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Tempeh with Iron Fortification to Overcome Iron Deficiency Anemia

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Abstract: Prevalence of iron deficiency anemia in Indonesia is still high. Iron fortification in food may contribute to addressing iron deficiency anemia. This study was conducted to determine the effect of NaFeEDTA fortification on iron levels in tempeh and some organoleptic properties of fortified tempeh (TemFe), as well as to determine the effect of TemFe in hemoglobin levels and the bioavailability of iron on Wistar rats anemia. This study is divided into two phases, so there are two research design. The first phase is purely experimental with completely randomized design. The second phase is quasi experimental with pre and post control group design. Fortification of tempeh with NaFeEDTA significantly increased the iron levels in tempeh ($p<0.05$). The organoleptic test showed that there is no significantly difference either tempeh nor TemFe ($p>0.05$). There are significantly differences in hemoglobin levels in tempeh and TemFe administration at a dose of 24 ppm compared with $FeSO_4$ with same dose ($p<0.05$). The relative bioavailability (RBV) value for TemFe and tempeh was 100.10 and 33.65%, respectively. The fortification of NaFeEDTA in tempeh can increase iron levels and did not alter the organoleptic properties. There is a significant increase of hemoglobin levels in anemic groups of Wistar rats that received TemFe with dose of 24 ppm. The bioavailability due to iron fortification in TemFe is higher than that of the tempeh and $FeSO_4$ as a gold standard.

Key words: Iron deficiency anemia, Fe fortification, NaFeEDTA, tempeh

INTRODUCTION

Prevalence of Iron Deficiency Anemia (IDA) in Indonesia is still in a high rate. Basic Health Research (known as *Riset Kesehatan Dasar*) in 2007 showed that 60% of iron deficiency anemia occurred in women in child bearing age and 59% occurred in pregnant women. The high incidence of anemia in Indonesia shows that a low intake of iron or low iron bioavailability, and is unable to meet increased of iron requirements (WHO, 1992; Cusick *et al*., 2008; de Benois *et al*., 2008).

IDA may increase risk of death and morbidity in pregnant women, low birth weight, infection in children, low level of IQ and school activities of students, causes physical and mental damage in children and also decrease the productivity of adults (Lozoff, 2000; Haas and Brownline, 2001; Angulo-Kinzler *et al*., 2002; de Benois *et al*., 2008). In addition, IDA also causes loss of healthy life stated in Disability-Adjusted Life Years (DALY). It is estimated that IDA contribute to 2.4% from world total DALY or 25 millions (Allen *et al*., 2006). Over the years, the intervention program of IDA in women of child bearing age, pregnant women and children in Indonesia, is done through iron supplementation. Besides a positive effect, sometimes supplementation have a negative effect on the digestive tract such as nausea, pain, vomiting, constipation and diarrhea (Sulastri *et al*., 1999).

The other efforts which are effective and cheap (cost effective) to treat IDA are iron fortification in food. One of the substances that are proven effective for fortified food is Sodium ferric ethylenediaminetetraacetic acid (NaFeEDTA). NaFeEDTA is selected as fortificant agent because the substance has a 2-4 times better absorption compared with ferrous sulphate, (Hurrell *et al*., 2000) and does not cause oxidation in the product during storage (Hurrell, 1997). Several studies also showed that food fortification using NaFeEDTA is effectively able to reduce the incidence of anemia (Huo *et al*., 2000; Chen *et al*., 2005; Thuy *et al*., 2005; Andang'o *et al*., 2007; Sun *et al*., 2007). In addition, WHO and FAO also recommend NaFeEDTA as an iron source in food (Allen *et al*., 2006). However, iron fortification in foodstuffs faces some problems, namely: iron is poorly soluble in water, iron changes the color and taste of the fortified foods and some iron absorption inhibitors in foods such as phytic acid, phenol and soy protein can be present (Hurrell, 1997). One of the foods that have been made for iron fortification program is wheat flour (Allen *et al*., 2006). Unfortunately, this program encountered some problems because not all Indonesian people are familiar with the consumption of flour-based foods such as bread and noodles.

