrit; NaFeEDTA; Platelet; Susu kedelai; Zat besi*

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INTRODUCTION

Iron deficiency anemia (IDA) is one of the micronutrient deficiencies that remains a problem in developing countries such as Indonesia, and is a significant cause of stunted growth cases. Almost 40% of cases of the disease occur in children and women (Ernawati et al., 2021). Indonesian people generally consume iron from vegetable sources (*non-heme*) compared to animal sources of iron (*heme*). Soy-based *non-heme* source foods, such as tempe, tofu, and soy milk, have a high level of consumption in the community because they are easily accessible, affordable, and reach all economic levels, and also have a high protein content (Pusat Data dan Sistem Informasi Pertanian, 2015). Soy milk is a product of soybean extraction processing that has a nutritional source equivalent to cow's milk. The amino acid composition of soy milk is similar to that of cow's milk, but it does not contain lactose, so it can be consumed by people who have lactose intolerance. In addition, the similarity of the components that make up soy milk with breast milk can be an alternative source of protein without cholesterol, with low saturated fat for breast-dependent infants (Olías et al., 2023)

Based on Pusat Data dan Sistem Informasi Pertanian data, the average soybean consumption of the Indonesian people reaches 2.2 million tons per year, but this consumption has not been able to reduce the number of IDA cases in Indonesia (Pusat Data dan Sistem Informasi Pertanian, 2015). This condition is caused by several factors. The first factor is that heme iron in the form of ferrous (Fe^{2+}) will be absorbed directly into the enterocytes as a whole porphyrin complex with the help of heme carrier protein (HCP1) while ferry-form non-heme iron (Fe^{3+}) must undergo a reduction process from ferrite form (Fe^{3+}) to ferrous form (Fe^{2+}) with the help of ascorbic acid so that it is easily absorbed. The absorption of heme iron in the body can reach 20–30%, while the absorption of non-heme iron in the body is 5% (Kalman et al., 2025; Basrowi & Dilantika, 2021)

The second factor is related to the mechanism of iron absorption. The absorption of iron in the body is affected by facilitators and inhibitors. Iron absorption facilitators are compounds that can increase iron absorption. Some examples of compounds that facilitate iron absorption include vitamin C (ascorbic acid), lactoferrin, and several amino acids in food, as well as synthetic compounds EDTA and Na. In addition, there are also compounds that can inhibit iron absorption or what is called iron absorption inhibitors. Some examples of these inhibitor compounds include phytic acid and polyphenols. Inhibitor compounds are abundant in plant foods and tend to form insoluble Fe complexes so that Fe cannot be absorbed. Iron absorption factors, in addition to coming from the outside, also come from the internal body, namely the alkaline atmosphere in the small intestine, and the condition of achlorhydria (Kalman et al., 2025; Basrowi & Dilantika, 2021).

Phytic acid, which is abundant in soybean-based food, causes iron absorption to still be low, and IDA cases in Indonesia are still often found (World Health Organization (WHO), 2008). Soy milk contains iron in the form of Fe^{3+} which will form bonds with phytic acid contained in soybeans. This causes iron to be unable be reduced to the form of ferrous ions Fe^{2+}. Phytic acid will also bind iron and form salts that settle in the intestines, so that the absorption of iron is disrupted and the iron cannot be absorbed by the body. Therefore, efforts to fortify food ingredients to increase the availability and absorption of iron need to be carried out (Kalman et al., 2025; Basrowi & Dilantika, 2021).

Fortification is an effort to add fortifications that contain one or more essential vitamins or minerals to food. The type of fortification and the carrier food to be fortified are the determining factors for the success of fortification. The purpose of fortification is to improve the quality of food nutrition as an effort to prevent deficiencies and provide benefits for public health with minimal risk (Ashraf, 2025). Sodium iron (Fe^{3+}) ethylene diamine tetra acetic acid (NaFeEDTA) consists of iron chelated by sodium EDTA which has the potential to be a fortifican. The advantages of NaFeEDTA are that it has water-soluble properties, high Fe bioavailability, minimal changes in organoleptic properties, and is stable in food processing and storage. Fe bioavailability is the proportion of iron that can be used by the body in the food consumed (Ashraf, 2025; Hurrell, 2022).

Soy milk meets the characteristics of being a NaFeEDTA food vehicle that can support the success of fortification efforts. The criteria for carrier food include being economically affordable, easy to obtain, the food chosen is food for people's daily consumption, does not change people's eating

behavior, and the bioavailability of micronutrients added to food is stable both in storage conditions and when consumed (Hurrell, 2022).

In vitro studies conducted by the Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Indonesia (FMIPA UI), demonstrate that NaFeEDTA is the most effective fortificant for enhancing iron bioavailability in soy-based food products compared to Fe succinate, Fe sulfate, Fe fumarate, and Fe bisglycinate (Darlan, 2012; Faradiba, 2014; Nurwulan, 2014). Furthermore, *in vivo* research by Imantaka et al. (2020) (Table 1, 2, & 3; Figure 1), and Wulandari et al. (2020) (Table 4, 5, & 6; Figure 2) regarding the effects of NaFeEDTA on hemoglobin and erythrocyte levels indicates that administering NaFeEDTA-fortified soy milk at a dosage of 5.4 mg Fe/kg BW significantly increases hemoglobin levels and red blood cell counts in rats over a 21-day treatment period. However, an excessive increase in erythrocyte count can lead to polycythemia, which impairs blood microcirculation and elevates the risk of thrombosis in rats (Fang et al., 2015; Zhou et al., 2023). Consequently, it is essential to identify blood hematocrit levels, which represent the proportion of erythrocytes relative to the total blood volume. Hematocrit values serve as an indicator to assess conditions of polycythemia or anemia. Normal hematocrit levels in rats range from 37% to 53% (Valenzuela-Briseño et al., 2022).

