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Immunological 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 in the immune stimulation of 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, white blood cell count, neutrophil percent, CD4 and CD8 were assessed. This study proved that there was immune stimulation to thalassemic children Infected with Hepatitis C Virus after 6 months from uptaking Spirulina.

1. Introduction

Thalassemia is a quantitative problem of too few globins synthesized, whereas sickle-cell disease (a hemoglobinopathy) is a qualitative problem of synthesis of an incorrectly functioning globin. Thalassemias usually result in underproduction of normal globin proteins, often through mutations in regulatory genes. Hemoglobinopathies imply structural abnormalities in the globin proteins themselves. Both the two conditions may overlap, however, since some conditions that cause abnormalities in globin proteins (hemoglobinopathy) also affect their production (thalassemia). Thus, some thalassemias are hemoglobinopathies, but most are not. Either or both of these conditions may cause anemia (Modiano, 2011).

People with thalassemia have an increased risk of infection. This is especially true if the spleen has been removed. Study showed that haemodialysis patients and thalassemia sufferers were at higher risk of having HCV infection; the prevalence being 55.9% and 63.8% respectively in comparison to the prevalence of blood donors (0.5%). A confirmatory immunoblotting was employed using HCV-positive cases (54 thalassemia sufferers and 19 blood donors) (Triantos et al., 2013).

Hepatitis C virus (HCV) is a small (50 nm) in size, enveloped, single-stranded, positive sense RNA virus. It is the only known member of the *hepacivirus* genus in the

family *Flaviviridae*. There are six major genotypes of HCV, which are indicated numerically (e.g., genotype 1, genotype 2, etc.). Based on the NS5 gene there are three major and eleven minor genotypes. A seventh genotype — 7a — has also been described (Nakano et al., 2011).

An estimated 180 million people worldwide are infected with hepatitis C. Hepatitis C is not known to cause disease in other animals. No vaccine against hepatitis C is currently available. The existence of hepatitis C (originally "non-A non-B hepatitis") was postulated in the 1970s and proven in 1989 (Hanafiah et al., 2013).

Spirulina is the common name for human and animal food supplements. *Spirulina* comes from two different species of cyanobacteria: *Arthrospira platensis*, and *Arthrospira maxima*. These and other *Arthrospira* species were once classified in the genus *Spirulina*. There is now agreement that they are a distinct genus, and that the food species belong to *Arthrospira*; nonetheless, the older term *Spirulina* remains the popular name. *Spirulina* is cultivated around the world, and is used as a human dietary supplement as well as a whole food and is available in tablet, flake, and powder form. *Spirulina* are free-floating filamentous cyanobacteria characterized by cylindrical, multicellular trichomes in an open left-hand helix. *Spirulina* occurs naturally in tropical and subtropical lakes with high pH and high concentrations of carbonate and bicarbonate. *A. platensis* occurs in Africa, Asia and South America, whereas an *A. maximum* is confined to Central America (Vonshak, 1997).

Spirulina contains an unusually high amount of protein, between 55% and 77% by dry weight, depending upon the source. It is a complete protein, containing all essential amino acids, though with reduced amounts of methionine, cysteine, and lysine when compared to the proteins of meat, eggs, and milk. It is, however, superior to typical plant protein, such as that from legumes (Babadzhanov et al., 2004).

Spirulina is also rich in gamma-linolenic acid (GLA), and also provides alpha-linolenic acid (ALA), linoleic acid (LA), stearidonic acid (SDA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA). *Spirulina* contains vitamin B1 (thiamine), B2 (riboflavin), B3 (nicotinamide), B6 (pyridoxine), B9 (folic acid), vitamin C, vitamin D, and vitamin E. *Spirulina* is a rich source of potassium, and also contains calcium, chromium, copper, iron, magnesium, manganese, phosphorus, selenium, sodium, and zinc (Tokusoglu and Uunal, 2003).

The pharmacokinetics and pharmacodynamics of *Spirulina* in humans have not been thoroughly investigated. However, the proteins, lipids, and carbohydrates in *Spirulina* are digested, absorbed, and metabolized by the humans upon oral consumption. *Spirulina* can be consumed at a dose of 3–20 g/day without manifestation of any adverse effects. Studies on the acute, subchronic, and chronic toxicity and mutagenicity of *Spirulina* have revealed no specific body or organ toxicity or genotoxicity (Chamorro et al., 1996).

