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Research Article

Ferroportin Upregulation For Iron Deficient Anemia- A New Research Insight

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Abstract

The present study discuss about a test formulation containing iron and its usefulness in treating iron deficiency anemia. The formulation was tested by CaCo2 cell line using TEER and found superior release of iron from the formulation than from equal strength of ferrous sulphate solution. The clinical evaluation on clinically diagnosed iron deficiency anemia patients has shown statistically significant improvement in hemoglobin, iron, and ferritin, packed cell volume (PCV), total RBC (T.Rbc), mean corpuscular volume (MCV), mean corpuscular hemoglobin after 30 and 90 days of treatment. Details of the study are presented in the article.

Keywords: Phyto-boosters, Iron deficient anemia, Phyllanthus embilica, Iron tonic

INTRODUCTION

Iron is the most essential element for the formation of hemoglobin in the blood that carries oxygen (1). Further iron is also necessary element to keep the cells of the skin and hair remain active and healthy (2).

The major source of iron that comes to our body is from the food that we eat. Iron is absorbed by the gastrointestinal tract and only a small portion of the iron is absorbed by the intestine which is being absorbed into the body. The iron thus absorbed by the body is then released into the blood, the protein transferrin binds to the iron and then release the same into the liver (3). Iron is stored in the liver as ferritin and is then released for the formation of new red blood cells in the bone marrow. When red blood cells are no longer able to function (after about 120 days in circulation), they are re-absorbed by the spleen. Iron from these old cells can also be recycled by the body. Hepcidin regulate how much iron to be absorbed by acting as s switch to on and off to keep the iron balance while ferritin act as iron reservoir (4).

The deficiency of iron would result in decreased oxygen delivery into the body and that may cause the following medical indication such as pale/yellow sallow skin colour, fatigue or lack of energy, shortness of breath or chest pain, generalized weakness, rapid heartbeat, whooshing in the ears, headache, picophagia, sore or smooth tongue, brittle nails and hair loss.

Increased iron absorption can be achieved through the help of certain herbal actives and vitamin substance like ascorbic acid (5). In the present study we have evaluated the absorption level of iron from a formulation by Caco-2 cells. TEER (Trans Epithelial Electric Resistance) using EVOM2 with STX2 electrodes was used for the above purpose (6,7).

The formulation contains iron in the form of ferrous sulphate along with the herbal actives such as Phyllanthus embilica, Phyllanthus amarus, and Moringa oleifera. After establishing the superior iron absorption efficacy by Caco-2, the formulation was further evaluated clinically for its effect in improving haemoglobin, ferritin, iron, along with various associated parameters linked directly to iron deficiency such as Polymorphonuclear neutrophils (PMN) , packed cell volume (PCV), total RBC (T.Rbc), mean corpuscular volume (MCV), mean corpuscular hemoglobin in clinically diagnosed iron deficiency anemia subjects. The present study report the findings in detail.

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MATERIALS AND METHODS

Cell culture assay

Preparation of cells Caco-2 cell lines

Thaw the frozen Caco-2 cells and culture them in DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin in a T-75 flask. The flask was incubated at 37°C with 5% CO^2 until cells reach 80-90% confluence. Then the cells were washed in PBS and the cells were detached using Trypsin-EDTA solution. Then fresh DMEM with FBS was added to neutralize Trypsin, then the cells were centrifuged at 1500 rpm for 5 minutes. The cell pellet thus obtained were re-dissolved in DMEM and counted the cells using a haemocytometer (7).

Seeding Cells in Transwell Inserts

The cells were adjusted to the concentration of 1×10^6 cells/mL and then 500 µL of cell suspension was added to each Transwell insert (apical side) and then 1.5 mL of DMEM was added to the basolateral side. Then the entire setup was incubated at 37°C with 5% CO_2 for 21 days, to allow the formation of a monolayer of Caco2.

Principle of the test

The present experiment is designed to evaluate the absorption of ferrous sulphate and such preparations across a Caco-2 cell monolayer, which the most opted cell culture models of human intestinal barrier studies. The integrity of the monolayer is monitored using Transepithelial Electrical Resistance (TEER), a measure of ionic flow across the cell layer that reflects tight junction integrity. A decrease in TEER after treatment with ferrous sulphate may indicate changes in barrier function.

