Study of ERYTHROPOEITIN Effect ON IgM serum levels IN HCV positive patients on regular HD

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Abstract: Background: Both uremia and HD process cause immunosuppression in HD patients. There was significant increase of total serum IgG and IgM levels found in patients with chronic HCV compared with healthy controls. There is evidence pointing to direct effect of rHuEPO upon B cells. High doses of rHu EPO enhanced in vitro Ig production and proliferation of various plasma cell lines, as well as human plasma cells generated in vitro. Patients and methods: Study was conducted at hemodialysis Unit of Shubra Municipal hospital between August 2010 to February 2011. 30 HCV positive patients on regular hemodialysis were included in study, using bicarbonate dialysate and polysulfone membrane dialyser, for 4 hours 3 times weekly. Patients were divided into 2 groups: first group: 15 patients on EPO therapy. 4000 IU/week and second group not taking EPO ,for all patients full clinical examination was done, CBC, BUN, serum creatinine, ALT, AST, serum albumin and serum IgM by ELISA (quantitative assay), were done. Results: There was no significant difference between 2 groups as regards age, sex distribution, WBC count, ALT, AST, serum creatinine, BUN and IgM serum level. First group had borderline significant higher Hgb and Hct than second group ($p = 0.056$). Females didn't have higher serum IgM level than males ($p = 0.403$). All correlations of IgM serum level to other parameters of study were irrelevant. Conclusion: Uremia seems to protect ESRD patients on regular HD from complications of HCV and also EPO effect on Ig serum levels.

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1. Introduction

Uremia is associated with a state of immune dysfunction characterized by immunodepression that leads to high prevalence of infections as well as by immune activation resulting in inflammation (kiechl *et al.* , 2002)

Improper immunological parameters of both humoral and cellular immunity in CKD patients seem to be deepened by hemodialysis (HD) process (Liwosca *et al.* , 2011b). Patients with renal disease have been at increased risk of acquiring HCV because of prolonged vascular access as well as the potential for exposure to infected patients and contaminated equipment (Fabrizi *et al.* , 2007).

Several studies have provided experimental evidence of disorders of both cellular and humoral immunity in chronic hepatitis C patients (Lotfy *et al.* , 2006).

HCV infection is strongly associated with mixed cryoglobulinemia (MC), a benign disorder characterized by the proliferation of B lymphocytes producing polyclonal IgG or monoclonal IgM with rheumatoid factor (RF) activity that characteristically may precipitate at low temperatures (Fazi *et al.*, 2010).

Besides B-cell activation (non-antigen-specific and antigen-specific), HCV seems to infect B lymphocytes directly (Bokle and Sepp , 2010).

Correction of anemia and maintenance of stable hemoglobin levels using erythropoesis stimulating agents (ESA) is an important aspect of ESRD management (Kalantar-Zadeh and Aronoff, 2009).

Epo therapy leads to improved humoral immune response, either directly or via T-cells help (Prutchi-Sagiv *et al.* , 2005). Epo treatment was associated with enhanced lymphocyte activity of both T-and B-cells (Lifshitz *et al.* , 2010).

Erythropoietin-receptor (EPO-R) presence on all populations of immune cells implies that EPO/rhEPO can influence lymphocytes, monocyte san granulocytes directly and somehow modulate their immunological responses(Liwoska *et al.* , 2011 a).

High doses of rHu EPO enhanced *in vitro* immunolglobulin production of various plasma cell lines, as well as human plasma cell generated *in vitro* (Prutchi-Sagiv *et al.* , 2005).

The uremic patient on regular hemodialysis (HD) is subjected to a wide range of immune modulators including the uremic sate per se, multiple transfusions and exposure to bio incompatible materials and endotoxins. Erythropoietin (EPO) therapy may raise concern about its potential

influence on this complex Scenario(William *et al.* , 1998).

Aim of the Work

Is to determine the effect of erythropoietin on IgM level in ESRD patients infected with HCV on regular hemodialysis as IgM is one of the markers of cryoglobulinemia.

2. Patients and Methods

This study was conducted at hemodialysis unit of Shubra Munieipal Hospital between august 2010 to February 2011. It was conducted on 30 ESRD hepatitis C positive patients on regular hemodialysis with bicarbonate dialysate and polysulfone membrane dialyser, three times per week. All patients had chronic hepatitis C infection for less than 10 years with liver enzymes less than two fold increase above normal (specially ALT) and last blood transfusion more than 30 days ago.

These patients were divided into 2 groups

First group: Includes 15 ESRD hepatitis C positive patients on regular HD and on erythropoietin therapy. Patients of this group were administered erythropoietin dose of 4000 IU/week.

Second group: Includes 15 ESRD hepatitis C positive patients on regular HD and not on erythropoietin therapy.

We excluded from the study patients with history of DM, autoimmune and allergic diseases.

