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Prevention and amelioration of erythrocyte instability observed under deficiency of vitamin B_{12} alone or combined with micronutrient limitation through dietary supplementation with *Chlorella* and *Spirulina*

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Micronutrient rich microalgae, *Chlorella* and *Spirulina*, could be natural food supplements to overcome the micronutrient deficiency, increasingly recognised as a global health issue. In two independent experiments, the *Spirulina* and *Chlorella* were evaluated as prophylactic and ameliorative dietary supplements of vitamin B_{12}. Erythrocyte stability (relative osmotic fragility and haemolysis percentage), haematological parameters, micronutrient deficiency (serum levels of iron, zinc), plasma vitamin B_{12} and vitamin B_{12} biomarker (methylmalonic acid) were analysed. The deficient groups receiving *Spirulina* and *Chlorella* as prophylactic dietary supplements showed a 1.34 to 1.41 folds increase in serum iron and a 2.13 to 2.19 folds increase in plasma vitamin $B_{12,}$ compared to B_{12} deficient group. Supplementation of *Spirulina* to ameliorate vitamin B_{12} deficiency combined with micronutrient limitation showed an increase of 1.14 folds and 1.2 folds in serum iron and zinc respectively and 1.51 folds in plasma vitamin B_{12} compared to the deficient group. The relative osmotic fragility of erythrocytes in deficient experimental animals was 17 to 45% higher compared to the control. The osmotic fragility and deformation in the morphology of erythrocytes observed under vitamin B_{12} deficiency, alone or in combination with micronutrient limitation, were prevented and ameliorated on dietary supplementation with the microalgal biomass.

Keywords: Microalgae, Micronutrients, Relative osmotic fragility, RBC stability

One of the major public health crises affecting more than 2 billion people worldwide is micronutrient (vitamins and minerals) deficiency[1]. Though required in small amounts, micronutrients are essential for the proper growth and development of human beings[2], as they serve as cofactors and coenzymes[3] in various metabolic processes. The erythropoietic micro-nutrients, mainly vitamin B_{12}, iron, folate, and zinc, are required for active erythropoiesis. Erythropoiesis under the deficiencies of these erythropoietic micronutrients is termed ineffective, leading to decreased erythrocyte production and subsequent decline in the number of circulating erythrocytes manifested as anaemia. The deficiencies of these erythropoietic micronutrients can also adversely affect the stability of erythrocytes[4].

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The erythrocytes/red blood cells (RBC) (the terms erythrocyte and RBC have been used interchangeably throughout the manuscript) play an essential role in transporting oxygen to meet the oxygen requirement of various tissues. The integrity maintenance of the RBC membranes is essential for their normal function. Any variations in the physicochemical properties of the membrane or changes in surrounding microenvironmental composition can make the RBC dysfunctional, thereby hampering the oxygen transport to various tissues[5].

The membrane stability is also crucial for RBC as these cells continuously circulate through capillaries much smaller than their cellular dimensions. For example, when RBCs flow through the blood vessels of only about 20 microns diameter in the brain, they must stretch into bullet shape to squeeze through and return to their native form after leaving the vessel[6]. Well-known factors that affect RBC stability are diet, temperature, age, use of drugs, and some physiological and pathological conditions[7]. Lack of membrane

stability can lead to a change in morphology of RBCs resulting in anaemia. Supplementation of micro-nutrients has been reported to enhance the fluidity of the RBC membranes[8], which plays a vital role in determining membrane stability. Micronutrients, i.e., zinc, and iron, have a significant role in metabolic function and tissue maintenance and act as essential cofactors for developing, maintaining, and expressing the natural body defence system[9]. Iron is an integral component of the haem for the structural integrity of the RBC, and it gives a reliable and stable shape to RBC[10]. Zinc is an antioxidant that plays an essential role in the membrane stability of erythrocytes[11].

Vitamin B_{12} plays a critical role in erythrocyte formation[4], and its deficiency may potentially affect the RBC stability. However, vitamin B_{12} deficiency influence on erythrocytes stability has not been reported. The microalgae *Spirulina* and *Chlorella* have been recognised as nutraceuticals by Food Safety and Standards Authority of India (FSSAI)[12]. Both *Spirulina* and *Chlorella* are well known for their micronutrient content,[13,14] and further clinical studies have shown *Spirulina* and *Chlorella* to overcome micronutrient deficiency[15] and promote the health[16]. These microalgae have been reported to contain natural forms of vitamin B_{12}, i.e., Methylcobalamin[17,18,] which has been shown to be bioavailable *in vivo* model[19,20]. Therefore these microalgae can be used as a natural supplement to meet the dietary requirement of erythropoietic micronutrients *viz*., iron, zinc and vitamin $B_{12.}$ The effect of microalgal biomass as a dietary supplement on erythrocyte stability has not been reported.

