

Original Communication

# Impact of a Multiple-micronutrient Fortified Salt on the Nutritional Status and Memory of Schoolchildren

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**Abstract:** *Objective:* This study was conducted to test the efficacy of a multiple micronutrient-fortified cooking salt. *Methods:* A randomized controlled trial with a pre- and post-test design was used to study children 5 to 18 years of age, with an experimental (n=213) and control group (n=189). The children were sampled from 3 residential schools and were studied for 9 months. The experimental group received a multiple micronutrient-fortified salt containing vitamins A, B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub>, B<sub>12</sub>, as well as folic acid, niacin, iron, iodine, and zinc. The control group received iodized salt. Biochemical measurements [hemoglobin, serum ferritin (SF), serum transferrin receptor (sTfR), C-reactive protein (CRP), alpha-1 acid glycoprotein (AGP), serum retinol, serum vitamin B<sub>12</sub>, serum folic acid, serum zinc, and urinary iodine (UI)] were measured at baseline and post-intervention. Hemoglobin was measured in all the children three times during the study period, while the remaining biochemical measurements were performed in a subsample of children. Children between 11 and 18 years of age were given cognitive tests to assess memory and attention. *Results:* There was a significant improvement (p<0.05) in all the biochemical measurements and memory tests in the experimental group when compared with the control group. Post-intervention in the experimental group, the increase in hemoglobin was 0.67 g/dL (p<0.05). Iron status and body iron stores increased significantly (p<0.05) in the experimental group

compared to the control group, while serum zinc increased by 50 µg/dL ( $p < 0.05$ ), and the prevalence of retinol deficiency decreased from 57.1 % at baseline to 16 % post-intervention ( $p < 0.05$ ). *Conclusion:* The multiple micronutrients from the multiple micronutrient-fortified cooking salt were absorbed in the children and helped in combating micronutrient deficiencies.

**Key words:** Multiple micronutrient salt fortification, children, memory, vitamin A, iron, iodine, B complex vitamins, zinc

## Introduction

Micronutrient deficiencies in developing countries are a consequence of the plant-based cereal diets typically consumed in these areas [1,2]. Dietary phytate inhibits the absorption of many micronutrients, notably iron and zinc. Micronutrient deficiencies in infancy can cause impairments in physical development and cognition that may be irreversible [3–5]. Iron and iodine deficiencies affect more than 30 % of the global population [6]. It has been suggested that supplementation with multiple micronutrients may be the best way to improve the nutritional status of malnourished populations [7]. There have been other studies wherein multiple-micronutrient fortification of beverages have been studied [8–10] or where multiple micronutrient-fortified foods [11] or multiple micronutrient-fortified biscuits [12] have been used. In most of the studies reviewed in the literature, iron was administered in the form of ferrous sulfate tablets for periods ranging from 2 to 8 months [13–16]. Multiple-micronutrient fortification of salt has been studied by these authors in an earlier study [17]. For salt fortification to be successful, the micronutrients should not change the color, odor, or taste of the food, should be stable at cooking temperatures, and should be bioavailable. With these concepts kept in mind, a multiple micronutrient-fortified salt was developed that contained vitamin A, vitamin B<sub>1</sub>, vitamin B<sub>2</sub>, vitamin B<sub>6</sub>, vitamin B<sub>12</sub>, folic acid, niacin, iron, iodine, and zinc.

This study tested the stability of the fortified salt during storage and the absorption of iron, vitamin A, vitamin B<sub>12</sub>, folic acid, zinc, and iodine in the target group of children in residential schools.

## Materials and methods

### Subjects

The study was a randomized controlled trial which had a pre- and post-test design with experimental and control groups. Three residential schools were randomly selected as the experimental schools and three other residential schools as the controls in the city of Chennai, Tamilnadu, South India. After the random selection, we then verified whether the children in the experimental and control schools were matched for age, dietary pattern, and socioeconomic status. The diets of the children in all the schools were compared and we found that the dietary patterns in all the schools were similar. All the children consumed 3 meals and an afternoon snack each day. Before the randomization of the schools, we chose only those schools wherein, as a policy, the schools did not routinely accept outside cooked (unfortified) food, and where the children go home only once a year for the summer holidays, as both these factors could cause an interruption in the study. The experimental schools were supplied with the multiple micronutrient-fortified salt every month. The control schools were provided with iodized salt every month. Verification by weighing the salt left over from the previous month confirmed the daily usage of the salt in all the schools. From our earlier study [17] and from this study, by observing the actual consumption of the salt provided in each school, we concluded that the actual consumption of salt per child per day was 10 grams. The main outcome indicators were the biochemical tests and the memory tests.

The study started in July 2005 and concluded in April 2006. The study began when the schools reopened after the summer vacation and continued for 9 months until the schools closed again for the next summer vacation.