Tempeh (soybean) is selected as foodstuffs fortified with iron because tempeh is an authentic Indonesian food

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and one of the favorite foods in Indonesia. Tempeh is made through a fermentation process using the fungus *Rhizopus sp* assistance (*Rhyzopus oryzae* or *Rhyzopus oligosporus*) or a mixture of fungi in the form of tempeh (Handjani, 2001). Tempeh is easily accessible by people both in rural and urban areas. In addition, tempeh is a good source of protein foods widely consumed by the people of Indonesia. Result of National Socio-Economic Survey 2008-2012, showed that the average per capita consumption of tempeh has increased from 6.935 kg up to 7.300 kg.

A study showed that the fermentation process in soybean could reduce the levels of phytic acid in soy as high as of 65% that are expected to prevent IDA (Van der Riet, 1987). The purpose of this study was to determine the effect of NaFeEDTA fortification on the levels of iron and organoleptic properties of tempeh, to determine the level of hemoglobin in anemic female Wistar rats and to measure the bioavailability of iron in tempeh compared to the fortified tempeh (called TemFe) with $FeSO_4$ in experimental animals.

MATERIALS AND METHODS

Soybeans, yeast, NaFeEDTA (Ferrazone from Akzo Nobel Chemical b.v, Amhem, Netherland), nitric acid, standard feed (AIN 93), $FeSO_4$, hemoglobin (Hb) kit from Diagnostic System (Diasys) GmbH kit from Germany.

Production of fortified tempeh (TemFe): The production of Tempeh used traditional method. Soy from the local market was washed thoroughly in order to remove dirt present on soybeans. Then, soybean is soaked for 1 night (24 h) and heated for 30 min. After heating 30 min, then soybean husk was peeled and dried. During the drying process, NaFeEDTA (Ferrazone from Akzo Nobel Chemical b.v, Arnhem, Netherland) was added to the soybeans at a dose of 28 mg/kg for the group-28, 56 mg/kg to groups of 56 and 112 mg/kg for group-112. This mixture was stirred until spread evenly and subsequently coupled with yeast fermented at 32°C for 16-32 h. Tempeh is declared successful when fungal mycelia grow in soybean and when pressed, the mixture is not crush.

Analysis of Iron content in tempeh: The iron content in tempeh was determined using atomic absorbtion spechtrophotometer (AAS) according to the method reported by Hernandez *et al.* (2004). In general, total of 5 g of sample was accurately weighed and ashed by heating. Then the ash was dissolved with 3 mL of concentrated nitric acid and diluted to a volume of 25 mL in the AAS instrument. The test solution was determined using AAS, based on calibration curve of iron standard (Sigma, USA) prepared previously.

Organoleptic test: A total of 48 expert panelists were recruited to determine the organoleptic properties of tempeh fortified with NaFeEDTA (TemFe) at a dose of 28, 56 and 112 mg/kg. Panelists did the organoleptic test in a room and observed the tempeh that has been presented. After trying and observing, panelists subsequently provide an assessment on the questionnaire that has been provided previously.

Animal experimental study: The animals used (weight of 160-180 g) of female Wistar rats aged 2 months were obtained from the Inter-University Gadjah Mada (known as *Pusat Antar Universitas* (PAU)-UGM). Rats were adapted for 3 days before treatment through standard feeding AIN 93 and taken for drinking in *ad libitum*. Rats were caged individually in stainless stell cage under the controlled room temperature and 12 h of lighting for 4 weeks.

Depletion phase: A total of 50 rats were given a low iron diet by removing of Fe in feed that has been modified from AIN 93 (Table 1) for 10 days in order to induce IDA. Rats with hemoglobin levels of <6 mg/dL were used in this study.

Repletion phase: After 10 days of induction, the rats were divided into 10 groups consisting of a normal group and three treatment groups. Each treatment group will receive tempeh, TemFe and $FeSO_4$ at a dose of 6, 12 and 24 ppm.Tempeh, TemFe and $FeSO_4$ were given using enteral method to the experimental animals. Each animal will receive treatment according to each dose group, i.e. 6, 12 and 24 ppm of iron, respectively.

