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ARTICLE

Interventions and public health nutrition

Impact of daily-supervised administration of a package of iron and folic acid and vitamin B_{12} on hemoglobin levels among adolescent girls (12–19 years): a cluster randomized control trial

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Abstract

Objective The prevalence of anemia has remained high among Indian adolescent girls over the past decade, despite the ongoing iron and folic acid (IFA) supplementation program. This study was conducted to assess the impact of daily supplementation of a package of IFA with vitamin B_{12} on hemoglobin levels among adolescent girls.

Methods A community-based cluster-randomized trial was conducted in the rural block of Faridabad District, Haryana, India in the year 2017. A total of 760 adolescent girls in the age group of 12–19 years with mild and moderate anemia were selected from government schools. Daily-supervised administration of iron and folic acid was conducted for 90 days: experimental group—IFA (iron (60 mg), folic acid (500) mcg), and cyanocobalamin (1000 mcg), control group—IFA and placebo. Hemoglobin, serum ferritin, and vitamin B_{12} levels were assessed at baseline and endline.

Results Two-hundred adolescent girls completed 90 doses of daily supplementation. The mean hemoglobin (experimental group: 1.3 ± 1.0 g/dL, control group: 1.6 ± 1.2 g/dL, $P = 0.004$) and ferritin levels (experimental group: 18.6 ± 31.5 ng/mL, control group: 18.8 ± 35.0 ng/mL, $P = 0.188$) increased in both the control and experiment groups. Serum vitamin B_{12} deficiency significantly reduced to 2.5% in the experimental group and ferritin deficiency alleviated in more than 96% of the girls post intervention.

Conclusions Daily supplementation of IFA with/without vitamin B_{12} for 90 days eliminated iron, vitamin B_{12} deficiency and reduced the overall proportion of anemia by 53.5%. However, addition of vitamin B_{12} to IFA supplementation had no impact on improving the hemoglobin levels among adolescent girls. The present study does not recommend provision of vitamin B_{12} for prevention and treatment of anemia in this population group.

Introduction

Anemia is a major public health problem in India with more than 40% of adolescent girls being anemic [1–4]. Anemia among adolescents contributes to long-term adverse health consequences on cognition and work productivity [5]. Presently, iron and folic acid (IFA) supplementation is provided to combat anemia [6]. However, recent evidence suggests that only 50% of anemia responds to iron supplementation [7].

Vitamin B_{12} is a cofactor for the enzyme methionine synthase, which is required for the conversion of homocysteine to methionine. Deficiency of vitamin B_{12} results in accumulation of homocysteine and interferes with formation of pyrimidine bases and DNA synthesis. Since DNA synthesis becomes impaired, the cell cycle cannot progress and the cell continues to grow without division into

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oversized and malformed red blood cells [8–10]. Deficiency of vitamin B_{12} therefore leads to macrocytic anemia [11–13] and may significantly contribute to the burden of anemia [9, 14].

High prevalence of vitamin B_{12} deficiency of more than 25% has been documented among anemic adolescent girls [12, 4]. Predominantly vegetarian Indian diets contribute to its deficiency as they lack vitamin B_{12}-rich animal foods, including meat, fish, eggs, milk, and other dairy products [12, 15]. High cost of animal products and socio-cultural factors further leads to inadequate dietary intake of vitamin B_{12}.

Earlier evidence has suggested that supplementation of vitamin B_{12} along with IFA increased the hemoglobin level and reduced anemia among pregnant women [16] and children aged 6 months to 5 years [17]. The recent World Health Organization (2016) guideline on prevention of anemia among adolescent girls recommends daily supplementation of IFA for 90 days rather than earlier advocated WIFS (weekly IFA supplementation) program [18]. Evidence on the impact of daily administration of IFA supplementation along with vitamin B_{12} among Indian adolescent girls is limited. Therefore, to fill the gap in the existing knowledge, a cluster-randomized control trial was conducted to assess the impact of daily administration of a package of IFA with vitamin B_{12} on increase in hemoglobin levels among adolescent girls (12–19 years).

Methdology

Study site and participants

The study was conducted in a rural block of Faridabad District, Haryana, India from July to December 2017. A list of all government schools in Ballabgarh block was developed. With the help of random number table, three schools in Dayalpur, Fatehpur Billoch, and Chhainsa were selected. A total of 1051 adolescent girls in the age group of 12–19 years, studying in class 6–12th standard, were included from the government schools after obtaining written consent. The flowchart of the methodology is presented in Fig. 1.