Following absorption phase, the concentration of iron in the basolateral chamber was determined by colorimetric assay. The assay typically involves the formation of a coloured complex between ferrous ions and a chromogenic reagent, with the intensity of the colour being proportional to the iron concentration, which can be quantified by measuring absorbance using a spectrophotometer and the value is compared with standard curve of known concentrations of iron samples.

TEER measurement to confirm monolayer integrity

On Day 21, TEER measurement was taken and the TEER value of 500 Ω·cm² was considered for the formation of monolayer and this value was recorded as initial value (pre-treatment) (6).

Preparation of ferrous sulphate solution and the formulation

A stock solution of ferrous sulphate and test formulation in DMEM was prepared at a concentration of 0.8 % solution and 10 µl/ml of sample was used for the test. Then the preparation was filtered using 0.22 µm syringe filter to sterilize the sample.

Treatment with ferrous sulphate and the formulation

The medium was aspirated from both the apical and basolateral compartments and then the cells were washed with warm PBS. Then 500 µL of ferrous sulphate solution and the formulation solution were added to the apical side and then 1.5 mL of fresh DMEM to the basolateral side. The setup was incubated at 37°C for different time points (e.g., 30 minutes, 1 hour, 2 hours).

TEER measurement post-treatment

TEER measurement at each time point was taken to calculate the impact of ferrous sulphate and the formulation on monolayer integrity and the value was compared with initial value to understand the percentage difference.

Colorimetric evaluation of iron was performed by using the standard procedure (8)

The following chemicals were used for the assay

1. Ferrozine reagent (chromogenic reagent for iron)
2. Iron standard solution (for calibration curve)
3. Acetate buffer (pH 4.5)
4. 96-well microplate
5. Spectrophotometer (capable of reading at 562 nm)
6. Samples from the basolateral chamber (after ferrous sulphate and test formulation)

Preparation of iron standards

Series of iron standard solutions was prepared in DMEM ranging from 0 µg/mL to 100 µg/mL and was used for creating a standard curve.

Preparation of Ferrozine reagent

The Ferrozine reagent was prepared in acetate buffer (pH 4.5) to obtain a 1% solution and care was taken to use freshly prepared solution for analysis.

Sample Collection

Collected 100 µL of the medium from the basolateral chamber after the final time point of ferrous sulphate and the test formulation treatment. And further dilution of the sample was followed if necessary, using DMEM to match the standard curve.

Colorimetric assay

100 µL of each standard solution, sample solution, and blank (DMEM without iron) into separate wells of a 96-well microplate. Then to each well, 100 µL of Ferrozine reagent was added. Then the plate was mixed well and incubated at room temperature for 10 minutes to allow colour to develop.

Measurement of absorbance

The absorbance value of each well was measured at 562 nm using a spectrophotometer. And similarly a standard curve

using known concentrations of iron was prepared. And the iron value in the test was compared with the concentration of iron in the standard curve using the absorbance value and the result was expressed in µg/mL.

Clinical evaluation

Iron-deficiency anemia is known to alter the complete blood count (CBC) and serum ferritin. It is recommended that 150 to 200mg per day intake of elemental iron for managing iron deficiency anemia. Among the elemental iron recommended are ferrous sulfate, fumarate or gluconate.

The clinical evaluation was aimed at the following objectives

1. Hemoglobin improvement in blood after daily consumption of 15 ml the test formulation for 90 days along with iron deficiency linked parameters such as PMN- Polymorphonuclear neutrophils, PCV – packed cell volume, T.Rbc – total RBC, MCV- mean corpuscular volume, Mch - mean corpuscular hemoglobin
2. Palatability and acceptability of the test formulation
3. The side effects such as

1. abdominal discomfort,
2. nausea,
3. vomiting,
4. diarrhea,
5. constipation, and dark stools

Age group of subjects
Subjects of either gender between 20 to 60 years of age
Number of patients – 25
Male – 12
Female - 13

Inclusion criteria

1. Subjects with all or any of the signs and symptoms of iron deficiency anemia
2. Meeting the age group
3. Hemoglobin value below 12 for female and below 14 for male
4. Pregnant women
5. Subjects who does not suffer from any of the medical conditions that overlap with the side effects of iron supplementation

Exclusion criteria

1. Subjects who are already taking iron supplementation
2. Subjects who have other medical condition where iron supplementation contravene with the treatment
3. Subjects whom iron supplementation is not recommended

IEC approval

The study was performed after obtaining IEC approval and the study details are registered at CTRI. CTRI/2025/01/079792 dated 30-01-2025.