The treatment was given for 17 days. The weight and residual feed were weighed every day. Blood was taken at day 10 after the induction of anemia as a pre test and Spechtrophotometer (AAS) according to the method

Table 1: Research diet composition per 100 g (Martino *et al.*, 2011)

	------------------$FeSO_4$----------------			----------------Tempeh------------			-------------TemFe-------------		
Computation	6	12	24	6	12	24	6	12	24
Casein	20	20	20	20	20	20	20	20	20
Cornstarch	39.8	39.8	39.8	39.8	39.8	39.8	39.8	39.8	39.8
Sucrose	10	10	10	10	10	10	10	10	10
Cellulose	5	5	5	5	5	5	5	5	5
Soy oil	7	7	7	7	7	7	7	7	7
Mineral mix (Fe free)	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Vitamin mix	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
L-cysteine	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Choline bitartrate	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25

after 17 day of treatment administration as a post test. Blood was drawn from the sinus orbitalis for hemoglobin analysis.

Hemoglobin analysis: Hemoglobin analysis was performed using the photometric method by using Diagnostic System (Diasys) GmbH kits (Diasys, Germany). Hemoglobin analysis was adjusted with protocol that printed in the kits.

Statistical analysis: Iron measurement data and respondents acceptability were expressed as Mean±SEM (Standard Error of Mean). To determine whether there are differences in iron levels in tempeh, hemoglobin levels and RBV values, in each group, One Way ANOVA test was done. The organoleptic tests were analyzed using the Kruskal-Wallis.

RESULTS

Iron content in tempeh: The analytical results on iron levels in tempeh is presented in Table 2. The analysis shows that TemFe has significantly different in iron levels compared with normal tempeh. TemFe tend to have higher iron levels, especially tempeh fortified with NaFeEDTA at a dose of 112 mg.

Organoleptic test: Kruskal-Wallis analysis results for organoleptic test showed that there were no significant differences among treatment groups in the color ($p = 0.331$), flavor ($p = 0.079$), taste (0.438) and texture (0.417). This suggests that the three-dose fortification does not provide a big impact to the change of color, flavor, taste and texture of the tempeh fortified (TemFe).

Blood hemoglobin levels: Table 3 shows the effect of tempeh and TemFe to the hemoglobin levels after 17 days of treatment. Highest levels of hemoglobin found in the normal control group (12.04±0.09). Group receiving 24 ppm TemFe have hemoglobin levels which are not statistically different between 24 ppm $FeSO_4$ group and normal control. However, the group receiving 24 ppm $FeSO_4$ showed different results with normal control group. These results indicate that TemFe is very effective in increasing hemoglobin levels in Wistar rats anemia.

Iron bioavailability of TemFe: Table 4 shows linear equation for hemoglobin levels by doses of 6, 12 and 24 ppm among treatment materials. Relative bioavailability value (RBV) is calculated by comparing the level of the slope (gradient) between groups with tempeh or TemFe with degree slope (gradient) $FeSO_4$. The bioavailability of iron on TemFe can be calculated by using the ratio of the gradient line graph linear equations of $FeSO_4$ as the gold standard. The linear equation in

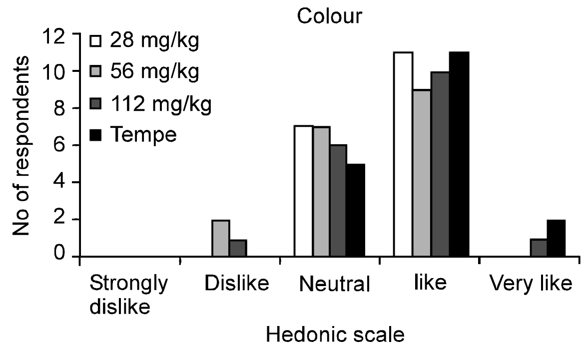

Fig. 1: Organoleptic test results for tempeh and TemFe with various levels of iron concentration on color

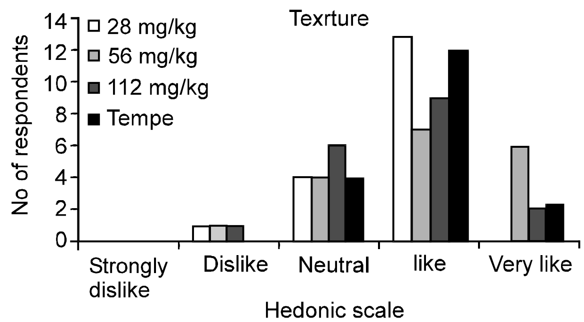

Fig. 2: Organoleptic test results for tempeh and TemFe with various levels of iron concentration on taste

