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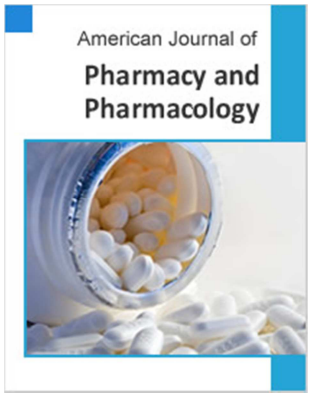

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Hematological Studies in Egyptian Thalassemic Children Infected with Hepatitis C Virus

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Abstract
The present study was conducted to evaluate the efficiency of natural *Spirulina* preparation on some hematological parameters in thalassemic children infected with hepatitis C virus (HCV). Twenty five (25) Egyptian thalassemic children infected with HCV (12 male, and 13 female children), aged from 3 to 8 years were subjected in this study. Volunteers were supplied with dried *Spirulina* (250 mg /kg /day) by oral uptake with their usual thalassemic drugs. Throughout 6 months treatment, Red blood cell count, hemoglobin level, serum ferritin and blood transfusion intervals were assessed. This study proved that there was improvement of the treated thalassemic children after 6 months from uptaking Spirulina

1. Introduction

Thalassaemia is an inherited autosomal recessive blood disease that originated in the Mediterranean region. In thalassemia, the genetic defect which could be either mutation or deletion, results in reduced rate of synthesis or no synthesis of one of the globin chains that make up hemoglobin. This can cause the formation of abnormal hemoglobin molecules, thus causing anemia, the characteristic presenting symptom of the thalassemias [1].

The two major forms of the disease, alpha and beta are prevalent in discrete geographical clusters around the world, it is presumed associated with malarial endemicity in ancient times. Alpha is prevalent in peoples of Western African and South Asian descent. It is nowadays found in populations living in Africa and in the Americas. It is also found in Tharus in the Terai region of Nepal and India. It is believed to account for much lower malaria morbidity and mortality, accounting for the historic ability of Tharus to survive in heavily malarial areas where others could not. Beta thalassemia is particularly prevalent among Mediterranean peoples, and this geographical association is responsible for its naming: Thalassa is Greek for the sea, Haema is Greek for blood. In Europe, the highest concentrations of the disease are found in Greece, coastal regions in Turkey. The thalassemia trait may confer a degree of protection against malaria, which is

or was prevalent in the regions where the trait is common, thus conferring a selective survival advantage on carriers (known as heterozygous advantage), and perpetuating the mutation. In that respect, the various thalassemias resemble another genetic disorder affecting hemoglobin, sickle-cell disease [2].

Normal hemoglobin is composed of four protein chains, two α and two β globin chains arranged into a heterotetramer. Thalassemia patients produce a deficiency of either α or β globin, unlike sickle-cell disease, which produces a specific mutant form of β globin. The thalassemias are classified according to which chain of the hemoglobin molecule is affected. In α thalassemias, production of α globin chain is affected, while in β thalassemia production of the β globin chain is affected [3].

The β globin chains are encoded by a single gene on chromosome 11; α globin chains are encoded by two closely linked genes on chromosome 16. Thus, in a normal person with two copies of each chromosome, there are two loci encoding the β chain, and four loci encoding α chain. Deletion of one of α loci has a high prevalence in people of African or Asian descent, making them more likely to develop α thalassemias. β Thalassemias are not only common in Africans, but also in Greeks and Italians [4].

The α thalassemias involve the genes HBA1 and HBA2, inherited in a Mendelian recessive fashion. There are two gene loci and so four alleles. It is also connected to the deletion of the 16p chromosome. α Thalassemias result in decreased alpha-globin production, therefore fewer alpha-globin chains are produced, resulting in an excess of β chains in adults and excess γ chains in newborns. The excess β chains form unstable tetramers (called Hemoglobin H or HbH of 4 beta chains), which have abnormal oxygen dissociation curves [5].

