Effects of NaFeEDTA in tofu flour on blood hemoglobin levels in male Sprague-Dawley rats (*Rattus norvegicus* L.)

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Abstract. This study examined the effects of the fortificant NaFeEDTA in tofu flour on blood hemoglobin levels in rats. Twenty-five rats were divided into five groups, namely a normal control group (NC) fed standard chow, water, and 0.5% carboxymethyl cellulose (CMC); a treatment control group (TC) given standard chow, water, 0.5% CMC and 0.45 g of tofu flour without NaFeEDTA; and three treatment groups (T1, T2, T3) that were given standard chow, water, 0.5% CMC, and 0.45 g of tofu flour containing 2.7, 5.4 or 10.8 mg Fe/kg bw of NaFeEDTA for 14 days. Hemoglobin levels were measured on days 0 and 4. One-way ANOVA illustrated that hemoglobin levels were higher in the treatment groups. A least significant different test revealed that blood hemoglobin levels in the treatment group were significantly different from those in the NC and TC groups, and those in the T2 group were also significantly different from those in groups T1 and T3.

Keywords: NaFeEDTA, tofu flour, hemoglobin levels, Sprague-Dawley rats

1. Introduction

Soy is a commonly consumed foodstuff in Indonesia. The average consumption of soy in Indonesia has reached 2.2 million tons/year. One of the most commonly consumed soy products in the country is tofu [1]. In addition to its high protein content, tofu is a source of iron, as each 100 g of tofu contains 2.2 mg of iron [2].

There are two different types of iron in foodstuffs, namely heme and nonheme iron [3]. Heme iron, which is in the ferrous (Fe²⁺) form, can be directly absorbed by the duodenum [4]. Meanwhile, nonheme iron, in the ferric form (Fe³⁺), must be reduced in the duodenal mucosa prior to absorption. However, nonheme iron tends to bind phytic acid, which is widely found in soy [3]. The bond between phytic acid and nonheme iron is strong and insoluble, hence explaining why nonheme iron cannot be absorbed [5]. Low iron absorption leads to decreased iron bioavailability.

Fortification is one strategy for increasing iron bioavailability [6]. The most effective fortificant for increasing blood iron levels is NaFeEDTA [7]. Increasing iron bioavailability is important because there...
is a significant relationship between iron intake and blood hemoglobin levels [8]. In the process of hemoglobin synthesis, iron is one of the major components of the heme cluster [9]. The intake of 0.45 g of tofu flour containing 5.4 mg Fe/kg bw of NaFeEDTA for 14 days can increase blood iron levels by as much as 53.18 % [10]. To determine the optimal concentration of the fortificant, this study examined the effects of three concentrations of NaFeEDTA, namely 2.7, 5.4, and 10.8 mg Fe/kg bw in 0.45 g of tofu flour, on hemoglobin levels in the rat *Rattus norvegicus*. This animal model was selected because it is easily maintained, easily adaptable, tolerant of the treatment, and shares a similar physiological response and characteristics with humans [11]. Based on our knowledge, this is the first study of the effects of an iron fortificant (NaFeEDTA) in tofu flour on rat hemoglobin levels.

2. Materials and method

2.1. Materials
The study included 25 male Sprague-Dawley rats (*Rattus norvegicus* L., Kemenkes RI, Jakarta, Indonesia) approximately 2-3 months in age and 180-200 g in weight. The animals were fed boiled water and food pellets obtained from CV PD Kasman, Sunter Jaya, North Jakarta, Indonesia. The chemicals used in the study were aquades, picric acid, carboxymethyl cellulose (CMC), 1.1 % vinegar, ether, and disinfectant.

2.2. Animal maintenance
The rats were equally divided into five cages and placed in a plastic tray. The rats were permitted to acclimatize to the environment for 14 days prior to experimentation. The rats were given access to food and water *ad libitum*, weighed daily, and observed for general signs. Animal cages were placed in the animal facility of the Faculty of Mathematic and Natural Sciences, Universitas Indonesia. A lamp tube was set up in the room to achieve 12 h/12 h light/dark cycles. Air circulation was assisted by the use of an exhaust fan, and room temperature was maintained at approximately 27 °C.

2.3. Production of tofu flour
Soybeans weighing 15 g were washed, soaked in water for 10-12 h, and filtered with 100 mL of water until they were smooth. The filtrate was boiled on a hot plate with stirring, and 1.1 % vinegar was added to the filtrate with slow stirring until tofu formed [10]. The tofu was further dried in an oven and blended into flour.

2.4. Addition of tofu flour
Tofu flour was divided into 0.45 g samples, then supplemented with NaFeEDTA. The amount of NaFeEDTA added to the tofu flour was determined using the following formula:

\[
\text{The amount of fortificant added (mg)} = \text{dose of Fe} \text{ in vivo} \times \frac{\text{Mr NaFeEDTA}}{\text{Ar Fe}}
\]  

(1)

where Mr is the relative molecular mass and Ar is the relative atomic mass [12].

