Food & Function

PAPER

View Article Online
View Journal | View Issue

Cite this: *Food Funct.*, 2014, **5**, 390

Effects of heme iron enriched peptide on iron deficiency anemia in rats

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Received 23rd July 2013
Accepted 2nd November 2013

DOI: 10.1039/c3fo60292c

www.rsc.org/foodfunction

The present study aims to investigate whether a daily intake of heme iron enriched peptide obtained from bovine hemoglobin is effective in alleviating iron deficiency anemia (IDA). Wistar rats were randomly divided into six groups: a control group, an anemic group not treated, and anemic groups treated with $FeSO_4$ or with the heme iron enriched peptide at low, moderate or high doses. The rats in the anemic groups were fed on a low-iron diet to establish the iron deficiency anemia model. After the model had been established, different doses of heme iron enriched peptide were given to the rats once a day *via* intragastric administration. After the iron supplement administration, it was observed that heme iron enriched peptide had effective restorative action returning the hemoglobin, red blood cells, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration and serum iron in IDA animals to normal values or better. In addition, compared with $FeSO_4$, higher Fe bioavailability and fewer side effects were observed. The rats in the moderate dose group had the highest apparent Fe absorption. Moreover, *in vivo* antioxidant activity was also observed, enhancing the activities of antioxidant enzymes and reduced malondialdehyde levels in IDA rats. Furthermore, the heme iron enriched peptide also exhibited strong *in vitro* antioxidant activities. In conclusion, heme iron enriched peptide significantly alleviated iron deficiency anemia, and exhibited strong *in vitro* and *in vivo* antioxidant activities. This suggests that heme iron enriched peptide might be exploited as a safe, efficient new iron supplement.

1 Introduction

Iron is an essential trace element for most life forms and is involved in many biological processes.[1] A deficiency in iron will lead to mental development problems in infants,[2] a poor pregnancy outcome[3] and decreased immunity.[4] However iron deficiency is the most common nutritional disorder, affecting people of all ages worldwide,[5] approximately 30% of the world's population are suffering from this disorder, especially in developing countries.[6] The prevalence of iron deficiency anemia can be reduced by supplementation with pharmaceutical preparations, iron fortification of foods and dietary diversification.[7] Iron supplements are useful for producing a rapid improvement in the iron status of anemic individuals. Electrolytic iron and ferrous sulfate are widely used, but depending on dietary bioavailability, the bioavailability of non-heme iron is between 2% and 20% and is influenced by a variety of inhibiting components in the diet.[7,8] Due to poor bioavailability and some other reasons, inorganic iron supplements have limitations for reducing the prevalence of iron deficiency anemia.

Heme iron is absorbed *via* heme transporters that are predominantly expressed in the brush border membranes of duodenal enterocytes and hepatocytes.[9] In contrast, non-heme iron is transported into the body through nonselective iron channels[10] which are easily influenced by other metal ions. Therefore, in humans, the absorption of heme iron is more complete than that of non-heme iron .[11] And it is used extensively as an iron supplement in the food industry.[12] Heme iron, however, is insoluble at neutral pH, and its absorption and bioavailability have been investigated in some studies.[13] The presence of amino acids in the intestines has been reported to increase iron absorption.[14] Therefore, the use of suitable proteins in the diet may increase the absorption rate of dietary iron. Animal blood contains about 18% protein, with hemoglobin, which is present in the red blood cells, accounting for more than half of the proteins present.[15] The most abundant protein in blood is hemoglobin, representing 12–18% of the total composition of blood. Hemoglobin can be easily purified and processed to produce several products, heme iron supplements,[16,17] peptides with different applications such as antioxidant,[18] antihypertensive,[19] antimicrobial[20–22] and iron-binding peptides[23] or simply used as an ingredient to enhance the protein and amino acid content of food products. However, of all the unwanted animal by-products, blood causes the most problems as a result of the huge volume produced and its high pollutant load. For many years, blood has been discarded as an unwanted by-product by slaughter houses in many countries.

Therefore, the present study is designed to utilize wasted bovine blood to prepare a heme iron enriched peptide. The *in*

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vitro antioxidant activities of the obtained heme iron enriched peptide are investigated. And a rat model is established to evaluate the toxicity and *in vivo* activities of the obtained heme iron enriched peptide. Although there are numerous reports about the production of various peptides from food proteins, there are few studies on the possible side effects that might be exerted by the peptides or their metabolites. Toxicology studies and *in vivo* experiments are necessary to assess the safety of a functional food prior to commercialization.

2 Materials and methods

2.1 Preparation of heme iron enriched peptide

Bovine hemoglobin was hydrolyzed by treatment with neutral protease for 60 min followed by treatment with flavourzyme for another 60 min according to the method previously developed by our team.[24] Briefly, the hemoglobin in water (4% v/v) was adjusted to pH 7.5 by adding 0.2 M NaOH. The neutral protease (10% w/v) was added to hydrolyze the hemoglobin for 60 min. After heated in boiling water for 10 min and adjusting to pH 7, the flavourzyme (4% w/v) was added to hydrolyze the hemoglobin for a further 60 min. The temperature was kept at 55 °C or 50 °C, and pH was kept at 7.5 or 7 for hydrolysis with neutral protease or flavourzyme, respectively. At the end of the hydrolysis period, the mixture was heated in boiling water for 10 min to inactivate the proteases. Then the hydrolysates were centrifuged at 10 000*g* for 10 min at 4 °C. The obtained supernatant was sequentially separated *via* ultrafiltration membranes with molecular weight cut-offs of 10, 3 kDa to obtain the peptides with a molecular weight less than 3 kDa. Then the peptides were chelated with ferrous chloride, then spray dried and stored at −20 °C. The major ingredients of the obtained heme iron enriched peptide are as following: polypeptide (<3 kDa) (71.21%), heme (8.49%), iron (10.32%), sodium chloride (2.78%) and moisture (5.57%).

