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In Vitro Study on the Role of Natural Iron Chelators Incorporated to the Diet on the Control of the Dietary Iron Contribution in the Iron Overload Condition

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***Abstract*—** *The iron overload condition is rising in the world especially in industrial counties. The use of natural iron chelators is proposed as a cheap and safer therapeutic alternative for the treatment of iron overload. The present study was carried out to investigate the effect of the natural iron chelating sources added to the diet on the in vitro bioavailability of dietary iron. Fifteen diet formulations were prepared based on four ingredients namely, red rice, fresh milk, turmeric and black tea against a control diet. The designed diets were subjected to simulated digestion and their effect on bio-availability of iron was tested using the Atomic Absorption Spectrometric method. The results revealed that the bio-availability of the iron had reduced within the range of 14.40% to 69.69% compared to that of the control. The diet containing combinations of turmeric, black tea, and fresh milk showed the highest decrease (69.69%) in the iron bio-availability compared to the control. The results indicated that polyphenols in turmeric and black tea reduced the iron bioavailability in a higher extent in the presence of lactoferrin in fresh milk, as lactoferrin has a protective effect towards the polyphenols. As the incorporation of natural iron chelators significantly reduced the in vitro iron bioavailability in the diets (p values<0.05), it can be concluded that contribution of dietary iron can be controlled by incorporating natural iron chelators to the diet given for individuals having iron overload conditions*.

***Keywords*—** *Dietary intervention: In vitro bio-availability: Iron overload: Natural iron chelators: Potential iron intake.*

I. INTRODUCTION

Iron overload has become a global health problem in recent years especially in industrialized countries (1) and in the older generation (2), (3). The iron overload disease where the absorption of the dietary iron is higher than that of a healthy individual is termed as 'Hemochromatosis'. There are two forms of 'Hemochromatosis' as primary or genetic iron overload and secondary or acquired iron overload (3). Genetic mutations in the gene that causes hereditary hemochromatosis called the HEF gene are found to be the main causative agents in primary iron overload condition while certain non HEF related causes such as hepcidin are also found to be causing genetic hemochromatosis (4), (5). Secondary iron overload conditions are results of pathophysiological conditions such as thalassemia and sickle cell anaemia. Transfused iron and ineffective erythropoiesis that downgrades the action of hepcidin are considered as the leading causes of secondary iron overload (6-8). In addition to that general habits such as heavy consumption of alcoholic drinks (9), iron-fortified foods and red meat (1) are identified as some of the risk factors behind 'hemochromatosis'.

The human body is not equipped to control the excess iron in the body. Thus, excess iron can be accumulated in the body causing damages to vital tissues and organs in both primary and secondary iron overload conditions. Some of the adverse conditions are cardiac complications, liver complications, endocrine complications (diabetes mellitus, hypothyroidism, hypogonadotropic hypogonadism and growth hormone deficiencies), bone complications (osteopenia and osteoporosis) and complications related with fertility and pregnancy (10-12). In addition, when excess iron is found in the body they tend to produce reactive oxygen species via Fenton reactions increasing the oxidative stress in the body (5), (13-15) and depleting the antioxidant stores (16). Therefore it is important to take measures for controlling the iron overload condition to avoid possible consequences which otherwise may occur.

The iron chelation treatments were introduced for the management of iron overload conditions and giving synthetic chelating drugs are common treatments used at present (17). The synthetic chelating drugs are expensive (18-20) and continuous use may result in adverse side effects (21–23). Hence, natural iron chelators are proposed as a cheaper and safe alternative method (24-27). In this context, the use of dietary constituents that possess a strong iron binding ability to suppress the Fenton reactions and other unfavourable effects due to iron overload conditions is being proposed (27). Food sources such as rice bran, turmeric, fresh milk, wheat grass, grapes, berries, pomegranate, green tea and black tea that are rich in phytochelators are considered as suitable good dietary sources with iron chelating properties (26), (27). Nevertheless, devising a dietary intervention for the management of complications of iron overload based on natural iron chelating sources is a novel concept to be introduced for the management of complications associated with iron overload.

In the investigations for alternative and novel iron chelating agents, natural or otherwise, the effect of iron chelators on the dietary iron intake is mostly overlooked. But as hemochromatosis results in an increase in the gastrointestinal absorption of iron(7),(8) it is essential to investigate on the effect of incorporation of natural iron chelators to the diet on the potential dietary intake of iron. This study was conducted to investigate the role of natural

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chelators incorporated into the diet towards the control of dietary iron contribution in the iron overload condition.