After informed consent was obtained from the subject who met the norms of inclusion criteria were given the test formulation 15 ml per day for 90 days.

The hemoglobin value in each patient was measured on day zero, day 30, day 90 and the hemoglobin value was recorded. The difference in the value during the treatment period from day zero to day 90 in each patient was analyzed for statistical significance to understand the clinical efficacy.

Similarly various other linked parameters of iron deficiency were also examined in each subject before and during treatment to understand how the test formulation intervention has improved the iron deficiency. The parameters include PMN- Polymorphonuclear neutrophils, PCV – packed cell volume, T.Rbc – total RBC, MCV- mean corpuscular volume, Mch - mean corpuscular hemoglobin.

Details of the investigational product

The test formulation (JRK's Ferro bekay tonic) is a proprietary Siddha medicine licensed to manufacture and market in India by State drug licensing authority – Indian medicine

Composition

Each 10 ml contains
Moringa oleifera – 150 mg
Annabethi (Processed ferrous sulphate) – 100mg
Phyllanthus emblica – 100 mg
Phyllanthus amarus- 100mg
Excipients- QS

RESULTS

Cell culture assay

Table 1. TEER measurement and iron absorption from ferrous sulphate.

Sample ID	Time Point	Initial TEER (Ω·cm²)	Final TEER (Ω·cm²)	% Change in TEER	Iron Concentration (µg/mL)
1	0 min	550	550	0	0.00
2	30 min	550	522	5.45	2.50
3	60 min	550	519	5.63	4.10
4	120 min	550	500	9.09	8.10
Control	120 min	550	540	1.81	0.00

Table 2. TEER measurement and iron absorption from test formulation.

Sample ID	Time Point	Initial TEER (Ω·cm²)	Final TEER (Ω·cm²)	% Change in TEER	Iron Concentration (µg/mL)
1	0 min	550	550	0	0.00
2	30 min	550	512	6.90	3.1
3	60 min	550	500	9.09	6.0
4	120 min	550	460	16.3	13.3
Control	120 min	550	540	1.81	0.00

Iron absorption from the test formulation was significantly higher from the test formulation than from ferrous sulphate solution despite all cell culture conditions and Caco2 permeability were same, **Table 1 and Table 2**.

Clinical evaluation

Table 3. Clinical evaluation of the test formulation in improving hemoglobin, iron, and ferritin after 30 days

Volunteers	Hemoglobin		Iron		Ferritin	
	12 to 15		50-170µg/dl		10 to 291 ng/ml	
	Before	After	Before	After	Before	After
1	11.1	11.8	52	54	54	54
2	12.1	13.5	69	87	37.8	36.4
3	10	12.1	51	69	36	51.4
4	6	7	36	37	11	22
5	11.7	11.9	47	64	16	25
6	8.2	8.6	42	45	18	27
7	11.5	12.3	74	60	14.9	22.7
8	9	11	51	67	17	25.6
9	13.5	13.8	100	109	45.9	50.4
10	11	12.5	80	100	41	58
11	10	11.8	45	69	14	22
12	9.8	10.5	42	74	18	28
13	10	11.9	48	78	15	21
14	8.5	9	46	74	20	27
15	10	11.2	49	72	21	25
16	10	11.1	50	77	24	29
17	9	10.2	40	60	14	26
18	9.5	10.5	42	65	28	35
19	8	9.1	41	55	21	31
20	8.8	9.9	39	49	24	28
21	8	8.8	42	58	26	29
22	8.5	9.2	41	64	19	21
23	9	9.8	43	69	24	27
24	9	10	41	70	31	42
25	9.1	11	40	67	18	27

All the three parameters such as hemoglobin, iron content in blood and ferritin showed a significant increase in all the volunteers treated with the test formulation for 30 days and the difference post treatment is statistically significant with P value $p < .05$.