Physiologically, iron plays an important role in hematopoiesis, including thrombopoiesis; however, the relationship between iron and platelets is poorly understood, and research on the effects of iron on platelet levels is still very limited. Excess platelets in the body can form clots that block and inhibit blood flow to vital organs such as the brain, heart, and lungs. Therefore, platelet levels must be within the normal range (Brissot et al., 2021; Jimenez et al., 2021; Scridon, 2022). The normal platelet counts in rats range from 600–1,500 ($\times 10^3/\mu L$) (Mourão et al., 2023). Rats with a deficiency in platelet levels will experience prolonged bleeding, even from minor wounds. A drastic decrease in platelets can lead to fatal internal hemorrhaging and death (Rudmann et al., 2011). Therefore, through *in vivo* (preclinical) research conducted by Imantaka et al. (2020) and Wulandari et al. in 2020, it must be complemented with data on platelet and hematocrit levels in rat blood to obtain initial data that the administration of NaFeEDTA iron fortification in soy milk can increase the availability of plasma iron, which has an effect on increasing hemoglobin and red blood cell levels, but can also maintain blood platelet levels in rats within the normal range.

Testing hematocrit and platelet levels is also one of the quality and safety checks for the use of the iron fortification NaFeEDTA in soy milk before it is used by the public as a product capable of increasing the number of red blood cells as a solution to address anemia, which contributes to the increase in stunting cases in Indonesia (Yang et al., 2011; Barth-Jaeggi et al., 2015; Teshome et al., 2017). Based on this, this study aims to analyze hematocrit and platelet levels to obtain initial data regarding the potential of NaFeEDTA as a booster for iron absorption in food fortification technology applications.

MATERIALS AND METHODS

Hematology analysis (hemoglobin, RBC, hematocrit, and platelets) can be measured using a hematology analyzer. Hematology analyzers are commonly used in rat research because they are automatic, rapid, and objective in counting blood cells, including platelets, in whole blood samples. It employs electrical or optical signal detection based on cell size characteristics, thereby reducing subjectivity and high coefficients of variation associated with manual methods (Moroff et al., 2005; Ameri et al., 2011; Kampfmann et al., 2012).

The research was experimental using a Complete Random Design (CRD) with 5 treatment groups within 5 repetitions. The number of repetitions refers to Frederer's formula, namely: (t-1) (n-1) ≥15 (where t= number of treatments and n= number of repetitions. The rat strain (*Rattus norvegicus*) used is Sprague-Dawley with characteristics: male, weight between 180–200 g, and about 2–3 months old. The experimental animals were obtained from the Badan Penelitian dan Pengembangan Kesehatan, Kementerian Kesehatan, Republik Indonesia, DKI Jakarta. The acclimatization of maintenance and treatment on trial animals was carried out at the Animal House of the Department of Biology, FMIPA UI, with a research period of 3 months. Rat food in the form

of pellets with a composition per 100 g is 4% crude fiber; protein 20–22%; fat 2–4% and ash content 7–9%. Test animal drinks are water that is put into an animal's bottle.

The preparation of NaFeEDTA at doses of 2.7 mgFe/kgBW, 5.4 mgFe/kgBW, and 10.8 mgFe/kgBW, incorporated into 2 mL of soy milk, was conducted in the Inorganic Chemistry Laboratory of the Department of Chemistry, FMIPA UI. Soy milk was prepared by extracting dried yellow soybean seeds. Oral administration of soy milk with NaFeEDTA was performed on test animals for 14 days according to the specific dose for each treatment group. The normal control group (KK1) received only pellets and standard beverages without soy milk, while the treatment control group (KK2) was given pellets and standard beverages along with 10 mL/kgBW of soy milk without NaFeEDTA. The treatment groups KP1, KP2, and KP3 were each administered standard food and beverages with 10 mL/kgBW of soy milk containing NaFeEDTA at doses of 2.7 mgFe/kgBW, 5.4 mgFe/kgBW, and 10.8 mgFe/kgBW, respectively.

Blood samples were collected before treatment (t_0) and after 14 days of treatment (t_{14}) using the venipuncture method via the retro-orbital plexus. Hematocrit and platelet levels were measured using a hematology analyzer (Nihon Kohden Celltac α MEK-6450) at the Laboratory of the Center for Primate Studies (PSSP), Institut Pertanian Bogor (IPB). The hematocrit and platelet levels of the test animals at t_0 and t_{14} were presented in tables and histograms. The data were statistically analyzed using the Statistical Product and Service Solutions (SPSS) for Windows version 22, employing a one-way ANOVA followed by the multiple comparison test (LSD).

RESULTS

Hemoglobin and Red Blood Cell (RBC) Levels

Based on research by Imantaka et al. (2020) and Wulandari et al. in 2020, the results of initial hemoglobin levels (t_0) and t_{21} in all treatment groups can be seen in Table 1 and Figure 1. The results of the one-way ANOVA test on hemoglobin levels at t_0 showed that there was no significant difference in hemoglobin levels between treatment groups (α= 0.05). Sequentially, (KK1-KK2-KP1-KP2-KP3) the average hemoglobin levels at t_0 were 13.58 ± 0.46 g/dL; 13.36 ± 0.56 g/dL; 13.34 ± 0.61 g/dL; 13.18 ± 0.37 g/dL; and 12.98 ± 0.50 g/dL, respectively. Meanwhile, for t_{21}, the average hemoglobin levels were 14.02 ± 0.34 g/dL; 14.22 ± 0.41 g/dL; 14.62 ± 0.41 g/dL; 15.54 ± 0.39 g/dL; and 14.88 ± 0.62 g/dL, respectively.

Table 1. The hemoglobin levels at t_0 and t_{21} in each treatment group (Imantaka et al., 2020)

Replicate	Initial hemoglobin levels (t_0) (g/dL)				
	KK 1	KK 2	KP 1	KP 2	KP 3
1	14.20	13.10	13.20	13.10	13.20
2	13.00	13.40	14.20	12.60	13.30
3	13.30	14.30	13.70	13.50	13.50
4	13.60	13.10	12.90	13.20	12.40
5	13.80	12.90	12.70	13.50	12.50
Average	13.58[a]	13.36[a]	13.34[a]	13.18[a]	12.98[a]
SD	0.46	0.55	0.61	0.37	0.50
Replicate	Hemoglobin levels on day 21 (t_{21}) (g/dL)				
	KK 1	KK 2	KP 1	KP 2	KP 3
1	14.10	13.60	14.00	15.90	13.90
2	13.60	14.40	14.80	15.50	15.50
3	14.10	14.50	14.40	15.80	15.20
4	13.80	14.00	15.00	15.60	15.10
5	14.50	14.60	14.90	14.90	14.70
Average	14.02[a]	14.22[b]	14.62[b]	15.54[b]	14.88[b]
SD	0.34	0.41	0.41	0.39	0.62