Beta thalassemias are due to mutations in the HBB gene on chromosome 11, also inherited in an autosomal-recessive fashion. The severity of the disease depends on the nature of the mutation. Mutations are characterized as either β^o or β thalassemia major if they prevent any formation of β chains, the most severe form of β thalassemia. Also, they are characterized as β^+ or β thalassemia intermedia if they allow some β chain formation to occur. In either case, there is a relative excess of α chains, but these do not form tetramers: Rather, they bind to the red blood cell membranes, producing membrane damage, and at high concentrations they form toxic aggregates [6].

As well as alpha and beta chains present in hemoglobin, about 3% of adult hemoglobin is made of alpha and delta chains. Just as with beta thalassemia, mutations that affect the ability of this gene to produce delta chains can occur [7].

Spirulina is the common name for human and animal food supplements. *Spirulina* comes from two different species of cyanobacteria: *Arthrospira platensis*, and *Arthrospira maxima* [8].

Persons suffering from anemia, oral cancers, and elevated cholesterol, may also derive benefit from the nutritional supplementation of *Spirulina* [9]. A preliminary report of the preventive effect of *Spirulina maxima* on the fructose-induced increase of the liver triglycerides level was observed together with an elevation of the phospholipids concentration in this tissue in the rat [10]. On the other hand, *Spirulina maxima* produced a plasma cholesterol level even lower than that observed in the control group [11].

A significant increase in blood hemoglobin levels was seen in the adolescent girls after 1 month of *Spirulina* supplementation. On an average the hemoglobin levels increased by 1.17 g/dL. This data indicates the beneficial effect of *Spirulina* in improving the hemoglobin levels in anemic girls. The supplementation of *Spirulina*, which contains a highly available form of iron, has led to the significant increase in hemoglobin levels in anemic adolescent [12].

2. Materials and Methods

(a) Application on human volunteers

This study was done according to guidelines of Egyptian ministry of Health and Population decree 95/year 2005 for medical research, good clinical practice, Declaration of Helsinki and World Health Organization Guidelines [13]. Spirulina was approved by the FDA (Food Drug Administration) by the issuance of a GRAS (generally recognized as safe) certificate. The FDA has stated that Spirulina can be legally marketed as a food or food supplement without risk to human health [14].

Follow up of 25 (12male, and 13 female) Egyptian thalassemic children infected with HCV, aged from 3 to 8 years was performed, the laboratory tests were performed at start before uptaking Spirulina and after 6 months from uptaking Spirulina. Volunteers were supplied with dried Spirulina (250 mg /kg/day) by oral uptake.

(b) Laboratory equipments

1- Neubauer hemocytometer, HBG Company, Germany.

2- Red blood cells reagent, Egyptian Diagnostic Media Company, Egypt.

3- Microscopic slides and cover glasses, Sail brand Company, China.

4- China light Microscope model XSZ-N107, Shenzhen Uni-Tech Instrument Company, China.

5- Micopette pipettes, Dragon Lab Company, China.

6-Ferritin kits and ELISA Reader

7- Micopette pipettes, Dragon Lab Company, China.

8-Prietest Eco Automatic Biochemistry Analyzer, Robonik Company, India.

(c) Blood sampling and serum preparation:

Blood samples were taken from patients and healthy children at the Hematology & Oncology unit, Tanta University Hospital**.** All samples were directed for the following tests:

(d) Measuring of red blood cell count (Egyptian Diagnostic Media Company, Egypt)

Procedures:

1. Add 4 ml of R.B.Cs counting soluting solution into small stoppered tube
2. Using Automatic pipette draw 0.02 (20 micron) capillary or venous blood
3. Add the blood into 4 ml of R.B.Cs counting soluting solution and mix well
4. Securely attach to counting chamber the special cover glass provided
5. Using a fine bore Pasteur pipette, fill the chamber with the well mixed diluted blood
6. Allow the cells to settle for 5 minutes
7. Using a 40 X objective.count the number of cells in 1/5 sq.mm; using 5 of the small squares of the large center square.

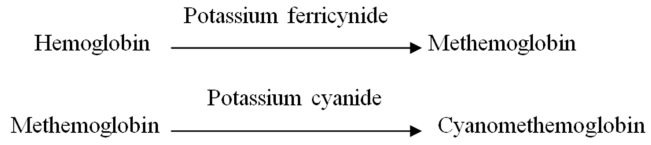
Number of R. B. C s /mm^3= number of R.B.Cs counted in five small squares x 10.000

(e) Measure of Hemoglobin [15]

Specimen: Capillary blood or EDTA anticoagulated venous blood can be used.