Statistical Analysis – t test

Hemoglobin

Difference Scores Calculations

Mean: 1.09

$\mu = 0$

S2 = SS⁄df = 6.93/(25-1) = 0.29
S2M = S2/N = 0.29/25 = 0.01
SM = √S2M = √0.01 = 0.11

T-value Calculation

t = (M - μ)/SM = (1.09 - 0)/0.11 = 10.13
The value of t is 10.126299. The value of p is < .00001. The result is significant at p < .05.

Iron

Difference Scores Calculations
Mean: 17.7
μ = 0
S2 = SS⁄df = 2852/(25-1) = 118.83
S2M = S2/N = 118.83/25 = 4.75
SM = √S2M = √4.75 = 2.18
T-value Calculation
t = (M - μ)/SM = (17.7 - 0)/2.18 = 8.12
The value of t is 8.118469. The value of p is < .00001. The result is significant at p < .05.

Ferritin

Difference Scores Calculations
Mean: 6.4
μ = 0
S2 = SS⁄df = 579.19/(25-1) = 24.13
S2M = S2/N = 24.13/25 = 0.97
SM = √S2M = √0.97 = 0.98
T-value Calculation
t = (M - μ)/SM = (6.4 - 0)/0.98 = 6.52
The value of t is 6.518033. The value of p is < .00001. The result is significant at p < .05.

Table 4. Clinical evaluation of test formulation in modulating various iron deficiency linked parameters after 30 days.

Volunteers	Poly (PMN)		PCV		T.Rbc		MCV		Mch	
	45-75%		36-48%		3.8-4.8%		83-101 fl		27-32 pg	
	Before	After	Before	After	Before	After	Before	After	Before	After
1	58	50	28	37	3.5	4	71	85	22	29
2	59	49	29	36	2.8	4.1	78	88	25	28
3	57	50	27	34	2.5	4.2	70	89	21	31
4	62	53	30	41	2.4	3.9	69	90	21	30
5	63	59	27	39	3	4.1	68	89	26	29
6	61	52	25	38	2.8	4.4	70	87	27	27
7	52	45	21	37	2.6	3.2	71	86	24	28
8	57	43	23	39	2.5	3.8	60	92	25	31
9	59	50	26	41	2.7	3.6	64	94	22	30
10	64	53	23	31	2.6	3.4	66	91	26	29
11	61	57	22	38	2.8	3.7	69	92	27	27
12	60	55	31	34	3	4.1	72	89	21	30
13	66	52	29	38	3.1	4.5	69	88	21	27
14	67	53	20	37	3.5	3.9	60	89	20	29
15	59	58	27	38	3.3	4.5	68	90	19	31
16	62	55	29	41	2.8	3.6	66	79	26	30
17	54	49	23	42	2.9	3.9	71	89	22	27
18	59	43	29	40	2.4	3.7	79	88	20	29
19	67	60	25	39	3	3.9	77	92	27	31
20	70	62	27	38	2.9	3.6	69	91	28	30
21	62	55	21	35	2.7	3.8	70	90	21	27
22	68	50	24	38	3.6	4.6	68	88	25	28
23	61	52	28	41	3.4	4.5	72	80	22	32
24	55	54	30	43	2.9	3.9	70	80	27	29
25	53	46	25	39	2.8	3.7	74	81	23	31

PMN- Polymorphonuclear neutrophils, PCV – packed cell volume, T.Rbc – total RBC, MCV- mean corpuscular volume, Mch - mean corpuscular hemoglobin All the three parameters such as PCV, T.Rbc, MCV, Mch showed a significant increase in all volunteers treated who received the test formulation and the difference post treatment is statistically significant with P value p < .05.The PMN levels showed decrease from the initial value.

Statistical analysis t-test

Poly (PMN)

Difference Scores Calculations
Mean: -8.44
μ = 0
S2 = SS⁄df = 438.16/(25-1) = 18.26
S2M = S2/N = 18.26/25 = 0.73
SM = √S2M = √0.73 = 0.85
T-value Calculation
t = (M - μ)/SM = (-8.44 - 0)/0.85 = -9.88
The value of t is -9.876469. The value of p is < .00001. The result is significant at p < .05.

PCV

Difference Scores Calculations
Mean: 12.2
μ = 0
S2 = SS⁄df = 318/(25-1) = 13.25
S2M = S2/N = 13.25/25 = 0.53
SM = √S2M = √0.53 = 0.73
T-value Calculation
t = (M - μ)/SM = (12.2 - 0)/0.73 = 16.76
The value of t is 16.757989. The value of p is < .00001. The result is significant at p < .05.