Note: KK1= normal control group; KK2= soy milk 10 mL/kgBW; KP1= soy milk 10 mL/kgBW + NaFeEDTA 2.7 mg Fe/kgBW; KP2= soy milk 10 mL/kgBW + NaFeEDTA 5.4 mg Fe/kgBW; KP3= soy milk 10 mL/kgBW + NaFeEDTA 10.8 mg Fe/kgBW. Different letters indicate a significant difference in the means based on the ANOVA and LSD tests (P <0.05)

The data also showed that there were significant differences between treatment groups at the end of the study (t_{21}) (α= 0.05). The results of the LSD test showed that the significant differences were between KK 1 to KK2, KP 1, KP 2 and KP 3; while there were no significant differences between KK 2 and KP1, 2 and 3. Descriptive data show that the highest increase in average hemoglobin levels in KP compared to KK1 and KK2 was in KP2, namely 10.84% compared to KK 1 and 9.28% compared to KK 2 (Table 3). The increase in average hemoglobin levels in KP2 between t_0 and t_{21} was 17.91% (Table 2).

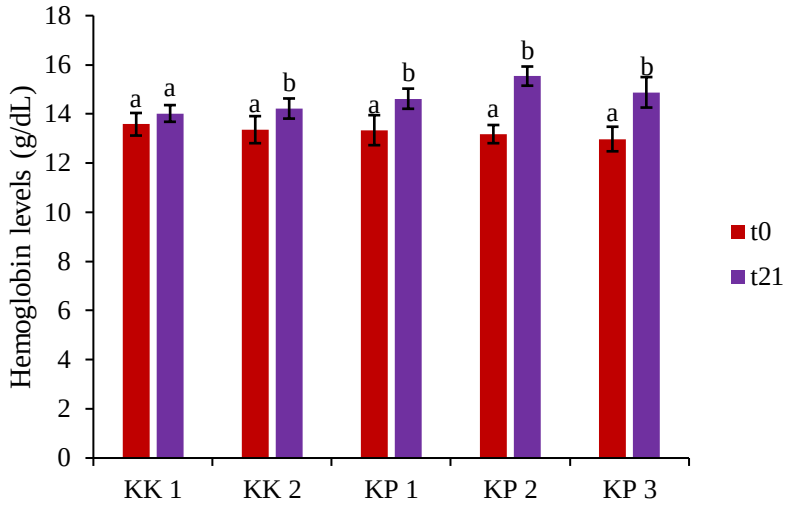

Figure 1. Histogram of average hemoglobin levels at t_0 and t_{21} (g/dL). Different letters indicate a significant difference in the means based on the ANOVA and LSD tests (P <0.05) (Imantaka et al., 2020)

Table 2. Percentage increase in the average hemoglobin levels between groups from t_0 to t_{21} (Imantaka et al., 2020)

Group	t_0	T_{21}	Increasing (%)
KK1	13.58	14.02	3.24
KK2	13.36	14.22	6.44
KP1	13.34	14.62	9.60
KP2	13.18	15.54	17.91*
KP3	12.98	14.88	14.64

Note: KK1= normal control group; KK2= soy milk 10 mL/kgBW; KP1= soy milk 10 mL/kgBW + NaFeEDTA 2.7 mg Fe/kgBW; KP2= soy milk 10 mL/kgBW + NaFeEDTA 5.4 mg Fe/kgBW; KP3= soy milk 10 mL/kgBW + NaFeEDTA 10.8 mg Fe/kgBW. *= indicates the highest value

Table 3. Percentage increase in the average hemoglobin levels between treatment groups on day 21 (t_{21}) (Imantaka et al., 2020)

Group	KK1 (%)	KK2 (%)
KK2	1.43	-
KP1	4.28	2.81
KP2	10.84*	9.28*
KP3	6.13	4.64

Note: KK1= normal control group; KK2= soy milk 10 mL/kgBW; KP1= soy milk 10 mL/kgBW + NaFeEDTA 2.7 mg Fe/kgBW; KP2= soy milk 10 mL/kgBW + NaFeEDTA 5.4 mg Fe/kgBW; KP3= soy milk 10 mL/kgBW + NaFeEDTA 10.8 mg Fe/kgBW. *= indicates the highest value

The results of the initial red blood cell (RBC) count measurements at the beginning of the study (t_0) and t_{21} can be seen in Table 4 and Figure 2. The average range of RBC levels at t_0 was 6.91 (× 10^6/µL)–7.48 (× 10^6/µL) and did not differ significantly between groups (α= 0.05). The data in Table 4 also show that the average RBC levels at the end of the study (t_{21}) respectively between treatment groups were 7.26 ± 0.16 (× 10^6/µL); 7.41 ± 0.12 (× 10^6/µL); 8.10 ± 0.17 (× 10^6/µL); 8.69 ± 0.11 (× 10^6/µL); and 8.29 ± 0.14 (× 10^6/µL). The results of the one-way ANOVA test (α= 0.05) showed a

significant difference between the average RBC levels in KP against KK1, but there was no significant difference between KP and KK2. However, the highest increase in the average RBC level at t_{21} was in KP2 against KP1 and KP3 (Table 5). When compared with the control group, the percentage increase in the average RBC level of KP2 against KK1 was 19.70% and 17.27% against KK 2 (Table 6).