Spirulina may also provide for an increase of immunological activity within the body. In comparative animal study, *Spirulina* increased natural killer (NK) cell and phagocytic activity (Hayashi et al., 1993). *Spirulina* is most effective at improving T-cell and thymus functioning. It has also been shown to increase the number of macrophages, or primary immune cells, which provide our bodies with a first line of defense. By increasing helper cells, killer cells, and antibodies, *Spirulina* assist our immune system in the eradication of specific microbes that cause infection (Kehrer and Smith, 1994).

Spirulina may provide protection against harmful free radicals. Extracts of *Spirulina* have been shown to scavenge peroxyl radicals' *in vitro* and *in vivo* study (Miranda et al., 1998). *Spirulina* is algae comprised of naturally-occurring, antioxidant-rich nutrients including beta-carotenes, phenolic acids, and tocopherols. *Spirulina* also contains the pigment phycocyanin, which has exhibited even greater antioxidant activity in preliminary studies. Phycocyanin may also provide a mechanism of protection and treatment for persons suffering from liver damage caused by various disease conditions. By increasing the plasma antioxidant capacities within the body, *Spirulina* may provide some antioxidant activities (Gorban et al., 2000).

Spirulina also harnesses the potential to reduce the incidence of any mast-cell mediated immediate-type allergic reactions by preventing the release of histamines. Histamines are important vaso-active proteins which contribute to the various symptoms associated with an allergic reaction. Symptoms associated with the release of histamines include; runny noses, watery eyes, hives, soft-tissue swelling, and in severe cases, smooth-muscle contraction. This may prove especially relevant for individuals at risk for the onset of anaphylactic shock (Yang et al., 1997).

Experimental works have shown that *Spirulina* products positively affect innate immune functions and build up both the humoral and the cellular immune system (Lee and Werth, 2004).

Polysaccharides and phycocyanin from *Spirulina* enhance bone marrow reproduction, thymus growth, and spleen cell proliferation, increasing immunity in the animal model, such as mice. Studies have also demonstrated that *Spirulina* up-regulates the immune system by improving their ability to function in spite of stress from environmental toxins, bacteria, and virus (Grzanna et al., 2006).

Sulfated-polysaccharides isolated from a water extract of *Spirulina*, called calcium spirulan (Ca-Sp), exhibit immunomodulatory and antiviral activities. Furthermore, immolina, a high-molecular-weight polysaccharide fraction of *Spirulina*, promotes chemokine expression in human monocytic THP-1 cells. Other investigations have studied the use of the *Spirulina* in improving immune response (Mao et al., 2005).

Moreover, the water-soluble extract of *Spirulina* caused the secretion of interleukins, such as IL-1, from murine peritoneal macrophages, and the proliferation of thymocytes. In addition, the effect of *Spirulina* on nonspecific immunity

has been measured at the level of natural killer (NK) cell activity (Al-Batshan et al., 2001).

2. Materials and Methods

2.1. 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 (Health Systems Profile- Egypt, 2006). 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 (Costa, et al., 2013).

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.

2.2. Laboratory Equipments

1- Neubauer hemocytometer, HBG Company, Germany.

2- White 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-Giemsa stain for blood film

7-Reagent used in detection CD4 and CD8, Becton Dickinson, USA

8- Flow cytometr (EPICS-XL).

9- Micopette pipettes, Dragon Lab Company, China.

2.3. 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:

2.4. Count of White Blood Cells (Cheesbrough, 2005)

Reagent:

WBC diluting fluid: This is a weak acid solution to which gentian violet is added which stains the nucleus of white cells

Principle of test:

Whole blood is diluted 1 in 20 in an acid reagent which haemolyzes the red cells (not the nucleus of nucleated red cells), leaving the white cells to be counted. White cells are counted microscopically using an Improved Neubauer ruled counting chamber (haemocytometer) and the number of WBCs per litre of blood calculated.

Blood sample:

EDTA anticoagulated blood or capillary blood can be used for counting white cells. Heparin or sodium citrate anticoagulated blood must not be used. The count should be performed within 6 hours (blood should not be refrigerated).