2.2 *In vitro* antioxidant activities

DPPH radical scavenging activity. The DPPH radical scavenging activity of heme iron enriched peptide was measured using a modified method by Shimada *et al.*[25] 2 mL of 0.1 mM DPPH dissolved in 95% ethanol was added to 2 mL of heme iron enriched peptide solution. The mixture was shaken and left for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm. For the blank, 2 mL of distilled water was used instead of the sample. The radical scavenging activity was calculated as follows:

$$\text{DPPH scavenging activity (\%)} = (\text{blank absorbance} - \text{sample absorbance})/\text{blank absorbance} \times 100$$

Hydrogen peroxide scavenging activity. The hydrogen peroxide scavenging activity of heme iron enriched peptide was determined according to the method by Ruch *et al.*[26] 3.4 mL of heme iron enriched peptide was added to 0.6 mL of 40 mmol L^{-1} hydrogen solution dissolved in 0.1 mol L^{-1} phosphate buffer (pH 7.4). The absorbance of hydrogen peroxide at 230 nm was determined after 15 minutes. For the blank, 3.4 mL of phosphate buffer was used instead of the sample. The hydrogen peroxide scavenging activity was calculated as follows:

$$H_2O_2 \text{ scavenging activity (\%)} = (\text{blank absorbance} - \text{sample absorbance})/\text{blank absorbance} \times 100$$

Reducing power assay. The reducing power was measured according to the method by Zhu *et al.*[27] with some modifications. 1 mL of heme iron enriched peptide sample was mixed with 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min followed by the addition of 1 mL 10% (w/v) trichloroacetic acid. 2 mL of the incubation mixture was mixed with 2 mL of distilled water and 0.4 mL of 0.1% ferric chloride in test tubes. After 10 min the absorbance of the resulting solution was measured at 700 nm. An increased absorbance by the reaction mixture indicates increased reducing power.

Total antioxidant capacity. The total antioxidant capacity of heme iron enriched peptide was evaluated by the method from Prieto *et al.*[28] with some modifications. 0.1 mL of sample solution was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture was incubated in a boiling water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of each resulting solution was measured at 695 nm against a blank. The blank solution contained 3 mL of reagent solution and 0.3 mL of distilled water. An increased absorbance of the reaction mixture indicates increased total antioxidant capacity.

2.3 Animal care and treatment

Female Wistar rats ($n = 60$) weighing 80 ± 6 g after behavioral examination were purchased from the Experimental Animal Center, Jilin University at the beginning of the experiment. The rats were acclimated for 7 days prior to experiments with an evaluation of their health status. All rats were housed in metabolic cages with sawdust bedding and maintained in an environmentally controlled room (22 ± 3 °C and 50% ± 10% relative humidity) with a 12 h light/dark cycle. Sawdust bedding was renewed every three days. Food (AOAC diet) and water were available ad libitum.

After acclimation, the rats were randomly divided into six groups. The control group was still fed the AOAC diet with a normal Fe level for the entire experimental period. The other five groups were fed the AOAC diet with a low Fe level (6.7 mg kg^{-1} diet, Fe) to induce anemia in the rats over 4 weeks. On the 30th day, blood was collected by blood letting *via* the caudal vein, and routine blood tests were carried out. Iron deficiency anemia was defined as a hemoglobin level below 100 g L^{-1}.

After the iron deficiency anemia rat model was established, the five groups of rats were continuously fed on a low iron diet for six weeks. During this period, the rats in control group and anemia model group were administrated deionized water everyday, the rats in positive group were administrated ferrous sulfate solution (2 mg kg^{-1} body weight, Fe),

while the rats in the low, middle and high dose groups were administrated different doses of heme iron enriched peptide solution that provided them with 1 mg kg^{-1}, 2 mg kg^{-1} and 3 mg kg^{-1} Fe, respectively. The body weight was monitored once a week and the doses of daily intragastric administration were administered once a day according to the body weight of the rats. All animal experiments were conducted in compliance with the Guide of the Care and Use of Laboratory Animals.[29]

2.4 Clinical observations

All animals were observed twice daily for general appearance, behaviour, signs of morbidity and mortality (once before treatment and once daily thereafter). Rats were observed for their general condition and the condition of the skin and fur, eyes, nose, oral cavity, abdomen. Body weight, food and water consumption were measured once a week.