Procedures

The research team consisting of six field investigators and two research assistants were given 2 weeks of formal training, which was repeated for 2 days at the end of each month during the study period. The investigators provided supportive supervision and continued education to the research team. The research team briefed the study participants about the objectives of the study and the duration of intervention. A medical doctor briefed the participants about the dosage, frequency, possible side effects, and health benefits of IFA consumption. Blood samples were collected for estimation of hemoglobin, ferritin, and vitamin B_{12} levels at baseline by a trained phlebotomist. Written informed consent was obtained from a total of 904 adolescent girls. Participants found with moderate anemia (8.0–10.9 g/dL) and mild anemia (11.0–11.9 g/dL) anemia were enrolled in the study. The following exclusion criteria were adopted: (i) participants with severe anemia (Hb < 8 g/dL), (ii) participants with hemoglobin level of 12 g/dL and more, (iii) participants who are already taking vitamin and mineral supplements, (iv) participants who refused to provide parental consent to take part in the study, and (v) participants with any major chronic illness. The presence of a chronic illness was based on each adolescent's self-report. The information on consumption of any form of medications was substantiated with available medical records. In case of doubt, the medical doctor was requested to medically examine the participants during their supervisory visits.

For ethical reasons, non-anemic participants were advised to check their hemoglobin every 3 months and were counseled to consume iron and vitamin C-rich diet. Severely anemic girls were referred to the nearest primary health center for treatment.

Deworming tablets containing 400 mg of Albendazole were given to all the participants 1 week before initiation of the supplementation of IFA. Adolescent girls studying at the government school of Dayalpur and Fatehpur billoch were assigned to the experimental group and those studying at the government school of Chhainsa were assigned to the control group. The research team was aware of the group to which they were assigned, but the participants were blinded to their allocation. To avoid contamination, the school in village chhainsa was selected as the control group area as it was geographically apart from the experimental group areas, Dayalpur (15 km) and Fatehpur Billoch (20 km). The experimental group was supplemented with 60 mg of ferrous sulfate, 500 mcg of folic acid along with 1000 mcg of vitamin B_{12} (cyanocobalamin). The control group was supplemented with the same dose of IFA along with placebo (lactose IP). Daily supplementation was done under direct supervision of the research team member. Those participants experiencing side effects were treated and counseled daily by the research team members. The teachers and school staff were oriented about the IFA supplementation, possible side effects, and trained on reporting and assessment of the side effects at school. This step greatly improved the adherence to IFA supplementation. In order to reduce the risk of infection and inflammation due to water-borne diseases like diarrhea, cholera, and viral hepatitis, study participants were motivated to bring their water bottles from home for consuming the tablets.