Principle of test:

Hemoglobin (oxyhemoglobin, methemoglobin, and carboxy-hemoglobin) is converted to cyanomethemoglobin according to following reactions:



Reagents composition:

Drabkin reagent contain

Potassium ferricyanide 30 mmol/L & Potassium cyanide 38 mmol/L & Monopotassium phosphate 50 mmol/L

Procedure:

This method is done by spectrophotometer

Wavelength: 540 nm & Temperature 37 c

Cuvette: 1 Cm light path

Read against reagent blank

Table 1. *Steps of Hemoglobin test.*

Working reagent	5 µL
Sample	20 µL

Mix and read the optical density within 1 hour

(f) Serum Ferritin ELISA test:

Principle:

Ferritin is an iron storage protein. It consists of 24 subunits with combined molecular weight of 474,000 Da. Serum Ferritin level is related to body iron stores and is influenced by several diseases. A low level of Ferritin is an indication of iron deficiency anemia.

A Ferritin specific antibody has been precoated onto 96-well plates and blocked. Standards or test samples are added to the wells and subsequently a Ferritin specific biotinylated detection antibody is added and then followed by washing with wash buffer. Streptavidin- Peroxidase Complex is added and unbound conjugates are washed away with wash buffer. TMB is then used to visualize Streptavidin- Peroxidase enzymatic reaction. TMB is catalyzed by Streptavidin-Peroxidase to produce a blue color product that changes into yellow after adding acidic stop solution. The density of yellow coloration is directly proportional to the amount of Ferritin captured in plate.

Ferritin Human in vitro ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of Ferritin concentrations in plasma, serum, milk, and cell culture supernatants.

Table 2. *Reagents of Ferritin test.*

Item	Amount
Ferritin Microplate (12 x 8 well strips)	96 wells
Ferritin Standard	1 vial
10X Diluent M Concentrate	30 mL
Biotinylated Human Ferritin Antibody	1 vial
100X Streptavidin-Peroxidase Conjugate (SP Conjugate)	80 µL
Chromogen Substrate	8 mL
Stop Solution	12 mL
20X Wash Buffer Concentrate	2 x 30 mL
Sealing Tapes	3

Procedure [16]

1- Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3,000 x g for 10 minutes and remove serum. Dilute samples 1:10 into 1X Diluents M and assay. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

2- Equilibrate all materials and prepared reagents to room temperature (18 - 25°C) prior to use. It is recommended to assay all standards, controls and samples in duplicate.

3- Prepare all reagents, working standards and samples as instructed. Equilibrate reagents to room temperature before use. The assay is performed at room temperature (18-25°C).

4- Remove excess micro plate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in vacuum desiccators.

5- Add 50 µL of Ferritin Standard or sample per well. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.

6- Wash five times with 200 µL of 1X Wash Buffer manually. Invert the plate each time and decant the contents; tap it on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 µL of 1X Wash Buffer and then invert the plate, decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid.

7- Add 50 µL of 1X Biotinylated Ferritin Antibody to each well and incubate for one hour.

8- Wash microplate as described above.

9- Add 50 µL of 1X SP Conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.

10- Wash microplate as described above.

11- Add 50 µL of Chromogen Substrate per well and incubate for about 15 minutes or till the optimal blue color

density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.

12- Add 50 µL of Stop Solution to each well. The color will change from blue to yellow.

13- Read the absorbance on a microplate reader at a wavelength of 450 nm immediately. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Calculation:

Calculate the mean value of the triplicate readings for each standard and sample. To generate a Standard Curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit. Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

3. Results

(a) Effect of Spirulina on red blood cell count

The results in Table 3 showed that red blood cell count at start before treatment by Spirulina was ranging from 2.1 (x 10^6 / cmm) to 3.9 (x 10^6 / cmm) but after 6 months from treatment by Spirulina was ranging from 2.3 (x 10^6 / cmm) to 5.1 (x 10^6 / cmm). The results also revealed that, there were significant increase in red blood bell count between at start before treatment by Spirulina and after 6 months from treatment by Spirulina (P value <0.0001).

