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Effect of Hydrolysed and Unhydrolysed Whey Protein Bound Iron Supplement on Wistar Rats

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Abstract

The study evaluated the body stores of iron in anaemic wistar rats after feeding with iron-fortified, whey protein concentrate (WPC)-based supplements. Both hydrolysed and unhydrolysed WPC were used for preparing WPC-iron complexes. Thirty-six male Wistar rats of three weeks of age, divided into six groups, were fed on standard diet, iron-deficient diet, diet with added mineral form of iron, diet with iron as unhydrolysed protein-iron complex ,diet with hydrolysed protein-iron complex , and diet with hydrolysed protein-iron complex with added Vitamin C respectively. Wistar rats that were made anaemic initially by feeding iron-deficient diet were fed the experimental diets with iron supplements later as a part of study. The study indicated that all the three whey based supplements had better iron regeneration capacity than the positive control. The maximum effect was indicated in hydrolysed protein iron complex with Hb of 14.57 g/dL and 48% increase on replenishing. The average Hb of control group was 13.1 g/dL. Vitamin C was not found to have any additional effect on iron bioavailability than that for hydrolysed protein –iron complex.

Key words: Whey protein concentrate (WPC), Haemoglobin regeneration efficiency (HRE), Relative biological value (RBV), Hydrolysis

Micronutrients are essential for a range of body functions, and their deficiency can lead to critical health conditions, decreased work productivity, and increased risk to infection (Gombart *et al*., 2020). The micronutrient deficiencyy can cause modifications in the elementary building blocks of the body and so is a significant public health concern (Shenkin 2006). One of the most common nutrient deficiency disorder is anaemia which is due to iron deficiency (Savarino *et al.,* 2021). Iron deficiency anaemia is one of the leading contributors to the global disease burden, specifically affecting 42 percent of children below five years of age and 40 percent of pregnant and non-pregnant women (Shubham *et al*., 2020). According to the World Health Organization, anaemia results due to lower haemoglobin levels (lower than 12.0 and 13.0 g/dL in females and males, respectively), and the red blood cells (RBCs) are unable to carry sufficient oxygen required to meet the physiological requirements of the body (Cappellini and Motta, 2015; Turawa *et al*., 2021). Iron deficiency usually results from inappropriate

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dietary intake or poor nutrient absorption. A balance in iron intake and utilisation is critical in maintaining optimum blood levels of iron.

Food fortification is a cost-effective, complementary technique with potential to address many dietary problems (Kiani *et al.*, 2017). Though micronutrient deficiency can be addressed by food fortification, studies on bioavailability and bioaccessibility need to be done for claiming the advantages (Sabatier *et al*., 2020). Bioavailability studies using animal models help in devising food fortification strategies to address nutrient deficiencies such as anaemia. A reliable conclusion can be drawn on the effectiveness of iron supplement based on assessment of the haemoglobin content, liver iron, and haemoglobin regeneration efficiency.

Supplementation of iron is advisable in explicit cases of iron deficiency (Savarino *et al*., 2021). However, the direct addition of iron to food in metal form affects its taste and so new strategies need to be developed for supplementation of iron (Miano and Rojas, 2023). Proteins have a high ability to bind to iron, and bovine whey proteins are often recommended. But bovine whey protein contains allergens like ß-lactoglobulin. Enzymatic hydrolysis of bovine whey protein renders it less allergic and suitable as a hypoallergenic protein-based fortificant for allergic subjects (Lukose *et al*., 2018).

In this study, wistar rats were made anaemic by feeding an iron deficient diet and the iron status of anaemic animals were assessed after feeding a newly developed iron-complexed protein supplement. Further, a comparative evaluation was done on the anti anaemic effects of hydrolysed and unhydrolysed protein-bound iron supplements compared to the standard diet and deficient diet.