Two independent experiments *in vivo* were carried out. The first experiment evaluated the prophylactic effect of *Spirulina* and *Chlorella* biomass as a dietary supplement to prevent vitamin B_{12} deficiency and erythrocyte instability. The second experiment evaluated the ameliorative effect of *Spirulina* biomass as a dietary supplement to overcome the vitamin B_{12} deficiency and erythrocyte instability under micronutrient limitation. The haemoglobin and serum iron levels were measured to assess the iron deficiency. The serum zinc levels were analysed to assess the zinc deficiency. Plasma vitamin B_{12} and Methyl Malonic Acid (MMA) levels were measured to assess the vitamin B_{12} deficiency. The osmotic fragility test was used to evaluate the RBC stability.

Materials and Methods

Chemicals, and microalgal biomass

Standard vitamin B_{12} (cyanocobalamin) was procured from Sigma-Aldrich (India). NaCl and other chemicals were supplied by Himedia, India. *Spirulina* biomass was supplied by Wellisen Nutraceuticals, Mysuru, India. *Chlorella* biomass was produced in outdoor open raceway ponds at CSIR-CFTRI, Mysuru, India.

Extraction and estimation of vitamin B_{12}

Vitamin B_{12} content of *Spirulina* and *Chlorella* biomass was estimated after extraction using standardized microbiological assay protocol[17,18].

Animals

Forty-eight healthy male albino Wistar rats weighing in the range of 50-60 g were used for the study. The experimental study was approved by the CSIR-CFTRI Institutional Animal Ethical Committee (IAEC No: 59), which follows the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Registration No: 49/Go/ReBi/s/1999/CPCSEA), Government of India, New Delhi, India.

Experimental protocol

Evaluation of Spirulina and Chlorella biomass as a prophylactic dietary supplement to prevent vitamin B_{12} deficiency (*Experimental condition 'B'*)

The rats were separated into four groups (Gr.), with six animals in each group. Rats in Gr. I served as control (basal diet AIN-93)[21], and Gr. II as B_{12} deficient group (AIN-93 diet devoid of vitamin B_{12}). Rats in Gr. III and IV received vitamin B_{12} deficient AIN-93 diet supplemented with *Chlorella* and *Spirulina* biomass, respectively, as the prophylactic dietary supplement of vitamin $B_{12.}$ The *Chlorella* and *Spirulina* biomass concentration used to supplement the deficient diet was 83 g/kg diet and 65 g/kg diet, respectively, as optimised in our previous studies[19,20]. These concentrations of the microalgal biomass provide 25 µg vitamin B_{12} /kg of diet equivalent to the vitamin B_{12} content in the control diet (Basal AIN 93 diet)[21]. All other conditions were maintained as detailed in earlier studies[19,20].

Evaluation of Spirulina biomass as an ameliorative dietary supplement to overcome vitamin B_{12} deficiency combined with micronutrient limitation (*Experimental Condition 'M'*)

The rats were separated into four groups (Gr.), with six animals in each group. Rats in Gr. I served as control and received basal diet AIN-93[21]. Gr. II, III and IV were subjected to vitamin B_{12} deficiency combined with micronutrient limitation (i.e., fed with

the diet which was completely devoid of vitamin B_{12} and contained 50% of minerals and other vitamins compared to the control diet) for 90 days. Hence, Fe and Zn levels in the deficient diet were 0.5 g/kg and 0.43 g/kg of diet. The micronutrient deficiency in experimental animals was confirmed by analysing Fe, Zn, and vitamin B_{12} levels in the serum and plasma at the end of the 90 days. The Gr. II served as the deficient group and continued to receive the same deficient diet, Gr. III received the deficient diet supplemented with *Spirulina* biomass (65 g/kg diet) and the Gr. IV received a standard AIN-93 diet for the next 45 days. The Fe and Zn levels in the *Spirulina* supplemented deficient diet in Gr. III remained comparable to deficient diet (Gr. II) with a minor increase of 3% in iron content and a 0.3% increase in zinc content. However, the vitamin B_{12} levels in the *Spirulina* supplemented deficient diet in Gr. III was equivalent to Gr. I & IV i.e., 25 µg/kg diet. No substantial change in the proximate composition of diet upon the inclusion of *Spirulina* biomass, except for a 2-3% decrease in carbohydrates and a 1-2% increase in protein, was observed. All other conditions were maintained as detailed in earlier studies[19,20].