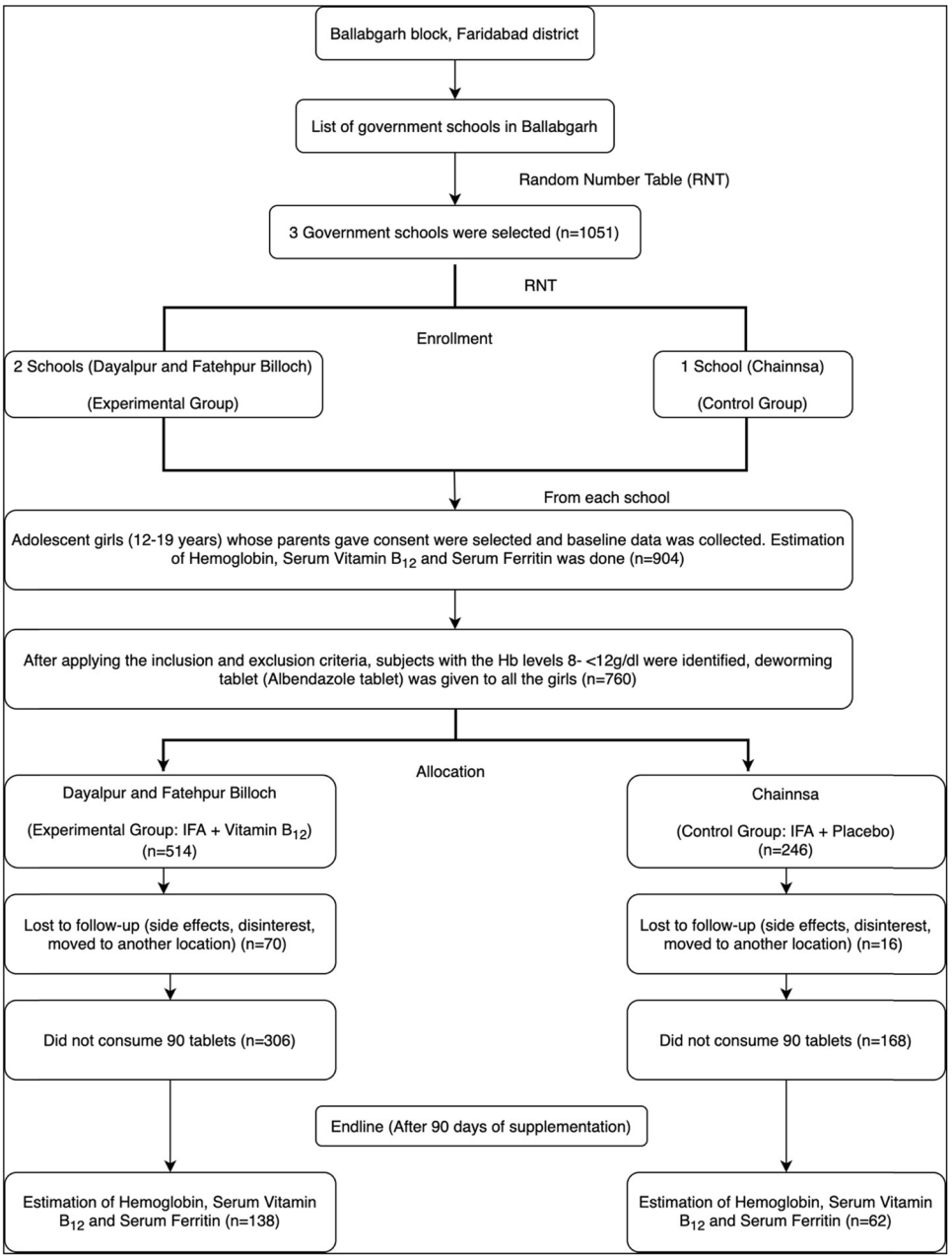

Fig. 1 CONSORT flow diagram depicting overall recruitment, retention, follow-up and endline status of the participants. Consort flowchart: methodology.

The research team members prepared attendance registers and monitoring charts with the details of study participants such as names, address, and phone number of parents for monitoring the daily IFA consumption. The research assistants validated 5% of data through supervisory visits.

During holidays and vacations, daily-supervised IFA supplementation was undertaken through house-to-house visits by the research team members to all study participants with the help of village- level functionaries like Anganwadi workers (AWW) and Accredited Social Health Activists (ASHA). AWW are responsible for providing care and support to children at the village level and ASHA are the village-level health educators and promoters. The AWW and ASHA did not participate in the supplementation; however, they facilitated in identifying the houses for undertaking supervised administration of IFA during the home visits. If the study participant was unavailable at their home on a holiday, extra IFA tablets were given to the participant and they were counseled about the importance of continuing the tablets. Extra care was undertaken for supervised administration of the tablets during the festival days. SMS and phone calls were sent to all the parents of children during holidays. The participants were requested to send their video or picture while consuming IFA and vitamin B_{12} supplements through networking platforms. To

ensure consumption of IFA and vitamin B_{12}, participants were asked to bring an empty wrapper of IFA and vitamin B_{12} on the following day of holiday.

At the completion of 90 doses of administration of intervention, the endline blood samples were collected for assessing the change in biochemical parameters of hemoglobin, ferritin, and vitamin B_{12} levels. A participant who had consumed 90 tablets in 110 days was considered to have completed the intervention.

Data collection

Sociodemographic profile

A questionnaire was administered orally for eliciting information regarding the sociodemographic profile of the participants on age, sex, educational qualification, parent's education and occupation, and family income.

Anthropometric profile

Height and weight of the study participants were measured using standard procedures. Weight was recorded using SECA model-813 weighing scale, to the nearest 100 g. Standing height was recorded using the SECA-213 stadiometer, to the nearest 0.1 cm.

Biochemical estimation

Blood samples were collected at the initiation (baseline) and after 90 days of intervention (endline) of the study from all the adolescent girls.

Venous blood (5 ml) was drawn in plain vacutainers (Beckton Dickinson, Franklin Lakes, NJ, USA) for the biochemical investigations of serum ferritin and vitamin B_{12} utilizing standard operating procedures. Blood samples were centrifuged within 2 h of collection. The serum samples were transported to the central laboratory for biochemical estimations and thereafter kept at −80 °C. Internal quality control was maintained using standards/controls with every batch of samples. Vitamin B_{12} estimation was done by competitive immunoassay using direct chemiluminescent technique (Immulite-1000, Siemens, Berlin, Germany). Serum ferritin was done by two-site sandwich immunoassay using direct chemiluminometric technique (Immulite-1000, Siemens, Berlin, Germany). Hemoglobin estimation was undertaken by cyanmethhemoglobin method. Twenty microliters of capillary blood were spotted on prelabeled Whatman Filter paper no. 1. The filter paper was then allowed to dry and packed in zip pouch and transported at 4 °C to the central laboratory for analysis. Indirect cyanmethemoglobin method using filter paper was used, as it was not feasible to transport whole venous blood within an hour under suitable conditions to the central laboratory.

