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Combined Inorganic Iron ($FeSO_4$) and Steamed Pumpkin (*Cucurbita moschata*) Fortification in Fresh Noodles Can Increase Hemoglobin Levels in Iron Deficient Mice

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Abstract: Noodles are important and popular diet in and outside Asian countries and therefore it is a potential iron carrier food which can provide wide geographical acceptance and coverage. Noodles which had been fortified with inorganic or organic iron only or combined with steamed pumpkin were fed to mice to study its ability to increase Hb level. Thirty 3 weeks old Balb/c mice (*Mus musculus*) with body weight of 22.9-24.8 g were allocated randomly into five treatment groups: (1) control unfortified wet noodles (unfortified), (2) inorganic iron fortified noodles ($FeSO_4$), (3) organic iron fortified noodles (Bioplex), (4) combined inorganic iron-steam pumpkin fortified noodles ($FeSO_4$+P), (5) combined organic iron-steam pumpkin fortified noodles (Bioplex+P). Each group consisted of six replicate mice. Iron depletion period by feeding unfortified noodles was performed for 14 days followed by treatment noodles for 14 days. The results showed that fortified noodles could increase serum iron levels ($p = 0.0001$) and transferrin saturation ($p = 0.0001$) in all groups. However, it could increase Hb levels ($p = 0.0001$) only in fortified but not unfortified groups. Body weight ($p = 0.965$), liver weight ($p = 0.086$) and liver iron levels were not affected by the treatments. Our studies demonstrated that noodles which have been fortified with combined inorganic iron and steamed pumpkin can serve as food carriers to intervene iron deficiency anemia in mice.

Key words: $FeSO_4$, pumpkin, fresh noodle, hemoglobin, anemia

INTRODUCTION

Anemia is a global health problem that afflicts 1.6 billion people with the greatest contribution factor is iron deficiency anemia. The most affected population group of 468.4 million are non pregnant woman, while 315 million of pregnant and non pregnant woman and preschool-age children is found in South East-Asia (de Benoist *et al*., 2008). In Indonesia, the prevalence of anemia in school-age children, puberty and adult are 28.1, 18.4 and 16.9%, respectively (Indonesian Ministry of Health, 2013). This global health problem must be prevented or corrected because iron deficiency anemia can lead to stunted growth, impaired mental development, poor school performance, reduced productivity, increased morbidity and mortality and lower self-esteem (Grantham-McGregor and Ani, 2001; Batra and Sood, 2005).

Iron food fortification has been recommended as one of the preferred approaches for preventing and eradicating iron deficiency (Baltussen *et al*., 2004). However, successful iron fortification depends on several factors i.e., the types of food carrier and the bioavailability of iron fortificants (Hurrel, 2002; Mehansho, 2006). Food carrier should also be acceptably and widely consumed to enable wide coverage and efficient delivery. Noodles are part of important and popular Asian diet including Indonesian people and also gaining popularity in countries outside Asia (Hou, 2010). Noodles also become food choice in time of economic crisis or emergency. Ease in preparation or cooking as well as its sensory acceptance have made noodles a potential iron carriers for vulnerable groups of iron deficiency anemia.

The bioavailability of iron fortificants are affected by the forms of iron fortificants i.e., organic and inorganic iron. Organic iron is iron that linked to organic ligand such as amino acid mixture or peptides. Some studies showed that organic iron bioavailability was better than inorganic iron (Murwani, 2008). However, the bioavailability of inorganic iron fortificant can be enhanced by combination with another nutrients such as vitamin A or the provitamin A (beta-carotene) (Domke *et al*., 2005). Enhanced inorganic iron bioavailability was thought to be due to iron protection from inhibitors during intestinal absorption (Layrisse *et al*., 1997; Garcia-Casal *et al*., 1998). Pumpkin (*Cucurbita moschata*) is a potential source of naturally occurring provitamin A as well as other micronutrients such as vitamin B and microminerals. It is locally available and well grown vegetable in many areas and therefore can be used in

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combination with inorganic iron fortificant to enhance its bioavailability. We hypothesize that combined inorganic iron-pumpkin fortified noodles can prevent iron deficiency in laboratory mice (*Mus musculus*) which would be reflected by an increase of anemic Hb level into normal level. We hope the fortified noodle can serve as an effective food carrier to intervene iron deficiency anemia in vulnerable groups.