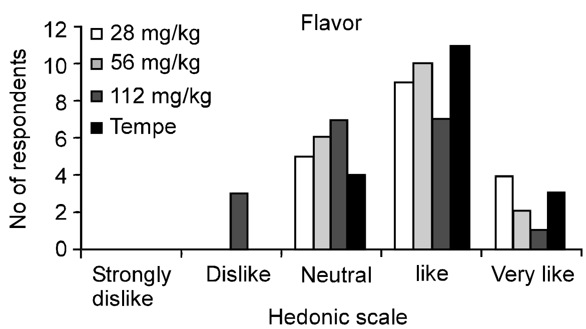

Fig. 3: Organoleptic test results for tempeh and TemFe with various levels of iron concentration on taste

$FeSO_4$ is $Y = 1.896X+5.037$, (Y and X are the hemoglobin levels of iron in the diet dose). While, the linear equation for ordinary tempeh is $Y = 0.638+6.248X$ and TemFe is $Y = 1.898X+5.671$. From these equations and comparing the value of the gradient line, it is known that the bioavailability of tempeh has low value, i.e., 33.65%. While, TemFe has the bioavailability equivalent value equal to 100.10% $FeSO_4$.

DISCUSSION

Iron content of tempeh and TemFe: The test results of iron levels in tempeh and TemFe showed a significant difference ($p<0.05$). The higher dose fortification

Table 2: Analytical results iron levels in soybean*

Variable	Group 1 Tempeh	Group 2 TemFe 28 mg	Group 3 TemFe 56 mg	Group 4 TemFe 112 mg	p
Iron	56.944±0.291[a]	60.472±0.467[b]	75.518±1.895[c]	79.595±0.491[d]	0.000

*Data are expressed in Mean±SEM (Standard Error of Mean). [abc]Different notation indicates p<0.05

Table 3: Effect of TemFe on hemoglobin levels *

Groups	Intervention Duration: Pre Test (10 days)	Post Test (17 days)
Normal control	12.23±0.08[a]	12.04±0.09[a]
$FeSO_4$		
6 ppm	5.82±0.03[b]	7.19±0.07[bc]
12 ppm	5.89±0.07[b]	8.30±0.19[de]
24 ppm	5.78±0.08[b]	10.99±0.20[f]
Tempeh		
6 ppm	5.82±0.07[b]	6.81±0.11[b]
12 ppm	5.81±0.09[b]	7.68±0.18[cd]
24 ppm	5.87±0.09[b]	8.08±0.09[de]
TemFe		
6 ppm	5.84±0.08[b]	7.88±0.24[cd]
12 ppm	5.70±0.11[b]	8.83±0.17[e]
24 ppm	5.84±0.07[b]	11.68±0.21[af]
P	0	0

*Data are expressed in Mean±SEM (Standard Error of Mean)
[abc]Different notation indicates p<0.05 by Tukey-HSD test

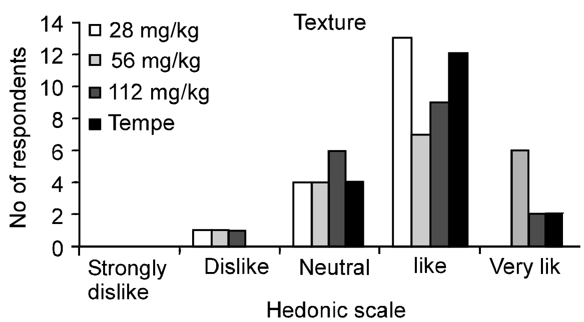

Fig. 4: Organoleptic test results for tempeh and TemFe with various levels of iron concentration on texture

given in tempeh, the higher levels of iron contained in the tempeh. According to Akhtar *et al.* (2008), NaFeEDTA is a material that is not sensitive to iron inhibiting substances in foods like phytic acid, because NaFeEDTA has the ability to maintain Fe, therefore, it does not bind to phytic acid present in cereals or legumes (SandstrÖm and Walter, 1998). In addition, NaFeEDTA is classified as substances that stable during processing and storage (Navarrete *et al.*, 2002).

Organoleptic of tempeh dan TemFe: On the 1st organoleptic test round, 30 respondents participated. However, the test did not passed because, on the dose of 224 mg/kg of tempeh, respondents felt dry in the throat and on the tongue, as a consequence, they were excluded from the study. There was also a difference in tempeh weight given to each respondent so that the organoleptic test was repeated using 3 doses, namely 28, 56 and 112 mg/kg of tempeh.