II. Methodology

A. Preparation of Diet Formulations

The four dietary sources rich in natural iron chelators namely, turmeric, red rice, fresh milk, and black tea were selected for the analysis. The control diet per day was prepared without including any of known dietary sources rich in natural iron chelators. Then 15 diet formulations were formulated by incorporating four dietary sources to the standard diet plan singly, in combinations of two, three and four. All the food items were purchased from local markets and supermarkets in Malabe, Colombo, Sri Lanka. The meal plans were cooked following general household recipes in Sri Lanka. All the components of the diet formulations, one meal at a time, were combined and homogenized in the food blender to a creamy consistency. After blending each diet was stored at -18°C in separate containers until further analysis (all the diets were subjected to *in vitro* digestion within one week of preparation).

B. In Vitro Gastrointestinal Digestion

The diet formulations that were homogenized to stimulate mastication were subjected to simulated gastrointestinal digestion according to the method published in the previous study(28) and as modified in the previous study(29). In brief, firstly the homogenized samples were completely thawed by keeping them at room temperature and then in 250 ml glass beakers 10g of the homogenized test meals were weighted using analytical balance. Then the samples were mixed with 50ml of 0.9% sodium chloride and 4.0ml of pepsin solution (40mg/ml in 0.1M HCl). The pH of the mixture was adjusted to 2.0 with an addition of 8 ml of 0.1M HCl. After that, the mixture was incubated for 1 h in shaking water bath at 37°C and 100rpm.

For the intestinal phase with dialysis, the dialysis tubing cellulose membrane was cut into 15.0 cm segments, both outer and inner surfaces were rinsed with 0.9% NaCl solution and one end was sealed with clips. After that, the prepared dialysis bags were filled with 5.5 ml 0.5M $NaHCO_3$ and the other end of the dialysis bag were sealed with clips without leaving any air bubbles inside. Then the sealed dialysis bags were immersed into gastric digested samples immediately after digestion. Then the samples were incubated for 45 min in the shaking water bath at 37°C and 100 rpm. After this step, the pH was brought to 6.5 with the addition of $NaHCO_3$ to reflect the transition from gastric phase to the intestinal phase. The pancreatin- bile mixture was prepared by dissolving 2mg/ml pancreatin and 12mg/ml bile extract in 0.1M $NaHCO_3$. Then 18 ml of the prepared pancreatin-bile mixture was added to each digesta and was further incubated in shaking water bath for 2 h at 37°C and 100 rpm. At the end of the incubation period, the dialysis bags were removed from the beakers and were carefully rinsed with water and dried using a paper towel. After that, the content of each dialysis bag was transferred quantitatively into measuring cylinders and diluted to a final volume of 14 ml with 0.9% NaCl. Finally diluted dialyzed fractions were filtered through Whatman filter paper and were stored at -18°C until analysis.

C. The Total Phenolic Content

The total phenolic content of the dialyzed fraction was analyzed using the Folin- Ciocalteu method as published in previous work (30) and modified in another work (31). Firstly 0.5ml of the test sample and 0.1 ml of Folin- Ciocalteu (FC) reagent (1:10 diluted V/V using deionized water) were mixed in glass test tubes and were incubated at dark in room temperature for 15 min. Then 7.5% sodium carbonate was added to the mixture and was incubated again at dark in room temperature for 2 h. The absorbance was measured at 760nm using UV/VIS spectrometer (Thermo Scientific GENESYS 10S Series). The standard curve of gallic acid was prepared at the concentration range 0-250 ppm and the total phenolic content was expressed as µg gallic acid equivalent (GAE) per gram of diet.

D. Iron Bio-Accessibility

The iron contents of the dialyzed fraction of the test meals were estimated following the previously published procedure(32) using Atomic Absorption Spectrometric method after subjecting to wet ashing. Concisely, 0.5ml of the test sample was introduced to the 50ml beaker and then to that 5ml of 65% nitric acid was introduced. After that, the samples were incubated in the water bath at 95°C for 2 h. Successively 2.5 ml of 65% HNO_3 was added to each sample and was incubated in the water bath at 95°C until a clear solution appears. Once a clear solution appeared the samples were removed from the water bath, cooled and were diluted 1:1 using deionized water. The diluted samples were filtered through syringe filters and were stored until analysis. The iron contents of the ashed samples were analyzed using Flame Atomic Absorption Spectrometer (Thermo Scientific iCE 3500). The iron standard curve was prepared at the concentration range 1-5 ppm using ammonium ferrous sulphate. The blank solution contained 0.5ml of deionized water instead of the test sample.