MATERIALS AND METHODS

Materials: Inorganic iron (20% Fe, Sigma USA), Bioplex organic iron (10.9% Fe, donated by Alltech, Indonesia), pumpkins, table salt and chicken eggs were obtained from local market, wheat flour (Cakra Twin, PT Indofood Sukses Makmur), drinking water and distilled water (devoid of iron and other elements).

Animals: Thirty, 3 weeks old all female Balb/c mice (*Mus musculus*) with body weight of 22.9-24.8 g. Animals experiments for this study was carried out following the principal stated in National Guides of Health Research Ethics-Ministry of Health RI 2007 and had been approved by Ethic Commission for Health Research-Faculty of Public Health, Diponegoro University with Ethical Clearance No.200/EC/FKM/2013.

Noodles: All materials used to make the wet noodles are from the same batch which was prepared prior to *in vivo* mice study and has been optimized in a separate experiment (Rustanti *et al*., 2011). Wet noodles were made from wheat flour, salt, egg, 200 ppm iron fortificant (inorganic or organic), 15% steamed pumpkin, fresh chicken egg and drinking water. The noodles were cooked in a boiling water for 2 min, drained and oiled. The resulting wet noodles were sampled for nutrient content analyses and the rest were stored in the fridge for feeding. The nutrient content of treatment noodles were given in Table 1.

Animals and treatment noodles: Thirty, 3 weeks old all female Balb/c mice (*Mus musculus*) with body weight of 22.9-24.8 g were allocated randomly into five treatment groups: (1) control unfortified wet noodles (unfortified), (2) inorganic iron fortified noodles ($FeSO_4$), (3) organic iron fortified noodles (Bioplex), (4) combined inorganic iron-steamed pumpkin fortified noodles ($FeSO_4$+Pumpkin), (5) combined organic iron-steamed pumpkin fortified noodles (Bioplex+Pumpkin). Each group consisted of six replicate mice and placed in a plastic housing (length: 38.4 x wide: 27.3 cm, height: 13.5 cm) with air ventilated top which can be used for climbing, wood shaved bedding, water and feed. The animal housing was placed in a 25-27°C room temperature, with 12 h light and dark lighting. Preliminary trials with *ad libitum* access to wet noodles showed that each animal consumed 5 g/day. Based on this feed intake, noodles were given 5 g/mouse/day. Distilled water (free of iron) was given as drinking water in free access. Iron depletion period was performed for 14 days by feeding unfortified noodles. At the end of depletion period, one mouse of each treatment group was sacrificed for blood and liver samples to determine initial iron status. Following the end of iron depletion, mice were fed treatment noodles for 14 days. Fortified noodles were fed first to provide an equivalent of 1.67 mg iron/kg body weight/day to meet iron requirement for each mouse (Rucker and Storms, 2002). After the fortified noodles were all consumed, the unfortified noodles were fed to meet total noodles intake of each animal. Fed noodles were well consumed and there was no remain. Total iron intake of each mouse of iron-fortified group was the same, except for control group (Table 2). Total intake of treatment noodles of each mouse was adjusted to an increase in body weight of each animal every two days, when body weight was measured (Table 3). At the end of treatments, blood samples were taken from each replicate mice for determination of serum iron, total iron binding capacity (TIBC), transferrin saturation (%) and Hb levels. After blood sampling, mice were sacrificed for determination of liver weight and iron levels. Animal experiments were summarized in Fig. 1.

Determination of hemoglobin levels: In principle blood was diluted with HCl solution so that the hemoglobin changed to acid hematin. It was further diluted with ddH_2O until the color was the same as the standard color. Haemoglobin levels was read on the scale.