Sample preparations

In experimental condition 'B', after 70 days and in experimental condition 'M', after 135 days, the blood was collected from rats by the retro-orbital method under anaesthesia with CO_2 for biochemical analysis. Heparin was used as an anticoagulant, and collected blood samples in heparin tubes were mixed gently to obtain a homogeneous suspension of the blood cells. The haematological parameters of the blood samples were analysed by a haematology analyser (Sysmex, USA). Blood collected without adding heparin was left for two hours to clot. Further, the unclotted and clotted blood was centrifuged at 2000 rpm for 20 min at 4°C (Hettich, Model: Universal-320R) to obtain plasma and serum. The obtained plasma and serum were used for further analysis.

Biochemical parameters

Plasma vitamin B_{12} content was measured using Rat vitamin B_{12} ELISA assay kit (KinesisDx, USA) according to the manufacturer instructions. Iron, zinc, IgA, and IgG levels were analysed in serum using a commercially available standard assay kit (Agappe Diagnostics Ltd., India).

Osmotic fragility test of erythrocytes

The resistance of RBCs to haemolysis under osmotic stress is determined by their osmotic fragility (OF). The osmotic fragility test is a useful tool to evaluate the sensitivity of RBC to environmental osmotic changes. The test is widely used to elucidate the mechanisms that affect the properties of red blood cell membranes[22]. The osmotic fragility test was used in the present study to evaluate the stability of the erythrocytes.

Relative osmotic fragility of erythrocytes was estimated by spectrophotometric measurements of the haemolysis, i.e., the release of the free haemoglobin when RBC was treated with water and different NaCl concentrations. Briefly, the RBC was washed and centrifuged three times with 5 mM phosphate buffer saline (pH 7.4) at 2000 rpm for 15 min. After washing, the RBC was re-suspended in phosphate buffer saline (0.9% NaCl) to obtain the same initial blood volume. The prepared RBC (10 µL) was added to water and 5 mM phosphate buffer (pH 7.4) having different NaCl concentrations (0.1-0.9%). The NaCl concentration of 0.9% was considered isotonic. After gentle mixing for a minute, the suspension was allowed to stand for 20 min at room temperature (25±2°C), then centrifuged at 2000 rpm for 20 min, and absorbance of the supernatant was measured at 540 nm[23]. The supernatant haemoglobin content was used as an indicator for determining relative osmotic fragility. The relative osmotic fragility of erythrocytes of each group of rats was calculated as per equation (1) by considering fragility in water as 100%.

$$Relative\ osmotic\ fragility = \left[\frac{Hb\ Trt}{Hb\ water}\right] * 100 \quad \ldots (1)$$

where, Hb_{Trt} is the average free haemoglobin concentration in the supernatants of the saline-treated samples. Hb_{water} is the average haemoglobin concentration of the RBC when suspended in water. Relative osmotic fragility of rat erythrocytes at each NaCl concentration was plotted for control, deficient diet, and microalgae supplemented diet groups of rats.

Haemolysis assessment and percentage calculation

The percentage of haemolysis was calculated by considering haemoglobin content of the supernatants (obtained after treating at different NaCl concentrations) and haematocrit (obtained through haematology analyser) values using equation (2) as reported earlier[24].

$$\%\ \text{Haemolysis} = \frac{[(100 - Haematocrit) * Hb_{sample}]}{Hb_{isotonic}} \quad \ldots (2)$$

$Hb_{isotonic}$ is the average haemoglobin concentration of RBC in supernatant obtained in isotonic solution (0.9% NaCl). Hb_{sample} is the Haemoglobin

concentration of the supernatant obtained in the sample (different NaCl concentrations). Haematocrit is the volume percentage of RBC in the blood.

Statistical analysis

All the data values were represented as Mean ± Standard deviation (n=3); all values were analysed using Minitab 18 software. One-way ANOVA followed by multiple comparison using Tukey's post hoc test was conducted. P <0.05 (n=3) was considered as statistically significant.

Results

***Spirulina* and *Chlorella* biomass as a prophylactic dietary supplement to prevent vitamin B_{12} deficiency (Experimental condition 'B')**

The vitamin B_{12} content of the *Chlorella* and the *Spirulina* biomass used in the study was estimated as 30.66±2.08 µg/100 g (w/w), and 38.16±1.75 µg/100 g (w/w), respectively. The vitamin B_{12} deficient group showed a two-fold increase in plasma MMA levels, about 50% decrease in plasma vitamin B_{12} levels and about 25% decrease in haemoglobin and serum iron levels compared to control (Table 1). However, as shown in the Table 1, these parameters were comparable to control in the groups receiving vitamin B_{12} deficient diet supplemented with the optimised levels of *Chlorella* and *Spirulina* biomass i.e., 83 g/kg diet[20] and 65 g/kg diet[19] respectively, as a prophylactic source of vitamin B_{12}. These results are in accordance with the results reported earlier[19,20].