Table 4. The red blood cell (RBC) levels at t_0 and t_{21} in each treatment group (Wulandari et al., 2020)

Replicate	Initial RBC levels (t_0) ($\times 10^6/\mu L$)				
	KK 1	KK 2	KP 1	KP 2	KP 3
1	7.29	6.96	7.39	7.19	7.18
2	7.15	7.36	7.21	7.27	6.97
3	7.34	7.26	7.34	7.45	7.42
4	7.26	6.91	7.48	7.38	7.31
5	7.06	7.29	7.22	7.26	7.46
Average	7.22[a]	7.16[a]	7.33[a]	7.31[a]	7.27[a]
SD	0.11	0.21	0.12	0.10	0.20
Replicate	RBC levels on day 21 (t_{21}) ($\times 10^6/\mu L$)				
	KK 1	KK 2	KP 1	KP 2	KP 3
1	7.20	7.56	7.94	8.69	8.23
2	7.08	7.48	8.21	8.81	8.29
3	7.41	7.29	7.89	8.79	8.10
4	7.46	7.45	8.28	8.54	8.48
5	7.17	7.28	8.16	8.62	8.34
Average	7.26[a]	7.41[b]	8.10[b]	8.69[b]	8.29[b]
SD	0.16	0.12	0.17	0.11	0.14

Note: KK1= normal control group; KK2= soy milk 10 mL/kgBW; KP1= soy milk 10 mL/kgBW + NaFeEDTA 2.7 mg Fe/kgBW; KP2= soy milk 10 mL/kgBW + NaFeEDTA 5.4 mg Fe/kgBW; KP3= soy milk 10 mL/kgBW + NaFeEDTA 10.8 mg Fe/kgBW. Different letters indicate a significant difference in the means based on the ANOVA and LSD tests (P <0.05)

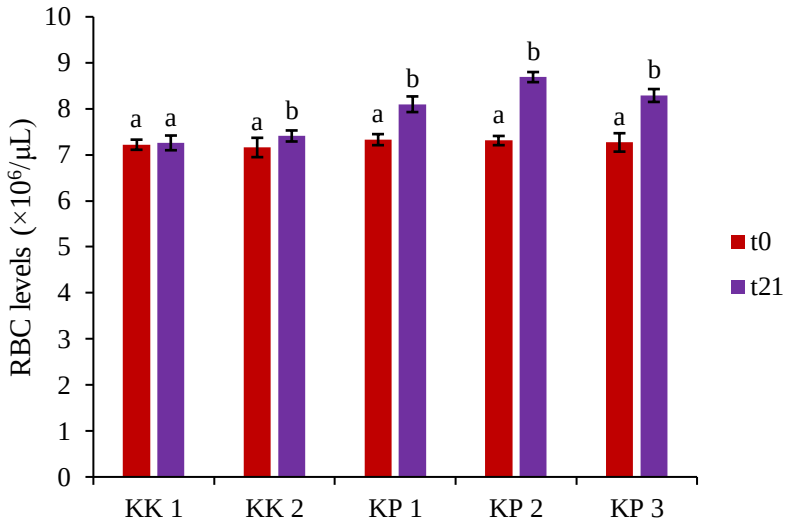

Figure 2. Histogram of average red blood cell (RBC) levels at t_0 and t_{21} ($x10^6/\mu L$). Different letters indicate a significant difference in the means based on the ANOVA and LSD tests (P <0.05) (Wulandari et al., 2020)

Table 5. Percentage increase in the average red blood cell (RBC) levels between groups from t_0 to t_{21} (Wulandari et al., 2020)

Group	t_0	T_{21}	Increasing (%)
KK1	7.22	7.26	0.55
KK2	7.16	7.41	3.49
KP1	7.33	8.10	10.50
KP2	7.31	8.69	18.88*
KP3	7.27	8.29	14.03

Note: KK1= normal control group; KK2= soy milk 10 mL/kgBW; KP1= soy milk 10 mL/kgBW + NaFeEDTA 2.7 mg Fe/kgBW; KP2= soy milk 10 mL/kgBW + NaFeEDTA 5.4 mg Fe/kgBW; KP3= soy milk 10 mL/kgBW + NaFeEDTA 10.8 mg Fe/kgBW. *= indicates the highest value

Table 6. Percentage increase in the average red blood cell (RBC) levels between treatment groups on day 21 (t_{21}) Wulandari et al., 2020)

Group	KK1 (%)	KK2 (%)
KK2	2.07	-
KP1	11.57	9.31
KP2	19.70*	17.27*
KP3	14.19	11.88

Note: KK1= normal control group; KK2= soy milk 10 mL/kgBW; KP1= soy milk 10 mL/kgBW + NaFeEDTA 2.7 mg Fe/kgBW; KP2= soy milk 10 mL/kgBW + NaFeEDTA 5.4 mg Fe/kgBW; KP3= soy milk 10 mL/kgBW + NaFeEDTA 10.8 mg Fe/kgBW. *= indicates the highest value

Hematocrit Level

The increase in hemoglobin and RBC levels in the treatment groups (KP1, 2, & 3) may affect blood hematocrit levels. Table 7 and Figure 3 present the average initial hematocrit levels (t_0) for the groups KK1, KK2, KP1, KP2, and KP3, which were 37.56 ± 0.93%, 39.40 ± 0.60%, 38.76 ± 2.32%, 38.70 ± 1.84%, and 39.48 ± 1.61%, respectively. The results of the one-way ANOVA parametric test (α= 0.05) on the average hematocrit data indicated that there was no significant difference between the groups at baseline, suggesting uniformity across the treatment groups. The average hematocrit levels at the end of the study (t_{14}) are shown in Table 7, with values for KK1, KK2, KP1, KP2, and KP3 being 38.26 ± 0.82%, 39.70 ± 0.82%, 40.16 ± 0.74%, 40.64 ± 0.70%, and 40.46 ± 0.73%, respectively, and there is a significant difference in the average hematocrit level between KK1 and KK2, KP1, 2, and 3. Descriptively, the data in Tables 8 and 9 show that KP2 had the highest increase in hematocrit levels in the treatment group, as well as compared to KK1 and KK2. The highest percentage increase in the average hematocrit level compared to KK1, KK2, and KP was also found in KP2.

Table 7. The hematocrit levels at t_0 and t_{14} in each treatment group

Replicate	Initial hematocrit levels (t_0) (%)				
	KK 1	KK 2	KP 1	KP 2	KP 3
1	37.30	40.10	37.80	38.50	40.70
2	36.80	40.00	40.50	40.80	37.00
3	37.70	39.00	41.90	39.80	41.10
4	39.10	38.90	36.70	35.90	39.20
5	36.90	39.00	36.90	38.50	39.40
Average	37.56[a]	39.40[a]	38.76[a]	38.70[a]	39.48[a]
SD	0.93	0.60	2.32	1.84	1.61
Replicate	Hematocrit levels on day 14 (t_{14}) (%)				
	KK 1	KK 2	KP 1	KP 2	KP 3
1	39.70	40.60	41.00	41.60	41.50
2	37.90	39.30	39.50	39.80	40.40
3	38.10	38.50	39.30	40.40	40.00
4	37.60	40.20	40.30	41.10	40.80
5	38.00	39.90	40.70	40.30	39.60
Average	38.26[a]	39.70[b]	40.16[b]	40.64[b]	40.46[b]
SD	0.82	0.82	0.74	0.70	0.73