The experimental and control groups of children were homogenous in terms of age and socioeconomic status; the families of all the children had a monthly income of less than Rs2000 (\$US50).

*Table I: Stability of the multiple micronutrient fortified salt during storage at 30 degrees C and Relative Humidity 45 % for 10 months*

Micronutrient in 10 grams salt	Initial concentration	Concentration after 10 months of storage	% loss
Vitamin A (3000 IU)	2915±52.3	2298±90.68	21.18
Vitamin B1 (1 mg)	1.013±0.009	0.966±0.013	4.64
Vitamin B2 (1 mg)	1.015±0.013	0.941±0.042	7.28
Vitamin B6 (1 mg)	1.02±0.017	0.946±0.015	7.32
Niacin (5 mg)	5.058±0.079	4.78±0.054	5.48
Iron (1000 ppm)	1012.7±15.3	991.9±9.91	2.05
Iodine (40 ppm)	41.57±1.96	35.62±1.97	14.31
Folic acid (100 mcg)	107.38±2.28	98.66±2.42	8.12
Vitamin B <sub>12</sub> (4 mcg)	4.077±0.023	3.939±0.08	3.38
Zinc (10 mg)	10.121±0.078	9.7±0.089	4.12

Data given as Mean ±SD of 10 batches prepared for the study

### Sample size

We have considered a p-level of 0.05 and power of 80 % with a two-tailed test for all sample size calculations. Our earlier experiences with the use of fortified salt in children showed a mean increase in hemoglobin from 0.4 to 0.7 g/dL, with a standard deviation of about 1 to 1.25 [17,18]. If a similar increase is assumed in this study, a sample size of 154 children in each group is required. With respect to serum vitamin A, our earlier studies [17] had seen a mean increase of 5.56 µg/dL with a standard deviation of 12.9. If a similar increase is assumed in this study, a sample size of 85 children in each group is required. Similarly observing the increase in the serum level of the micronutrients folic acid and vitamin B<sub>12</sub> in our studies [19], and other studies involving zinc fortification and iron fortification, we arrived at a sample size of 42 children for serum B<sub>12</sub>, folate, zinc, and other parameters for iron status, namely serum ferritin (SF) and serum transferrin (sTfR).

### Manufacture of the fortified salt

The multiple micronutrient-fortified salt was manufactured in a ribbon blender (Pragmatic Engineering, Chennai, India) at 50 rpm. The homogeneity of the salt's micronutrient content was established at the manufacturing stage by assessing the micronutrient content of the fortified salt in different parts of the blender. Six samples were taken from the 4 corners (samples A,B,C,D), and the center from the top (sample E) of the blender. The 6<sup>th</sup> sample (sample F)

was taken from the discharge unit at the bottom of the blender. It was determined that all of the micronutrients were uniformly and homogeneously distributed within the product. The salt was produced on a monthly basis and distributed to the schools within 2 days of manufacture.

### Dosage of micronutrients

The salt was used in all the meals prepared for the children. It was found out that the average consumption of salt was 10 g per child per day. Therefore the fortified salt was prepared such that 10 g of the fortified salt contained about one RDA [20] of the micronutrients; the exception was iron, which was given at a dosage of 10 mg per day (30 % RDA) as the iron was chelated, instead of 28 mg iron, which was the RDA (Table I). The cooking staff of all the six schools certified that the fortified salt did not change the color or taste of any food. Each school has a central kitchen where the food is prepared and a central dining room where the resident children eat.

It was generally observed that there was no wastage of the food prepared in the schools; all prepared food was consumed. The children were served the quantity of food required by them, and there was no food left over on the plate.

### Blood collection and storage

Venous blood samples (5 mL) were drawn from each child and 500 µL were transferred into vials with ethylenediaminetetraacetate (EDTA) as an antico-

agulant. The hemoglobin measurements were performed on these samples within a few hours of blood collection at the laboratory. The remaining 4.5 mL of blood was transferred into vials covered with black paper to prevent exposure to light, and the blood was allowed to clot. Zinc-free tubes were used for collecting blood. Tests were conducted on samples of needles, cotton, and the alcohol with which the skin was cleaned before pricking, ensuring that none of these items had zinc contamination. The blood samples were transferred to the laboratory within 2 hours of collection. Serum separation was performed in the laboratory and the samples were frozen at  $-20^{\circ}\text{C}$  within a few hours after collection of blood. In those children where only hemoglobin measurement was performed, only 0.5 mL of venous blood was drawn and transferred into vials with EDTA as an anticoagulant. During vitamin A estimations, the samples were processed in a dark room with yellow lighting to prevent retinol isomerization. SF, sTfR, C-reactive protein (CRP), and alpha-1 acid glycoprotein (AGP) measurements were performed in a laboratory in Germany. The serum samples were transported on dry ice from India to Germany.