The results of the osmotic fragility (OF) test conducted to evaluate the stability of rat erythrocytes are presented in Fig. 1. In general, RBC fragility decreases with increasing NaCl concentration from

Table 1 ─ Haemoglobin, serum iron, plasma vitamin B_{12} and MMA profile under experimental condition 'B'

Parameters	Groups			
	Control	Vitamin B_{12} Deficient	*Chlorella* supplemented	*Spirulina* supplemented
Plasma vitamin B_{12} conc. (ng/L)	520.20±11.01^{b}	252.69±1.46^{c}	537.82±4.68^{a}	552.10±4.48^{a}
Haemoglobin (g/dL)	20.80±0.69^{a}	14.83±1.19^{b}	21.10±0.28^{a}	21.06±0.41^{a}
Serum Iron (mg/dL)	209.50±14.67^{a}	160.64±44.21^{b}	226.49±33.3^{a}	214.95±15.46^{a}
Plasma MMA (nmol/L)	51.23±10.01^{b}	118.01±0.28^{a}	55.66±1.85^{b}	56.83±1.59^{b}

[Values are presented as mean ± SD, ^{abc} superscript indicate the values with significant difference (P <0.05)]

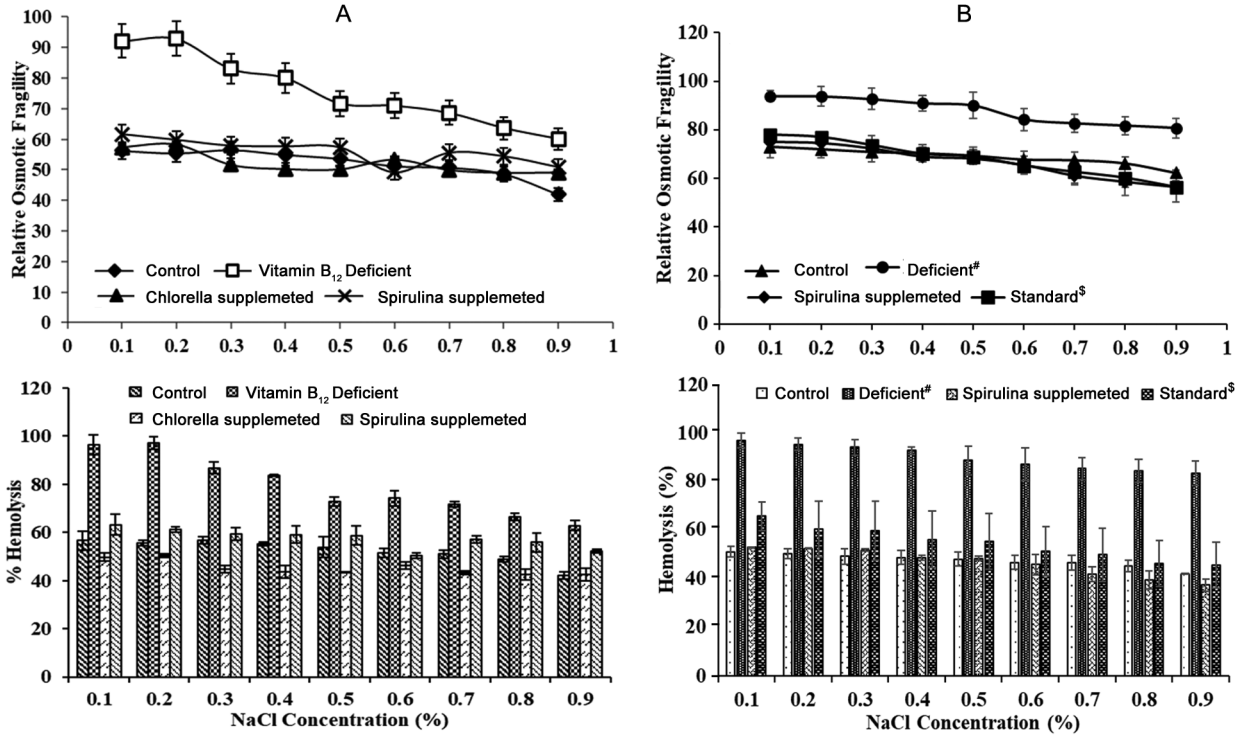

Fig. 1 — Relative osmotic fragility and Hemolysis percentage at various saline concentrations of erythrocytes from rats fed with (A) vitamin B_{12} deficient and microalgae supplemented diet; and (B) micronutrient deficient (including vitamin B_{12}) and *Spirulina* supplemented diet. [Note: #indicates diet which was completely devoid of vitamin B_{12} and contained 50% of minerals and other vitamins compared to control; $^{\$}$indicates deficient group fed with AIN-93 diet. Error bars indicates standard deviation, n=3; P <0.05]

0.1 to 0.9 %. Further, the vitamin B_{12} deficient group showed higher relative osmotic fragility at all the NaCl concentrations than the control group, with highest osmotic fragility of 93% at lower NaCl concentrations (0.1 and 0.2%). The osmotic fragility of RBC of vitamin B_{12} deficient groups receiving *Spirulina* and *Chlorella* biomass supplemented deficient diet were comparable to the control group.