Table 3. *Effect of Spirulina on red blood cell Count.*

Groups	Red blood cell count by (x 10^6 / cmm)						T-Test	
	Range			Mean	±	SD	t	P-value
AT Start	2.1	-	3.9	3.06	±	0.469929	5.90	<0.0001*
After 6 months	2.3	-	5.1	3.632	±	0.583609		

P value is statistically highly significant at< 0.05 level

(b) Effect of Spirulina on hemoglobin level

The results in Table 4 showed that hemoglobin level at start before treatment by Spirulina was ranging from 5.5 (g/100 ml) to 10.6 (g/100 ml) but after 6 months from treatment by Spirulina was ranging from 6.8 (g/100 ml) to 14 (g/100 ml). The results also revealed that, there were significant increase in hemoglobin level between at start before treatment by Spirulina and after 6 months from treatment by Spirulina (P value <0.001).

Table 4. *Effect of Spirulina on hemoglobin level.*

Groups	Hb (g/100 ml)						T-Test	
	Range			Mean	±	SD	t	P-value
AT Start	5.5	-	10.6	7.9	±	1.2	7.1	<0. 001*
After 6 months	6.8	-	14	9.5	±	1.47		

P value is statistically highly significant at< 0.05 level

(c) Effect of *Spirulina* on serum ferritin

The results in Table 5 showed that serum ferritin at start before treatment by Spirulina was ranging from 1327 (ng/L) to 14000 (ng/L) but after 6 months from treatment by Spirulina was ranging 633 (ng/L) to 5465 (ng/L). The results also revealed that, there were significant decrease Serum Ferritin between at start before treatment by Spirulina and after 6 months from treatment by Spirulina (P value = 0. 01).

Table 5. *Effect of Spirulina on serum ferritin.*

Groups	Serum Ferritin (ng/L)						T-Test	
	Range			Mean	±	SD	t	P-value
AT Start	1327	-	14000	3787.08	±	3011.94	2.64	0.01*
After 6 months	633	-	5465	2326.64	±	1174.22		

P value is statistically highly significant at< 0.05 level

(d) Evaluation of blood transfusion intervals as an indicator for improvement of the treated thalassemic children

Another promising result for the improvement of thalassemic syndrome was recorded in table 6, and figure 1, as the blood transfusion intervals were highly prolonged in the improved patients, as there were 17 cases needed blood transfusion with intervals less than 15 days at the start of treatment; while there were 19 cases needed blood transfusion with intervals more than 15 days, which reached 45 days in some cases at the end of treatment.

Table 6. *Evaluation of blood transfusion intervals as an indicator for improvement of the treated thalassemic children.*

Blood transfusion intervals	at start		after 6 months	
	No. of cases	(%)	No. of cases	(%)
≤ 15 days	17	68	6	24
> 15 days	8	32	19	76
Chi square	X^2		76.004	
	P value		< 0.001*	

* P value is statistically highly significant at the 0.001 level.