The WHO cutoff (2011) for hemoglobin of <12 g/dL was utilized to assess the status of anemia [19]. The WHO cutoff (2008) for defining vitamin B_{12} deficiency was <203 pg/ml [9]. Iron deficiency was defined as serum ferritin level of <15 ng/ml [20].

Sample-size calculation

The sample size was calculated keeping in view the difference of 0.5 g/dL between the baseline and endline hemoglobin level assuming standard deviation (SD) of 1 g/dL with 80% power and 95% confidence interval. The estimated sample size was 63 participants each for the experimental and control group of the study. A design effect of 1.5 was applied to account for the clustering effect. The total sample size was calculated to be 264 participants. However, we enrolled 514 participants in the experimental group and 246 participants in the control group after applying the inclusion and exclusion criteria.

Ethical clearance

The study was approved by the Institutional ethical committee of All India Institute of Medical Sciences, New Delhi, India. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. After describing the objectives and risks of the study to the adolescent girls, they were provided with the participant information sheet and an informed consent form for obtaining the consent of parents/guardians. The adolescent girls were requested to bring the signed consent form on the following day. A written assent was obtained for children between 12 and 18 years. The consent was written for those parents/caretakers who are literate and thumb impression for those who were illiterate.

Statistical analysis

Data were entered using Microsoft Office Excel 2007 and were then exported to SPSS Statistics 20 (IBM, New Delhi, India) for analysis. Quantitative data were expressed in mean and standard deviation for normally distributed variables, median (25th percentile, 75th percentile) for non-normally distributed continuous variables, and frequency (percentage, %) for categorical variables. The distribution for serum ferritin was found to be skewed, and thus, the values were log-transformed and reported as geometric mean and interquartile ranges.

Table 1 Baseline measurement of different parameters.

	All enrolled subjects with mild and moderate anemia ($n = 760$)			Subjects who completed 90 days of supplementation ($n = 200$)		
	Experimental group ($n = 514$)	Control group ($n = 246$)	*P* value	Experimental group ($n = 138$)	Control group ($n = 62$)	*P* value
Age (years)	14.5 ± 1.8	13.9 ± 1.5	<0.001	14.4 ± 1.9	14.1 ± 1.5	0.223
Per capita income (in rs.)[a]	1486.7 ± 1404.8 1200 (714.3–1875)	1593.0 ± 1072.0 1336.7 (875–2000)	0.294	1794.6 ± 1545.8 1500 (1083.3–2000)	1635.9 ± 952.8 1519.2 (928.6–2000)	0.465
Height (cm)	150.8 ± 7.6	147.6 ± 9.0	<0.001	150.8 ± 7.9	149.9 ± 8.9	0.483
Weight (kg)	40.9 ± 8.2	37.9 ± 8.0	<0.001	39.9 ± 8.8	39.8 ± 8.0	0.932
BMI (kg/m^2)	13.5 ± 2.3	12.8 ± 2.2	<0.001	13.2 ± 2.5	13.2 ± 2.1	0.909
Hemoglobin (g/dL)	10.3 ± 0.9	10.5 ± 0.8	0.002	10.4 ± 0.9	10.6 ± 0.7	0.102
Serum ferritin (ng/ml)[b]	29.6 ± 35.2 21 (10.5–35.5)	35.1 ± 35.1 26.1 (14.8–41.6)	0.053	30.6 ± 28.6 22.3 (12.3–37.6)	34.5 ± 23.9 26.3 (17–52.7)	0.369
Serum vitamin B_{12} (pg/ml)	256.3 ± 73.5	226.3 ± 77.3	<0.001	247.5 ± 66.8	207.9 ± 48.1	<0.001

[a]Per capita income is expressed as the household's monthly income divided by the number of family members.

[b]Values are expressed as p_{50} (p_{25}–p_{75}).

A paired *t* test was used to compare the hemoglobin, serum ferritin and vitamin B_{12} levels before and after the intervention within the groups. Independent *t* test was used to compare the biochemical levels between the groups. Statistical analysis was conducted for all particpants who completed the study (per- protocol analysis ($n = 200$) and repeated using intention-to-treat principle on all 760 participants randomized into the study at baseline.

Results

Hemoglobin estimation was undertaken in a total of 904 adolescent girls, out of which 88.3% ($n = 798$) girls were found to be anemic. Mild, moderate, and severe anemia was reported among 25.7% ($n = 232$), 58.4% ($n = 528$), and 4.2% ($n = 38$) study participants, respectively. After applying the inclusion and exclusion criteria, 760 participants with mild and moderate anemia were allocated into experimental and control groups. The sociodemographic profile of the participants has been given in Table 1. The mean age of adolescent girls in the experimental group was 14.5 ± 1.8 years and control group in 13.9 ± 1.5 years (Table 1).