Method:

1. Measure 0.38 ml of diluting fluid and dispense it into a small container or tube.

2. Add 20 μL (0.02 ml, 20 cmm) of well-mixed EDTA anticoagulated venous blood or free flowing capillary blood and mix. Important: The volume of blood used in the test must be correct.

3. Assemble the counting chamber:

– Make sure the central grid areas of the chamber and the special haemocytometer cover glass are completely clean and dry.

– Slide the cover glass into position over the grid areas and press down on each side until rainbow colors (Newton's rings) are seen. Prior moistening of the chamber surface on each side of the grid areas will help the cover glass to adhere to the chamber.

4. Re-mix the diluted blood sample. Using a capillary, Pasteur pipette, or plastic bulb pastette held at an angle of about 45°, fill one of the grids of the chamber with the sample, taking care not to overfill the area.

5. Leave the chamber undisturbed for 2 minutes to allow time for the white cells to settle.

6. Dry the underside of the chamber and place it on the microscope stage. Using the 10 X objective with the condenser iris closed sufficiently to give good contrast, focus the rulings of the chamber and white cells. Focus the cells until they appear as small black dots.

7. Count the cells in the four large corner squares of the chamber marked W1, W2, W3, W4. Include in the count the cells lying on the lines of two sides of each large square.

8. Report the number of white cells per litre of blood using the following simple calculation:

– Divide the total number of cells counted by 2.

– Divide the number obtained by 10.

The number obtained x 10^9 is the white cell count

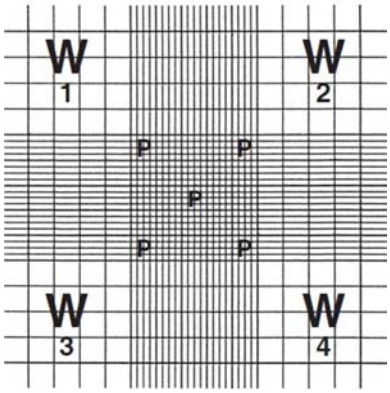

Figure 1. *Neubauer ruled counting chamber. The four large squares marked W1, W2, W3, W4 are used for counting WBCs and the five small squares marked P are used for counting platelets.*

2.5. Calculation of Neutrophils Percent by Blood Films (Cheesbrough, 2005)

Technique of making a thin blood film

1. Make a blood spreader from a slide which has ground glass polished sides as follows:

-Examine each end of the slide and select the end which is completely smooth, i.e. no chips in the glass. If one end of the slide is frosted, use the non-frosted end (ensure it is smooth).

– Using a glass marker, etch across a corner of the slide.

– Holding the slide between a piece of cloth, break off the corner and discard safely the broken off piece of glass.

2. Place a drop of blood on the end of a clean dry slide. Avoid making the drop too large (if too large, use a drop from the excess blood to make the film).

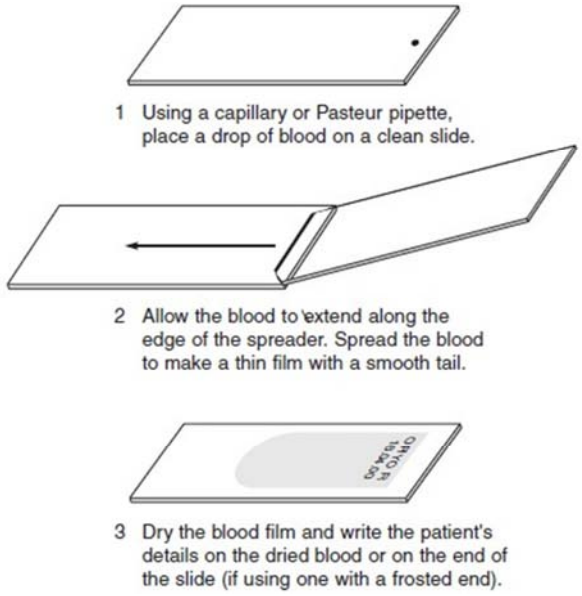

Figure 2. *Spreading a thin blood film.*

3. using a clean smooth edged spreader, draw the spreader back to touch the drop of blood and allow the blood to extend along the edge of the spreader. Holding the spreader at an angle of about 30°, spread the drop of blood to make a film about 40–50 mm in length (two thirds of the slide).