The Second organoleptic test was carried out by forming the tempeh into the same shape with the help of the cookie cutter. The cookie cutter equipment used is in the form of the star-shaped. The average tempeh has an average weight of 5 g with similar form among groups. This star-shaped tempeh is subsequently fried in oil for 5 min that has been previously heated. Tempeh was fried without flour to minimize research bias. Tempeh which lightly browned is then drained and put in plastic before being tested to respondents. The second organoleptic tests were performed in the Laboratory of Health and Nutrition, the Faculty of Medicine UGM with student as respondents. A total of 18 students were successfully recruited and did organoleptic test phase 2. Students were asked to give an assessment of the color, flavor, taste and texture of a variety of tempeh.

Organoleptic test results to the tempeh and TemFe revealed that there is no significant difference (p>0.05). This suggests that the three-dose NaFeEDTA fortification does not provide a significant difference to the taste, flavor, color and texture of TemFe.

NaFeEDTA is one of the fortificant substances having little organoleptic problems compared to other water-soluble iron compounds (Navarrete *et al.*, 2002). Anderson and Gordon (2012) also reported that NaFeEDTA has good organoleptic acceptance, has good bioavailability and is soluble in water. The study conducted by Fidler *et al.* (2003) showed that NaFeEDTA fortification in fish sauce and soy sauce was acceptable organoleptic, which indicates that these substances can be used as a good fortificant.

Blood hemoglobin levels: Low-iron diet for 10 days in experimental animals can significantly reduce the levels of hemoglobin (p<0.05). Result of this study revealed that 24 ppm TemFe have effectiveness in increasing hemoglobin levels compared with the gold standard ($FeSO_4$). This is according to research conducted by Huo *et al.* (2000) which give NaFeEDTA fortified soy sauce in anemic school children. The results showed that there is a significant increase in hemoglobin levels after intervention of soy sauce fortified with NaFeEDTA for 3 months. Based on these studies, it is known that NaFeEDTA could be used as fortificant compounds for foods containing high amounts of phytate. NaFeEDTA has the ability to keep the iron from binding to phytic acid contained in foods, especially in cereals and legumes.

Table 4: Linear equations hemoglobin levels by dose Iron (6, 12 and 24 ppm) amongst treatment materials*)

Fortification Material	Linear Equations	R^2	RBV (%)
$FeSO_4$ (gold standard)	Y=1.896.X+5.037	0.945	100
Tempeh	Y=0.638.X+6.248	0.955	33.65
TemFe	Y=1.898.X+5.671	0.924	100.10

*)Linear equation is Y = concentration of hemoglobin and X = iron doses

Food containing high amounts of phytate is then fortified with NaFeEDTA, will make these food 2-4 times better absorbed than other fortificant (Hurrell *et al.*, 2000).

Bioavailability is the proportion of a nutrient in food which is absorbed and utilized (O'Dell, 1984). The calculation of bioavailability is performed by measuring the absorption, the body storage, or appropriate response parameters, such as concentration in the tissue, bone mass, enzyme activity, etc., (Miller, 1989). In this study, the measurement of iron bioavailability in experimental animals use RBV methods. According to Forbes *et al.* (1989), RBV method is the recommended method for testing the bioavailability of tested substances using experimental animals when compared with hemoglobin regeneration efficiency (HRE).

The results showed that tempeh fortified with NaFeEDTA has higher value of bioavailability compared with the gold standard $FeSO_4$. NaFeEDTA is one of the water-soluble compounds widely used as fortificant in some foods. Generally, the water-soluble compounds have high bioavailability, thus NaFeEDTA fortificant is recommended as a substance that can be used to fortify foods such as soy sauce, fish sauce, flour, sugar and foods containing large amounts of phytate (Allen *et al.*, 2006).

Conclusion: Based on this study, it can be concluded that NaFeEDTA fortification in tempeh at a dose of 112 ppm can increase the iron levels in tempeh. NaFeEDTA fortification in tempeh with three dose levels (24, 56 and 112 ppm) did not alter the organoleptic properties of tempeh. There is a significant increase in hemoglobin levels in the group of female Wistar rats given with TemFe at a dose of 24 ppm. The bioavailability of iron fortification in TemFe is higher compared with tempeh only and $FeSO_4$ as the gold standard.

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