2.5 Organ coefficient

In this study, a gross pathology examination was conducted by visual inspection during the necropsy. The thymuses, hearts, livers, spleens, kidneys, bladders and ovaries of all rats were excised and weighed. Paired organs were weighed separately. The relative weight of each organ was calculated based on the final body weight measured on that day. The organ coefficient was calculated by the following:

$$\text{Organ coefficient} = \text{organ weight/rat body weight} \times 100.$$

2.6 Hematological test

Blood samples were collected in EDTA-K_2 solution tubes. Serum was obtained from blood samples after centrifugation and stored at −80 °C until analysis. Hemoglobin concentration (Hb), red blood cells (RBC), haematocrit (HCT), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were measured using an automated hematology analyzer (Sysmex, Tokyo, Japan). Serum iron concentration was measured using a serum iron reagent kit (Nanjing Jiancheng Bioengineering Inst., Nanjing, China) according to the instructions from the manufacturer.

***In vivo* antioxidant indicator determination.** The CAT, SOD, GSH, T-AOC activities in the serum and MDA contents were determined using diagnostic kits (Nanjing Jiancheng Bioengineering Inst., Nanjing, China) according to the instructions from the manufacturer.

2.7 Apparent Fe absorption

The dried feces were ground and the samples were used for mineral analysis. The apparent absorption of Fe was calculated according to the equation:

$$\text{Apparent Fe absorption (\%)} = (\text{Fe intake-fecal Fe})/\text{Fe intake} \times 100$$

2.8 Statistical analysis

The SPSS statistical software program was used for all data analysis. Results were expressed as the mean ± SD. Single factor analysis of variance was performed by ANOVA, followed by Dunnet's test, with an alpha value of $P < 0.05$ being regarded as statistically significant.

3 Results and discussion

3.1 *In vitro* antioxidant activities

In vitro antioxidant activities were determined to characterize the obtained heme iron enriched peptide. The DPPH radical with a single electron shows strong absorbance at 517 nm in ethanol solution, the absorbance reduces gradually as the free radicals are scavenged by accepting an electron or hydrogen radical and the colour of the solution changes from modena to yellow in the presence of a proton-donating substance.[30,31] The relatively stable DPPH radical has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and thus to evaluate antioxidant activity.[25,32] As can be seen from Fig. 1A, the heme iron enriched peptide exhibited dose-dependent DPPH radical scavenging activities. The scavenging activity increased with increasing concentration (25 to 125 μg mL^{-1}). The DPPH scavenging activity reached 79.1 ± 1.32% at 125 μg mL^{-1}. In this study, ascorbic acid was used as a positive control to compare the results at the same concentrations. As shown in Fig 1A, the scavenging activity of heme iron enriched peptide was significantly higher ($p < 0.01$) than ascorbic acid. The DPPH radical scavenging activity of ascorbic acid was only 32 ± 0.15%. This result indicates that heme iron enriched peptide possesses higher *in vitro* antioxidant activity than ascorbic acid in the DPPH radical scavenging activity assay. This may be due to the presence of ferrous ions in heme iron.

During various physiological and pathological processes, ROS are generated from hydrogen peroxide (H_2O_2) by mitochondria at both extra and intracellular levels.[33] This induces oxidative chromosomal damage as well as direct breakage of DNA.[34] H_2O_2 itself is not reactive enough, however, in association with superoxide anions it can damage many cellular components.[35] Thus antioxidants which possess the property of scavenging free radicals or ROS can protect from oxidative damage followed by decreasing the level of pro-oxidants such as H_2O_2. Therefore, the H_2O_2 scavenging activity of heme iron enriched peptide was also determined in our study. As presented in Fig. 1B, the H_2O_2 scavenging activity of heme iron enriched peptide was concentration-dependent since it increased significantly with the increasing concentration. The scavenging activity was 61.4 ± 1.19% at 125 μg mL^{-1}. However, the scavenging activity was lower than ascorbic acid (76.2 ± 1.73%) at the same concentration.

Reducing power is usually used as a measurement of the activity of peptide fractions and protein hydrolysate. Reduction of the Fe^{3+}/ferricyanide complex to the ferrous form was used to determine the reducing power of peptides.[36] Therefore, Fe^{2+} can be monitored by measuring the absorption of Perl's