In addition, the vitamin B_{12} deficient group showed 50% of the RBC with abnormal morphology *viz.* 3-6% half-moon shape, 14-17% elliptocytes, 1-4% acanthocytes, 18-20% teardrop shape, and 3-5% crescent shape. The groups receiving vitamin B_{12} deficient diet supplemented with *Spirulna* and *Chlorella* biomass as a prophylactic source of vitamin B_{12} showed 84-94% of the RBCs with normal morphology, which was comparable to the control group. The results indicate that the *Spirulina* and *Chlorella* biomass were effective as a prophylactic source of vitamin B_{12} and prevented the instability and deformation in the morphology of RBC seen under vitamin B_{12} deficiency.

***Spirulina* biomass as an ameliorative dietary supplement to overcome vitamin B_{12} deficiency combined with micronutrient limitation (Experimental Condition 'M')**

A 20.5% decrease in serum iron content was observed in the deficient group (Gr. II) subjected to the vitamin B_{12} deficiency combined with the micronutrient limitation compared to the control group. The serum iron content in Gr. III and Gr. IV, receiving the deficient diet supplemented with *Spirulina* biomass and the standard diet respectively after 90 days of administration of the deficient diet were restored to 90% level of the control group (Table 2).

Further, the deficient group (Gr.II) showed a 22% less serum zinc content than the control group. However, the Gr. III receiving the deficient diet supplemented with *Spirulina* biomass showed restoration of the serum zinc content to 95% level of the control group. (Table 2). IgA and IgG immunoglobulin levels increased in the deficient group (Gr.II) by 1.75 fold and 1.61 fold, respectively, compared to the control group (p=0.004, p=0016). On the other hand, the IgA and IgG immunoglobulin levels of the deficient groups receiving the deficient diet supplemented with *Spirulina* biomass (Gr. III), and the standard diet (Gr. IV) was comparable to the control group (Table 2).

The erythrocytes of the deficient group (Gr. II) showed 40% less haemoglobin content compared to the control group (p=0.033). The administration of *Spirulina* biomass supplemented deficient diet, or the standard diet, to the deficient groups (Gr. III and Gr. IV, respectively) resulted in a 1.5-fold increase in the erythrocyte haemoglobin content, thereby restoring the haemoglobin levels to 92-97% level (p=0.033) of the control group (Table 2).

Further, plasma vitamin B_{12} content in the deficient group (Gr. II) was 40% less than the control group. However, on the feeding of the *Spirulina* supplemented deficient diet or the standard diet, the plasma vitamin B_{12} levels of the deficient groups (Gr. III and Gr. IV) showed recovery with values being 92% of the levels of the control group (p=0.011) (Table 2).

The deficient group (Gr. II) showed 1.27 folds increase in relative osmotic fragility (93%) and 1.9 folds increase in haemolysis (97%) compared to the control group. In the case of deficient groups receiving the *Spirulina* supplemented diet or the standard diet (Gr. III and IV, respectively), the values were comparable to control, as shown in Fig. 1.

The morphology of the RBC in the deficient group (Gr. II) showed about 54% RBCs with abnormal morphology *viz.*, 9-12% half-moon shape, 13-15% elliptocytes, 2-4% acanthocytes, 14-17% teardrop shape, and 5-6% crescent shape. The control group showed about 90-98% RBC with normal morphology. The deficient group administered with a deficient diet supplemented with *Spirulina* biomass (Gr. III),

Table 2 — Serum micronutrients, immunoglobulins, haemoglobin and plasma vitamin B_{12} profile under experimental condition 'M'

Parameters	Groups			
	Control	Micronutrient Deficient[#]	*Spirulina* supplemented	Standard (AIN 93) diet
Serum Zinc (µg/dL)	92.48±10.04^{a}	72.71±4.40^{ab}	88.42±7.89^{ab}	83.73±2.34^{b}
Serum Ig A (mg/dL)	36.11±5.55^{b}	62.33±13.05^{a}	32.00±2.40^{b}	43.67±2.60^{ab}
Serum Ig G(mg/dL)	137.70±26.5^{ab}	222.10±40.3^{a}	127.60±29.1^{b}	116.40±33.9^{b}
Serum Iron (mg/dL)	131.33±3.51^{a}	104.38±5.93^{c}	119.62±2.84^{ab}	117.38±7.05^{bc}
Haemoglobin (g/dL)	12.23±0.80^{a}	7.97±1.85^{b}	11.96±0.85^{ab}	11.27±2.19^{ab}
Plasma vitamin B_{12} (ng/L)	844.80±127.2^{a}	511.50±64.1^{b}	774.90±79.5^{a}	776.40±94.8^{a}

[Values are presented as mean ± SD, [#]indicates group fed with the diet which was completely devoid of vitamin B_{12} and contained 50% of minerals and other vitamins compared to control; and ^{abc} superscripts indicate the values with significant difference (P <0.05)]

showed only about 20-25% of RBC with abnormal morphology. The RBC of the deficient group receiving standard AIN-93 (Gr.IV) diet also showed morphological recovery, with about 85% RBC showing normal morphology (Table 3 and Fig. 2).