2.5. Animal treatment
The rats were administered an oral treatment daily at 9:00-10:00 a.m. for 14 days. The treatment groups were as follows: normal control group (NC) given 0.5 % CMC; treatment control (TC) group given a suspension of 0.5 % CMC and 0.45 g of tofu flour without NaFeEDTA; treatment group 1 (T1), which was given a suspension of 0.5 % CMC and 0.45 g of tofu flour supplemented with 2.7 mg Fe/kg bw of NaFeEDTA; group treatment 2 (T2), which was given a suspension of 0.5 % CMC and 0.45 g of tofu flour supplemented with 5.4 mg Fe/kg bw of NaFeEDTA; and treatment group 3 (T3), which was given a suspension of 0.5 % CMC and 0.45 g of tofu flour supplemented with 10.8 mg Fe/kg bw of...
NaFeEDTA. The volume of the suspension administered was adjusted for the weight of the animals using the following formula [13]:

\[
\text{Oral treatment volume (mL)} = \frac{\text{weight of rats (g)}}{100 \text{ (g)}} \times 1 \text{ (mL)} \quad (2)
\]

2.6. Hemoglobin level measurement
Hemoglobin levels were measured before (t₀) and after treatment (t₁₄) using a hematology analyzer in the laboratory of Pusat Studi Satwa Primata, Bogor Agricultural University. Blood samples were obtained from rats via venipuncture and placed in 0.5-mL EDTA tubes. Blood samples were aspirated by the hematology analyzer, which was also used to complete a full blood hematology profile. The obtained data were presented on the analyzer’s screen.

2.7. Data analysis
The data were statistically analyzed using Statistical Product and Service Solutions program version 16. The normality and homogeneity of data were assessed using the Levene and Shapiro-Wilk tests. When the data were distributed normally and homogeneity was achieved, the analysis was continued via one-way ANOVA and a multiple comparison test (least significant difference) [14].

3. Results and discussion

3.1. Results

3.1.1. Initial hemoglobin levels (t₀). The mean hemoglobin levels in the five groups ranged 12.60–14.20 g/dL (table 1), in line with the normal range in white rats of 11.10–18.00 g/dL [15]. The results of ANOVA indicated that the initial hemoglobin levels in the five treatment groups were homogeneous. An initial homogeneous sample is needed to minimize errors in the final results of research [16].

3.1.2. Final hemoglobin levels (t₁₄). The final blood hemoglobin level in the NC group was 13.56 ± 0.32 g/dL (table 1), reflecting a 0.74 % increase over the initial level (t₀) (table 2). The increase in this group was attributable to the nutrient contents in the feed, including bran, tapioca, corn, groats, flour, fish, flour, meat, coconut oil, grass flour, coconut, and soy. These base materials can provide the components needed for hemoglobin synthesis, namely protein and iron [17]. Despite the increase, the hemoglobin levels at t₁₄ in the NC group remained in the normal range, permitting comparisons with the other groups. The final blood hemoglobin level in the TC group was 13.72 ± 0.33 g/dL (table 1), reflecting a 1.18 % increase versus the final level in the NC group (table 2). However, the statistical

| Repetition | Initial hemoglobin levels (t₀) (g/dL) | Final hemoglobin levels (t₁₄) (g/dL) |
|------------|---------------------------------------|-------------------------------------|
|            | NC T1 T2 T3                             | NC T1 T2 T3                          |
| 1          | 13.50 13.30 13.00 13.20                | 13.60 13.50 14.70 15.60              |
| 2          | 12.70 13.50 12.90 12.60                | 13.20 13.70 14.50 15.30              |
| 3          | 14.00 13.60 13.30 13.40                | 14.00 14.10 14.70 15.40              |
| 4          | 13.80 13.80 13.00 13.50                | 13.70 14.00 13.90 14.40              |
| 5          | 13.30 13.00 13.10 13.20                | 13.30 13.30 14.20 15.10              |
| Mean       | 13.46 13.42 13.52 13.18                | 13.56 13.72 14.40 15.16              |
| SD         | 0.50 0.32 0.54 0.24                    | 0.32 0.33 0.35 0.46                  |
Table 2. Percentage increase of hemoglobin levels at t14.

| Treatment | NC (%) | TCv (%) |
|-----------|--------|---------|
| TC        | 1.18   | -       |
| T1        | 6.19   | 4.96    |
| T2        | 11.80  | 10.50   |
| T3        | 7.67   | 6.41    |

analysis revealed that the levels were not significantly different between the NC and TC groups. This finding is possibly associated with the presence of phytic acid, which inhibits heme absorption [3]. The final hemoglobin levels in the T1, T2 and T3 groups were 14.40 ± 0.35, 15.16 ± 0.46, and 14.60 ± 0.49 g/dL, respectively (table 1). The statistical analysis revealed significant increases in hemoglobin levels in all three treatment groups compared to the findings in the NC and TC groups. The largest increase occurred on the T2 group (11.80 % versus the NC group and 10.50 % versus the TC group) (table 2). The increases in the treatment groups were apparently due to the presence of NaFeEDTA in tofu flour.