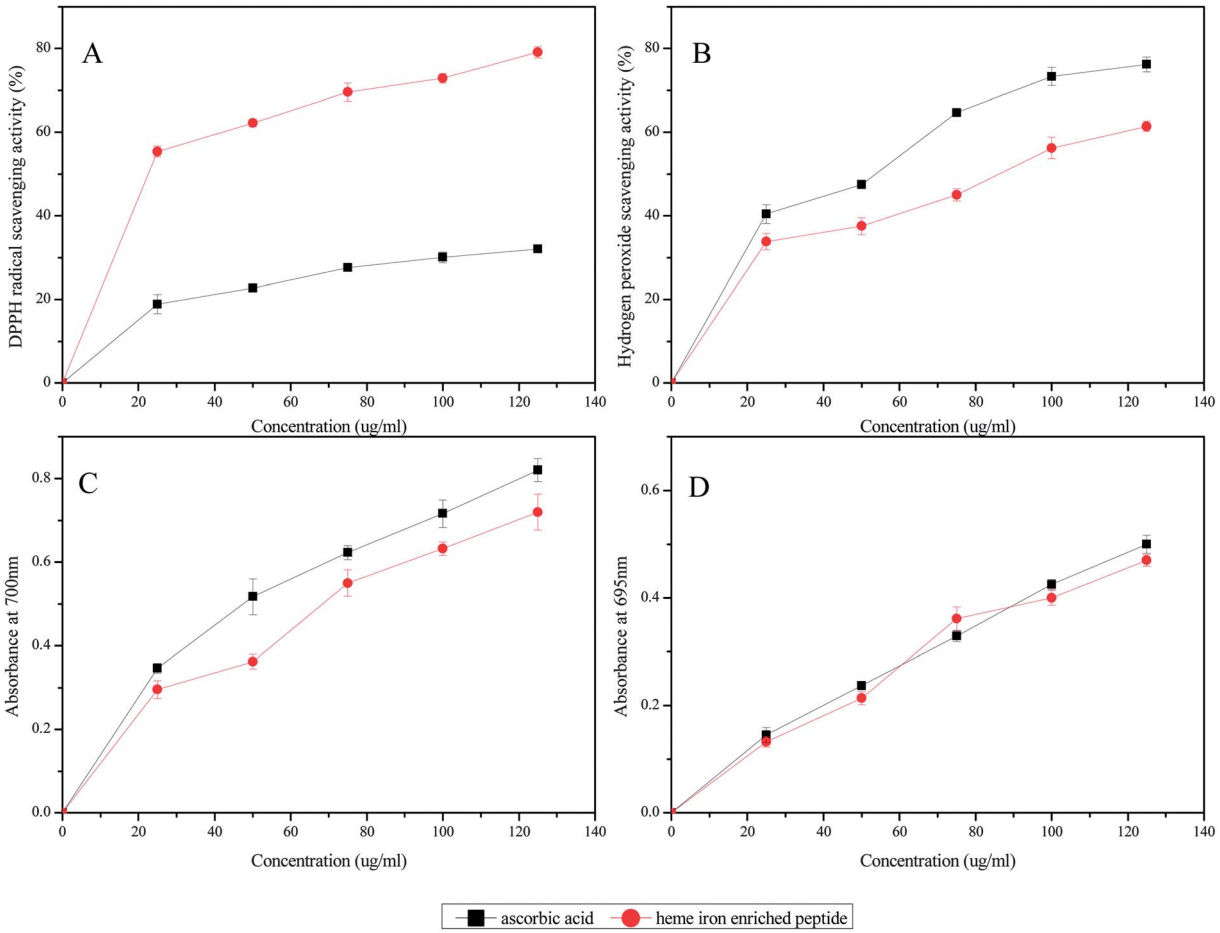

Fig. 1 *In vitro* antioxidant activities of heme iron enriched peptide.

Prussian blue at 700 nm.[37] From the obtained results (Fig. 1C), the increase in concentration of heme iron enriched peptide, increased the reducing power activity. The absorbance reached 0.72 ± 0.043 at 125 μg mL^{-1}. However, the reducing power was still lower than ascorbic acid at the same concentration. Antioxidants have been found to exhibit their activity by various mechanisms at different stages of the oxidation reaction. The phosphomolybdenum assay, initially developed by Prieto *et al.*[28] was used in this study for the evaluation of the total antioxidant capacity of heme iron enriched peptide. This method is based on the reduction of Mo(VI) to Mo(V) by the antioxidant compound and the formation of a green phosphate–Mo(V) complex, which is formed under acidic pH conditions and can be monitored at 695 nm with a spectrophotometer. The increase in the absorbance indicated the increase in the total antioxidant capacity. Fig. 1D shows that heme iron enriched peptide exhibited significant activity. In addition, the total antioxidant activity of heme iron enriched peptide was still concentration-dependent. The absorbance increased with increasing concentration. The absorbances of heme iron enriched peptide and ascorbic acid were 0.47 ± 0.011 and 0.5 ± 0.017 respectively at 125 μg mL^{-1}. Although its reducing power was still lower, it showed no significant difference from ascorbic acid.

3.2 Clinical observations

During the heme iron enriched peptide supplementation, no abnormal clinical signs or macroscopic findings were observed. All animals that received heme iron enriched peptide survived the study period. The behaviour of the animals was normal, and the condition of their skin and fur, eyes, nose, oral cavity and abdomen were normal. There were no statistical differences in body weights between the heme iron enriched peptide supplementation groups and the control group. Similarly, no statistically significant differences in body weight gain, food and water consumption were noted (data not shown). However, the general status of the animals with induced iron-deficiency anemia was poor. They lost their appetite and were apathetic.

3.3 Body weight changes

The growth curves and body weight gain for the rats are presented in Fig. 2. The body weight of the experimental rats at the beginning showed no significant differences ($p > 0.05$). After the iron deficiency anemia model was established, the anemic rats showed a significant decrease ($p < 0.01$) in body weight compared with the normal control group and supplementation groups. Moreover, the rats with induced iron-deficiency anemia a exhibited dull and rough coat, unresponsiveness and