Discussion

Vitamin B_{12} is involved in the processes which are essential in the erythroblast maturation i.e., DNA synthesis, essential amino acid methionine synthesis, and one-carbon metabolism[25]. Further, micronutrients play an essential role in metabolism, development, maintenance, and defence systems[26]. The consumption of a diet with insufficient vitamins and minerals leads to micronutrient deficiency[27] affecting the biological processes. A single micronutrient deficiency may coexist with other micronutrients, and evaluating concurrent deficiencies is important as coexisting deficiencies of other micronutrients may lead to worse consequences than with a single micronutrient deficiency as observed with anaemia[28]. Therefore, it is relevant to assess the effect of deficiency of one micronutrient when other micronutrients are limited.

In general, it is challenging to achieve induction of 100% vitamin B_{12} deficiency in rats because of the endogenous storage of vitamin B_{12}[29]. In the present study, feeding a vitamin B_{12} deprived diet resulted in approximately a 40 to 52% decrease in plasma vitamin B_{12} levels in rats, under both the experimental conditions 'B' and 'M', compared to the control group. Homocysteine and methylmalonic acid (MMA) are the functional biomarkers indicating a vitamin B_{12} deficiency. A two-fold elevation of the plasma MMA levels observed in the vitamin B_{12} deficient group under experimental condition 'B' also confirmed the vitamin B_{12} deficiency. It has been reported that vitamin B_{12} deficiency inhibits purine and thymidylates synthesis[4] and causes ineffective erythropoiesis, which can lead to anaemia. The vitamin B_{12} deficient group in experimental condition 'B' also showed approximately 25% decrease in iron and haemoglobin (one of the iron deficiency indicators) contents compared to the control. In the experimental condition, 'M', the serum iron and zinc levels decreased by 20.5 and 22%, respectively, in the deficient group compared to the control group (Table 2).

Iron plays a vital role in haemoglobin biosynthesis, proper functioning of the immune system, and cellular metabolism[10]. Iron and vitamin B_{12} correlation has a significant role in erythropoiesis[4,30]. Haem is an integral part of the haemoglobin, and RBC is made up of haemoglobin. It has been reported that vitamin B_{12} deficiency has always been associated with iron deficiency[4]. Lowered levels of iron and haem contents could be due to the insufficient vitamin B_{12} availability in the body, which results in the ineffective synthesis of erythrocytes.

Table 3 — Morphological changes in RBC under different experimental conditions

Cell type	% RBC of various morphology under experimental conditions							
	B Condition groups				M Condition groups			
	Control	Vitamin B_{12} deficient	*Chlorella* supplemented	*Spirulina* supplemented	Control	Micronutrient Deficient#	*Spirulina* supplemented	Standard (AIN 93) diet
Half moon	<1%	3-6%	2-3%	-	<1%	7-9%	4-6%	5-7%
Normal cells	95-98%	40-50%	84-87%	90-94%	90-98%	54-56%	75-81%	85%
Elliptocytes	<1%	14-17%	4-5%	2-4%	<1%	6-8%	4-7%	3-5%
Acanthocytes	-	1-4%	1-2%	1-2%	-	4-5%	2-6%	-
Teardrop	<1%	18-20%	2-4%	1-2%	<2%	15-20%	5-8%	2-4%
Crescent	-	3-5%	<1%	-	1-2%	4-5%	1-2%	1-2%

[The percentage values represented in the table are the average of three representative photographs; and #indicates diet which wa completely devoid of vitamin B_{12} and contained 50% of minerals and other vitamins compared to control]

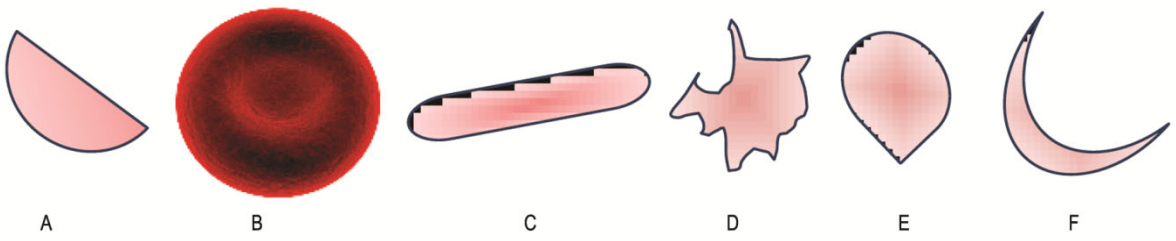

Fig. 2 — RBC morphology. Different cell types and their shape. (A) Half moon; (B) Normal cells; (C) Elliptocytes; (D) Acanthocytes; (E) Teardrop; and (F) Crescent