Materials and Methods

Whey Protein Concentrate (WPC 80) was procured from Mahaan Proteins, New Delhi. All chemicals used in the present study were of analytical grade. Ferrous sulphate ($FeSO_4.7H_2O$) was procured from Sigma Aldrich, St. Louis, Mo., USA. Trypsin (1:250), sodium hydroxide and L-Ascorbic acid, were all of analytical grade procured from Sisco Research Laboratories, Mumbai. Deionised water was used for all dilution purposes and as drinking water for wistar rats to avoid iron as a contaminant. All iron fortified protein samples were dried in a freeze drier (OPERON -70 °C, Korea) and stored under refrigeration at below 4 °C. A water bath (ROTEK, Ernakulam, Kerala) was used for sample incubation. Acid-washed glassware was used throughout the experiment. Haematology analyser (Orphee, Switzerland) was used for haemoglobin assessment. Iron content in feed, faeces and urine were digested (Microwave digester, Titan MPS, USA) and iron content was determined by Atomic Absorption Spectrometry with graphite furnace (Perkin Elmer, USA). Individual stainless steel cages were used for housing animals. AIN 93 G diet was procured from NIN, Hyderabad, India and proximate analysis was done in the Feed Analysis Laboratory , at Department of Animal Nutrition, Mannuthy, Kerala Veterinary and animal Sciences University (KVASU). The iron estimation was done at CentraI Instrumentation Lab, KVASU which was later confirmed at Radio Tracer Lab, Kerala Agricultural University (KAU).

Table I. Nutritional analysis of experimental diets procured from NIN, Hyderabad

Parameters (%)	AIN93G Purified diet	AIN93G Iron deficient diet
Moisture	2.99	2.98
Total Ash	3.14	3.2
Crude protein	19.39	20.00
Crude fibre	0.07	0.07
Ether Extract	6.20	6.67
Calcium	0.90	0.90
Iron (mg/kg)	70	9.0

All the values except moisture on a dry matter basis

Preparation of hydrolysed and unhydrolysed whey protein concentrate iron complex

WPC -iron (40:1) was incubated in a shaking water bath at 40°C for two hours to effect complex formation followed by freeze drying. Initially a four per cent solution of WPC was prepared on protein basis and ferrrous sulphate was added as iron source. Hydrolysed WPC-iron complex was prepared after enzymatic hydrolysis of WPC

(Lukose *et al*., 2018). Hydrolysis was arrested by heating at 85°C for 15 min before adding iron source. Whey protein concentrate hydrolysate-iron (WPCH-Fe) complex was prepared using the same procedure and WPC-Fe complex with added ascorbic acid was prepared by addition of ascorbic acid with 1:0.5 of iron to ascorbic acid during incubation (Venkatasubramanian *et al*., 2014). All the complexes were freeze dried, characterised for nutritional characteristics and kept under refrigeration until added to purified iron deficient diet to meet the minimum requirement of iron.

Animal models and treatments

This present experiment (Approval No. IAEC/22/04) was conducted in accordance with the recommendations of the Institutional Animal Ethical Committee (IAEC/CVASMTY 3/2022). The sample size was determined using G power software. Thirty-six weaned male Wistar rats of 16 days age were selected from the Small Animal Breeding Station (SABS) at the College of Veterinary and Animal Sciences, Mannuthy, Thrissur for a total experimental period of 35 days. During the initial seven days of acclimatisation, rats were fed on a purified diet (AIN 93 G, NIN, Hyderabad, India). After that, the experimental rats were randomly sub-grouped into two, a standard group (S) with six animals and a deficient group (D) with 30 animals and were fed *ad libitum* AIN 93G diets. For anaemic diet-fed rats (D), all animals were provided a low iron diet (Table 1) for 14 days to induce iron deficiency and were again sub-grouped into five groups as deficient (T2) and iron replenished groups with added $FeSO_4$ (as a positive standard) (T3), WPC-Fe (T4), hydrolysed WPC-Fe (T5) and hydrolysed WPC-Fe with added ascorbic acid (T6).Iron was added to the deficient diet in each treatment as follows