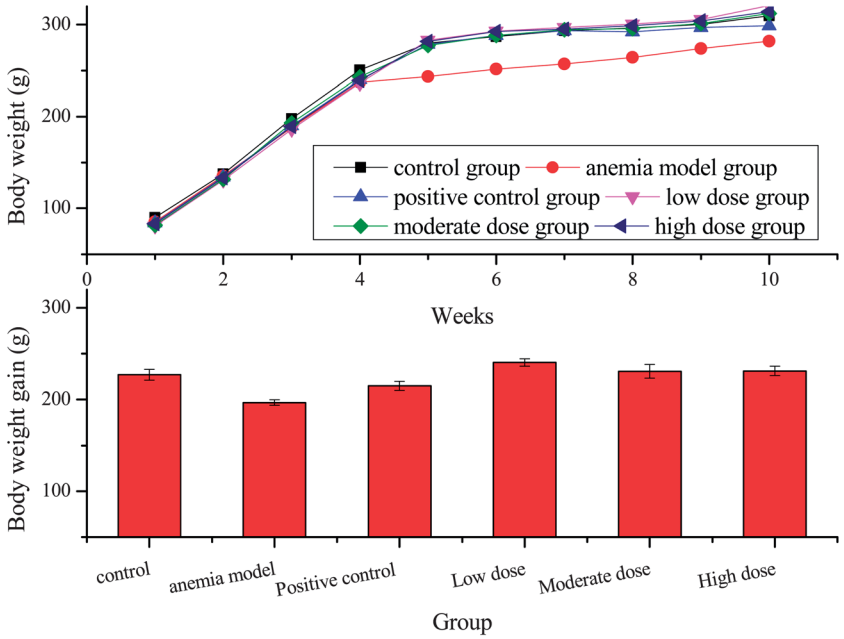

Fig. 2 The body weight changes of rats in different groups. Anemia model group none treated, positive group treated with $FeSO_4$ (2 mg Fe kg^{-1}), low dose, moderate dose and high dose group treated with the heme iron enriched peptide at 1 mg Fe kg^{-1}, 2 mg Fe kg^{-1} and 3 mg Fe kg^{-1} respectively.

irritability. The result indicated that iron deficiency anemia would affect the growth of the rats. As can be seen from Fig. 2, supplementation with iron increased the animals' growth in comparison with the anemia control group. Final body weights and weight gains did not differ for rats that received heme iron enriched peptide and showed no significant differences ($p > 0.05$) compared with the normal control group. However, body weight gain was significantly lower ($p < 0.01$) in anemic rats than in the normal control or treated groups.

3.4 Organ coefficient changes

There were no significant differences in heart weights among the rats in different groups, although the effect of cardiomyopathy in iron-deficient rats has been confirmed in earlier studies.[38] However, in this study, higher organ coefficients of the hearts in anemic rats were also observed. The organ coefficients of the livers in heme iron enriched peptide supplemented animals were lower than that of iron-deficient animals. But the reduction in liver volume induced by iron deficiency was observed in other studies.[39] Hyperplasia of the liver is quite often observed during iron supplementation.[39,40] This effect was not observed as the liver had recovered after the long term iron supplementation. Hypertrophy of the spleen was observed in iron-deficient animals. This result was also observed by Yun *et al.*[39] The highest organ coefficients of the kidney, thymus, bladder and ovaries in anemic rats were noted but there were no significant differences among the rats in different groups. The rats in the $FeSO_4$ group presented the highest organ coefficients among the iron supplemented rats. Also the rats in the moderate dose group had the lowest organ coefficients. Increased coefficients of these organs in anemic rats was probably due to the oedema induced by iron deficiency anemia. No general growth parameters in supplemented animals were significantly different from the normal control group, which proves the heme iron enriched peptide has no side effects on the rats' healthy state.

3.5 Hematological studies

Various conventional laboratory tests are available for successful diagnosis of iron deficiency anemia. In the present study, hematologic parameters were determined to confirm the induction of anemia and recovery following iron supplementation treatment. Due to the low iron diet, at the end of the experiment, the mean hemoglobin concentration (72.4 ± 8.8 g L^{-1}) of the rats in anemia model group was significantly lower ($p < 0.01$) than the normal control or iron supplemented groups. The groups that were supplemented with heme iron enriched peptide did not show significant difference in hemoglobin concentration when compared with the normal control group (140.7 ± 5.22 g L^{-1}). The animals that supplemented $FeSO_4$ had the lowest mean hemoglobin concentration (125 ± 4.78 g L^{-1}) among the iron supplemented rats. And the rats in the high dose of heme iron enriched peptide group had the highest mean hemoglobin concentration (150.8 ± 8.35 g L^{-1}). Moreover, the rats in the high dose group had the highest mean hemoglobin concentration gain, increased by 54.8 g L^{-1}, while the level of hemoglobin in anemic rats decreased by an average of 23.6 g L^{-1} (data not shown). The results indicated that heme iron enriched peptide had effective restorative action, returning the hemoglobin concentration to normal values. Also the iron bioavailability of the heme iron enriched peptide was higher than the inorganic iron. Red blood cells