3.2. Discussion

In the stomach, phytic acid can bind the iron in tofu flour and form an insoluble salt complex that cannot be absorbed by the duodenum [3]. This results in decreased blood hemoglobin levels because of the lack of absorbable iron [4]. This finding illustrates that the consumption of tofu flour alone cannot significantly elevate blood hemoglobin levels.

NaFeEDTA has high affinity for iron (Fe³⁺), and can form the Fe (III)-EDTA complex. This complex formation protects Fe³⁺ against binding by phytic acid present in soybeans [3]. Meanwhile, the iron absorbed by the intestines was derived from tofu flour opposed to NaFeEDTA [10]. NaFeEDTA has the ability to bind iron from food in the gastrointestinal lumen and transport it to enterocytes in the duodenum via a process termed the shuttle effect [18].

Previous research illustrated that 14 days of intake of 5.4 mg/ kg bw of NaFeEDTA in 0.45 g of tofu flour can increase blood iron levels by as much as 53.18 % [10]. Increased iron content in blood plasma can lead to significantly higher blood hemoglobin levels. The findings of the present study were in accordance with research conducted by Cendani and Murbawani, who found a positive correlation between iron intake and hemoglobin levels [8]. Meanwhile, iron is required for hemoglobin synthesis as a core component of heme [9]. Heme is composed of a porphyrin ring complex with ferrous ion (Fe²⁺) in the center of the ring (figure 1) [19].

Heme synthesis, which occurs in mitochondria, starts with the formation of aminolevulinic acid (ALA) induced by ALA synthase [9]. Synthesis continues with various reactions and molecular changes to form protoporphyrin IX [20]. Protoporphyrin IX requires iron to form heme; therefore, the final stage of heme formation is controlled by the availability of iron in cells [9].

Iron used to synthesize heme is bound by transferrin receptors in erythroid cells in the form of diferric transferrin and transported to the plasma via endocytosis (figure 1) [21]. Furthermore, iron is transported out of endosomes to mitochondria. In mitochondria, iron is complexed with protoporphyrin IX by ferrochelatase, thus permitting the heme cluster to form (figure 2) [22]. The synthesis of hemoglobin then continues to the process of configuration between the heme cluster and globin [20].

During hemoglobin synthesis, iron acts as a constituent component while also playing an active role in regulating the process itself [17]. Iron levels in cells affect the function of iron regulatory proteins (IRPs), which regulate the synthesis of transferrin receptors and ALA synthase [22]. If cellular iron levels are low, then IRPs bind to iron response elements (IREs) in ALA synthase and transferrin receptor mRNA. IRPs block the translation of ALA synthase mRNA [22].

ALA synthase catalyzes the condensation of glycine and succinyl Co-A during ALA synthesis in the early stages of heme cluster formation. If ALA synthase is not available, then heme synthesis is
disturbed [23]. Meanwhile, IRPs can bind to IREs in transferrin receptor mRNA, thus preventing the degradation of transferrin. This consequently decreases iron intake by erythroid cells and increases the supply of iron from transferrin (figure 2) [22].

As previously mentioned, the increase of hemoglobin levels was smaller in groups T1 and T3 than in T2 (table 1). The NaFeEDTA concentration of 2.7 mg/kg bw appears insufficient for maximizing the absorption of iron from tofu flour, resulting in reduced iron bioavailability. Meanwhile, the reduced hemoglobin levels in the T3 group might be attributable to a negative feedback mechanism activated by excessive iron levels, given that heme synthesis appeared to be maximized at the concentration of 5.4 mg/kg bw [17]. The negative feedback mechanism in erythroid cells prevents the production of transferrin receptors. If iron levels in cells are high, then IRPs release transferrin receptor mRNA, preventing the production of the receptors. Due to the absence of transferrin receptors, iron cannot enter cells (figure 2) [22]. In addition, if there is any excess heme in erythroid cells, heme degradation is activated by heme oxygenase [24], which transforms iron into biliverdin IX α. Furthermore, biliverdin IX α is converted to bilirubin by biliverdin reductase α IX and excreted [25].
4. Conclusion
Supplementation with tofu flour containing NaFeEDTA for 14 days increased blood hemoglobin levels in male rats. The largest increase of 15.15% was noted in the T2 group, which reflected increases of 11.8% versus the NC group and 10.5% versus the TC group.

Acknowledgments
This work was financially supported by Universitas Indonesia under research grant PUPT Batch 2 (Penelitian Unggulan Perguruan Tinggi).

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