WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 12, 1334-1348. Research Article ISSN 2277– 7105

AN IMMUNOLOGICAL EVALUATION AND STUDY OF DYSBIOSIS ASSOCIATED WITH THALASSEMIC PATIENTS IN BABYLON PROVINCE

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Article Received on
21 August 2017,
Revised on 11 Sept. 2017,
Accepted on 01 October 2017
DOI: 10.20959/wjpr201712-9794

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ABSTRACT

Thalassemia is one of the most common genetic diseases in the world and it is a major health problem. It is among the most common genetic disorders worldwide, occurring more frequently in the Mediterranean, Indian subcontinent, Africa and south East-Asia. In Iraq, thalassemia is a major health probl em with prevalence of carrier range from 4.4%-6.66%. Throughout the past century, there have been an emergence of a large number of diseases, including thalassemia, many of which have been recently associated with intestinal dysbiosis - that is, compositional and functional alterations of the gut microbiome. Dysbiosis may be responsible for a shift in the homeostatic healthy flora to detrimental proinflammatory microbial species, which can later predispose to intestinal inflammation and can drive an autoimmune disease – in particular, a non-gut autoimmune disorder. This study aimed at identifying a possible link between disturbed gut microbiota and the immunological status of patients with thalassemia in Babylon province. Eighty-eight children & adolecents were enrolled in this case – control study. Blood & stool samples were taken from all subjects in the study for biochemical, immunological & microbiological (bacteriological) studies. After demographic data analysis, results presented in the current study established a correlation between the frequency of blood transfusion and acquisition of hepatitis C. also, in the present study bacterial microbiota was assessed among the study groups to reveal a hypothetical association between disturbed microbiota and the immunological status among patients with thalassemia. Results got in our study stated that, there was a significant positive correlation between the distribution of gram

negative bacteria (E coli, Klebsiella and Enterobacter) in stool samples of thalassemic patients than controls.

KEYWORDS: thalassemia, microbiota, immune disturbance.

INTRODUCTION

Thalassemia is one of the most common genetic diseases in the world and it is a major health problem.[1] The precise complications of β-thalassemia's are diverse and they depend on the amount and identity of the globin chain accumulating in excess.[2] In thalassemia, the imbalance of globin chain synthesis leads to red blood cells damage, resulting in destruction of red cells in the bone marrow and peripheral circulation.[3] These important complications include iron overload, infections, splenectomy, hepatitis C, and immune disturbances.[4]

Thalassemia is among the most common genetic disorders worldwide, occurring more frequently in the Mediterranean, Indian subcontinent, Africa and south East-Asia.[5] Iraq is one of the countries in which 6-10% of the population have hemoglobinopathy of which thalassemia is a major part.[6] In Iraq, thalassemia is a major health problem with prevalence of carrier range from 4.4%-6.66%-in Bagdad (1996)-4.6% in Basrah(2003)-3.7% in Dohuk(2010)-4.14%, and in Sulaimania(2008).[7][8][9]

A wide range of abnormalities of the humoral and cell mediated immunity, along with other aspects of immune system have been reported in patients with thalassemia. The immune alterations concern both the innate and the adaptive immune systems (quantitative and functional).[10]

The susceptibility to infections in thalassemia is multifactorial and appears to be related to the disease itself, altered immune system secondary to blood transfusions, iron overload and splenectomy.[11]

The human microbiota is the aggregate of microorganisms that resides on or within any of a number of human tissues and biofluids, including the skin, mammary glands, placenta, seminal fluid, uterus, ovarian follicles, lung, saliva, oral mucosa, conjunctiva, and gastrointestinal tracts.[12] Throughout the past century, there have been an emergence of a large number of diseases, including thalassemia, many of which have been recently associated with intestinal dysbiosis - that is, compositional and functional alterations of the gut microbiome.[13]

The mammalian intestine is colonized by trillions of microorganisms, most of which are bacteria that have co-evolved with the host in a symbiotic relationship. The collection of microbial populations that reside on and in the host is commonly referred to as the microbiota. A principal function of the microbiota is to protect the intestine against colonization by exogenous pathogens and potentially harmful indigenous microorganisms via several mechanisms, which include direct competition for limited nutrients and the modulation of host immune responses.[14] Conversely, pathogens have developed strategies to promote their replication in the presence of competing microbiota. Breakdown of the normal microbial community increases the risk of pathogen infection, the overgrowth of harmful pathobionts and inflammatory disease. Understanding the interaction of the microbiota with pathogens and the host might provide new insights into the pathogenesis of disease, as well as novel avenues for preventing and treating intestinal and systemic disorders.[15]