Zinc has a significant role in maintaining DNA integrity, immune response, stabilisation of RBC membrane, and haem synthesis. Zinc deficiency leads to anaemia, inadequate immune response to antigens, chronic inflammation, and oxidative stress[31]. Zinc also affects RBC lifespan[11].

In the deficient group under experimental condition 'M', increased serum immunoglobulins, IgA (1.94 folds), and IgG (1.64 folds) were observed. A correlation between the increased levels of serum immunoglobulins and low zinc levels has been reported earlier[32] (Table 2). Previous reports have also shown elevated serum immunoglobulin levels in undernourished conditions[26].

The relative osmotic fragility and haemolysis tests were used in the present study to evaluate the stability of erythrocytes. The results showed higher relative osmotic fragility and haemolysis of RBC in the deficient groups in both the 'B' and 'M' experimental conditions. These results indicate that erythrocytes were more prone to lysis even to a slight change in the osmotic environment under deficiency conditions. It can be inferred that in their microenvironment, a deficiency in vitamin B_{12}, zinc and iron may significantly affect the membrane characteristics as previously reported for zinc deficiency[11]. Further, it was reported that accumulation of MMA in vitamin B_{12} deficient patients produce free radicals, which may increase oxidative stress[33] and cause RBC instability. Inefficient RBC synthesis caused by the deficiency of erythropoietic micronutrients (Iron, Zinc, and Vitamin B_{12}) could also be one of the reasons for RBC instability.

The morphology of RBC can also be used as an indicator of its stability. In the deficient groups of both 'B' and 'M' experimental conditions, 50-54% of RBC showed abnormal morphology (Table 3). The typical bi-concave structure of RBC provides a higher surface area to absorb more oxygen and exchange it with carbon dioxide through the haemoglobin. If there is a change in RBC morphology for any reason, it will reduce its oxygenation and deoxygenation capacity. Anaemia has been shown as one of the red blood cell diseases that cause changes in RBC morphology in various ways[34]. The changes observed in RBC morphology in the present study in the animals subjected to vitamin B_{12} deficiency, alone or combined with micronutrient limitation, suggest a link between the vitamin B_{12} deficiency and a loss of RBC stability.

In the experimental condition 'B' the plasma vitamin B_{12} levels and plasma MMA levels of the deficient groups receiving *Chlorella* and *Spirulina* biomass as a prophylactic dietary supplement of vitamin B_{12}, were comparable to the control group. In the experimental condition 'M', the plasma vitamin B_{12} levels of the deficient group fed with *Spirulina* biomass supplemented deficient diet normalised to 92% of the levels of the control group. These results were as expected since the vitamin B_{12} bioavailability from *Chlorella* and *Spirulina* biomass has been reported earlier[19,20].

The haemoglobin and serum iron levels of the *Chlorella* and *Spirulina* supplemented deficient groups were also comparable to control in the experimental condition 'B'. In the experimental condition 'M', the *Spirulina* supplemented and standard diet fed deficient groups showed an increase of 14 and 12% in serum iron, respectively, compared to the deficient group. This led to the normalisation of the serum iron levels to about 91% of the control in the *Spirulina* supplemented diet fed deficient group, which was equivalent to the serum iron levels observed in the deficient group fed with the standard diet.

Similarly, under experimental condition 'M', the *Spirulina* supplemented diet and standard diet fed deficient groups showed an increase of 21 and 15% in serum zinc, respectively, compared to the deficient group. This led to the normalisation of the serum zinc levels in the *Spirulina* supplemented diet fed deficient group to about 96% of the control group, whereas the normalisation of the serum zinc levels in the standard diet fed deficient group was about 91% of the control.

It was noted that the quantity of *Spirulina* biomass used as a dietary supplement of vitamin B_{12} in the present study would not alter the Fe and Zn content of the deficient diet, which would remain at about 47 and 50%, respectively, of the control diet. The improvement in haemoglobin and serum iron levels of deficient groups receiving the deficient diet supplemented with *Spirulina* biomass appears to be linked to the normalisation of the plasma vitamin B_{12} levels. Earlier studies have shown that iron deficiency is mostly associated with vitamin B_{12} deficiency[35]. However, the mechanism is still unknown. The improvement in serum zinc content of deficient groups fed with *Spirulina* supplemented diet indicates that incorporating *Spirulina* biomass might have improved the availability of zinc from the diet.