## Laboratory analyses

The biochemical estimations done were for hemoglobin, serum ferritin, sTfR, CRP, AGP, serum vitamin B<sub>12</sub>, serum folic acid, serum zinc, serum retinol, and urinary iodine. Hemoglobin was measured in all the children in both groups (n=213 in experimental group and n=189 in control group) three times during the study, at baseline, after 4 months, and post-intervention after 9 months. Serum vitamin A was measured by high-performance liquid chromatography (HPLC) only in those children who were identified as having vitamin A deficiency by a physician who checked the eyes of the children for clinical signs of vitamin A deficiency, such as Bitot's spots or xerosis. One-hundred nineteen children in the experimental group and 87 children in the control group had clinical signs of vitamin A deficiency, and serum retinol was measured in these children at baseline and post-intervention.

Serum folic acid, vitamin B<sub>12</sub>, serum zinc, sTfR, serum ferritin, CRP, and AGP were measured in the 50 children who had the lowest hemoglobin levels in the experimental group and 50 children with lowest hemoglobin in the control group at baseline and post-intervention. Urinary iodine was measured in a random sub-sample of 73 children in the experimen-

tal group and 70 children in the control group at baseline and post-intervention.

Hemoglobin was estimated by the cyanmethemoglobin method with a spectrophotometer (UV double-beam model, Shimadzu, Japan) [21]. Serum vitamin A was measured by a rapid, reverse-phase HPLC method (HPLC-Shimadzu, Japan). NIST serum sample standards (SRM968c- lyophilized frozen serum sample with certified retinol values) were used to calculate the retinol values in the children. Vitamin B<sub>12</sub> and folic acid assays were performed with a paramagnetic particle, chemiluminescent immunoassay for the quantitative determination of levels in human serum using the Advia Centaur immunoassay system (Siemens, Germany). Serum zinc was determined by atomic absorption spectroscopy. Serum ferritin, sTfR, AGP, and CRP were determined by sandwich ELISA method [22]. Urinary iodine was measured by using the Sandell-Kolthoff reaction as modified by Pino *et al.* [23].

Iron deficiency (ID) was defined as SF  $<15\ \mu\text{g/L}$  or sTfR concentration  $>7.6\ \text{mg/L}$  [7]. Anemia was defined as a hemoglobin concentration  $<13\ \text{g/dL}$  in boys aged  $\geq 15$  years; a hemoglobin concentration  $<12\ \text{g/dL}$  in children aged  $\geq 12$  years and in girls aged  $\geq 15$  years; and a hemoglobin concentration  $<11.5\ \text{g/dL}$  in children aged 5–11 years [7]. Iron deficiency anemia (IDA) was defined as simultaneous presence of ID and anemia. Body iron stores were estimated by the method of Cook *et al.* [24]. Infection was defined as CRP greater than  $10\ \text{mg/L}$  [25] and such data was removed before statistical analysis. Serum vitamin A deficiency was defined when serum vitamin A was less than 20 micrograms/dL.

## Validation of biochemical measurements

For hemoglobin, serum vitamin A, urinary iodine, serum zinc, serum vitamin B<sub>12</sub> and serum folic acid, estimations were measured in duplicate in 10 % of the samples. The coefficient of variation (CV) for estimation of serum vitamin A was 4.1 %. The ELISA tests for ferritin, sTfR, AGP, and CRP were done in duplicate for every sample; the CV for ferritin was 7.5 %; sTfR, 6.3 %; CRP, 12.4 %; and AGP, 7.8 %.

## Cognitive test

*Memory test:* The investigators who carried out the memory tests were blinded to the group assignment for the schools and did not know which were the experimental and control schools.

The children were divided into 2 groups based on their ages. Sixth through eighth grade children (11- to 13-year-olds) were in one group and 9<sup>th</sup>-12<sup>th</sup> grade children (14- to 18-year-olds) were in another group. The younger children were given a test wherein an audio tape with 15 words was played to them. The 15-word sequence was repeated 10 times. The children had to memorize the words in the same sequence and write them down at the end of the test. The older children were given a test with a 20-word sequence. The test analyzed the ability of the children to memorize the words in the same sequence. One mark was allotted to each word written in the correct sequence. The words were chosen from the Shellenberg's list and there was no connection or correlation between the words. The memory tests were given at baseline and post-intervention to 92 children in the experimental group and 70 children in the control group, in the age group 11–18 years. Memory tests were not given to younger children 5–10 years of age.

## De-worming

Both the experimental and the control children were given a tablet of albendazole (400 mg) at baseline, at 4 months, and post-intervention after 9 months. De-worming was carried out to ensure that there were no worms competing for the micronutrients and that the intestinal tract was clear for absorption of the micronutrients [26,27].

## Clinical assessment

Clinical assessment of angular stomatitis, a condition caused by deficiencies of B-complex vitamins, and clinical vitamin A deficiencies such as xerosis and the presence of Bitot's spots was conducted by a physician before the start of the study and after 9 months of intervention.