Determination of serum iron (CAB/Chromazurol B method): Serum iron was determined by the CAB method with the reagent kit no. 122 992 (PT Rajawali Nusindo). In principle, Fe(III) will react with chromazurol B (CAB) and cetyltrimethylammonium bromide (CTMA) to form a colored complex which can be read in a spectrophotometer at a wavelength of 623 nm. Serum iron level was calculated according the following formula:

$$\text{Serum iron level} = \text{A sample}/\text{A standard} \times 100\ (\mu g/dL)$$

Determination of total iron binding capacity (TIBC): TIBC are proteins that bind iron-transferrin in serum. They were mixed with saturated Fe (III) iron. Excess iron or un-bound iron was absorbed by aluminum oxide. Thus TIBC levels in the supernatant can be determined. TIBC levels were determined as iron levels in supernatant.

Statistical analysis: This experiment was performed as a single factor experiment (iron fortificant) and completely randomized design with 6 replicates. Data

Table 1: Nutrient contents of fortified noodles

Treatment groups	Moisture (%)	Carbohydrate (%)	Protein (%)	Fat (%)	Ash (%)	Fibre (%)	Fe (mg/kg)	beta-carotene (mg/kg)
Unfortified	62.86±0.95	26.37±0.93	6.83±0.29	3.54±0.16	0.40 ± 0.03^{bc}	0.68±0.24	14.09 ± 2.03^{c}	26.20 ± 0.20^{b}
$FeSO_4$	64.26±1.86	24.86±2.22	6.95±0.38	3.55±0.30	0.39 ± 0.04^{c}	0.50±0.06	38.10 ± 2.74^{a}	26.05 ± 0.33^{b}
Bioplex	61.74±1.45	27.08±1.60	7.09±0.26	3.68±0.25	0.42 ± 0.01^{bc}	0.72±0.18	29.26 ± 2.82^{b}	27.28 ± 0.98^{b}
$FeSO_4$+Pumpkin	63.66±0.80	25.77±1.04	6.54±0.33	3.59±0.26	0.44 ± 0.01^{ab}	0.53±0.03	41.08 ± 2.58^{a}	34.18 ± 4.35^{a}
Bioplex+Pumpkin	62.14±3.23	27.11±3.61	6.66±0.29	3.63±0.22	0.47 ± 0.04^{a}	0.56±0.05	30.04 ± 3.30^{b}	35.65 ± 4.70^{a}

Table 2: Iron and beta-carotene content of unfortified noodles for 14 days iron depletion period dan total intake of iron and beta-carotene of each mouse per day in each treatment groups

Treatment groups	Iron content of unfortified noodles fed for iron depletion period (mg/kg)	beta-carotene content of unfortified noodles (mg/kg)	Total noodles intake (mouse/day) (g)	Total iron intake (mouse/day) (mg/kg)	Total beta-carotene intake (mouse/day) (mg/kg)
Unfortified	14.09	26.2	5.00	0.07	0.13
$FeSO_4$	14.09	26.2	5.00	0.07	0.13
Bioplex	14.09	26.2	5.00	0.07	0.13
$FeSO_4$+Pumpkin	14.09	26.2	5.00	0.07	0.13
Bioplex+Pumpkin	14.09	26.2	5.00	0.07	0.13