T.RBC

Difference Scores Calculations
Mean: 1.04
μ = 0
S2 = SS⁄df = 2.54/(25-1) = 0.11
S2M = S2/N = 0.11/25 = 0
SM = √S2M = √0 = 0.07
T-value Calculation
t = (M - μ)/SM = (1.04 - 0)/0.07 = 16.04
The value of t is 16.040671. The value of p is < .00001. The result is significant at p < .05.

MCV

Difference Scores Calculations
Mean: 18.24
μ = 0
S2 = SS⁄df = 1084.56/(25-1) = 45.19
S2M = S2/N = 45.19/25 = 1.81
SM = √S2M = √1.81 = 1.34
T-value Calculation
t = (M - μ)/SM = (18.24 - 0)/1.34 = 13.57
The value of t is 13.566683. The value of p is < .00001. The result is significant at p < .05.

Mch

Difference Scores Calculations
Mean: 5.68
μ = 0
S2 = SS⁄df = 263.44/(25-1) = 10.98
S2M = S2/N = 10.98/25 = 0.44
SM = √S2M = √0.44 = 0.66
T-value Calculation
t = (M - μ)/SM = (5.68 - 0)/0.66 = 8.57
The value of t is 8.572019. The value of p is < .00001. The result is significant at p < .05.

Table 5. Clinical evaluation of test formulation in improving hemoglobin, iron, and ferritin after 90 days.

Volunteers	Hemoglobin		Iron		Ferritin	
	12 to 15		50-170μg/dl		10 to 291 ng/ml	
	Before	After	Before	After	Before	After
1	11.8	12	54	55	54	57
2	13.5	13.8	87	88	36.4	36.7
3	12.1	12.5	69	70	51.4	54.6
4	7	8	37	42	22	25.1
5	11.9	12	64	70	25	29.3
6	8.6	8.8	45	55	27	29
7	12.3	12.5	60	63	22.7	25.1
8	11	11	67	69	25.6	29
9	13.8	13.8	109	110	50.4	52
10	12.5	12.4	100	101	58	61
11	11.8	12.1	69	69	22	27
12	10.5	11.2	74	76	28	32
13	11.9	12	78	80	21	25
14	9	10.5	74	79	27	37
15	11.2	11.9	72	77	25	33
16	11.1	11.3	77	81	29	34
17	10.2	10.3	60	65	26	30.5

18	10.5	10.8	65	69	35	39
19	9.1	9.7	55	57	31	33.2
20	9.9	10.0	49	51	28	37.9
21	8.8	9.1	58	63	29	31
22	9.2	9.5	64	67	21	22
23	9.8	10.4	69	72	27	28
24	10	10.1	70	75	42	42.3
25	11	11.1	67	69	27	28

All the three parameters such as hemoglobin, iron content in blood and ferritin showed a significant increase in all the volunteers treated with the test formulation for 90 days and the difference is statistically significant with P value $p < .05$.

Statistical Analysis – t test

Hemoglobin

Difference Scores Calculations

Mean: 0.33

$\mu = 0$

$S2 = SS/df = 3.03/(25-1) = 0.13$

$S2M = S2/N = 0.13/25 = 0.01$

$SM = \sqrt{S2M} = \sqrt{0.01} = 0.07$

T-value Calculation

$t = (M - \mu)/SM = (0.33 - 0)/0.07 = 4.67$

The value of t is 4.668499. The value of p is .00005. The result is significant at $p < .05$

Iron

Difference Scores Calculations

Mean: 3.2

$\mu = 0$

$S2 = SS/df = 118/(25-1) = 4.92$

$S2M = S2/N = 4.92/25 = 0.2$

$SM = \sqrt{S2M} = \sqrt{0.2} = 0.44$

T-value Calculation

$t = (M - \mu)/SM = (3.2 - 0)/0.44 = 7.22$

The value of t is 7.215802. The value of p is < .00001. The result is significant at $p < .05$.