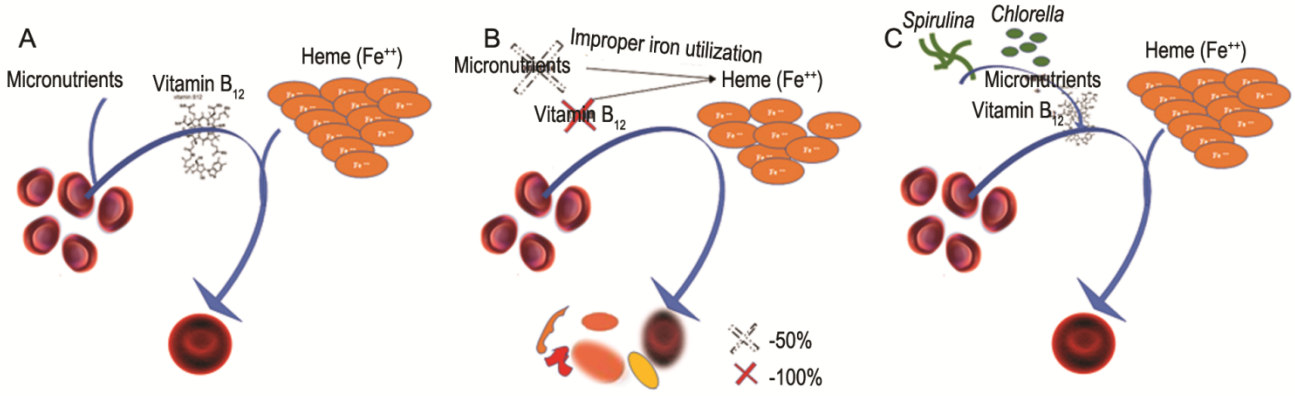

Fig. 3 — Hypothetical representation of the present study. (A) In normal conditions vitamin B_{12} and micronutrients help in proper utilization of heme; (B) Absence of B_{12} causes improper iron utilization that leads to membrane deformation of RBC and changes in its morphology; and (C) its recovery with supplementation of algal biomass

The normalisation of immunoglobulin levels in the deficient rats after feeding with the *Spirulina* supplemented deficient diet also indicates an improved availability of micronutrients from the diet on *Spirulina* incorporation (Table 2).

The relative osmotic fragility and haemolysis of RBC in *Spirulina* and *Chlorella* biomass supplemented deficient diet groups under the 'B', and 'M' experimental conditions were comparable to the control group. In experimental condition ' B ', the deficient groups receiving deficient diet supplemented with *Chlorella* and *Spirulina* biomass showed 84-94% of RBC with normal morphology. Similarly, the deficient group receiving the *Spirulina* supplemented deficient diet in experimental condition 'M' showed 75-81% of RBC with normal morphology. The result suggests that the incorporation of (i) *Chlorella* and *Spirulina* in vitamin B_{12} deficient diet as a prophylactic dietary supplement of vitamin $B_{12,}$ and (ii) *Spirulina* in deficient diet (vitamin B_{12} deficiency combined with micronutrients limitation) as an ameliorative dietary supplement of vitamin B_{12} maintained or improved the RBC stability.

Based on the results obtained, our proposed hypothesis is presented in Fig. 3. In the absence of vitamin $B_{12,}$ improper utilisation of iron in haem synthesis might occur, which may influence the integrity of the RBC leading to its structural instability and deformation. The *Spirulina* and *Chlorella* biomass supplementation in the diet provides the required amount of vitamin B_{12}. It improves the haemoglobin and serum iron content, and further and also improves the availability of micronutrients like Zn from the diet, which helps in the maintenance of the structural integrity of the RBC.

Conclusion

The erythrocytes of the groups of experimental animals in the present study, deficient in vitamin B_{12}, either alone or combined with micronutrient limitation, showed higher relative osmotic fragility, suggesting the important role of vitamin B_{12} in their stability. The incorporation of *Chlorella* and *Spirulina* effectively provided the required levels of vitamin B_{12} to deficient groups of animals and maintained or improved the haemoglobin, serum iron and plasma vitamin B_{12} levels and reduced the plasma MMA levels. Incorporating *Spirulina* as a dietary supplement also improved the serum zinc levels in the deficient group of animals. The incorporation of *Chlorella* and *Spirulina* biomass in the vitamin B_{12} deficient diet maintained and normalised the erythrocyte stability and morphology. The present study is possibly the first report on the potential of *Chlorella* and *Spirulina* as a dietary supplement in the prevention and amelioration of erythrocyte instability observed under vitamin B_{12} deficiency.

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Conflicts of interest

Authors declare no competing interests.

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