4. Wipe clean the end of the spreader.

5. Immediately air dry the film by waving the slide back and forth. Protect the dried film from dust and insects.

6. When completely dry and within a few minutes of making the blood film, fix it in absolute methanol.

2.6. Measuring CD4, and CD8 indicators (Uppal et al., 2003)

Principle:

The enumeration of CD4 and CD8 positive cells is helpful in management and follow up of immuno compromised patients. In assessing the degree of immune deficiency in patients of a particular region, knowledge of reference range of T-cell subset counts is essential.

Flow cytometry is an accepted standard method for determination of absolute count of CD4+, CD3+ and CD8+ T- lymphocytes. Absolute T-lymphocyte subset counts are preferred over percentages by both clinicians and laboratory personnel, as the percentages are relative values and involve the use of multiplatform methods, which are prone to errors and analytical bias, while single platform methods have the potential to yield a less variable analysis.

Collection of blood:

The blood samples of the patients were collected between 9.00 to 12.00 h with all bio-safety precautions; in K-3 liquid EDTA vacutainer (Becton Dickinson, Mountain View CA) for CD4/CD8 testing.

Reagents:

Ready to use paired reagent tubes contained known number of fluorochrome labeled reference beads and fluorochrome conjugated monoclonal antibodies in a buffered solution (Becton Dickinson). These beads functioned as fluorescence and quantitation standard for determining absolute counts of CD4+, CD8+ and CD3+ cells. Reference bead concentration differed from lot to lot.

Controls:

Ready to use control kit consisted of paired control bead sets containing fluorochrome integrated (2 µm) polysterene beads at four levels of concentration (zero, low= 50 beads/µl, medium= 250 beads/µl and high= 1000 beads/µl) (Becton Dickinson). The bead concentration differed slightly from lot-to-lot, except the size which was constant.

CD4/CD8 testing:

The protocol adopted by Ray (2004) was followed. The blood samples were processed immediately within 2 h of collection, for determining the absolute counts of CD4+, CD8+, CD3+ cells and their ratios by two colour immuno phenotyping on the single platform fluorescence activated cell sorting (FACS) count system (Becton Dickinson Pvt. Ltd., Mountain View, CA), using fluorochrome labeled monoclonal antibodies to CD4+/CD3+ and CD8+/CD3+ T-cells, strictly following manufacturer's instructions. The same batches of antibodies were used for testing the samples. The batches, however, differed from time to time. FACS count protocol software versions 1.2 (3/95), 1.3 (2/00) and 1.4 (4/02) (Becton Dickinson) were used for data acquisition and analysis.

2.7. Statistical Analysis

Data were presented as the mean ± standard deviation (SD) values. Paired t test was carried out, and the statistical comparisons among the groups were performed using a statistical package program (SPSS version 14). $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Effect of *Spirulina* on White Blood Cell Count

The results in Table 1 showed that white blood cell count at start before treatment by Spirulina was ranging from 3.7

(x 10^3/cmm) to 20.1 (x 10^3/cmm) but after 6 months from treatment by Spirulina was ranging from 3.3 (x 10^3/cmm) to 36.3(x 10^3/cmm). The results also revealed that, there were no significant difference in white blood bell count between at start before treatment by Spirulina and after 6 months from treatment by Spirulina (P value =0.086).

Table 1. *Effect of Spirulina on White Blood Cell Count.*

Groups	White Blood Cell Count (x 10^3/cmm)					T-Test	
	Range		Mean	±	SD	t	P-value
AT Start	3.7	20.1	10.088	±	4.877527	1.78	0.086
After 6 months	3.3	36.3	14.528	±	10.25299		

P value is statistically highly significant at< 0.05 level

3.2. Effect of *Spirulina* on Neutrophils Percent

The results in Table 2 showed neutrophils percent at start before treatment by Spirulina was ranging from 28% to 78% but after 6 months from treatment by Spirulina was ranging from 26% to 68%. The results also revealed that, there were no significant difference in neutrophils percent between at start before treatment by Spirulina and after 6 months from treatment by Spirulina (P value =0.054).