III. Statistical Analysis

All data from the study were presented as mean ±SD of two replicates and the difference between the control and the test meals were analyzed in duplicates using two-sample t-test at 95% confidence interval. Pearson correlation coefficient analysis was used to test the relationship between total phenolic and the iron bio-accessibility. All the analysis were conducted using MINITAB 17 and MS Excel 2013

IV. Results and Discussion

In order to understand the dietary iron contribution from diets, it is important to define mineral bioavailability and bio-accessibility separately as two terms. Mineral bioavailability is defined as the measure of the proportion of the total minerals in food or diet that is digested, absorbed and metabolized by normal pathway and the mineral bio-accessibility as the fraction of the total mineral in the food or diet that is available for uptake by the intestinal brush border cell membrane(33).

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I*n vitro* methods are used to assess the bio-accessibility but the values obtained can establish the trends in bioavailability(33). The *in vitro* digestion of the present study was conducted with the use of dialysis tubing cellulose membranes that represent a simplified model of the epithelial barrier(29). Thus, the content inside the dialysis bag, that is referred as 'digesta' represent the fraction that is potentially bioavailable after the digestion of the diet. Further in the analysis, it was assumed that the increase of iron content due to the addition of dietary iron chelating sources is negligible. Therefore the results represented in table I were inferred under the assumption that undigested test meals contain the same amount of iron as that of the control meal.

As indicated in table I, potential iron intake in all the diet plans, except plan 15 (P15), have decreased compared to that of the control diet. Despite the fact that diet 15 showed an increase in potential intake of iron, the increase was found to be statistically insignificant (p value>0.05) against the control (Table I). Conversely, there was no particular pattern observed in the decrease of the potential iron intake with the incorporation of the food sources rich in iron chelators. The effect due to in cooperation of red rice to the diet was not detectable under the conditions of the present analysis and as such a more sophisticated analysis using ICP-MS may provide a better explanation.

TABLE I. The mean potential intake of iron and the percentage increase/ (decrease) of the potential iron intake in each diet plan and its statistical significance.

No	Food type	Mean potential iron intake in ppb per gram of the diet Mean ± SD	Percentage Increase/ (decrease) of the potential iron intake	Statistical significance against the control
C1	Control	31.64±1.16	-	-
P15	Turmeric +red rice +fresh milk+ black tea	43.58±0.53	37.77%	P value>0.05
P14	Turmeric +red rice + black tea	19.18±1.01	(39.88%)	P value<0.05
P13	Turmeric+ red rice + fresh Milk	25.69±0.88	(18.78%)	P value>0.05
P12	Red rice + fresh milk + black tea	13.70±0.64	(56.69%)	P value<0.05
P11	Turmeric + fresh milk +black tea	9.59±0.87	(69.69%)	P value<0.05
P10	Red rice + fresh milk	11.70±0.16	(62.84%)	P value<0.05
P9	Red rice + black tea	18.70±1.56	(40.92%)	P value<0.05
P8	Turmeric + black tea	27.08±0.85	(14.40%)	P value<0.05
P7	Fresh milk + turmeric	13.45±0.40	(57.49%)	P value<0.05
P6	Red rice + turmeric	22.76±0.65	(28.04%)	P value>0.05
P5	Fresh milk + black tea	12.47±1.59	(60.57%)	P value<0.05
P4	Turmeric	19.86±0.55	(37.24%)	P value>0.05
P3	Red rice	ND	ND	ND
P2	Black tea	16.64±0.25	(47.42%)	P value<0.05
P1	Fresh milk	11.70±0.03	(63.04%)	P value<0.05

The results indicate that at 95% confidence level the potential iron intake had decreased within the range of 14.40% to 69.69% compared to that of the control. The decrease of potential intake of iron in the diet containing only fresh milk (63.04%) was found higher than all the other diet plans that contained one source of chelators. This could be attributed to the presence of both calcium and lactoferrin in fresh milk. Calcium can compete with iron and inhibit the release of iron from the food systems in the lumen of the small intestine (34), (35) and lactoferrin in milk and in synovium fluid exhibit the ability to decrease the potential iron intake in adults (36), (37). But studies have stated that the role of lactoferrin in iron absorption is doubtful and the mode of action of lactoferrin in the decrease of potential iron intake to be unknown (36), (37).