Loss to follow-up (due to side effects ($n = 21$), disinterest ($n = 50$), and relocation ($n = 15$)) was recorded to be 13.6% in the experimental group and 6.5% in the control group. A total of 138 participants in the experimental group and 62 participants consumed 90 tablets in 110 days and completed the study. The average number of IFA tablets consumed by this subsample in the experimental group was 93 and 94 by control group after the completion of 110 days of study period.

Mean hemoglobin level of the control group was significantly higher than the experimental group at baseline (IFA alone: 10.5 ± 0.8 g/dL and IFA + vitamin B_{12}: 10.3 ± 0.9 g/dL, $P = 0.002$). We found that the hemoglobin status of 39.5% participants in the experimental group and 67.5% participants in the control group improved to normal, post intervention (Table 2). According to the per-protocol analysis among participants who consumed 90 tablets, a higher proportion of participants (47.1%) attained normal hemoglobin status in the experimental group ($n = 200$) (Table 3). High reduction in the proportion of moderate anemia was observed in both experimental (49.2%) and control group (50.5%). Hemoglobin status did not improve in 28.4% and 21.9% of the adolescent girls in the experimental and control group, respectively. The experimental group reported high improvement (33%) in ferritin levels and deficiency (<15 ng/ml) reduced to 3.9% at the endline (Table 2). Ferritin deficiency was eliminated in the control group and reduced to 0.8% in the experimental group among participants who completed 90 days of supplementation (Table 3). Significantly higher improvement in the serum vitamin B_{12} was documented in the experimental group (20.5%) as compared to the control group (11.4%) ($P < 0.001$). At endline, 97.5% of the participants in the experimental group attained sufficient vitamin B_{12} (≥ 203 pg/ml) (Table 2).

Mean change in hemoglobin, serum vitamin B12, and serum ferritin levels was similar in both per- protocol and intention-to-treat analysis (Tables 4 and 5). The mean hemoglobin increased in both the groups post intervention; however, the control group had significantly higher improvement ($P < 0.001$) (Table 4). After adjusting the potential confounders, the hemoglobin level was 0.4 g/dL lower in the experimental group as compared to the control

Table 2 (a, b) Change in the prevalence of hemoglobin, serum vitamin B12 and serum ferritin post intervention in control and experiment group (Intention to treat analysis).

(a) Biochemical parameters	Experimental group ($n = 514$)				Control group ($n = 246$)				P value
	n	Improvement, n (%)	No change, n (%)	Decline, n (%)	n	Improvement, n (%)	No change, n (%)	Decline, n (%)	
Hemoglobin	514	355 (69.1)	146 (28.4)	13 (2.5)	246	189 (76.8)	54 (21.9)	3 (1.2)	0.074
Serum ferritin	466	154 (33.0)	310 (66.5)	2 (0.4)	219	55 (25.1)	162 (74.0)	2 (0.9)	0.057
Serum vitamin B_{12}	464	95 (20.5)	365 (78.7)	4 (0.9)	219	25 (11.4)	166 (75.8)	28 (12.8)	<0.001

(b) Biochemical parameters	Experimental Group ($n = 514$)		Control group ($n = 246$)	
Hemoglobin	Baseline	Endline	Baseline	Endline
No anemia (≥12 g/dl)	0	203 (39.5)	0	166 (67.5)
Mild anemia (11.0–11.9 g/dL)	152 (29.6)	202 (39.3)	84 (34.1)	42 (17.1)
Moderate anemia (8.0–10.9 g/dL)	362 (70.4)	109 (21.2)	162 (65.9)	38 (15.4)
Serum ferritin[a]				
Above cutoff	301 (63.0)	471 (96.1)	168 (74.7)	233 (97.5)
Below cutoff	177 (37.0)	19 (3.9)	57 (25.3)	6 (2.5)
Serum vitamin B_{12}[b]				
Above cutoff	373 (78.2)	477 (97.5)	122 (54.2)	127 (53.1)
Below cutoff	104 (21.8)	12 (2.5)	103 (45.8)	112 (46.9)

[a]Cutoff of 15 ng/ml was utilized for defining serum ferritin deficiency.

[b]Cutoff of 203 pg/ml was utilized for defining vitamin B_{12} deficiency.

Table 3 (a, b) Change in the prevalence of anemia, serum vitamin B_{12}, and serum ferritin post intervention in control and experimental group (per-protocol analysis).