## Administration of iron

In our study, the experimental group received 10 mg of elemental iron through the multiple micronutrient-fortified cooking salt every day for 9 months. The iron was in the form of chelated ferrous sulfate. Chelated iron compounds have a much higher bioavailability than inorganic iron compounds. The studies reviewed in the literature used ferrous sulfate, ferrous fumarate, iron dextran and other iron compounds, but did not use chelated iron compounds [28–31].

## Measurement of stability of the multiple micronutrient-fortified salt

To determine the stability of the multiple micronutrient-fortified salt, its composition was analyzed initially, and every month for a storage period of 10 months at 30 degrees Celsius, humidity 45 %. Micronutrient composition was analyzed by methods described in the Indian Pharmacopoeia [32] (Table I).

## Statistical analysis

Statistical analysis was performed with SPSS 11.0 software (SPSS Inc., Chicago IL, USA) and Microsoft Excel 2000 (Microsoft Corp., Seattle WA, USA). Repeat measures of analysis of variance were used to compare the effects of group  $\times$  time for hemoglobin, serum ferritin, sTfR, body iron stores, CRP, AGP, serum vitamin B<sub>12</sub>, serum folic acid, serum zinc, and serum retinol. If the interaction effect of group  $\times$  time was significant ( $p < 0.05$ ),  $t$ -tests between groups and paired  $t$ -tests within groups were done. If there were significant  $p < 0.05$  differences in biochemical parameters at baseline between the experimental and control groups, then analysis of covariance (ANCOVA) was performed to adjust the baseline values to a common mean and then calculate the adjusted endpoint values. When ANCOVA was done, the baseline parameters were added as covariates. When there were 3 time points –baseline, midpoint, and endpoint as in hemoglobin analysis, after ANCOVA analysis, a *post hoc* Bonferroni test was done. Proportions were compared by using Chi-square tests. If data was not normally distributed, statistical analysis was done after log transformation. Binary logistic regression was done to compare the effects of group  $\times$  time for the binary variables of anemia, iron deficiency (ID), iron deficiency anemia (IDA),

vitamin A deficiency, and angular stomatitis. Significance was set at  $p < 0.05$ . The Mann-Whitney test and Wilcoxon's signed-rank test were used to analyze urinary iodine (UI).

## Ethical issues

The study was approved by the institutional review board of the Sundar Serendipity Foundation. Informed written consent was obtained from the school directors, and informed oral consent was obtained from the parents or legal guardians of all of the children. The parents of the children in the experimental schools were informed about the use of the fortified salt in all meals cooked in the schools and about the blood tests to be performed. The parents of the children in the schools were informed that blood tests would be performed on all of the children and those children with severe anemia (hemoglobin  $< 8$  g/dL) would be treated with ferrous sulfate tablets and excluded from the study. Children found to be anemic at the end of the study in both the experimental and control groups were treated with ferrous sulfate tablets (60 mg elemental iron) for a period of 3 months.

## Results

### Stability of the multiple micronutrient-fortified salt

The percentage loss of the micronutrients over a period of 10 months is given in Table I. It can be seen that the micronutrients are extremely stable on storage. In this study the salt batches were prepared every month and delivered to the schools on a monthly basis. The data given in Table I represents the mean  $\pm$  SD of the 10 batches of salt prepared for the study.

### Efficacy trial

There were 213 children in the experimental group (96 in school A, 50 in school B, and 67 in school C) and 189 children in the control group (21 in school D, 110 in school E, and 58 in school F) who completed the study (see Table II). Twenty-three children in the experimental group and 24 children in the control group were absent during phlebotomy at baseline,

midpoint after 4 months, or post-intervention after 9 months and therefore did not complete the study and were not included in the statistical analysis. In the subsample of 50 children analyzed for CRP, AGP, serum ferritin, and sTfR, 7 children in the control group had CRP greater than 10 mg/L and were not included in the statistical analysis. In the subsample of 50 children analyzed for CRP, AGP, ferritin and sTfR, 5 children in the experimental group had taken a long leave and gone home between the midpoint and post-intervention phlebotomies. They were also excluded from statistical analysis. Three children in the experimental group and 4 children in the control group had severe anemia with hemoglobin less than 8 g/dL at baseline. They were given ferrous sulfate tablets for 3 months and excluded from the study.

The mean baseline age of the experimental group was 12.31 years and that of the control group was 12.27 years; there was no significant difference in the age between the two groups. At baseline, the mean hemoglobin value in children in the experimental schools was significantly lower than the hemoglobin value of the children in the control schools (see Table II). In the subgroup of children in which serum ferritin, sTfR and body iron store measurements were performed, in the experimental group of children body iron stores were significantly lower than the control group and sTfR in the experimental group was significantly higher than the control group, showing that the experimental group had a higher prevalence of anemia at baseline. With respect to serum retinol, serum vitamin B<sub>12</sub>, serum folic acid, and serum zinc, there was no difference between the two groups at baseline. Therefore ANCOVA was done to adjust the baseline hemoglobin of the whole group and to adjust the sTfR and body iron stores in the subsample of the children on whom these tests were done (see Table III). ANCOVA analysis showed that there was a significant ( $p=0.0001$ ) group  $\times$  time interaction with respect to hemoglobin and sTfR, and a significant ( $p=0.014$ ) group  $\times$  time interaction with respect to body iron stores (see Table III).