(RBC) play a crucial role in the support of tissue metabolism and enough RBC must be available to maintain tissue oxygenation and sustain a normal acid–base balance in the system.[41] However, if there is not enough iron available, hemoglobin production is limited, which in turn affects the production of red blood cells. In iron deficiency anemia, the RBC decreases in proportion to the decrease in hemoglobin concentration.[41] As shown in Table 1, the RBC counts of anemic rats were significantly lower ($P < 0.01$) than the normal rats or iron supplemented rats, with a mean RBC count of 3.1 $\times$ 10^{12} L^{-1}. However, the RBC numbers were increased dramatically after administrated the heme iron enriched peptide for six weeks. The rats that received different doses of heme iron enriched peptide had higher RBC numbers than the rats in $FeSO_4$ group. And the rats in the moderate dose group had the highest RBC numbers (5.25 $\times$ 10^{12} L^{-1}). This result also indicated that the heme iron enriched peptide was more efficient at ameliorating the anemia symptoms. Hematocrit (HCT) indicates the proportion of the whole blood occupied by the red blood cells and depends on the hemoglobin concentration in red blood cells.[42] As can be seen from Table 1, the hematocrit value was also significantly lower ($p < 0.01$) in anemia rats compared with normal rats or iron supplemented rats. The hematocrit value increased significantly after iron supplementation. The hematocrit values of rats in three heme iron enriched peptide supplemented groups were all higher than that of the $FeSO_4$ group, and showed no significant difference when compared with the normal rats. The rats in the moderate group had the highest hematocrit value (43.2 $\pm$ 2.1%). Other hematological parameters are also presented in Table 1. The mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) in iron supplemented rats showed no significant difference when compared with normal rats, but differed from the levels observed in iron deficiency anemia animals ($p < 0.01$). Also the rats in the $FeSO_4$ group had the lowest level among the iron supplemented rats. The results mentioned above show that there were significant differences in the blood indices response when administrating the iron supplement to iron deficiency anemia rats, both inorganic iron and heme iron can significantly enhance Hb, RBC, HCT, MCV and MCHC in iron deficiency anemia rats. In general, treatment with heme iron enriched peptide improves the iron levels of iron deficiency anemia rats to normal or near normal better and more quickly than the traditional $FeSO_4$ treatment.

Serum iron concentration is a medical laboratory test that measures bound iron . As can be seen from Fig. 3, our observation showed that iron deficiency anemia significantly ($p < 0.01$) affected the serum iron concentration in rats, with the lowest serum iron concentration (5.5 $\pm$ 0.53 μM). However, the heme iron enriched peptide can significantly ($P < 0.01$) increase the serum iron concentration of iron deficiency anemia rats. The rats in the high dose group had the highest serum iron concentration (13.75 $\pm$ 0.32 μM) and the rats in $FeSO_4$ group had the lowest serum iron concentration (9.1 $\pm$ 0.74 μM) among the iron supplemented rats. Many factors affect absorption of minerals and one of the most important ones is the interaction of one mineral with another to the extent that absorption and utilization are reduced or enhanced.[43] According to Ebihara and Okano,[44] sufficient iron is absorbed *via* the large intestine for recovery from iron deficiency anemia. In the present study, iron deficiency anemia rats showed a significant decrease in serum iron concentration when compared with the control group or iron supplemented groups, and this confirms the findings of Milne *et al.*[45] and Sundaram *et al.*[46] Besides the serum iron concentration, the apparent Fe absorption was also determined. As shown in Fig. 3, the rats in the three heme iron enriched peptide groups showed higher apparent Fe absorption compared with the rats in the $FeSO_4$ group. And the rats in the moderate dose group had the highest apparent Fe absorption (34.1 $\pm$ 1.27%), which was significantly higher ($p < 0.01$) than that in the $FeSO_4$ group. This may be due to heme iron obtained from meat and blood-derived foods being absorbed more efficiently than non-heme iron. Its absorption rate is approximately three times higher than inorganic iron and is not easily affected by the digestive system.[47] Moreover, the presence of the amino acids promoted non heme Fe absorption.[48] However, the rats in the moderate dose group did not show the highest serum iron concentration, this may be because the serum iron concentration could affected by many factors such as a meal, the presence of infections or inflammations and the existence of diurnal variations.[42] The moderate dose seems to be a suitable dose.

Table 1 Hematological parameters of rats in different groups[a]

Group	Hb (g L^{-1})	RBC (10^{12} L^{-1})	HCT (%)	MCV (fL)	MCHC (g L^{-1})
Control	140.7 $\pm$ 5.22[a]	5.01 $\pm$ 0.46[a]	42.33 $\pm$ 3.4[a]	63.6 $\pm$ 2.57[a]	335.4 $\pm$ 3.5[a]
Anemia model	72.4 $\pm$ 8.8	3.1 $\pm$ 0.3	27.5 $\pm$ 2.5	41.3 $\pm$ 1.53	262.27 $\pm$ 4.32
Positive control	125 $\pm$ 4.78[a]	4.37 $\pm$ 0.24[a]	37.5 $\pm$ 3.7[a]	53.1 $\pm$ 4.88[a]	330.6 $\pm$ 4.28[a]
Low dose	137 $\pm$ 3.2[a]	4.75 $\pm$ 0.29[a]	40.5 $\pm$ 4.2[a]	59 $\pm$ 2.78[a]	338.2 $\pm$ 3.78[a]
Moderate dose	145.6 $\pm$ 4.55[a]	5.25 $\pm$ 0.5[a]	43.2 $\pm$ 2.1[a]	64.7 $\pm$ 3.2[a]	340.5 $\pm$ 3.96[a]
High dose	150.8 $\pm$ 8.35[a]	5.1 $\pm$ 0.37[a]	42.6 $\pm$ 3.4[a]	62 $\pm$ 4.6[a]	349.3 $\pm$ 4.87[a]

[a] Anemia model group not treated, positive group treated with $FeSO_4$ (2 mg Fe kg^{-1}), low dose, moderate dose and high dose group treated with the heme iron enriched peptide at 1 mg Fe kg^{-1}, 2 mg Fe kg^{-1} and 3 mg Fe kg^{-1} respectively, Hb: hemoglobin concentrations, RBC: red blood cell, HCT: hematocrit, MCV: mean corpuscular volume, MCHC: mean corpuscular haemoglobin concentration. [a] indicates a significant difference from the negative control group, [a]$P < 0.01$.