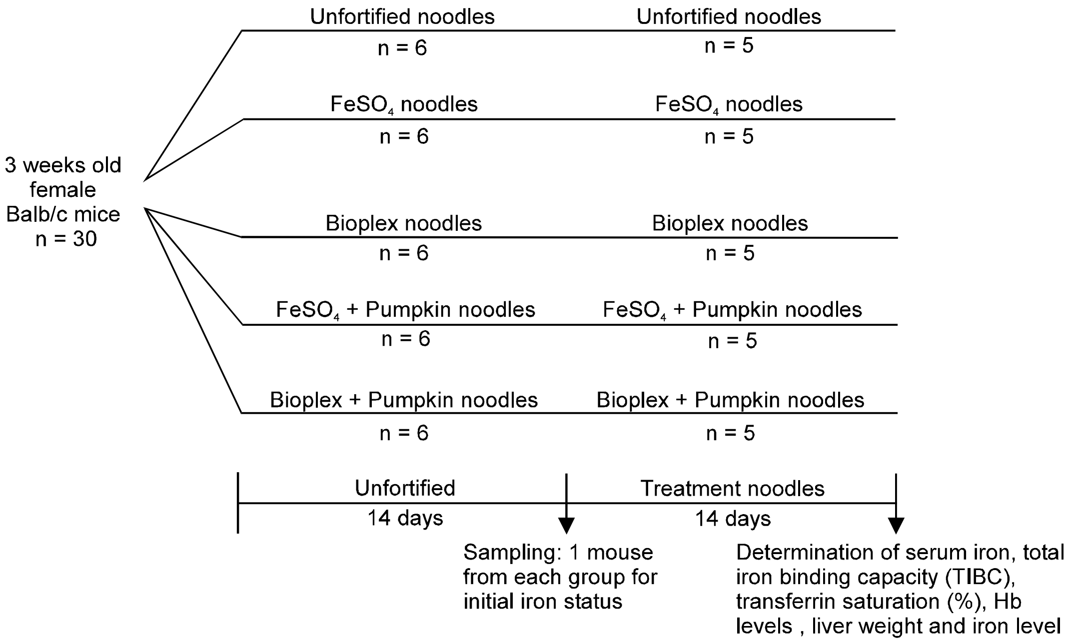

Fig. 1: Summary of iron-fortified noodles experiment in Balb/c mice

were analyzed by Analyses of Variance (ANOVA). When there was a significant effect of treatment, further analyses was performed.

RESULTS

Table 4 showed body weight of experimental mice at the end of 14 days iron depletion and after 14 days treatment with fortified noodles. At the end of 14 days iron depletion, all mice had similar body weight. Feeding fortified noodles for the following 14 days had no significant effect on body weight.

Table 5 showed that fortified noodles did not affect liver weight, liver iron and TIBC level. However, the fortified noodles increased significantly Hb, serum iron and transferrin saturation level. Hb level at the end of iron-deficient period was 10.44 g/dl and increased significantly to 10.16-14.92 g/dL in fortified groups ($p = 0.0001<0.05$). Inorganic fortified group was not significantly different to unfortified group. When inorganic iron was combined with pumpkin there was a significant increase in Hb which was similar to Bioplex and combined Bioplex and pumpkin groups.

Serum iron level at the end of iron-deficient period was 36.67 µg/dL and the level after 14 days increased to 183.76-289.50 µg/dL. The highest level was found in $FeSO_4$ group but it was not different than Bioplex group. Combined $FeSO_4$+pumpkin decreased significantly serum iron level. The same pattern was found when Bioplex was combined with pumpkin, serum iron level dropped significantly compared to Bioplex group.

Total Iron Binding Capacity (TIBC) at the end of iron deficient period was 361.28 µg/dL. After feeding iron

Table 3: Total intake of fortified and unfortified noodles and total intake of iron, beta-carotene, carbohydrate, protein, fat and fibre during 14 days iron fortified noodles treatment

Treatment groups	Iron content in treatment noodles (mg/kg)	Beta-carotene content in treatment noodles (mg/kg)	Treatment noodle intake (g)	Unfortified noodle intake (g)	Total noodles intake (treatment plus unfortified) (g)	Total iron intake of each mouse (mg/kg)	Total beta-carotene intake of each mouse (mg/kg)	Total intake of protein (g)	Total intake of carbohydrate (g)	Total intake of fat (g)	Total intake of fibre (g)
Unfortified	14.09	26.20	0.00	5.00	5.00	0.07	0.13	0.34	1.32	0.18	0.03
$FeSO_4$	38.10	26.05	1.14	3.86	5.00	0.10	0.13	0.34	1.30	0.18	0.03
Bioplex	29.26	27.28	1.42	3.58	5.00	0.09	0.13	0.35	1.33	0.18	0.03
$FeSO_4$+Pumpkin	41.08	34.18	1.03	3.97	5.00	0.10	0.14	0.34	1.31	0.18	0.03
Bioplex+Pumpkin	30.04	35.65	1.36	3.64	5.00	0.09	0.14	0.34	1.33	0.18	0.03

fortified noodles it was elevated to 374.65-487.04 µg/dL. ANOVA test showed that there was no effect of fortified noodles on TIBC (p = 312>0.05) of all groups.