Table 2. *Effect of Spirulina on neutrophils percent.*

Groups	Neutrophils (%)					T-Test	
	Range		Mean	±	SD	t	P-value
AT Start	28	78	49.28	±	12.22538	2.02	0.054
After 6 months	26	68	44.04	±	12.66057		

P value is statistically highly significant at< 0.05 level

3.3. Effect of *Spirulina* on CD8

The results in Table 3 showed that CD8 at start before treatment by Spirulina was ranging from 6 (x10^3cells/100ml) to 30 (x10^3cells/100ml) but after 6 months from treatment by Spirulina was ranging from 11 (x10^3cells/100ml) to 48 (x10^3cells/100ml). The results also revealed that, there were significant increase in CD8 between at start before treatment by Spirulina and after 6 months from treatment by Spirulina (P value <0. 001*).

Table 3. *Effect of Spirulina on CD8.*

Groups	CD8 (x10^3cells/100ml)					T-Test	
	Range		Mean	±	SD	t	P-value
AT Start	6	30	17.68	±	6.88	11.4	<0. 001*
After 6 months	11	48	26.44	±	9.08		

P value is statistically highly significant at< 0.05 level

3.4. Effect of *Spirulina* on CD4

The results in Table 4 showed that CD8 at start before treatment by Spirulina was ranging from 9 (x103cells/100ml) to 33 (x10^3cells/100ml) but after 6 months from treatment by Spirulina was ranging from 13 (x10^3cells/100ml) to 53 (x10^3cells/100ml). The results also revealed that, there were significant increase in CD4 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 CD4.*

Groups	CD4 (x10^3cells/100ml)					T-Test	
	Range by%		Mean	±	SD	t	P-value
AT Start	9	33	19.56	±	7.8	9.8	<0. 001*
After 6 months	13	53	32.2	±	13.5		

P value is statistically highly significant at< 0.05 level

4. Discussion

In the present study, white blood cell count was determined as immune indicator, which was increased from 10.08± 4.87 to 14.5± 10.25 after 6 months of the treatment by Spirulina. Neutrophils percent also was determined as immune indicator, which was decreased from 49.28± 12.2 to 44.04± 12.66 after 6 months of the treatment by Spirulina.

These findings were in agreement with other studies Qureshi, and Ali, (1996) who approved that Phycocyanin from Spirulina stimulates hematopoiesis, and especially erythropoiesis, by inducing the release of erythropoietin hormone (EPO). Phycocyanin and polysaccharides from *Spirulina* promote antibody and white blood cell production.

The percentage of phagocytic macrophages in cats increased when they were administered a water-soluble extract of *Spirulina*

These findings were in agreement with other studies dealing with more detailed immunological parameters of health improvement throughout the course of HCV thalassemic patients; as Mederacke et al. (2012) recorded the increase of absolute differential neutrophil count to 10000 cells/μL after 4 months of treatment with *Spirulina*. This can be confirmed with similar records by Fytili et al. (2007) in 23 cases out of 25 cases.

In the present study CD4 was determined as immune indicator, which was significant increased from 19.56 ± 7.8 to 32.2 ± 13.5 after 6 months of the treatment by Spirulina. CD8 also was determined as immune indicator, which was significant increased from 17.68 ± 6.88 to 26.44 ± 9.08 after 6 months of the treatment by Spirulina.

These findings were in agreement with other studies; as CD4 and CD8 were considered as very important indicator for immune status of thalassemic patients, Coppola et al. (2007) observed a significant increase in concentration of CD4 and CD8 to 50 mg/100 ml in 28 cases out of 40 cases under study throughout the course of treatment. Echevarria et al. (2006) recorded similar results in another study on 50 cases of thalassemic patients with HCV infection, treated with *Spirulina*.

5. Conclusion

The thrust of this study to measure white blood cell count, neutrophil percent, CD4 and CD8 in thalassemic Children Infected with Hepatitis C Virus for evaluation the effect of Spirulina on immune system. This study proved that there was immune stimulation to thalassemic children Infected with Hepatitis C Virus after 6 months from uptaking Spirulina.

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