Changes in biochemical parameters over the study period in the experimental and control groups are shown in Table II. Although mean hemoglobin values of the experimental group were significantly lower than the control group at baseline, at the end of 9 months, the experimental group hemoglobin was significantly higher than that of the control group ( $p=0.0001$ ; ANOVA group  $\times$  time interaction). There was a significant increase in the hemoglobin ( $p=0.0001$ ) of the experimental group over the study period, whereas there was no statistically

Table II: Biochemical parameters in the experimental and control group over 9 months

	Experimental group				Control group				
	N	Baseline	Post Intervention	p value Paired t-test	N	Baseline	Post Intervention	p value Paired t-test	p value Group x time ANOVA
Hemoglobin g/dL *	213	11.94 ±1.4	12.61 ±1.3	0.0001	189	12.22 ±0.99	12.12 ±0.96	0.099	0.0001
Ferritin µg/L*+	45	8.43 ±18.06	8.31 ±42.12	0.889	43	12.17 ±19.3	8.13 ±15.75	0.001	0.013
sTfR mg/L*	45	12.86 ±5.9	11.74 ±5.25	0.005	43	9.77 ±2.48	10.31 ±3.27	0.226	0.005
Body iron stores mg/Kg*	45	-2.68 ±5	-2.4 ±5.23	0.489	43	-0.55 ±4.7	-2.14 ±4.5	0.002	0.004
CRP mg/L	45	0.47 ±0.92	0.41 ±0.72	0.620	43	0.66 ±1.24	0.57 ±1.1	0.707	0.913
AGP g/L	45	0.84 ±0.18	0.84 ±0.23	0.987	43	0.82 ±0.23	0.77 ±0.22	0.285	0.447
Serum vitamin A µg/dL *	119	20.68± 8.23	25.34 ±5.74	0.0001	87	19.08 ±6.6	19.21 ±5.24	0.872	0.0001
Serum vitamin B <sub>12</sub> pg/mL*	45	5612± 7624	15741 ±10979	0.0001	50	4707 ±5950	557 ±366	0.0001	0.0001
Serum folic acid ng/mL*	45	16.4 ±11.6	10.12 ±7.02	0.004	50	17.88 ±11.98	5.08 ±2.46	0.0001	0.018
Serum zinc µg/dL *	45	92.5 ±39.6	142.5 ±132	0.010	50	101.22 ±48.3	102.73 ±88.78	0.916	0.042
Angular stomatitis † Prevalence %	213	12.8	4.2		189	14.8	16.4		0.0228

Data given as mean ±SD; + Geometric mean ±SD; \* Significant group x time interaction ANOVA repeat measures; † By Binary logistic regression, there was a significant time x group interaction for angular stomatitis.

Hemoglobin was done 3 times, at baseline, midpoint, and post-intervention. The midpoint values of hemoglobin in the experimental group were 12.27 ±1.42 g/dL and in the control group it was 12.04 ±1.06.

significant change in the control group hemoglobin. All indices of iron status (serum ferritin, sTfR, and body iron stores) improved significantly in the experimental group when compared with the control group at the end of the study ( $p < 0.05$ ) (Table II). There was no significant difference between the groups in the mean CRP and AGP concentrations at baseline or at the end of the study in the children who were included after excluding the children who had CRP greater than 10 mg/L. Serum retinol, serum vitamin B<sub>12</sub>, serum folic acid, and serum zinc improved significantly over the study period ( $p < 0.05$ ) in the experimental group when compared with the control group (Table II). At baseline 12.8 % of the children in the experimental group and 14.8 % of the children in the control group had angular stomatitis, a condition attributable to B-complex deficiencies. At the end of the study the prevalence of angular stomatitis in the experimental group had reduced

significantly from 12.8 % to 4.2 %, whereas in the control group the prevalence of angular stomatitis increased marginally from 14.8 % to 16.4 % (Table II).