T2-Iron deficient diet continued

T3- Iron added as $FeSO_4$

T4-Iron added as unhydrolysed WPC-Fe complex

T5-Iron added as hydrolysed WPC-Fe complex

T6-Iron added as hydrolysed WPC-Fe complex with added ascorbic acid

Blood was collected from the experimental subsets by retro-orbital plexus puncture on the first day, 14th and 28th day as approved by IAEC. The animals were maintained in individual stainless steel cages. Feed and deionised water were provided *ad libitum*. The feeding trial was conducted for a period of four weeks after the initial adaptation period. Daily feed intake and body weight gain were recorded for the entire period. All the animals were euthanized at the end of the study as per CPCSEA recommendations to remove the liver. The iron content in the liver was estimated by the AAS method (Lobo *et al*., 2011). The blood hemoglobin level was analysed by a hematology analyzer. Hb iron pool and haemoglobin regeneration efficiency were calculated using the equations (1) and (2) given below (Zhang *et al*., 1989). The Relative biological value was calculated from the HRE of different groups compared to that of the standard diet.

Haemoglobin-Fe pool (mg) = body weight

x ml blood/g body weight (assumed to be 0.067 ml)

x g haemoglobin/ml blood

x mg Fe/g haemoglobin (assumed to be 3.35 mg) (1)

HRE = [Haemoglobin-Fe pool (final) - Haemoglobin-Fe pool (initial)]/mg Fe in feed (2)

RBV= (HRE of treatment group/HRE standard group) X 100

Statistical analysis

Comparison between the standard and deficient groups was done by using an independent t-test. Variables like Hb, HRE and liver iron content between six groups were compared by using one-way ANOVA followed by Duncan Multiple Range Test (DMRT) to find out the homogeneity of the groups. Statistical analysis was done using SPSS software version 24.

Results and Discussion

The success of food fortification with iron depends on the factors like medium of fortification, bioavailability of iron, the form and amount of iron compound used, the sensory impact, and presence of inhibitors or enhancers

Table II. Initial haemoglobin level of experimental rats on 14th day

Variable	Standard Diet	Deficient Diet	t-value	P-value
Hb (g/dL)	11.76 ± 0.17	9.83 ± 0.21	4.067**	<0.001

*** Significant at 0.01 level (P<0.01)*

in the fortified medium (Largueza *et al*., 2023). In the present study ferrous sulphate was used as fortificant in all diets as it was reported to have maximum bioavailability by Wu *et al*. (2012). They also studied the effect of different enzymes on iron chelating activity by anchovy muscle protein on hydrolysis and proved that the trypsin hydrolysate possessed the greatest iron-chelating activity among papain, trypsin, pepsin, alcalase, neutrase and flavourzyme. Trypsin enzyme was used for enzymatic hydrolysis and a maximum of five per cent degree of hydrolysis was used based on literature reports on better functional properties and reduced allergenicity (Lukose *et al*., 2018). Ascorbic acid is a known bioavailability enhancer and its effect while complexng protein with iron (T6) was evaluated to study its effect in a complex food system.

Iron absorption in the intestine is reported to be increased by the presence of amino acids in the intestine (Allen, 2006). This led to the possibility that use of a suitable protein in the diet may increase dietary iron absorption. Whey protein concentrate, which is known for functional properties was used as iron binder in this study .Walczyk *et al*. (2014) and Hurrell *et al*. (1989) reported that the relatively high concentrations of calciumand casein inhibits iron absorption and hence WPC which is a concentrated form of whey proteins in milk, free from casein was identified as fortification vehicle in this study. When compared to iron salts, iron–peptide complexes are proven as a source of more bioavailable iron with reduced side effects. Caetano-Silva *et al*., 2015 observed that the iron-binding capacity when evaluated for different enzymes, pH was a critical factor in deciding solubility of iron with an optimum of 7.0 .

The wistar rat (*Rattus norvegicus*) is the most widely used model for studies on iron absorption as it was proven that the absorption mechanism is affected similarly as in humans (Susanti, 2017). The normal Hb values in wistar rats at the particular age could be used as a useful tool to study the effect of different diets used as iron source, since there is a direct relationship between haematological parameters and the iron status of the animal.