Because commensal microbes produce numerous active metabolites, the microbiome is essential for several aspects of host health, such as metabolism, immune response, and physiology. Several studies have shown the role of specific and combined microorganisms in the severity of gut inflammation. All of these studies point to a substantial loss of bacterial diversity as a dominant factor in disease pathogenesis.[16]

This reduction is characterized as dysbiosis, which may be responsible for a shift in the homeostatic healthy flora to detrimental proinflammatory microbial species, which can later predispose to intestinal inflammation. The commensal microbe-derived nutrient, butyrate, also plays an immune regulatory function, because it can drive the differentiation of regulatory T (Treg) cells in vivo and in vitro. Recently, a specific member of gastrointestinal microbiota was identified, that is a critical inducer of Th17 cell proliferation in the intestine. It is known that this population of effector CD4 T helper lymphocytes is one of the major contributors to gut inflammation. Altogether, these studies confirm the key importance of gut microbiota in the immunological balance that underlies the development of intestinal inflammation.[17]

Members of the E. coli family represent normal constituents of a healthy intestinal microbiota. E. coli can acquire virulence factors and become pathogenic; AIEC are an example of such a pathogenic subtype. AIEC are able to adhere to, and invade, the gut epithelium and provoke a chronic inflammatory response. Their presence is frequently observed in thalassemiac patients and is suspected to play a role in the initiation and/or

maintenance of gut inflammation. This infection results in ileal and colonic inflammation involving Th1 and Th17 immune responses and protection by CD8+ cells. Thus potentiating the important contributions of the gut microbiota, through their critical mechanisms during the natural course of thalassemia over time.[18]

The gut flora has the largest numbers of bacteria and the greatest number of species compared to other areas of the body. In humans the gut flora is established at one to two years after birth, and by that time the intestinal epithelium and the intestinal mucosal barrier that it secretes have co-developed in a way that is tolerant to, and even supportive of, the gut flora and that also provides a barrier to pathogenic organisms. The composition of human gut flora changes over time, when the diet changes, and as overall health changes.[19]

Th17 cells define a subset of T helper cells that mainly produce IL-17A, but also IL-17F, IL-21, and IL-22, and are increasingly recognized as paramount in several chronic inflammatory diseases. Recent studies utilizing novel mouse models highlight the significance of the gut microbiota and link components of bacterial sensing, to IL-17/Th17 immune responses. This support its role in the intestine, linking IL-17/Th17 immune responses and other associated mediators to disturbed gut microbiota.[18]

Gut microbiota can drive an autoimmune disease – in particular, a non-gut autoimmune disorder. Antibiotics treatment can modulate arthritis development and TH17 expression in gut. Gut microbiota testing is important in several clinical research approaches. Given the great microbiota variation between individuals, dysbiosis has been difficult to define. However, the largest and most important part of the study was to identify which bacteria are most important in relation to normal and healthy intestinal flora. Dysbiosis is associated with many diseases, including autoimmune diseases. Potential clinical impact of imbalance in the intestinal microbiota suggests need for new standardized diagnostic methods to facilitate microbiome profiling.[20]

The regulation of these host defense mechanisms largely depends on the activation of innate immune receptors by microbial molecules. Under steady-state conditions, the microbiota provides constitutive signals to the innate immune system, which helps to maintain a healthy inflammatory tone within the intestinal mucosa and, thus, enhances resistance to infection with enteric pathogens. During an acute infection, the intestinal epithelial cell barrier is

breached, and the detection of microbial molecules in the intestinal lamina propria rapidly stimulates innate immune signaling pathways that coordinate early defense mechanisms.[21]

Intestinal microbiota is generally comparable for individuals comprising the general adult population, with recent evidence supporting the gut microbiota as representing a healthy state defined as normobiosis. Notably, deviations from normobiosis can result in a transient or permanent microbiotic imbalance known as dysbiosis, which has been linked to several disorders, including autoimmune disorders. Analysis of faecal samples from individuals with dysbiosis is anticipated to enable characterization of the bacterial profile associated with different pathological conditions, thus aiding clinical diagnosis of pathological conditions and improving therapeutic regimens.[20]

The intestinal immune system remains unresponsive to beneficial microbes and dietary antigens while activating pro-inflammatory responses against pathogens for host defence. In intestinal mucosa, abnormal activation of innate immunity, which directs adaptive immune responses, causes the onset and/or progression of inflammatory bowel diseases. Thus, innate immunity is finely regulated in the gut. Multiple innate immune cell subsets have been identified in both murine and human intestinal lamina propria. Some innate immune cells play a key role in the maintenance of gut homeostasis by preventing inappropriate adaptive immune responses while others are associated with the pathogenesis of intestinal inflammation through development of Th1 and Th17 cells. In addition, intestinal microbiota and their metabolites contribute to the regulation of innate/adaptive immune responses. Accordingly, perturbation of microbiota composition can trigger intestinal inflammation by driving inappropriate immune responses.[22]

This study aimed at identifying a possible link between disturbed gut microbiota and the immunological status of patients with thalassemia in Babylon province, no data are available about this project in Babylon province to date.