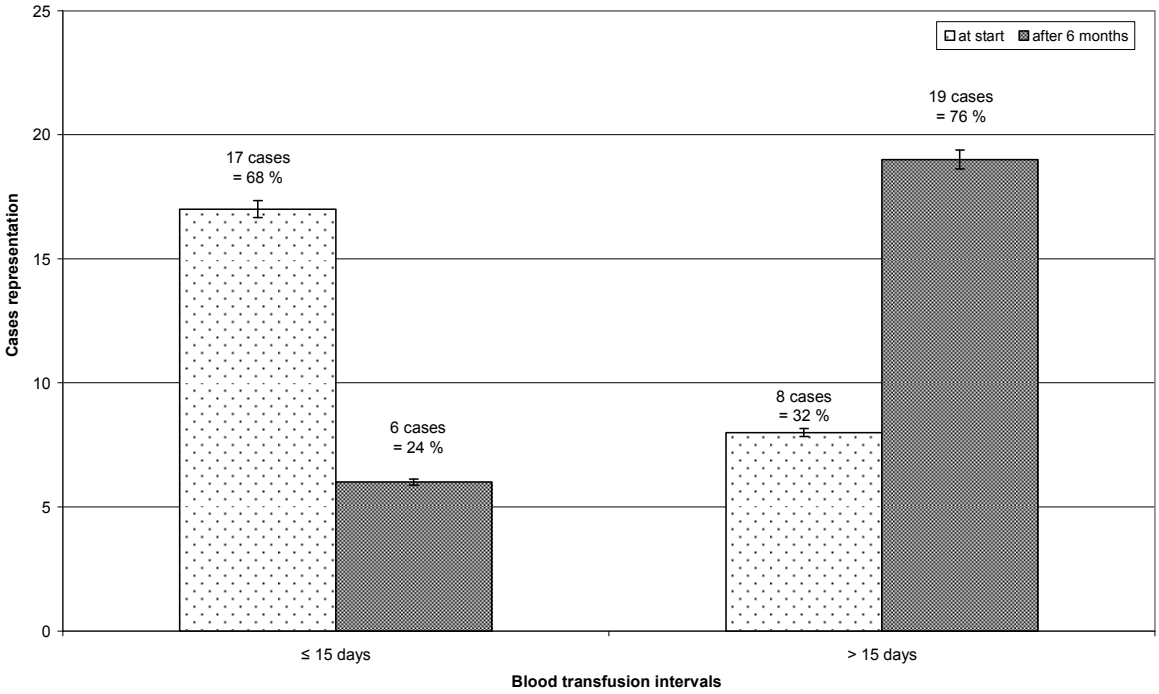


Figure 1. *Evaluation of blood transfusion intervals as an indicator for improvement of the treated thalassemic children.*

4. Discussion

In the present study, red blood cell count was determined as hematological indicator, which was increased from 3.06 ± 0.4699 to 3.632 ± 0.583 after 6 months of the treatment by Spirulina., Hemoglobin level also was determined as hematological indicator, which was decreased from 7.9 ± 1.2 to 9.5 ± 1.47 after 6 months of the treatment by Spirulina.

These findings were in agreement with other studies; as a modern study of HCV treatment in thalassemic patients with different doses of *Spirulina* recorded an improvement in RBCs count in 35 cases out of 40 cases under study [17]. Another study recorded the decreased severity of HCV infection, and the increased count of RBCs in 28 cases out of 40 cases of thalassemic cases [18].

In the present study, serum Ferritin was determined as hematological indicator, which was increased from 3787.08 ± 3011.94 to 2326.64 ± 1174.22 after 6 months of the treatment by Spirulina.

Ferritin levels can be discussed in other studies as an important parameter for health indicator through out the course of the treatment. 36 cases out of 50 cases were improved for more than 2500 ng/L of ferritin during the treatment of HCV thalassemic cases [19]. The ferritin levels were considered as an important health parameter, which was significantly improved during the treatment of thalassemic cases to more than 2500 ng/L in 41 cases out of 60 cases [20].

Another promising result for the improvement of thalassemia was the blood transfusion intervals, blood transfusion intervals were highly prolonged in the improved patients, as there were 68% cases needed blood transfusion with intervals less than 15 days at the start of treatment; while there were 76% cases needed blood transfusion with intervals more than 15 days, which reached 45 days in some cases at the end of treatment.

These findings were in agreement with anther study that recorded prolonged transfusion intervals as an indicator for health improvement in thalassemic patients treated with different natural products of plant origin [21]. Another study recorded a significant increase in blood transfusion intervals up to 40 days in 16 cases out of 20 cases of HCV thalassemic patients treated with algal extracts [22].

5. Conclusion

The thrust of this study to measure red blood cell count, hemoglobin level, serum ferritin and blood transfusion intervals in thalassemic children infected with Hepatitis C

Virus for evaluation the effect of Spirulina on hematological parameters. This study proved that there were improvements in the results of hemoglobin level, serum ferritin and blood transfusion intervals in thalassemic children infected with Hepatitis C Virus after 6 months from uptaking Spirulina.

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