Ferritin

Difference Scores Calculations

Mean: 3.53

$\mu = 0$

$S2 = SS/df = 161.33/(25-1) = 6.72$

$S2M = S2/N = 6.72/25 = 0.27$

$SM = \sqrt{S2M} = \sqrt{0.27} = 0.52$

T-value Calculation

$t = (M - \mu)/SM = (3.53 - 0)/0.52 = 6.8$

The value of t is 6.803715. The value of p is < .00001. The result is significant at $p < .05$

Table 6. Clinical evaluation of the test formulation in modulating various iron deficiency linked parameters after 90 days.

Volunteers	Poly (PMN)		PCV		T.Rbc		MCV		Mch	
	45-75%		36-48%		3.8-4.8%		83-101 fl		27-32 pg	
	Before	After	Before	After	Before	After	Before	After	Before	After
1	50	45	37	39	4	5	85	87	29	30
2	49	48	36	38	4.1	5.1	88	90	28	31
3	50	45	34	36	4.2	4.8	89	92	31	32
4	53	51	41	44	3.9	4.2	90	91	30	31
5	59	56	39	41	4.1	4.4	89	90	29	33
6	52	49	38	45	4.4	5.0	87	88	27	31
7	45	43	37	42	3.2	3.8	86	88	28	30
8	43	41	39	44	3.8	4.0	92	94	31	31
9	50	48	41	45	3.6	3.8	94	95	30	31
10	53	50	31	36	3.4	4.0	91	92	29	31
11	57	55	38	40	3.7	4.2	92	95	27	29
12	55	52	34	38	4.1	4.5	89	93	30	30
13	52	49	38	42	4.5	4.8	88	90	27	31
14	53	49	37	40	3.9	4.3	89	91	29	30
15	58	51	38	41	4.5	4.7	90	94	31	31
16	55	53	41	43	3.6	4.4	79	82	30	32
17	49	40	42	45	3.9	4.5	89	90	27	28

18	43	41	40	42	3.7	4.6	88	89	29	30
19	60	52	39	41	3.9	4.1	92	93	31	31
20	62	55	38	44	3.6	4.2	91	92	30	30
21	55	50	35	40	3.8	4.6	90	93	27	28
22	50	46	38	42	4.6	4.7	88	90	28	29
23	52	51	41	45	4.5	4.6	80	84	32	31
24	54	50	43	48	3.9	4.1	80	82	29	30
25	46	42	39	43	3.7	4.0	81	83	31	31

PMN- Polymorphonuclear neutrophils, PCV – packed cell volume, T.Rbc – total RBC, MCV- mean corpuscular volume, Mch -mean corpuscular hemoglobin All the three parameters such as PCV, T.Rbc, MCV, Mch showed a significant increase in all the volunteers treated with the test formulation and the difference is statistically significant with P value $p < .05$.
The PMN levels showed decrease from the initial value.

Statistical analysis t-test

Poly (PMN)

Difference Scores Calculations
Mean: -3.72
$\mu = 0$
$S2 = SS/df = 111.04/(25-1) = 4.63$
$S2M = S2/N = 4.63/25 = 0.19$
$SM = \sqrt{S2M} = \sqrt{0.19} = 0.43$
T-value Calculation
$t = (M - \mu)/SM = (-3.72 - 0)/0.43 = -8.65$
The value of t is -8.647266. The value of p is < .00001. The result is significant at $p < .05$.

PCV

Difference Scores Calculations
Mean: 3.6
$\mu = 0$
$S2 = SS/df = 50/(25-1) = 2.08$
$S2M = S2/N = 2.08/25 = 0.08$
$SM = \sqrt{S2M} = \sqrt{0.08} = 0.29$
T-value Calculation
$t = (M - \mu)/SM = (3.6 - 0)/0.29 = 12.47$
The value of t is 12.470766. The value of p is < .00001. The result is significant at $p < .05$.

T.RBC

Difference Scores Calculations
Mean: 0.47
$\mu = 0$
$S2 = SS/df = 1.83/(25-1) = 0.08$
$S2M = S2/N = 0.08/25 = 0$
$SM = \sqrt{S2M} = \sqrt{0} = 0.06$
T-value Calculation
$t = (M - \mu)/SM = (0.47 - 0)/0.06 = 8.55$
The value of t is 8.545641. The value of p is < .00001. The result is significant at $p < .05$.