PATIENTS AND METHODS

Eighty-eight children & adolecents were enrolled in this case – control study, including fifty-eight thalassemic patients (39 splenectomized thalassemic patients & 19 asplenectomized thalassemic patients) & thirty healthy individuals matched as control group. All patients in the study were referred & diagnosed in the thalassemia hemoglobinopathy center in Hilla hospital of pediatrics from September 2016 through March 2017.

A full questionnaire including demographic information's set, residence, family history of thalassemia, date and period of medical history including hepatitis – C, test results, and treatment. Control group subjects were selected from those family members & relatives of thalassemia patients who were diagnosed as non – thalassemic healthy subjects. All were matched regarding the age & set to the patients group. Blood & stool samples were taken from all subjects in the study for biochemical, immunological & microbiological (bacteriological) studies.

RESULTS AND DISCUSSION

In a case –control study design a total of (88) subjects (58 thalassemic and 30 healthy control) were joined in this study. All patients of thalassemia were referred & diagnosed in Thalassemia and Hemoglobinopathy Center for Hereditary Blood Diseases in Babylon Hospital for Maternity and Pediatrics from September 2016 till March 2017. Demographic data comprising age, gender, history of splenectomy, presence of hepatitis C, and frequency of blood transfusion were studied consequently.

The distribution of thalassemic patients and control according to age groups during this study was examined. Among a total of 88 subjects of the study, age was studied as a demographic factor and results presented that, the mean age of thalassemic patients was 9.07 years while the mean age of the control group (whom were selected to match the cases) was 9.9 years as shown in Figure (1). All thalassemic patients were presented with age groups ranging from minimal of 5 months to maximum of 17 years.

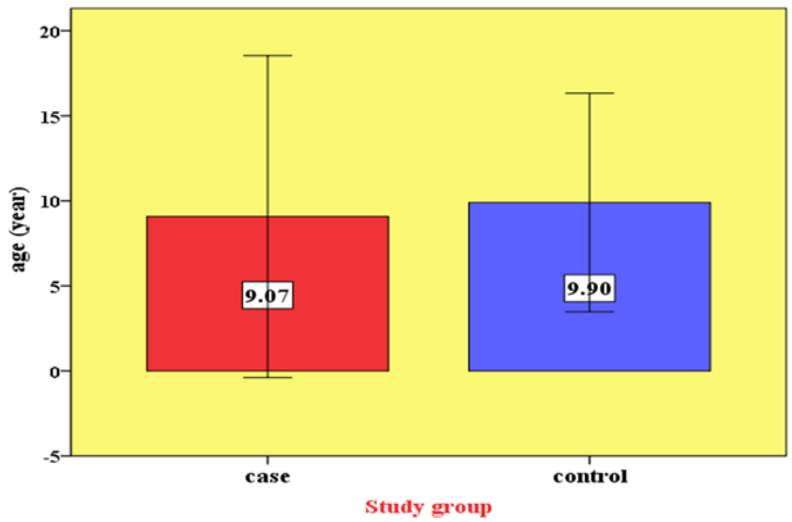

Figure (1): Mean and standard deviation of age of the study groups.

Results regarding age distribution of thalassemic patients in the current study was confirmed by an Indian study which stated that, the majority of thalassemic patients were diagnosed and registered about age of 10 years. Age of the youngest patient at diagnosis was 4 months and oldest patient of the study was 28 years old.[23]

Age at initiation of transfusion therapy, liver iron load, poor compliance with chelation, and male sex were found to be significant factors in the development of diabetes.[24][25]

The distribution of thalassemic patients and controls according to gender through this study is shown in Figure (2). Results indicated that, male gender was more detected as having thalassemia than female gender with a ratio of male: female about (1.16:1).