Note: KK1= normal control group; KK2= soy milk 10 mL/kgBW; KP1= soy milk 10 mL/kgBW + NaFeEDTA 2.7 mg Fe/kgBW; KP2= soy milk 10 mL/kgBW + NaFeEDTA 5.4 mg Fe/kgBW; KP3= soy milk 10 mL/kgBW + NaFeEDTA 10.8 mg Fe/kgBW. Different letters indicate a significant difference in the means based on the ANOVA and LSD tests (P <0.05)

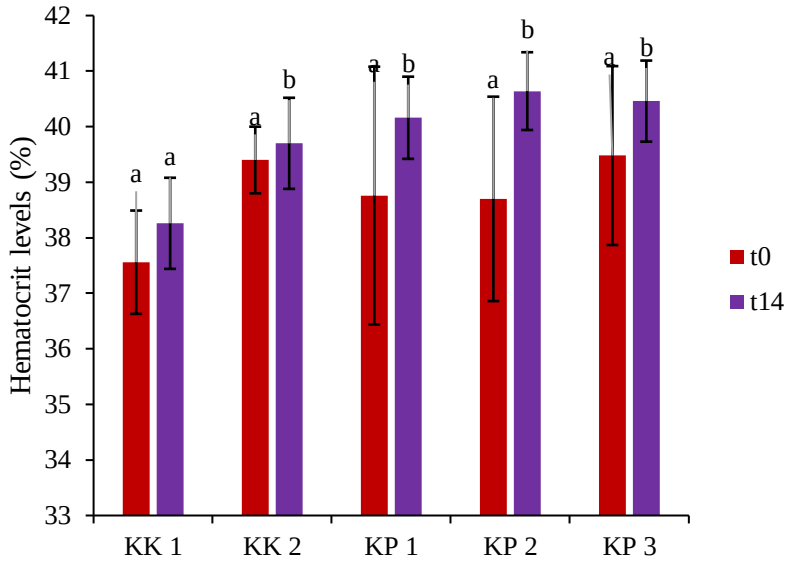

Figure 3. Histogram of average hematocrit levels at t_0 and t_{14} (%). Different letters indicate a significant difference in the means based on the ANOVA and LSD tests (P <0.05)

Table 8. Percentage increase in the average hematocrit levels between groups from t_0 to t_{14}

Group	t_0	t_{14}	Increasing (%)
KK1	37.56	38.26	1.86
KK2	39.40	39.70	0.76
KP1	38.76	40.16	3.61
KP2	38.70	40.64	5.01*
KP3	39.48	40.46	2.48

Note: KK1= normal control group; KK2= soy milk 10 mL/kgBW; KP1= soy milk 10 mL/kgBW + NaFeEDTA 2.7 mg Fe/kgBW; KP2= soy milk 10 mL/kgBW + NaFeEDTA 5.4 mg Fe/kgBW; KP3= soy milk 10 mL/kgBW + NaFeEDTA 10.8 mg Fe/kgBW. *= indicates the highest value

Table 9. Percentage increase in the average hematocrit levels between treatment groups on day 14 (t_{14})

Group	KK1 (%)	KK2 (%)
KK2	3.76	-
KP1	4.96	1.15
KP2	6.22*	2.36*
KP3	5.75	1.91

Note: KK1= normal control group; KK2= soy milk 10 mL/kgBW; KP1= soy milk 10 mL/kgBW + NaFeEDTA 2.7 mg Fe/kgBW; KP2= soy milk 10 mL/kgBW + NaFeEDTA 5.4 mg Fe/kgBW; KP3= soy milk 10 mL/kgBW + NaFeEDTA 10.8 mg Fe/kgBW. *= indicates the highest value

Platelet Count

Table 10 and Figure 4 show the results of platelet count measurements at the start of the study (t_0) and on day 14 (t_{14}) for each research group. The average initial platelet count (t_0) for KK1, KK2, KP1, KP2, and KP3 were 990.8 ± 7.09 (× 10^3/µL); 988.6 ± 7.73 (× 10^3/µL); 988.8 ± 9.07 (× 10^3/µL); 986.6 ± 8.36 (× 10^3/µL); and 987.4 ± 8.44 (× 10^3/µL), respectively. The initial platelet count measurements (t_0) across all treatment groups ranged from 976–999 (× 10^3/µL). The results of the one-way ANOVA parametric test (α= 0.05) indicate that there were no significant differences between the treatment groups. The average platelet counts at t_{14}, based on the LSD test (α= 0.05), results show a significant difference between the KK1 group and KP1, KP2, and KP3 groups, as well as between the KK2 group and KP1, KP2, and KP3 groups. The platelet counts at t_{14} for KK1, KK2, KP1, KP2, and KP3 were 928.8 ± 13.10 (× 10^3/µL); 926.4 ± 13.59 (× 10^3/µL); 906.4 ± 13.85 (× 10^3/µL); 900.4 ± 13.13 (× 10^3/µL); and 905.6 ± 13.50 (× 10^3/µL), respectively. When comparing the percentage change in platelet count between t_0 and t_{14} (Table 11) and across the research groups

(Table 12), the highest percentage decrease in platelet count occurred in the KP2 group, with a reduction of 3.06% compared to KK1 and 2.81% compared to KK2.