(a) Biochemical parameters	Experimental group ($n = 138$)				Control group ($n = 62$)				P value
	n	Improvement, n (%)	No change n (%)	Decline, n (%)	n	Improvement, n (%)	No change, n (%)	Decline, n (%)	
Hemoglobin	138	108 (78.3)	26 (18.8)	4 (2.9)	62	49 (79.0)	13 (21.0)	0	0.505
Serum ferritin	123	39 (31.7)	84 (68.3)	0	58	12 (20.7)	46 (79.3)	0	0.157
Serum vitamin B_{12}	122	31 (25.4)	91 (74.5)	0	58	11 (19.0)	38 (65.5)	9 (15.5)	<0.001

(b) Biochemical parameters	Experimental group ($n = 138$)		Control group ($n = 62$)	
Hemoglobin	Baseline	Endline	Baseline	Endline
No anemia (≥12 g/dl)	0	65 (47.1)	0	41 (66.1)
Mild anemia (11.0–11.9 g/dL)	38 (27.5)	50 (36.2)	22 (35.5)	13 (21.0)
Moderate anemia (8.0–10.9 g/dL)	100 (72.4)	23 (16.7)	40 (64.5)	8 (12.9)
Serum ferritin[a]				
Above cutoff	85 (68.5)	132 (99.2)	46 (79.3)	62 (100)
Below cutoff	39 (31.4)	1 (0.8)	12 (20.7)	0
Serum vitamin B_{12}[b]				
Above cutoff	91 (74.0)	131 (99.2)	27 (46.6)	30 (48.4)
Below cutoff	32 (26.0)	1 (0.8)	31 (53.4)	32 (51.6)

[a]Cutoff of 15 ng/ml was utilized for defining serum ferritin deficiency.

[b]Cutoff of 203 pg/ml was utilized for defining vitamin B_{12} deficiency.

Table 4 Change in mean hemoglobin, serum vitamin B_{12}, and serum ferritin post intervention in control and experiment group (intention-to-treat analysis).

Parameters	IFA + vitamin B12 (experimental group) ($n = 514$)			IFA only (control group) ($n = 246$)			Difference of difference (95% CI)[a] (95% CI)[b]	P value[a]; P adjusted[b]
	Baseline	Endline*	Change (E-B)	Baseline	Endline*	Change (E-B)		
Hemoglobin (g/dL)	10.3 ± 0.9	11.6 ± 0.9	1.3 ± 1.0	10.5 ± 0.8	12.1 ± 1.0	1.6 ± 1.2	−0.2 (−0.39, 0.08)	0.004
							0.4 (0.24, 0.51)	<0.001
Moderate anemia (8.0–10.9 g/dl)	9.9 ± 0.7	11.5 ± 0.8	1.6 ± 0.9	10.1 ± 0.6	11.9 ± 1.1	1.8 ± 1.2	−0.2 (−0.4, −0.06)	0.011
							0.4 (0.22, 0.58)	<0.001
Mild anemia (11.0–11.9 g/dL)	11.4 ± 0.2	12.1 ± 0.8	0.7 ± 0.9	11.4 ± 0.2	12.4 ± 0.8	1.0 ± 0.8	−0.3 (−0.6, −0.11)	0.004
							0.3 (0.09, 0.50)	0.007
Serum ferritin (ng/ml)[#]	29.6 ± 35.2	48.4 ± 28.6	18.6 ± 31.5	35.1 ± 35.2	52.8 ± 28.9	18.8 ± 35.0	−0.3 (−5.5, 5.0)	0.922
	21.0 (2.4–412.1)	42.1 (3.4–256.0)		26.1 (3.3–331.7)	45.3 (9.8–206.7)		2.9 (−1.4, 7.2)	0.188
Serum vitamin B_{12} (pg/ml)	256.3 ± 73.5	365.6 ± 105.0	109.0 ± 92.1	226.3 ± 77.3	219.8 ± 76.5	−10.1 ± 54.2	119.1 (105.9, 132.3)	<0.001
							−127.7 (−141.0, −114.5)	<0.001

IFA iron folic acid.

Values are expressed as mean ± SD.

*Endline mean levels are provided among those who had moderate or mild anemia at baseline (control group: moderate anemia ($n = 162$); mild anemia ($n = 84$); experimental group: moderate anemia ($n = 362$); mild anemia ($n = 152$)).

P value[a] signify the comparison between the changes in mean values in the experimental group vs control group. *P* value[b] = *P* value adjusted for confounders, like hemoglobin, vitamin B_{12}, and serum ferritin at baseline level, caste, and per capita income.

[#]values are expressed as p50 (p25-p75)

group ($P < 0.05$) (Table 4). Adolescent girls with moderate anemia had higher increase in the mean hemoglobin levels as compared to mild anemic participants in both the groups (Table 4).

Significant increase in the serum B_{12} levels in the experimental group ($P < 0.005$), did not correspondingly increase the mean levels of hemoglobin and serum ferritin as compared to the control group (Table 4).