In the subsample of the children where we tested for anemia, iron deficiency, and iron deficiency anemia, there was a significant ( $p < 0.005$ ) reduction in anemia and iron deficiency anemia, but not iron deficiency, in the experimental group compared to the control (Table IV). In the subsample of children on whom serum retinol measurements was performed, there was a significant reduction in the prevalence of retinol deficiency in the experimental group ( $p < 0.0001$ ) when compared to the control group. (Table V). When median urinary iodine is considered, there is a significant improvement from baseline to post-intervention levels in both experimental and control groups, showing that the absorption of iodine from the multiple micronutrient-

Table III: Analysis of Covariance (ANCOVA)

Parameter	Mean ±SD	Time	Time	p value ANCOVA Bonferroni Post Hoc test	p value ANCOVA Group x Time interaction
Adjusted Hemoglobin gram/dL Experimental group n=213	12.08±1.42	1	2	0.032	0.0001
			3	.0001	
	12.36±1.04	2	1	0.032	
			3	0.007	
	12.70±0.94	3	1	0.0001	
			2	0.007	
Adjusted Hemoglobin gram/dL Control group n=189	12.08±0.98	1	2	0.132	
			3	1.000	
	11.90±0.93	2	1	0.132	
			3	0.382	
	12.03±0.59	3	1	1.000	
			2	0.382	
Adjusted serum transferrin receptor mg/L Experimental group n=45	11.35	1			0.0001
	10.52	3			
Adjusted serum transferrin receptor mg/L control group n=43	11.35	1			
	11.4	3			
Adjusted body iron stores mg/kg Experimental group n=45	-1.64	1			0.014
	-1.46	3			
Adjusted body iron stores mg/kg control group n=43	-1.64	1			
	-2.93	3			

Time 1=baseline, Time 2= midpoint, and Time 3= post intervention

Table IV: Prevalence percentage of anemia, iron deficiency, and iron deficiency anemia in the 2 groups at baseline and post-intervention

	Experimental group – Multiple micronutrient-fortified salt n=45		Control group – Iodized salt n=43		p values
	Baseline	Post intervention	Baseline	Post intervention	
Anemia	100	60	86	69.8	0.0039
Iron Deficiency	91.1	84.4	83.7	86	0.6264
Iron Deficiency Anemia	91.1	51.1	74.4	58.1	0.0048

By binary logistic regression, there was a significant time x group interaction for anemia and Iron deficiency anemia but not for iron deficiency.

Iron deficiency (ID) was defined as SF <15 µg/L or sTfR concentration >7.6 mg/L. Anemia was defined as a hemoglobin concentration <13 g/dL in boys ages ≥15 years, a hemoglobin concentration <12 g/dL in children aged ≥12 years and in girls aged ≥15 years, and a hemoglobin concentration <11.5 g/dL in children aged 5–11 years. Iron deficiency anemia (IDA) was defined as simultaneous presence of ID and anemia.

fortified salt is similar to the absorption of iodine from iodized salt (Table VI). In subjects who were iodine-deficient at baseline, there was no significant difference (p<0.05) between experimental and con-

trol groups at baseline or post intervention; as both groups improved equally, it was shown that the absorption of iodine from the multiple micronutrient-fortified salt was similar to the absorption of

Table V: Prevalence percentage of serum vitamin A deficiency in the 2 groups at baseline and post intervention

	Experimental group- Multiple micronutrient fortified salt n=119		Control group- Iodized salt n=87		p value
	Baseline	Post intervention	Baseline	Post intervention	
Serum vitamin A deficiency	57.1	16	62.1	59.8	0.0001

By binary logistic regression, there was a significant time  $\times$  group interaction for vitamin A deficiency. Serum vitamin A deficiency was defined when serum vitamin A was less than 20 micrograms/dL.

Table VI: Median Urinary iodine excretion values mcg/L (Range) in the experimental and control group over 9 months

	Experimental group – Multiple micronutrient-fortified salt			Control group – Iodized salt			p values
	n	Baseline	Post intervention	n	Baseline	Post intervention	
Whole group	73	250 (20–500) <sup>ac</sup>	365 (220–550) <sup>bc</sup>	70	110 (20–500) <sup>ac</sup>	317.5 (50–570) <sup>bc</sup>	0.0001
Subjects with baseline median urinary iodine less than 100 mcg/L – iodine deficient	13	75 (20–95) <sup>dc</sup>	315 (275–450) <sup>ec</sup>	30	50 (20–90) <sup>dc</sup>	260 (50–570) <sup>ec</sup>	0.001
Subjects with baseline median urinary iodine more than 100 mcg/L – iodine sufficient	60	312.5 (105–500) <sup>ac</sup>	370 (220–550) <sup>bc</sup>	40	167.5 (100–500) <sup>ac</sup>	337.5 (80–570) <sup>bc</sup>	0.002

<sup>a</sup> Mann-Whitney test. Significant difference between the experimental and control group at baseline, the control group significantly lesser than the experimental group.

<sup>b</sup> Mann-Whitney test. Significant difference between the experimental and control group post-intervention, the control group significantly lesser than the experimental group.

<sup>c</sup> Wilcoxon Signed-rank test. Significant improvement from baseline to post-intervention in both experimental and control groups, showing that the absorption of iodine from the multiple micronutrient-fortified salt is similar to the absorption of iodine from iodized salt.