Table 10. Average platelet levels at t_0 and t_{14} in each treatment group

Replicate	Initial platelet levels (t_0) ($\times 10^3/\mu L$)				
	KK 1	KK 2	KP 1	KP 2	KP 3
1	999	978	988	980	989
2	983	991	979	996	982
3	990	984	981	986	997
4	985	992	999	977	976
5	997	998	997	994	993
Average	990.8[a]	988.6[a]	988.8[a]	986.6[a]	987.4[a]
SD	7.09	7.73	9.07	8.36	8.44
Replicate	Platelet levels on day 14 (t_{14}) ($x10^3/\mu L$)				
	KK 1	KK 2	KP 1	KP 2	KP 3
1	930	911	925	920	900
2	910	923	911	886	898
3	923	917	899	893	915
4	944	939	888	906	924
5	937	942	909	897	891
Average	928.8[a]	926.4[a]	906.4[b]	900.4[b]	905.6[b]
SD	13.10	13.59	13.85	13.13	13.50

Note: KK1= normal control group; KK2= soy milk 10 mL/kgBW; KP1= soy milk 10 mL/kgBW + NaFeEDTA 2.7 mg Fe/kgBW; KP2= soy milk 10 mL/kgBW + NaFeEDTA 5.4 mg Fe/kgBW; KP3= soy milk 10 mL/kgBW + NaFeEDTA 10.8 mg Fe/kgBW. Different letters indicate a significant difference in the means based on the ANOVA and LSD tests (P <0.05)

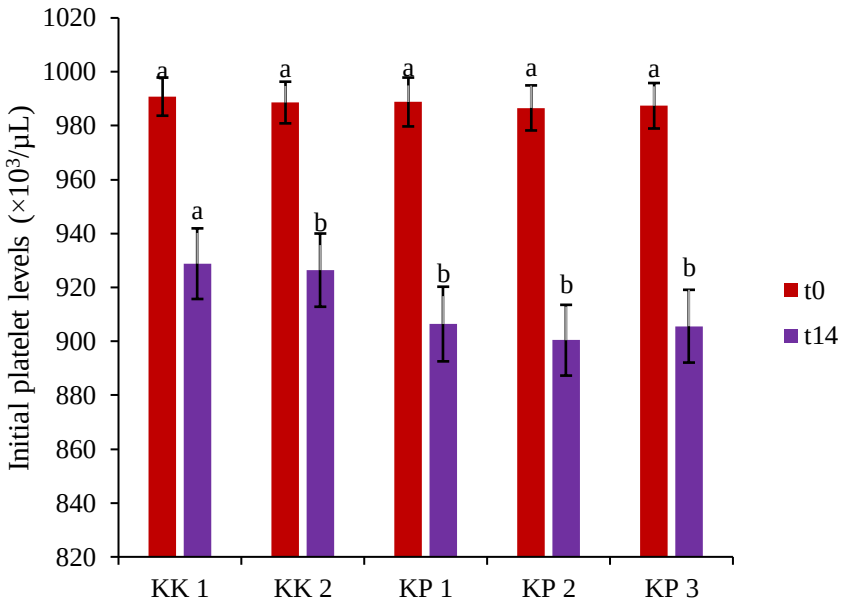

Figure 4. Histogram of the mean platelet count at t_0 and t_{14} ($\times 10^3/\mu L$). Different letters indicate significant differences in the means based on ANOVA and LSD tests (P <0.05)

Table 11. Percentage decrease in the mean platelet count between groups from t_0 to t_{14}

Group	t_0	t_{14}	Decreasing (%)
KK1	993.6	928.8	6.26
KK2	991.2	926.4	6.29
KP1	990.6	906.4	8.33
KP2	987.8	900.4	8.74*
KP3	989.6	905.6	8.28

Note: KK1= normal control group; KK2= soy milk 10 mL/kgBW; KP1= soy milk 10 mL/kgBW + NaFeEDTA 2.7 mg Fe/kgBW; KP2= soy milk 10 mL/kgBW + NaFeEDTA 5.4 mg Fe/kgBW; KP3= soy milk 10 mL/kgBW + NaFeEDTA 10.8 mg Fe/kgBW. *= indicates the highest value

Table 12. Percentage decrease in the mean platelet count between treatment groups on day 14 (t_{14})

Group	KK1 ($\times 10^3/\mu L$)	KK2 ($\times 10^3/\mu L$)
KK2	0.26%	-
KP1	2.41%	2.16%
KP2	3.06%*	2.81%*
KP3	2.50%	2.25%

Note: KK1= normal control group; KK2= soy milk 10 mL/kgBW; KP1= soy milk 10 mL/kgBW + NaFeEDTA 2.7 mg Fe/kgBW; KP2= soy milk 10 mL/kgBW + NaFeEDTA 5.4 mg Fe/kgBW; KP3= soy milk 10 mL/kgBW + NaFeEDTA 10.8 mg Fe/kgBW. *= indicates the highest value

DISCUSSION

Research on the potential of NaFeEDTA as an iron fortificant that can increase the availability of plasma iron began with identifying the effect of iron on hemoglobin and red blood cell (RBC) levels, followed by hematocrit and blood platelet levels in rats. KK1 is a normal control group used as a reference for normal hemoglobin and RBC levels without treatment of soy milk and iron fortification. KK2 was the control group of treatment given soy milk without the iron fortification NaFeEDTA. This control group was used as a reference that food products containing natural non-heme iron are not sufficient to increase plasma iron levels. The study used soy milk as a vehicle food for the iron fortificant NaFeEDTA. The selection of carrier food is one of the factors that determines the success of food fortification, and one of the criteria that must be considered is that the food product must be frequently and widely consumed by the community (Arisyi et al., 2016).

Soy milk contains iron, protein, copper, vitamin B6, vitamin B12, and folic acid, with respective contents of 0.7 mg, 3.27 g, 0.12 mg, 0.1 mg, 0.004 mg, and 0.0015 mg per 100 g of soy milk. These compounds may enhance the process of hemoglobin synthesis in the rats' bodies, thereby affecting their RBC levels. However, soy milk also contains iron absorption inhibitors, namely phytic acid. Phytic acid, found in legumes, contains 70% phosphate. The body lacks the enzyme phytase, making phytic acid difficult to digest, and its phosphate content cannot be utilized by the body. Phytic acid can bind to essential minerals such as calcium, zinc, iron, and magnesium, affecting hemoglobin synthesis, and can also bind to proteins, forming complexes that inhibit protein digestion by proteolytic enzymes (Dietitians of Canada, 2015; United States Department of Agriculture (USDA), 2016).