The above data clearly indicate that feeding of an iron-deficient diet for two weeks, had significantly reduced the haemoglobin content of the animals in the deficient group. Jacob *et al*. (2018) had noted that for growing wistar rats of one to two months the normal Hb was 11-12g/dL and that of two to three months old male wistar rats was 12-14g/dL.

Hydrolysed WPC iron complexes (T5 and T6) were found to be the most effective in regenerating the iron status, as evident from the HRE values, percentage increase in Hb on replenishing and Hb values of T5 as given in Table 4. When rats were fed with the hydrolysed WPC-Fe added diet as replenishing diet after induced deficiency, the iron absorption rate proved to be higher for animals on deficient diet (D) than that in rats on standard diet in the initial phase (S). A similar result was obtained when the rats were fed on unhydrolysed WPC-Fe diet as replenishing diet compared to the hemin diet (Nakano *et al*., 2007). The results obtained for relative biological value (RBV) and liver iron suggested that iron deposition in the liver of rats fed with the unhydrolysed WPC-Fe diet and hydrolysed WPC-is higher than that in the rats fed with the standard diet. The RBV value of hydrolysed WPC-Fe based diets with 130.22 ± 5.51 and 128.80 ± 8.25[a] (T5 and T6) and unhydro-

Table IIII. Effect of replenishing diet on haemoglobin increase, liver iron content and HRE of experimental rats

Parameter	Standard diet	Deficient group (D) replenished					F-value
	Group (S)	T2	T3	T4	T5	T6	(P-value)
Average Hb 0th day (g/dL)	9.97 ± 0.13						
Average Hb 14th day (g/dL)	11.76 ± 0.17	9.51 ± 0.43	10.07 ± 0.43	10.57 ± 0.59	9.54 ± 0.38	9.49 ± 0.48	4.067** <0.001
Average Hb final (g/dL)	13.1 ± 0.13	10.23 ± 0.34	14.12 ± 0.31	14.57 ± 0.19	14.14 ± 0.31	13.96 ± 0.28	36.531** (<0.001)
% increase in Hb (Total)	31.39	02.60	41.60	46.13	41.82	40.02	-
% increase in Hb on replenishing	11.39	07.57	40.21	37.84	48.21	47.10	-
HRE	0.098 ± 0.004^{bc}	0.081 ± 0.009^{c}	0.113 ± 0.007^{ab}	0.119 ± 0.005^{ab}	0.128 ± 0.005^{a}	0.126 ± 0.008^{a}	7.647** (<0.001)
Liver iron content (mg)	0.378 ± 0.03^{a}	0.159 ± 0.023^{b}	0.458 ± 0.091^{a}	0.375 ± 0.079^{a}	0.402 ± 0.047^{a}	0.403 ± 0.071^{a}	3.307* (0.016)
RBV (%)	100	82.38 ± 9.38^{b}	115.76 ± 7.36^{a}	121.10 ± 5.20^{a}	130.22 ± 5.51^{a}	128.80 ± 8.25^{a}	7.370**

** Significant at 0.01 level (P<0.01) for Average final Hb and HRE

* Significant at 0.05 level (P<0.05) for iron content in liver

Means having different letters as super script differ significantly

lysed WPC-Fe based diet (121.10 ± 5.20^{a}) was numerically greater than the standard diet (100) and ferrous sulphate added diet (115.76 ± 7.36^{a}) in T3 group. For the deficient group (T2), the RBV value of 82.38 ± 9.38^{b}was significantly lower than all other groups. Nakano *et al*. (2007) found that the solubility of iron in the small intestine of rats fed with the unhydrolysed WPC -Fe diet was approximately twice as high as that in rats fed with the hemin diet. The results of present study confirmed this and proved that the bioavailability of hydrolysed WPC-Fe diet was still better. However, there was no beneficial effect added due to ascorbic acid as evident from the HRE values and Hb values of T6 and T5.