Discussion

This study found that addition of vitamin B_{12} to IFA did not have an impact on hemoglobin level among adolescent girls as compared to IFA alone. At the endline, the control group had higher mean hemoglobin rise (1.6 ± 1.2 g/dL) and higher proportion of study participants with normal hemoglobin levels (67.5%) than the experimental group (1.3 ± 1.0 g/dL, 39.5%). Higher mean hemoglobin levels and the proportion of mild anemic participants at the baseline may have resulted in this finding. An earlier clinical trial study conducted among Indian adolescent girls also reported a similar increase in mean hemoglobin of 1.7 g/dL after daily administration of 90 IFA tablets (60 mg of elemental iron and 500 mcg of folic acid) [21].

Daily supplementation of IFA with or without vitamin B_{12} for 90 days reduced the overall proportion of anemia by 53.5% and moderate anemia by 50%. The significantly higher baseline serum vitamin B_{12} levels in the experimental group did not result in a notably increased rise in hemoglobin and ferritin levels, post intervention. In concordance to the present study, an earlier study conducted among adolescent girls also reported no benefit of vitamin B_{12} along with IFA tablets (iron (100 mg), folic acid (500 mcg), and vitamin B_{12} (500 mcg for 6 weeks and 15 mcg for 20 weeks)) supplemented weekly for 26 weeks as compared to IFA alone [22]. The authors reported a significantly higher serum ferritin rise and 23% decline in vitamin B_{12} deficiency in the experimental group (IFA + vitamin B_{12}). They recommended the use of a higher dose of vitamin B_{12} than 500 mcg for further studies. Our study used lower iron dosage of 60 mg as per the WHO guidelines [18] and a higher dose of vitamin B_{12} (1000 mcg). Higher dose and duration of vitamin B_{12} supplementation reduced deficiency and significantly improved the serum levels in the experimental group, but did not have the expected positive effect on the anemia status. Addition of a higher dose of vitamin B_{12} to IFA also did not further improve the ferritin absorption since the rise in serum ferritin levels was similar between the groups. Daily supplementation of IFA with/without vitamin B_{12} alleviated ferritin deficiency in 96.8% of the study participants. Hence, the role and mechanism of vitamin B_{12} in the etiology of anemia needs further investigation.

Table 5 Change in mean hemoglobin, serum vitamin B_{12}, and serum ferritin post intervention in control and experiment group (per-protocol analysis).

Parameters	IFA + vitamin B12 (experimental group) ($n = 138$)			IFA only (control group) ($n = 62$)			Difference of difference (95% CI)[a] (95% CI)[b]	*P* value[a]; *P* adjusted[b]
	Baseline	Endline*	Change (E-B)	Baseline	Endline*	Change (E-B)		
Hemoglobin (g/dL)	10.4 ± 0.9	11.7 ± 0.8	1.4 ± 0.9	10.6 ± 0.7	12.1 ± 0.9	1.5 ± 1.1	−0.2 (−0.45,0.13)	0.269
							0.3 (0.06,0.57)	0.015
Moderate anemia (8.0–10.9 g/dl)	9.9 ± 0.6	11.5 ± 0.8	1.6 ± 0.8	10.2 ± 0.5	11.9 ± 0.8	1.7 ± 1.0	0.05 (−0.4,0.3)	0.761
							0.2 (−0.08,0.59)	0.136
Mild anemia (11.0–11.9 g/dL)	11.4 ± 0.2	12.2 ± 0.7	0.8 ± 0.7	11.4 ± 0.2	12.2 ± 0.8	0.7 ± 0.9	0.06 (−0.4,0.5)	0.790
							−0.06 (−0.4,0.54)	0.005
Serum ferritin (ng/ml)[#]	30.6 ± 28.6	50.3 ± 26.4	20.1 ± 28.1	34.5 ± 23.9	58.6 ± 26.5	23.9 ± 27.0	−3.8 (−12.5, 4.9)	0.392
	22.3 (12.3–37.6)	45.7 (30.2–58.1)		26.3 (17–52.7)	45.9 (38.4–74.9)		3.0 (−5.2,11.22)	0.476
Serum vitamin B_{12} (pg/ml)	247.5 ± 66.8	390.9 ± 111.6	140.5 ± 93.1	207.9 ± 48.1	206.8.1 ± 56.9	0.6 ± 48.6	139.9 (114.2,165.6)	<0.001
							−133.0 (−160.0, −107)	<0.001

IFA iron folic acid.

Values are expressed as mean ± SD.

*Endline mean levels are provided among those who had moderate or mild anemia at baseline (control group: moderate anemia ($n = 162$); mild anemia ($n = 84$); experimental group: moderate anemia ($n = 362$); mild anemia ($n = 152$)).