Transferin saturation at the end of iron deficient period was 11.21%. Feeding iron fortified noodles increased transferin saturation to 43.56-68.07%. Increase transferin saturation was highest in $FeSO_4$ fortified groups and lowest in combined Bioplex and pumpkin (Table 5).

DISCUSSION

During depletion period, unfortified noodles served as iron deficient diet. In spite of this deficiency it did not affect the body weight of mice (p = 0.965>0.05). This result was similar to Setyani *et al.* (2004) who also found that a diet without iron administration for 15 days did not affect body weight of mice. Following 14 days feeding of fortified noodles, all groups experienced a body weight increase with an average final body weight of 31.2-32.5 g/mouse. Increase body weight indicated that the deficient diet had provided sufficient feed intake which had been adjusted for each mouse and therefore provided sufficient energy and nutrient for growth. At the end of experiment, experimental mice had reached 7 weeks of age and growth had ceased.

Fortified noodles did not affect liver weight and liver iron level indicating that iron deficiency in mice did not affect normal growth and liver as the central organ for iron metabolism and storage. Iron was stored as ferritin and hemosiderin and 30% was found in liver, 30% in bone marrow and the remainder was found in spleen and muscle (Gibson, 2005). Hepatic iron storage which remained constant in spite of iron fortification might also indicate that mobilization from this reserve could not be speculated and other iron pool such as circulating serum iron, TIBC, transferrin saturation and the final Hb level at the end of treatment must be observed and discussed together.

Hb levels at the end of iron depletion period i.e., 10.44 g/dL was slightly less than what was usually found in normal mice which was at least 10.7 g/dL (Kusumawati, 2004). Therefore mice only had a very mild anemia. Following treatment of fortified noodles, Hb level in Bioplex, combined $FeSO_4$-pumpkin or Bioplex-pumpkin groups increased significantly. On the contrary, unfortified or $FeSO_4$ fortified groups remains anemic, shown by Hb level of 8.24 and 10.16 g/dL, respectively. This was an interesting result as inorganic iron ($FeSO_4$) only fortification could not improve Hb level and produce Hb level the same as those of iron deficient group. Observing circulating serum iron level in $FeSO_4$ group which was higher than unfortified group could indicate that increase inorganic iron supply from the diet and transported by serum could not be used for Hb synthesis. When $FeSO_4$ fortified group was compared to combined $FeSO_4$-pumpkin group it could be seen that

Table 4: Body Weight of Mice at the end of 14 days iron depletion and after 14 days of iron fortified noodles treatment

	Body weight (g)								
	Days								
Treatment groups	At the end of 14 days iron-depletion	0	2	4	6	8	10	12	14
Unfortified	23.6±0.75	24.8±0.78	25.1±0.91	25.6±1.04	26.4±1.42	27.2±1.60	28.7±2.16	30.5±1.06	31.2±1.03
FeSO$_4$	24.8±1.00	26.1±1.25	26.8±1.07	27.4±0.88	29.0±1.06	30.0±1.35	31.5±1.46	32.0±1.39	32.5±1.06
Bioplex	23.8±1.33	24.9±1.34	25.2±1.54	25.9±1.33	27.5±0.98	27.8±0.83	29.0±1.20	31.1±1.03	31.9±1.67
FeSO$_4$+pumpkin	23.6±0.45	25.3±1.41	26.3±1.37	26.7±1.40	28.2±1.31	28.5±1.17	29.7±1.12	30.7±0.80	31.5±1.08
Bioplex+pumpkin	22.9±0.93	24.4±1.01	24.7±1.04	25.1±1.03	26.1±1.11	26.4±1.14	27.9±0.96	29.8±1.30	31.3±1.30