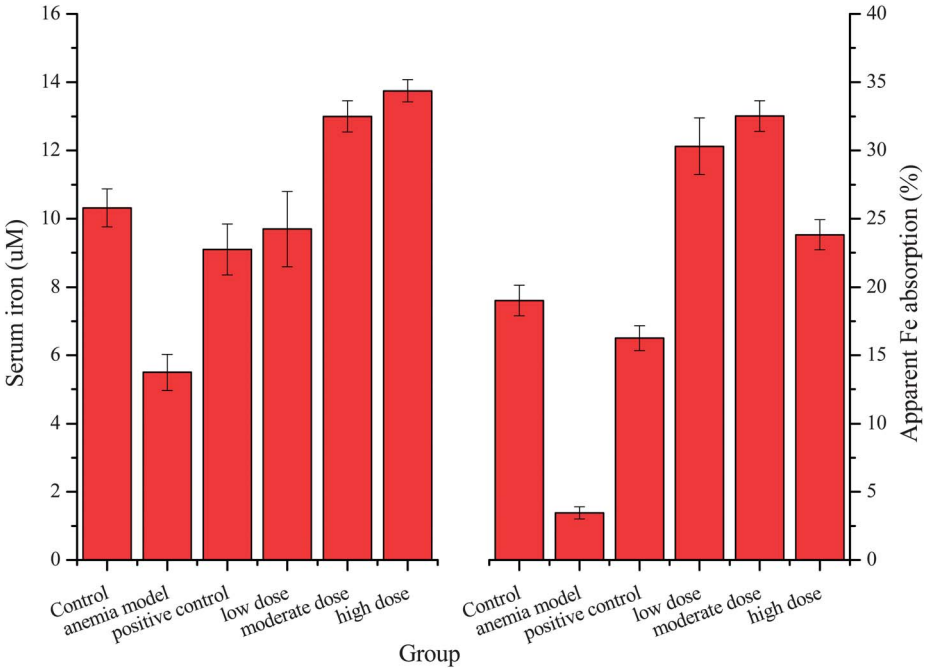

Fig. 3 The serum iron content and apparent Fe absorption of rats in different groups. Anemia model group not treated, positive group treated with $FeSO_4$ (2 mg Fe kg^{-1}), low dose, moderate dose and high dose group treated with the heme iron enriched peptide at 1 mg Fe kg^{-1}, 2 mg Fe kg^{-1} and 3 mg Fe kg^{-1} respectively.

3.6 *In vivo* antioxidant activities

Normal cell function and the integrity of cell structures may be disrupted by accumulation of reactive oxygen species (ROS). Catalase (CAT) is a common enzyme found in the cells of nearly all living organisms which acts as a catalyst for the decomposition of hydrogen peroxide to water and oxygen.[49] The enzyme superoxide dismutase (SOD) catalyzes dismutation of the superoxide anion into hydrogen peroxide (H_2O_2), which is then detoxified to H_2O by catalase.[50] Also glutathione peroxidase has a direct antioxidant function by reacting with superoxide radicals, peroxy radicals and singlet oxygen.[51] These enzymes in the organism play an important role in the regulation of a variety of cell functions and in the protection of cells against oxidative injury.[52] The antioxidant enzyme activities including SOD, CAT and GSH-Px in iron deficiency anemia rats have been evaluated in several studies.[53–58] Although conflicting results have been reported,[54,57] it is generally accepted that oxidative stress is increased through decrease in antioxidant enzyme capacities in iron deficiency anemia. Our results confirmed the argument that iron deficiency anemia can lead to nutritional decompensation and accumulation of reactive oxygen species (ROS), while heme iron enriched peptide can effectively reduce oxidation levels in iron deficiency anemia rats. As can be seen from Table 2, the SOD activity (130.29 ± 2.38 U mL^{-1}) of anemic rats was significantly lower ($p < 0.01$) than normal rats or iron supplemented rats. The rats in the $FeSO_4$ group had the lowest activity among the iron supplemented rats, but showed no significant difference when compared with normal rats. The rats in the moderate dose group showed the highest SOD activity (158.25 ± 12.89 U mL^{-1}), also higher than the normal rats. As shown in Table 2, iron deficiency anemia significantly decreased the CAT activity in rats, with the lowest activity reaching 1.34 ± 0.31 U mL^{-1}. Also the rats in the moderate dose group had the highest CAT activity (7.56 ± 0.07 U mL^{-1}). As presented in Table 2, the GSH-Px activities in anemic rats were the lowest, and showed a significant difference ($p < 0.01$) when compared with the normal rats or iron supplemented rats. The rats in the moderate dose group had the highest GSH-Px activity, while the rats that received $FeSO_4$ had the lowest GSH-Px activity among the iron supplemented rats. Generally, decreased GSH-Px activity along with normal or increased SOD and CAT activity are the main findings in iron deficiency anemia.[54] However, in some studies, decrease in all of these activities was also reported. Our results indicated that iron deficiency anemia could decrease the activities of these antioxidant enzymes. However, treatment with heme iron enriched peptide significantly increased the activity of these antioxidant enzymes to normal or better than normal. Compared with traditional $FeSO_4$ treatment heme iron enriched peptide was more effective. Moreover, the rats in the moderate dose group had the highest SOD, CAT and GSH-Px activities, also higher than the normal rats. This may due to the heme iron enriched peptide possessing *in vivo* antioxidant activity. Meanwhile, when the total antioxidant activity was determined, similar results were obtained. As Table 2 shows, the rats in the moderate dose group had the highest total antioxidant activity (9.58 ± 0.33 mL^{-1} serum).