In all the diet plans that contained red rice and black tea, a comparatively higher decrease in the potential iron intakes were observed as well. The fiber in red rice decreases the bioavailability of iron by entrapping iron molecules in fiber network (38). In addition to fiber, phytic acid, most prominent polyphenol in red rice, has the ability to bind with iron and forms insoluble phytate complexes reducing the bioavailability of iron (38), (39). This same action of phytic acid to bind with iron makes it one of the most effective iron chelators in nature (40), (41). The action of polyphenols in tea is same as that of polyphenol, phytic acid. The tea polyphenols limit the availability of iron by forming an iron-polyphenol complex (42), (43).

The diet plan that showed the highest decrease in the potential iron intake was the one to contain turmeric, black tea, and fresh milk. Turmeric, when added to the diet alone showed no significant decrease in the iron bioavailability when compared to that of the control diet. Curcumin, the active group in turmeric, demonstrate iron chelation ability by binding with Fe^{2+} and Fe^{3+} in the body fluids such as serum (44-46). But no sufficient evidences are available to explain the effect of curcumin on iron in the food matrix. The results from the present study showed that curcumin when present alone had little effect toward the decrease of iron in food matrix but once it was complemented with other sources rich in iron chelators it had an enhanced effect on the decrease of iron bioavailability. This enhanced effect on the decreased iron bioavailability in both turmeric and black tea can be attributed to the presence of lactoferrin in milk. Availability of polyphenols decreases considerably in the process of digestion (29). But lactoferrin has a protective effect towards polyphenols (47). Thus, in turn in the presence of lactoferrin, polyphenols in turmeric and black tea reduce the iron bioavailability in higher magnitude than when present alone.
Even though the reduction of the iron bioavailability was mostly attributed to the polyphenols present in the digesta

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there was no significant (p value>0.05) correlation observed between iron bioavailability and total phenolic content. Further at 5% level of significance, no significant linear, quadratic or cubic relationships were observed between the two variables. Based on the results of the correlation analysis it could be inferred that the decrease of the potential iron intake with the incorporation of the sources rich in iron chelators to be a combined function of many functional groups present in the diet not only a function of polyphenols.

Yet as the analysis was designed to identify most effective chelator or combination of chelators, the study design may not be adequate to model the relationship between the total phenolic content and the potential iron intake. There may be other nonlinear relationship between the two variables (e.g.- Fourier relationship). But the presence of many diverse functional groups in the digesta makes the modeling of the relationship between the two variables complicated. More sophisticated study designs based on the findings of the present study can be of importance in studying the relationship between polyphenols and the potential iron intake in the diet. In addition, Kim, Ham, Shigenaga, & Han, (2008) have found that polyphenols accumulated outside the intestine prevent the exit of iron through the cell line to the bioavailable fraction. Thus, the decrease of the iron bioavailability could be a function of polyphenols remained in the intestinal fraction without being absorbed. Hence it requires further investigations on polyphenol content and iron bioavailability at each stage of digestion in the presence of iron chelators to arrive at an appropriate conclusion.

V. Conclusion

In conclusion, it can be stated that the potential intake of dietary iron can be decreased through the incorporation of dietary sources to the diet such as fresh milk, turmeric and black tea that are rich in natural iron chelators. Natural iron chelators can reduce the bio-availability of the dietary iron subsequently declining the complications associated with the iron overload condition. Thus, the sources rich in natural iron chelators can be used in dietary interventions to control complications associated with iron overload conditions. Further, it can be concluded that the effect of the iron chelating sources on the dietary iron contribution can be a multi-factorial outcome of many functional components present in the food system alongside polyphenols. Additionally, further studies are recommended to investigate into the functional groups responsible for the decrease in the potential iron intake and the effect of polyphenols on the dietary iron contribution in each stage of digestion using cell lines and then successively using animal and human models.

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Liyanapathirana, Navoda Nirmani, Ranaweeraa, KKDS, and Gunathilake, KDPP, "*In vitro* study on the role of natural iron chelators incorporated to the diet for control of the dietary iron contribution in iron overload condition," *International Research Journal of Advanced Engineering and Science*, Volume 4, Issue 1, pp. 179-183, 2019.