P value[a] signify the comparison between the changes in mean values in experimental group vs control group. *P* value[b] = *P* value adjusted for confounders, like hemoglobin, vitamin B_{12}, and serum ferritin at baseline level, caste, and per capita income.

[#]Values are expressed as p_{50} (p_{25}–p_{75}).

Another randomized controlled trial was conducted in rural Querétaro, Mexico to assess the impact of daily administration of 500 mcg of vitamin B_{12} supplementation among women 20–59 years with no anemia [23]. They documented that normalization of vitamin B_{12} status as indicated by increased serum vitamin B_{12}, holotranscobalamin, and lowered methylmalonic acid and total homocysteine through supplementation had no effect on any hematological indicators, including hemoglobin, serum ferritin and folate, and bone-specific alkaline phosphatase.

Clinical trials conducted to assess the impact of daily administration of vitamin B_{12} along with IFA supplementation also reported no improvement in hemoglobin among pregnant women [24, 25–27], 6–24-month children [28], and the elderly [29].

Conversely, a hospital-based study conducted among 150 Indian children aged between 6 months and 5 years, concluded that the group supplemented with IFA and vitamin B_{12} had higher hemoglobin level and percentage hemoglobin rise from baseline assessed at 2 weeks, 4 weeks, and 8 weeks as compared with iron and folic acid alone [30]. However, the difference in the iron requirements between 6 months and 5 years may have resulted in such an effect.

Recent evidence suggests an association of low vitamin B_{12} status with high plasma folate insufficiency threshold and their further role in erythropoeisis [31]. Deficiency of vitamin B_{12} leads to functional folate deficiency as it is directly involved in folate metabolism and is required for folate retention during erythropoiesis [32]. Anemia due to vitamin B_{12} deficiency in turn is hypothesized to occur due to functional folate deficiency [33]. Yajnik et al. reported that daily supplementation of vitamin B_{12} provided to Indian adolescent girls for 11 months did not improve serum folate levels, post intervention [34]. The evidence on the association of vitamin B_{12} with folic acid and hemoglobin is inconclusive.

It was interesting to observe that anemia remained uncorrected among 50.8% adolescent girls in the experimental group and 36.2% girls in the control group, even though all adolescent girls attained sufficient ferritin and vitamin B_{12} levels, post intervention. Impaired improvement in the hemoglobin even after addition of vitamin B_{12} along with IFA supplementation may be due to the fact that only 26.8% and 22.2% of the anemia among adolescent girls is due to iron and folate or vitamin B_{12} deficiency, respectively, as per the recent Comprehensive National Nutrition Survey [35]. Around 31% of anemia among adolescent girls was due to other causes (27.9%) and inflammation (2.9%). The possible role of inflammation and other hemopoietic nutrients in the causation of anemia needs to be investigated.

The strength of this study is that it was a carefully conducted randomized controlled trial in which supervised administration of tablets was provided daily for 90 days to adolescent girls. The study had some limitations. We selected only one school as the control group due to operational issues. The inflammation markers (e.g., CRP and AGP) were not measured and thus iron deficiency among the study participants may be artificially low. Only serum ferritin was used to diagnose iron deficiency instead of a combination of markers due to budgetary constraints. High non-compliance and dropouts

were reported in both experimental and control groups due to frequent absenteeism of girls from school. However, interpersonal and group counseling sessions provided at the initial phase of the study about the adverse effects of anemia, benefits of IFA supplementation, and management of side effects at school and at home, helped in improving compliance to IFA supplementation.

In conclusion, provision of vitamin B_{12} with IFA did not increase the hemoglobin level significantly among adolescent girls as compared to IFA alone. However, the findings of this study are limited to the population included in the study. Multicentric study in varying population types, diet, cultural, and social practices needs to be undertaken in the different regions of the country to substantiate the present research findings. Contribution of other factors such as inflammation, hemoglobinopathies and other micronutrients to anemia requires exploration. The results of this study support the recent WHO guideline (2016) that daily supplementation of 60 mg of elemental iron and 500 mcg of folic acid supplementation for 90 days is effective in alleviating iron deficiency and reduction of anemia among adolescent girls [18].

Author contributions AG: literature review, content developing, writing the protocol and report, and paper development. SK: literature review, content developing, and writing the protocol. LR: biochemical analysis and interpretation content developing, writing the protocol and report, and paper development. RMP: writing the protocol, data analysis, and interpretation. RK: literature review, content developing, writing the protocol and report, and paper development. UK: literature review, content developing, writing the protocol and report, and paper development and finalization, and will act as guarantor for the paper. HSS: literature review, content developing, writing the protocol and report, and paper development and finalization.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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