MCV

Difference Scores Calculations
Mean: 2.04
$\mu = 0$
$S2 = SS/df = 24.96/(25-1) = 1.04$
$S2M = S2/N = 1.04/25 = 0.04$
$SM = \sqrt{S2M} = \sqrt{0.04} = 0.2$
T-value Calculation
$t = (M - \mu)/SM = (2.04 - 0)/0.2 = 10$
The value of t is 10.001923. The value of p is < .00001. The result is significant at $p < .05$.

Mch

Difference Scores Calculations
Mean: 1.28
$\mu = 0$
$S2 = SS/df = 43.04/(25-1) = 1.79$
$S2M = S2/N = 1.79/25 = 0.07$
$SM = \sqrt{S2M} = \sqrt{0.07} = 0.27$
T-value Calculation
$t = (M - \mu)/SM = (1.28 - 0)/0.27 = 4.78$
The value of t is 4.779137. The value of p is .00004. The result is significant at $p < .05$

DISCUSSION

Though the iron deficiency anemia is easily treatable but the medical condition still pose a major public health problem especially in rural India (9). Lack of absorbable iron in the food and possible loss of iron from body cause iron deficient anemia. Even when the common iron deficient anemia signs and symptoms are pronounced such as fatigue, nausea, generalized weakness, anxiety, irritability, hair loss, poor brain power, the treatment is never sought by those who suffer from iron deficient anemia making the medical condition difficult to be addressed, early (10).
Though plenty of iron supplements are available, often they cause various side effects such as stomach ache, constipation, nausea etc. Further, the absorption rate of iron from such supplements is relatively poor. The iron that get absorbed in the stomach is likely to get rejected in the intestinal region

and therefore the absorption of iron in the intestine also must be considered. Only from the intestinal region, the iron is transported to blood and then to liver.

In the present investigation, a test formulation that contain iron in the form of annabedi or processed ferrous sulphate, was incorporated besides certain select herbs. The herbal inclusion was done mainly to increase the iron absorption as several herbal preparations are known to increase iron absorption. Initially the formulation was tested at in vitro level in Caco2 cell line by TEER. The concentration of the processed iron in the formulation was tested against the same concentration of ferrous sulphate solution. The permeability of Caco2 used for testing both the test formulation and ferrous sulphate solution was ascertained to be similar. Then the absorption rate of iron from both system was tested by calorimetry. The findings show that, iron content absorbed by Caco2 cells from the test formulation treatment was several fold higher than from ferrous sulphate solution treatment of same concentration.

We questioned why despite the Caco2 cells having similar permeability, the iron absorption was greater from the test formulation than from ferrous suphate solution. The possible explanation could be, due to the role of herbal constituents in the test formulation that may aiding greater iron absorption than from ferrous suphate solution. The mechanism of action and the underlying science is yet to be established, the findings clearly show the superior iron deliverability of the test formulation.

Based on the findings obtained with Caco2 cell lines, the clinical evaluation of the test formulation was conducted in 25 clinically diagnosed iron deficiency anemia subjects. Both gender and diverse age groups were considered for the study. Various blood parameters directly linked to iron deficiency anemia were measured after 30 and 90 days of usage of the test formulation. Hemoglobin, ferritin and iron in the blood of all the 25 subjects showed significant increase after 30 and 90 days and the difference was statistically significant by students t test.

Further, the other parameters such as PVC, T.Rbc, MCV and Mch also showed improvement on day 30 and day 90 and the difference was statistically significant. The collective change from day zero to day 30 and then day 90 clearly show, the undeniable therapeutic effect of the test formulation in correcting the iron deficient anemia.

The question of why each parameter only showed improvement which was statistically significant level but never reached the standard level or base level to be present in our body, the possible explanation could be the fixed duration of the clinical evaluation we have conducted. For complete change to achieve, prolonged treatment may be required or the possible rate of excretion of iron must be diagnosed.

Findings of the study clearly show that the test formulation is effective in improving the iron content in blood which was confirmed by more than one parameters that are associated and linked to the medical condition. During the 90 day trial period, no probable side effects such as nausea, stomach ache, constipation, lingering iron taste in palate or any other complaints reported by the subjects.

The considerable safety and efficacy of the test formulation as established by modern research methods and by clinical trial clearly suggest the usefulness of the test formulation in the treatment of iron deficiency anemia.

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