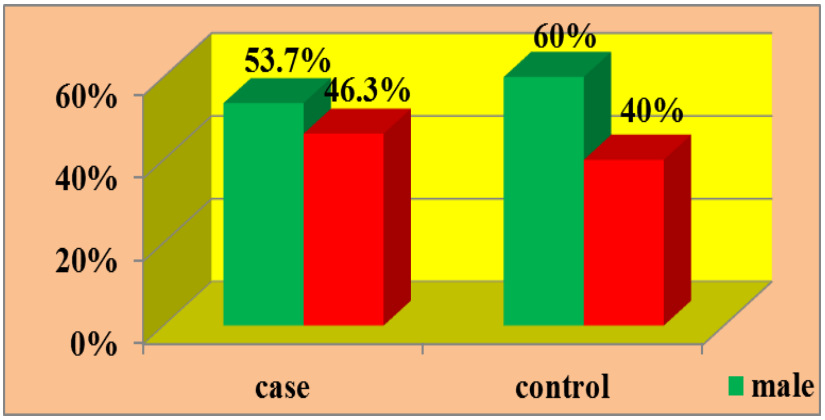

Figure (2): Distribution of the study groups by gender.

These results regarding the distribution of gender in thalassemia patients stated in the present study was approved by Kirti Grow and his colloquies (2013) who found a high ratio of male: female (2.6: 1). He also established that, statistical analysis using Chi square revealed significant values for the association of complications and sex ratio in beta thalassemia major patients.[26]

It seems that differing immunoglobulin serum levels in thalassemia could be due to heterogeneity of different studies in aspects including age groups, race, socioeconomic status, nutrition and difference in the care provided for the patient to control anemia and varied measures of ferritin, ignorance of the patient`s simultaneous affliction with hepatitis C and the failure to divide them into two groups of splenectomized and not splenectomized patients. Repetitive transfusions lead to continuous allo-antigenic stimulation and therefore

disturbance of the immune balance. Multiple transfusions have been associated with autoimmune hemolysis and B-lymphocyte changes.[27]

The distribution of thalassemic patients according to history of splenectomy during this study is presented in Table (1). Results showed that 67.3% of thalassemic patients had a positive history of splenectomy at time of the study, while only 32.7% didn't have splenectomy.

As stated at 2008 by Thalassaemia International Federation, many patients with thalassaemia major require splenectomy. However, optimal clinical management from the time of diagnosis may delay or even prevent hypersplenism, thereby increasing the efficiency of transfusion therapy and reducing the need for splenectomy.

Table (1): Distribution of thalassemic patients by history of splenectomy.

History of splenectomy	Number	Percentage (%)
Present	39	67.3
Absent	19	32.7
Total	58	100.0

Results established in the existing study concerning the distribution of splenectomy among thalassemic patients were further reinforced by Smith and his colleagues illustrating that, splenectomy eliminates the extracorpuscular mechanism responsible for the accelerated destruction of normal donor red cells in the patient's circulation. This represents a lasting improvement and accounts for an increased longevity of normal blood supplements and a striking reduction of transfusion requirements. Less blood was required to maintain at least as good, and at times improved, hemoglobin levels. Estimates of duration of survival of the patient's own erythrocytes suggest that the basic hemolytic defect is not altered significantly by this operative procedure.[28]

Splenectomy in thalassaemia increases the incidence and the severity of infections much more than in other diseases. Splenectomy is often carried out to avoid repeated blood transfusions and to minimize its need and frequency. However, many studies known that splenectomy increases the risk of sepsis and of thrombotic events.[29][30][31]

The distribution of hepatitis C among thalassemic patients in the existent study was investigated as demonstrated in Table (2). Results revealed that, 43.2% of thalassemic patients had a positive history of hepatitis C during the time of sample collection, while 56.8% had a negative history of hepatitis C.

Table (2): Distribution of thalassemic patients by history of HCV.

History of HCV	Number	Percentage (%)
Present	25	43.2
Absent	33	56.8
Total	58	100.0

The engagement of thalassemia patients according to history of HCV during this study was supported by a study done by UK researcher,[32] who established that, the second commonest cause of death in thalassemia major patients over 15 years of age is liver disease, due to blood borne viral hepatitis. Hepatitis C virus infection is a global health problem and is the main cause of chronic liver disease worldwide. Most persons who are infected will develop cirrhosis with liver failure and Hepatocellular Carcinoma.[33]

Strength came from similar studies concerning results of hepatitis C reached in the present study, which established that, the most recent estimates of disease burden show an increase in seroprevalence over the last 15 years to 2.8%, resulting in >185 million infections worldwide.[34][35]

In the past, patients with haemoglobinopathies represented a population at high risk of acquiring HCV as before 1992 there was no blood screening for HCV. As a result, the prevalence of antibodies to HCV (anti-HCV) in patients with thalassemia varies depending on the population studied between 12% and 85%.[36] Furthermore, chronic hepatitis C and iron overload were proved to be independent risk factors for liver fibrosis progression and their concomitant presence results in a striking increase of the risk.[37]

So, it is essential for patients with haemoglobinopathies to have a multidisciplinary approach, in order to achieve both effective chelation therapy and treatment of chronic hepatitis C, with a view to preventing liver complications and improve morbidity and mortality.[38][39]

As a demographic data, the history of blood transfusion was calculated among thalassemic patients as a frequency in this study. It was found that, about 10% of patients had 10 times of blood transfusions, 55.4% of patients in the study had 10-20 times of blood transfusion, 25.8% of patients in the study had 21-30 times of blood transfusion, and less than 10% experienced more than 30 times blood transfusions as indicated in Table (3).