In biological systems, the level of lipid peroxidation is often assessed by the measurement of lipid peroxidation breakdown products such as malondialdehyde (MDA).[59] Previously some

Table 2 The activities of *in vivo* antioxidant enzymes and MDA level of rats in different groups[a]

Group	SOD (U mL^{-1})	CAT (U mL^{-1})	GSH-Px (activity unit)	TAC (mL^{-1} serum)	MDA (nmol mL^{-1})
Control	150.71 ± 5.22[a]	5.53 ± 0.14[a]	627.14 ± 32.43[a]	6.37 ± 0.33[a]	1.07 ± 0.21[a]
Anemia model	130.29 ± 2.38	1.34 ± 0.31	409.41 ± 35.67	3.31 ± 0.17	2.71 ± 0.28
Positive control	146.14 ± 7.23[a]	4.72 ± 0.19[a]	536.47 ± 23.06[a]	6.04 ± 0.07[a]	2.58 ± 0.28
Low dose	148.66 ± 4.99[a]	6.78 ± 0.16[a]	707.65 ± 46.08[a]	6.49 ± 0.22[a]	1.23 ± 0.12[a]
Moderate dose	158.25 ± 12.89[a]	7.56 ± 0.07[a]	712.72 ± 29.35[a]	9.58 ± 0.33[a]	1.01 ± 0.04[a]
High dose	152.21 ± 4.97[a]	5.58 ± 0.09[a]	630.89 ± 59.78[a]	5.91 ± 0.17[a]	1.09 ± 0.17[a]

[a] Anemia model group not treated, positive group treated with $FeSO_4$ (2 mg Fe kg^{-1}), low dose, moderate dose and high dose group treated with the heme iron enriched peptide at 1 mg Fe kg^{-1}, 2 mg Fe kg^{-1} and 3 mg Fe kg^{-1} respectively, SOD: superoxide dismutase, CAT: catalase, GSH-Px: glutathione peroxidase, TAC: total antioxidant activity, MDA: malondialdehyde. [a] indicates a significant difference from the negative control group, [a]$P < 0.01$.

researchers reported higher lipid peroxide levels in anemic conditions. However, there are some conflicting reports which showed lower or unchanged lipid peroxide levels in anemic conditions.[43,46,56,60] Despite extensive literature reports on iron and lipid peroxidation, few studies have investigated the effects of oral iron supplements on lipid peroxidation. In our study, the mean concentration of MDA in normal rats was 1.17 ± 0.21 nmol mL^{-1}. There was a significant increase in the MDA concentration (2.71 ± 0.28 nmol mL^{-1}) of anemic rats, which agrees with reports that explain the generation of lipid peroxides in the case of iron deficiency anemia. However, the rats in the three heme iron enriched peptide treated groups had significantly lower ($p < 0.01$) MDA concentration than rats in the anemia model group, but showed no significant difference from normal rats. Our study agrees with Sundaram *et al.*[46] who found an increased level of MDA in anemic patients and a significant decrease in MDA concentration after treatment. According to numerous scientific reports, various physiological mechanisms might be responsible for the development of oxidative stress and lipid peroxidation in anemic animal models.[61–63] The main reason for our observation was speculated to be the enough compensatory capacity to keep antioxidant defenses high with adequate antioxidant enzyme activities from SOD, CAT and GSH-Px.

4 Conclusions

Heme iron enriched peptide was prepared from bovine hemoglobin. The obtained peptide exhibited strong *in vitro* antioxidant activities. In the DPPH radical scavenging activity assay, the scavenging activity of heme iron enriched peptide was significantly higher ($p < 0.01$) than ascorbic acid. In addition, heme iron enriched peptide significantly alleviated iron deficiency anemia by returning hemoglobin, red blood cell, hematocrit, mean corpuscular volume and mean corpuscular hemoglobin concentration, serum iron in IDA animals to normal values or better. Higher Fe bioavailability and fewer side effects were observed when compared with $FeSO_4$, moreover, *in vivo* antioxidant activity was also observed that enhanced the activities of antioxidant enzymes and reduced malondialdehyde levels in IDA rats. Therefore, heme iron enriched peptide obtained from bovine hemoglobin might be exploited as a safe, efficient new iron supplement.

Acknowledgements

This work was funded by the Key Projects in the National Science & Technology Pillar Program during the Twelfth Five-Year Plan Period (2012BAD33B03).

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