<sup>d</sup> Mann-Whitney test. In subjects who are iodine-deficient at baseline, there is no significant difference between experimental and control groups at baseline.

<sup>e</sup> Mann-Whitney test. In subjects who are iodine-deficient at baseline, there is no significant difference between experimental and control groups post-intervention so both groups have improved equally, showing that the absorption of iodine from the multiple micronutrient fortified salt is similar to the absorption of iodine from iodized salt.

iodine from iodized salt (Table VI). At the end of the study, in both the experimental and control groups, all the urinary iodine-deficient children had crossed the cutoff value (100 micrograms/L) for the risk of iodine deficiency and attained a sufficient status in urinary iodine values.

## Memory tests

At baseline, there was no significant difference in the memory scores of the experimental and control groups of children, implying that the 2 groups of children were homogenous with respect to memory skills and hence comparable for memory outcomes. At the end of the study after 9 months of intervention, the memory scores of the experimental group were significantly higher than those of the control group. ANOVA repeat measures showed significant

( $p=0.045$ ) group  $\times$  time interaction. If changes in the memory scores are considered (post-intervention scores minus baseline scores), the change in the experimental group is significantly higher than the change in the control group, showing that the use of the multiple micronutrient-fortified salt helped in increasing memory scores in children (Table VII).

## Discussion

There is strong evidence that among school-aged children, initially low scores on tests of cognition or school achievement due to iron deficiency anemia can be improved and in some cases reversed after iron treatment [33–36]. Most of the studies [33–36] showed significant improvement in cognitive function or educational achievement of the children who received iron supplements compared to those who

Table VII: Memory test Scores

	Experimental group					Control group					
	n	Baseline	Post inter- vention	Change (post inter- vention scores minus baseline scores)	p value Paired t-test	n	Baseline	post inter- vention	Change (post inter- vention scores minus baseline scores)	p value Paired t-test	p Value Group x time ANOVA
Mean	92	13.7±	16.22±	2.52±0.4	0.0001	70	12.94±	14.16±	1.20±	0.025	0.045
Memory test scores*		0.588	0.414				0.564	0.518	0.521		

Data given as Mean ±Standard Error of mean.

\*Significant group x time interaction ANOVA showing improvement of memory scores in the experimental group.

received the placebo. These results are in contrast with those obtained in infants where the benefits of treatment are rarely observed. The adverse effects on cognitive and educational test performance due to iron deficiency anemia in preschool and school-aged children appear more transitory in nature than the effects on development in infants, and imply that treatment of iron deficiency anemia in preschool and school-aged children through iron supplementation programs may have beneficial and immediate effects.

The memory scores obtained at the end of the study were subtracted from the initial scores to obtain the change in scores. A comparison was made between the experimental and control groups with regard to the change in scores to offset the increment due to familiarity with the re-test. In earlier studies in India [33], there also were increments in scores in arithmetic and digit span subtests in the placebo group. In a Thailand study [34] all the children improved their scores at follow-up regardless of their iron status. It can be reasoned that there is always a familiarity element when a re-test is given and this familiarity leads to improvement in scores in the control groups. In this study, an improvement in memory scores was also seen in the control group. To offset this improvement in scores due to familiarity, the post-intervention score was subtracted from baseline score and the change in scores was taken to consider whether there was an improvement of the experimental group over the control. We found a significant improvement in the change in memory scores in the experimental group compared to the control in this study.

In an earlier study [18] we tested the efficacy of the multiple-micronutrient salt in improving the iron status in school children, and also tested for improvement in cognition in children in the age group 7–11 years. We found an improvement in hemoglobin and

red cell count in these children who had been given the multiple micronutrient-fortified salt, as well as improvement in 4 out of the 7 memory tests and in the letter cancellation test in the experimental group of children when compared to the control. In this study therefore we decided to give the memory tests to older children in the 11–18 year age group, and in this study too a significant improvement ( $p < 0.05$ ) in memory scores in the experimental group of children could be attributed to the multiple micronutrients in the fortified salt.

A recent study in India [37] showed that even twice weekly supplementation with iron and folic acid tablets improved cognition in adolescent girls. Thus it may be theorized that the chelated iron in this study contributed substantially to the improvement in hemoglobin, decrease in anemia, and improvement in cognitive scores in the children. Other studies [8,11] which have given multiple micronutrients to children have shown improvements in cognition scores.