Data presented in the current study established a correlation between the frequency of blood transfusion and acquisition of hepatitis C. As it was clear that, hepatitis virus C seropositivity

was more commonly distributed among thalassemic patients who experienced frequent blood transfusion episodes than those patients with less frequent blood transfusion (P<0.05).

Table (3): Distribution of the frequency of blood transfusions among thalassemic patients.

Range of Blood transfusions	Number	Percentage(%)
<10	6	10.3
10-20	32	55.4
21-30	15	25.8
31-40	3	5.1
>40	2	3.4
Total	58	100.0

These results came in accordance with other studies, [40][41][42] affirming that, blood transfusion frequency was observed to be directly linked with seropositivity to hepatitis C. Poly-transfusion in thalassemic patients is considered one of the major risk factors to acquire hepatitis. And transfusion-related iron overload and hepatitis C are the main proposed causes of liver damage in thalassaemic patients.[43][44]

In the present study bacterial microbiota was assessed among the study groups to reveal a hypothetical association between disturbed microbiota and the immunological status among patients with thalassemia. Through the traditional laboratory tests and bacterial detection techniques, results got in our study stated that, there was a significant positive correlation between the distribution of gram negative bacteria (E *coli*, Klebsiella and Enterobacter) in stool samples of thalassemic patients than controls as presented in Table(4).

Table (4): Association between intestinal microbiota (E-coli, klebsiella and enterobacter) between the study groups.

Bacteria	Study groups		Total	P value
	Case Number (%)	Control Number (%)		
E –coli				
Positive	46(97.6%)	4(40.0%)	50(86.3%)	
Negative	12(2.4%)	26(60.0%)	38(13.7%)	**<0.001*[f]**
Total	58(100.0%)	30(100.0%)	88(100.0%)	
Klebsiella spp.				
Positive	25(46.3%)	17(70.0%)	42(51.0%)	
Negative	33(53.7%)	13(30.0%)	46(49.0%)	0.291[f]
Total	58(100.0%)	30(100.0%)	88(100.0%)	
Enterobacter spp.				
Positive	33(56.1%)	16(60.0%)	49(56.9%)	
Negative	25(43.9%)	14(40.0%)	39(43.1%)	1.0[f]
Total	58(100.0%)	30(100.0%)	88(100.0%)	

By using fisher exact test to examine the association between result of culture of (E-*coli*, Klebsiella and Enterobacter) among the study groups, there was a highly significant association in E-*coli* distribution among thalassemic patients according to study findings (*p* value <0.001).

Similar study done in 2003 in Taiwan[45], revealed that the major causative organisms were gram-negative bacilli, especially E *coli* and *K. pneumoniae.* Only 10% of the causative agents were gram-positive bacteria. These results were also similar to those described from another study, according to a review by Wanachiwanawin, (2000)[46], one-half of the major organisms responsible for severe infection in patients with thalassemia in Thailand were gram-negative bacilli, such as *E. coli* (26% of organisms) and *K. pneumoniae* (23% of organisms).

Our results about the significant association between E *coli* and thalassemic patients was confirmed by another study in Iran which was conducted on patients with thalassemia and HIV-infected patients to determine the frequency of diarrheagenic Escherichia coli. Statistical analysis was carried out to determine the correlation of diarrheagenic E coli among patients with thalassemia. The frequency of E coli was more common in patients with thalassemia (67.64%) than controls.[47]

This study result was also in accordance with other studies,[48][49][50], where the percentages of E *coli* among thalassemic patients significantly higher than controls. Reason came from the fact that, this bacterial virulence is increased in the presence of excess iron.

Agreement to our results about the high percentage of E *coli* detection among thalassemic patients that controls was came from another study[51]. Host factors in persons with iron overloud characteristic of thalassemia could increase the risk for serious E *coli* infections because this bacterium need iron for its growth. Accordingly, elevated ferritin level typical of multitransfused thalassemic patients could promote E *coli* growth.

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