Non-heme iron in Fe^{3+} present in soy milk can strongly bind with phytic acid, preventing its reduction to the ferrous form (Fe^{2+}), which is required for absorption by enterocytes in the intestine (Kalman et al., 2025; Basrowi & Dilantika, 2021). Therefore, adding soy milk alone may not be sufficient to increase hematocrit levels, and it is necessary to fortify the soy milk with additional iron to improve its bioavailability and enhance iron absorption for hemoglobin synthesis. NaFeEDTA undergoes breakdown in the stomach to form sodium (Na) and Fe (III)-EDTA (Hurrell, 2022). The bond between ferric iron (Fe^{3+}) and EDTA prevents the iron from binding with phytic acid, which acts as an inhibitor. The affinity of ferric iron (Fe^{3+}) for EDTA is higher than its affinity for inhibitors. The acidic conditions in the stomach further strengthen the bond between ferric iron (Fe^{3+}) and EDTA. Ferrous iron (Fe^{3+}) bound in the Fe(III)-EDTA complex then enters the duodenum and separates from the EDTA. This occurs due to an increase in pH in the duodenum, which causes the bond in the Fe(III)-EDTA complex to weaken. The released ferrous iron (Fe^{3+}) is reduced to the ferrous form (Fe^{2+}) by the enzyme ferrireductase. The ferrous iron (Fe^{2+}) is then absorbed by enterocytes, thereby increasing iron availability in the body (Gupta et al., 2020; Piskin et al., 2022).

Therefore, based on the research results in Table 1 and Figure 1, although the average hemoglobin level in KK2 was not significantly different from KP1, 2, and 3, descriptively, the average hemoglobin level in KP was higher than KK2, with the highest percentage increase in KP2, including the percentage increase in hemoglobin levels between t_0 and t_{21}. Research data also provides information that although soy milk contains iron, protein, copper, vitamin B6, vitamin B12, and folic acid which are needed in hemoglobin synthesis, the presence of an inhibitor in the form of phytic acid causes the absorption of iron which is the main component in hemoglobin synthesis to be suboptimal so that only giving soy milk is not enough to increase hemoglobin levels.

An increase in pH in the duodenum weakens the bond in the Fe (III)-EDTA complex, causing ferric iron to release from EDTA. Ferric iron (Fe^{3+}) is then reduced to ferrous iron (Fe^{2+}) by the enzyme ferrireductase. Ferrous iron is absorbed by enterocytes, thereby increasing iron bioavailability in the body (Gupta et al., 2020; Hurrell, 2022; Piskin et al., 2022)

Approximately 5% of EDTA is absorbed into the body and excreted via urine. Unabsorbed EDTA remains in the gastrointestinal lumen, where it binds with iron from soy milk, facilitating its absorption by enterocytes. This mechanism is known as the shuttle effect of EDTA, which enhances iron absorption efficiency in the body (Gupta et al., 2020; Hurrell, 2022; Piskin et al., 2022). Besides being used for hemoglobin synthesis, iron is also a factor in the formation of red blood cells (erythropoiesis). The iron used in erythropoiesis is transferrin iron. This is because erythroid cells in the bone marrow only have receptors for transferrin. Erythroid cells are precursor cells that will later form red blood cells. Iron that has been absorbed by intestinal enterocytes is passed to the basolateral intestine and will bind with apotransferrin to form transferrin. This transferrin then goes with the bloodstream to the bone marrow, where there are erythroid cells. Transferrin will then enter the erythroid cells, assisted by its receptors, and the iron it carries will be used to form heme, a component of red blood cells (Rishi & Subramaniam, 2017). Increasing iron levels by administering the iron NaFeEDTA fortificant causes an increase in hemoglobin levels, which affects RBC formation.

The mechanism for increasing ferritin iron levels by adding the iron NaFeEDTA fortificant begins with the breakdown of NaFeEDTA in the stomach. The acidic environment causes sodium (Na) to separate from Fe(III)EDTA, while the Fe(III)EDTA complex remains intact. This prevents iron from binding to inhibitor compounds. Upon entering the duodenum, an increase in pH occurs, which causes a decrease in the stability of the Fe(III)EDTA bond, allowing Fe^{3+} from the Fe(III)EDTA complex to be easily released. The ferric iron (Fe^{3+}) released from the Fe(III)EDTA complex is then reduced by the ferrireductase enzyme to ferrous iron (Fe^{2+}) and can then be absorbed by the intestine (Gupta et al., 2020; Hurrell, 2022; Piskin et al., 2022. Iron absorbed by intestinal enterocytes is then partially stored as ferritin, while the remainder is released via ferroportin to the basolateral intestine. This iron is then converted back to Fe^{3+}. The Fe^{3+} is then bound by apotransferrin to form transferrin and then distributed to all body tissues that require it (Rishi & Subramaniam, 2017).

The relationship between increased transferrin levels and increased hemoglobin levels as a component of RBC occurs when transferrin binds to ferric iron (Fe^{3+}), which then attaches to transferrin receptors (TfR1) on erythroblasts in the bone marrow. Ferric iron is released from transferrin due to the reduced pH in the erythroblast endosome, and it is subsequently reduced to ferrous iron (Fe^{2+}) by the enzyme Steap3. The iron exits the endosome, while transferrin is recycled for further iron transport. Mitoferrin 1, a receptor in the mitochondria, binds to ferrous iron (Fe^{2+}) brought by DMT-1 and transports it into the mitochondrial matrix. Within the matrix, ferrous iron (Fe^{2+}) is incorporated into protoporphyrin IX by the enzyme ferrochelatase, forming heme. This heme then binds to polypeptides to form hemoglobin. An increase in hemoglobin synthesis will impact the process of RBC formation (Rishi & Subramaniam, 2017).

The increase in the mean RBC levels was significantly different between KK2 and KP1-3 compared to KK1 (Table 4; Figure 2). Although there was no difference in the mean RBC levels between KK2 and KP, descriptively, the increase in RBC levels in KP was higher than in KK2, with the highest percentage increase in KP2, which was given the addition of NaFeEDTA iron fortificant at a dose of 5.4 mgFe/kgBW.