It is rather difficult to pinpoint which of the micronutrients, other than the iron in the multiple micronutrients, were responsible for the improvement of cognitive scores in children. There is a lot of interaction amongst micronutrients and several micronutrients work synergistically. It is well known that vitamin A improves iron status. Also, several B-complex vitamins are required during erythropoiesis. Riboflavin supplementation has been shown to improve hemoglobin in earlier studies [38]. It has been shown that riboflavin has a direct role in the release of iron from ferritin [39,40]. Animal studies have shown that riboflavin deficiency impairs iron absorption [41,42]. Moreover, other studies have shown [43,44] a better hematological response when riboflavin was given along with iron than when iron was given alone. A recent study in Bangalore, a

neighboring state, on 100 non-pregnant, non-lactating women [45], showed good correlations between blood hemoglobin and serum ferritin and dietary intakes of riboflavin. A study in Vietnam [46] showed that children who were given milk as an intervention improved their short-term memory scores. It is probable that the B-complex vitamins in the milk helped improve their cognitive performance. Though we did not carry out biochemical analyses of B-complex deficiency in this study, we observed clinical signs of angular stomatitis, which may indicate B-complex deficiency. Angular stomatitis may also be due to infection, as it responded to topical applications of antibiotics or gentian violet as in earlier studies, [47] or due to micronutrient deficiencies [48]. Because angular stomatitis was reduced significantly in our study when fortified salt was used, it may be assumed that the cause of angular stomatitis in our study might have been micronutrient deficiencies and not infection. The improved B-complex status in the children could have led to the improvement in cognition scores as well. We feel that it is the synergistic interaction of the multiple micronutrients that led to improved micronutrient status in the children, which in turn led to improvement in cognition scores.

We decided to measure serum ferritin, sTfR, serum vitamin B<sub>12</sub>, serum folic acid, and serum zinc only in a subsample of 50 children in each group with the lowest hemoglobin, since this is more than the minimum sample size needed with power of 80 % and alpha value of 0.05. This resulted in the baseline sTfR and body iron stores being significantly different in the experimental and control groups at baseline, therefore we used ANCOVA to adjust for the baseline differences. Baseline hemoglobin also was significantly different between the experimental and control groups and again we used ANCOVA to adjust for baseline differences. We also decided to measure serum vitamin A only in those children with physician-identified, clinical vitamin A deficiency, because this sample size is also more than the minimum sample size needed with power of 80 % and alpha of 0.05; we followed this procedure in our earlier study [17].

There was no significant change in serum ferritin in the experimental group over 9 months. However there was a significant decrease in sTfR in the experimental group over 9 months. Elevated sTfR (>7.6 mg/L.) is a sign of tissue iron deficiency and a significant decrease in sTfR in the experimental group shows that iron absorption took place over the study period. No significant change in serum ferritin

in the experimental group may also be due to the concurrent zinc present as a fortificant, which competes with iron for absorption. Serum ferritin <15 µg/L denotes depleted iron status. Another reason for no significant change in serum ferritin in the experimental group may be because at baseline only half the children in the experimental group had depleted iron status (serum ferritin <15 µg/L), but 91 % of the children at baseline had tissue iron deficiency (sTfR >7.6 mg/L.). Significant change was therefore observed in the experimental group in sTfR but not in serum ferritin.

The normal range of folic acid is 1.3 to 13.7 ng/mL in serum. In our study, both in the experimental and control group, at baseline or post-intervention there were no children who were folic acid-deficient; i.e. with folic acid values <1.3 ng/mL. In both the experimental and control groups there was a decrease in mean folic acid values, but the decrease in the control group was larger than the decrease in the experimental group. The study results demonstrate the fluctuating values of serum folic acid in non-deficient children.

The bioavailability of all the vitamins and minerals has been studied extensively in the past when they have been delivered as supplements in the form of tablets or syrups, but what is different in this study is that these fortificants must be stable at the high temperatures of cooking, and during storage in the harsh environment of salt. We found that all the fortificants except vitamin A are very stable during storage and cooking. Even with respect to vitamin A, its stability is considerably enhanced by microencapsulating it, as was done in this study. With appropriate overages as was added in this study, we find that the vitamin A is highly stable and bioavailable, and was able to decrease the prevalence of retinol deficiency from 57.1 % to 16 % in the experimental group. The B-complex vitamins and iodine were also very stable during cooking and storage.

Several factors contributed to the absorption of all the micronutrients in this study. The salt was used in cooking in all the meals. The children consumed 3 meals and an evening snack each day, therefore the micronutrients were delivered in small doses throughout the day. Malaria is not a problem in this area and hookworms too are normally not present in this region. Hookworms thrive in cool moist soil whereas the temperatures in Chennai are high for most parts of the year. *Ascaris* infection may be commonly present and we tackled that through periodic de-worming.

Global control of multiple micronutrient deficiencies requires an integrated approach of food fortification, targeted supplementation, and dietary diversification. A stable and efficacious multiple micronutrient-fortified salt could be useful in combating multiple micronutrient deficiencies in many developing countries. The multiple micronutrient-fortified salt will be especially useful because salt is a commodity consumed universally and in about the same amounts everyday. The multiple micronutrients in the salt were stable during storage and had good bioavailability. This study was carried out in the controlled environment of residential schools. It should be repeated in the form of larger field trials in various communities in many places all over the world.

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