Table 5: Liver weight, liver iron, serum iron, total iron binding capacity (TIBC) and transferrin saturation (%) level of mice at the end of 14 day a depletion and after 14 days of iron fortified noodles treatment

Treatment	Liver weight (g)	Liver iron (ppm)	Hemoglobin (g/dL)	Serum iron (µg/dL)	TIBC (µg/dL)	Transferrin saturation (%)
At the end of 14 days Fe-deficient period	0.80±0.12	81.12±10.14	10.44±0.99^{b}	36.67±10.57^{d}	361.28±155.39	11.21±4.62^{c}
Unfortified	1.21±0.41	111.14±28.26	8.24±3.02^{b}	194.21±36.66^{c}	374.65.±71.88	53.71±14.48^{ab}
FeSO$_4$	1.26±0.51	136.11±47.46	10.16±0.52^{b}	311.91±56.21^{a}	487.04±139.08	68.07±20.89^{a}
Bioplex	1.00±0.16	151.29±14.13	13.96±0.26^{a}	289.50±92.05^{ab}	451.19±51.53	64.14±19.90^{ab}
FeSO$_4$+pumpkin	1.22±0.19	151.51±61.33	13.48±0.87^{a}	229.74±65.52^{bc}	472.10±53.65	48.95±13.10^{ab}
Bioplex+pumpkin	1.36±0.23	138.19±41.68	14.92±3.90^{a}	183.76±35.67^{c}	443.72±97.96	43.56±13.85^{b}
ANOVA	0.086^{NS}	0.056^{NS}	0.0001*	0.0001*	0.312^{NS}	0.0001*

the Hb level of combined FeSO$_4$-pumpkin group was significantly higher reaching 13.48 g/dL. The circulating serum iron in combined FeSO$_4$-pumpkin group which was lower than that of FeSO$_4$ fortified group might indicate increase utilization or uptake of serum iron for Hb synthesis. This increase of iron utilization may indicate the contribution of micronutrients in pumpkin such as provitamin A, various vitamin B, trace minerals and amino acids such as glycine which were needed in Hb synthesis in bone marrow (Hoffbrand and Pettit, 1993; Allen and Casteline-Sabel, 2001; Garcia-Casal *et al*., 2003; Subagio, 2007). A study in mice deficient of vitamin A showed that iron transfer into marrow was disturbed so that the iron incorporation into erythrocytes also decreased. It was also shown that retinoids another form of vitamin A metabolite stimulated erythropoiesis directly in the final stages of the development of red blood cells by increasing the concentration of circulating erythropoietin and haemoglobin (Roodenburg *et al*., 1996; Zimmermann *et al*., 2006). In addition, vitamin A affects hormones and cytokines that affect erythropoiesis. Pumpkin contains 0.071 mg of vitamin B6, 0.128 mg riboflavin, 18.792 mg of folic acid and 0.147 mg copper (Cu), per 100 g (USDA, 2011). Pumpkin also contains carotenoids amounting to 1569 g whose activity is equivalent to 261.5 Vitamin A and in the form of dry flour contains the amino acid glycine of 19.97 to 27.12 mg/100 g (Widowati *et al*., 2003). It is very likely that these micronutrients help the utilization of iron in combined FeSO$_4$-pumpkin group in Hb synthesis and hence increase Hb level in this group (Joshi and Gumashta, 2013).

Another interesting result was found in Bioplex fortified group. Hb level of this group could match the Hb level of combined FeSO$_4$-pumpkin group. Organic iron fortification alone was sufficient to increase the Hb concentration (13.96 g/dL). Bioplex as organic minerals has been shown to have a higher availability and utility than inorganic minerals (Acda and Chae, 2002). When organic iron Bioplex was combined with pumpkin, it could improve the Hb levels further to 14.92 g/dL, although it was statistically insignificant. This again signified that pumpkin could provide micronutrients required in hemoglobin synthesis. As noodles are popular food not only in Asia Pacific region but also across the world, combined iron-pumpkin fortified noodle is a potential food carrier to reduce iron deficiency anemia in vulnerable group.

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