Iron fortificant dosage in KP3, which is higher than KP2, does not cause an increase in the average hemoglobin and RBC levels, but can lead to toxic effects. Excess hemoglobin can cause lipid peroxidation and the formation of reactive oxygen species (ROS), leading to cell damage and apoptosis (Sousa et al., 2020). The decrease in hemoglobin and RBC levels in KP3, with the addition of a higher NaFeEDTA dose than in KP2, is suspected to result from a negative feedback mechanism in hem synthesis, regulated by the heme itself. Heme inhibits the absorption of ferric iron (Fe^{3+}) from transferrin. This inhibition prevents the incorporation of iron into protoporphyrin IX by the enzyme ferrochelatase, thus preventing heme formation (Morciano et al., 2021; Ahmad et al., 2022)

After identifying hemoglobin and RBC levels during 21 days of treatment, hematocrit and blood platelet levels were then identified using the same rat. The difference in treatment time is 14 and 21 days based on the results of the pre-study using treatment periods of 7 days (t_7), 14 days (t_{14}), and 21 days (t_{21}). Changes in hemoglobin and RBC levels occurred on day 21 (t_{21}), while hematocrit and platelet levels occurred on day 14 (t_{14}). This is also in accordance with the results of research conducted by Italiano (2016); Hong et al. (2019); Chen et al. (2024) which stated that the formation of hemoglobin and RBC takes 3–8 weeks, while platelets are formed very quickly through megakaryocyte maturation for several days followed by proplatelet formation for several hours, while hematocrit in 2–3 weeks. (Italiano, 2016; He et al., 2019; Chen et al., 2024).

Hematocrit represents the volume of red blood cells (erythrocytes) after they have been separated from plasma, expressed as a percentage (El-Kady et al., 2025; Kalman et al., 2025; Ahmad et al., 2022; Basrowi & Dilantika, 2021). KP2 is the group that demonstrated the highest percentage increase in hematocrit levels compared to KK1, KK2, KP1, and KP3. These data correlate with the increase in hemoglobin and RBC levels that also occurred in KP2. It is hypothesized that the 5.4 mg Fe/kgBB dose of NaFeEDTA is the optimal dose that minimizes the inhibitory effects of phytic acid, allowing more iron from the soy milk to bind with EDTA. In KP2, EDTA not only chelates iron from the breakdown of NaFeEDTA but also chelates iron from the soy milk, thereby increasing the availability of iron in the blood necessary for hemoglobin synthesis. EDTA that is not excreted through urine is also believed to function maximally as part of the "shuttle effect" of EDTA, which chelates any remaining iron in the soy milk and facilitates its reabsorption by enterocytes, thus enhancing the effectiveness of iron absorption in the body. Sodium (Na) released from the NaFeEDTA complex will be used as one of the electrolytes in the blood, assisting in cell transport processes. This leads to more effective iron absorption in KP2 compared to the other treatment groups.

The normal platelet counts in rats range from 600–1500 ($\times 10^3/\mu L$) (Mourão et al., 2023). Therefore, although a decrease in platelet count was observed in all treatment groups, the platelet levels remained within the normal range. The decrease in platelet count in KK1, KK2, and KP1–3 is related to the administration of feed, soy milk, and NaFeEDTA, all of which contain iron. Although the relationship between iron and platelet formation is not yet known, it is suspected that Iron supplementation normalizes iron deficiency-induced thrombocytosis by inhibiting megakaryopoiesis (Brissot et al., 2021; Jimenez et al., 2021). The iron source for control group 1 (KK1) comes from the rat feed, which contains 2.3 mg of iron per 100 g from corn meal and 10 mg of iron per 100 g from soybean meal, while the KK2 iron source comes from both the feed and soy milk. Each 100 g of soy milk contains approximately 0.64 mg of iron. The treatment groups (KP) receive iron from the feed, soy milk, and NaFeEDTA, with doses of 2.7 mg Fe/kgBW for KP1, 5.4 mg Fe/kgBW for KP2, and 10.8 mg Fe/kgBW for KP3. Therefore, the increase in iron levels in the rats in the KP groups leads to a greater decrease in platelet count compared to KK1 and KK2, with the lowest percentage decrease observed in KP2.

The non-heme iron from the feed, soy milk, and NaFeEDTA is in the ferric (Fe^{3+}) form. EDTA, derived from the breakdown of NaFeEDTA, can prevent the formation of iron-phytate complexes in the feed and soy milk by forming Fe (III)-EDTA complexes. As a result, the availability of iron in the KP groups is higher than in KK1 and KK2. Ferric iron (Fe^{3+}) is converted into ferrous (Fe^{2+}) iron by the enzyme ferrireductase, making it available for absorption by enterocytes in the intestine. In addition to being essential for hemoglobin and erythrocyte synthesis, electron transport, forming Fe/S complexes, and protein formation, iron in the blood can also increase and inhibit megakaryocyte formation and prevent platelet aggregation, depending on the iron levels in the blood (Gupta et al., 2020; Kalman et al., 2025; Piskin et al., 2022)

A dose of 5.4 mg Fe/kgBW NaFeEDTA in KP2 resulted in the highest percentage decrease in platelet count compared to KP1 and KP3. This suggests that this dose is optimal for minimizing the binding of non-heme iron to phytate, which acts as an inhibitor of iron absorption from the rat diet and soy milk. This increases iron availability, which subsequently affects megakaryopoiesis.

Platelets are formed through a series of processes known as megakaryopoiesis. Megakaryopoiesis is a multistep process that begins with proliferation and leads to cytoplasmic maturation of megakaryocytes, which results in platelet formation. Increased iron levels are thought to cause a decrease in megakaryocyte synthesis, which is involved in platelet production, although the decrease in platelet levels remains within the normal range. While information explaining the mechanism by which iron affects platelet formation is limited, research data does indicate changes in platelet levels after administration of the NaFeEDTA iron fortificant.

CONCLUSION

Based on the results of the ANOVA and LSD tests ($P < 0.05$) and the percentage changes in hematocrit levels between t_0 and t_{14}, the addition of NaFeEDTA at doses of 2.7 mg Fe/kgBW, 5.4 mg Fe/kgBW, and 10.8 mg Fe/kgBW in KP1, KP2, and KP3 significantly increased hematocrit levels compared to KK1 and KK2. This suggests that NaFeEDTA has the potential fortificant for enhancing iron absorption. The optimal dose of NaFeEDTA, 5.4 mg Fe/kgBW (KP2), minimizes the binding of non-heme iron from soy milk with phytate, which inhibits iron absorption, thereby increasing iron availability for hemoglobin and red blood cell synthesis, key components in hematocrit levels. While the increase in iron levels was associated with a decrease in platelet count, the reduction remained within the normal range for rat platelet levels. Therefore, the findings of this study demonstrate the potential of NaFeEDTA as an iron absorption fortificant, and these results provide a basis for future research on the application of NaFeEDTA in soy milk fortification to improve hemoglobin and red blood cell levels,while maintaining platelet levels within the normal range.

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