From Table III it was clear that there was remarkable iron regeneration efficiency associated with an increase in haemoglobin levels on feeding these iron-supplemented diets. As the direct addition of iron in metal form leads to undesirable reactions to produce off-flavours, unacceptable colours, and metallic taste, it is not generally recommended. Hence, though group T3 exhibited good regeneration efficiency, practically, it cannot be recommended. Metallic or free iron can also cause an upset stomach, constipation, nausea, abdominal pain, vomiting, and diarrhoea (Jackson and Lee, 1991). Villal-pando *et al*. (2006) stated that ferrous sulfate along with vitamin C when added to powdered cow's whole milk reduced the prevalence of anemia but organoleptic alterations reduced the consumer acceptability and keeping quality. Eventhough studies proved the effectiveness of iron fortified milk and milk products in reducing prevalence of anaemia in United States, Chile and China, the negative sensory attributes reduced its acceptability (Mannar and Boy Gallego, 2002).

In the current research, iron was bound to both hydrolysed and unhydrolysed WPC to develop fortified iron supplements T4, T5 and T6. Iron in bound form gave promising results

with respect to flavour, colour and taste when unhydrolysed WPC was used (Shilpashree *et al*., 2016). The results of present study revealed that groups T4, T5 and T6 possessed good iron regeneration ability and the best results were obtained for group T5 . The possibility of using hydrolysed WPC as iron binder was not explored in any previous researches and was taken up in this study. The observations in this study indicated the advantageous effect of hydrolysis on iron absorption.

The addition of ascorbic acid to commercial powdered milk formulae (Stekel *et al*., 1986) or to dried cow milk fortified with ferrous sulfate or ferrous gluconate has been reported to overcome the inhibition and improve iron absorption slightly. The molar ratio at which vitamin C was added is important in deciding its effectiveness as a bioavailability enhancer.As at higher molar ratios (1 : 1 to 1 : 4), iron uptake was inhibited, a ratio of 1:0.5 of iron:ascorbic acid was used in the present study for maximum absorption (Venkatasubramaniam *et al*. 2014). In the present study, the treatment T6 with added ascorbic acid was not found to increase bioavailability and iron absorption as compared to T5 which was also hydrolysed WPC-iron complex but without added ascorbic acid . It might be inferred that impact of meal composition and the characteristics of food matrix must have contributed to the inertness of ascorbic acid with respect to iron absorption (Piskin *et al*., 2022).

Nakano *et al*. (2007) reported that just like haemoglobin, the liver iron contents were also directly affected by iron absorption. The total liver iron content was found to be more for T3,T4, T6 and T5 treatments without any significant difference among them, but the HRE and RBV need to be considered to evaluate the iron status of the animal. Hence, it could be concluded that an iron fortified supplement with better sensory attribute and higher regeneration efficiency could be developed using hydrolysed WPC (T5 and T6) followed by unhydrolysed WPC (T4) and the values were better than standard diet and positive control (T3). The addition of ascorbic acid while iron complexing of hydrolysed WPC was found to be ineffective in enhancing iron absorption. However, the effect of hydrolysis on allergy reduction makes addition of ascorbic acid acceptable for allergic subjects who were hypersensitive to whey proteins (unpublished data). Moreover, extensive study needs to be done on available supplements to find the minimum time span required for raising Hb levels to normal value.

Conclusion

The present study investigated the effect of hydrolysed and unhydrolysed whey protein bound iron supplements on induced anaemia in wistar rats. The results have shown that both supplements are effective in enhancing the iron status of the animal and hydrolysis of WPC significantly improved iron absorption. A 48 percent increase in haemoglobin was observed on replenishing, when hydrolysed WPC was supplemented compared to unhydrolysed WPC-Fe complex with 40 percent Hb increase. Further, the addition of ascorbic acid as a bioavailability enhancer to hydrolysed protein iron complex could not increase the iron bioavailability. A supplement with added ascorbic acid could be considered while formulating a fortified food for milk allergic subjects as they are reported to reduce allergy.

Ethics statement

Study protocols were approved by the Institutional Animal Ethical Committee 328/GO/ReBt-S/Re-L/01/CPCSEA for Research for Education Purpose on Small animals, and the animals were maintained as per the rules and regulations of the ethics committee.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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