Patients with hepatitis β virus confection, dialysis vascular access infection, history of paraproteinemia, systemic vasculitis, acute hepatitis liver cell failure or chronic infections other than HCV and chronic inflammatory diseases were excluded from the study.

All patients were subjected to full history and complete physical examination, complete blood count, blood urea nitrogen, serum creatinine, liver enzymes (AST and ALT), serum albumin, and serum IgM by ELISA (quantitative assay).

Methods

1- Creatinine

This assay is a kinetic method (Yatzidis , 1974).

Assay principle

Creatinine in alkaline solutions react with picrate to form a colored complex. The rate of complex formation is measured photometrically at 492 nm.

Calculations

A2-A2: A(specimen)-A (standard)

* Concentration of creatinine in serum or plasma

$$(\text{mg/dL}) = \frac{A\,Speacimen}{A\,s\tan dard} X\,2$$

2- Urea

This procedure is enzymatic-spectro-photometric (Tabacco *et al.* , 1979).

Assay principle

Urea in the sample originates by means of the coupled reactions described below, a colored complex that can be measured by spectrophotometry:

$$\text{urea} + H_2O \xrightarrow{Urea} 2NH_4 + CO_2$$

$$NH4+ \text{ salicylate} + NaCLO \xrightarrow{nitroprusside} \text{indophenol}$$

Calculations

The urea calculation in the sample is calculated using the following general formula:

$$\text{Urea in sample} = \frac{A\,Sample}{A\,s\tan dard} XC\,S\tan dard$$

X sample dilution factor

Where c= concentration

3- Albumin (BCG):

This assay is colori-metric method (Doumas et al. , 1971).

Assay principle

In a buffered solution bromo-cresol green forms with albumin, a green colour complex whose intensity is proportional to the amount of albumin present in the specimen calculations:

$$\text{Albumin Concnetration (g/dL)} = \frac{A\,Speacimen}{A\,s\tan dard} X\,4$$

4- ALT (SGPT)

Liqui-UV test (Schumann and Klauke , 2003).

Assay principle

Kinetic method for the determination of ALT activity according to the recommendations of the expert panel of the IFCC (International federation of clinical chemistry) without pyridoxal-phosphate activation.

Reaction principle:

$$\text{2- Oxo-glutarate+L-alanine} \xrightarrow{GPT} \text{L-glutamate+pyruvate}$$

$$\text{Pyruvate} + NADH+H+ \xrightarrow{LDH} \text{L-lactate+}NAD^+$$

5- AST (SGOT)

Liqui-UVtest(Schumann and Klauke, 2003).

Assay principle

Kinetic method for the determination of AST activity according to the recommendation of the expert panel of the IFCC (international federation of clinical chemistry) without pyridoxal-phosphate activation reaction principle: 2-Oxo-glutarate+L-aspartate $\xrightarrow{GOT}$ L-glutamate=oxaloacetate.

$$\text{Oxaloacetate} + NADH+H+ \xrightarrow{MDH} \text{L-malate+}NAD^+$$

6- Serum IgM by Elisa (Diagnostic Automatic Inc. , 2009).

Intended use : to quantitate total human immunoglobulin M (IgM).

Statistical analysis

Statistical presentation and analysis of the present study was conducted, using the mean, standard error, student t-test, chi-square and linear correlation coefficient by SPSS V17. We also used Analysis of variance (ANOVA) test to compare different items in the same group in quantitative data.

P-value ≤ 0.05 is considered significant

* *P* value = 0.05 to < 0.1 is considered borderline significance

* *P* value ≤ 0.01 is considered highly significant

* *P* value > 0.1 is considered non-significant

3.Results

On comparing first and second group as regards age, there was no statistically significant difference between 1st group (50.533±8.766 years) and 2nd group (51.133± 6.632 years) using unpaired student t-test (*p*-value = 0.834).

We didn't find a statistically significant difference between 1st and 2nd group as regards sex distribution, (*p*-value = 0.140) using chi-square test, while females constituted 40% of 1st group and 66.67% of 2nd group and the total number of females included in the study constituted 53.33% of all participants in the study. Males constituted 60% of 1st group and 33.33% of 2nd group with a total of 46.67% of all participants in the study.

Table (1): Comparison of first group and second group as regards serum creatinine

	S. creatinine (mg/dL)		T-test*	
	Range	Mean±SD	t	*p*-value
First group	6.100-12.500	10220±2.066	0.290	0.774
Second group	7.000-15.900	9.993±2.207		

* Unpaired student t-test

Table (2): Comparison of first group and second group as regards blood urea nitrogen

	Blood urea nitrogen (mg/dL)		T-test*	
	Range	Mean±SD	t	*p*-value
First group	112.000-184.000	148.400±21.596	0.164	0.871
Second group	107.000-200.000	146.800±31.122		

* Unpaired student t-test

Table (3): Comparison of first group and second group as regards AST level in serum

	AST Iu/L		T-test*	
	Range	Mean±SD	t	*p*-value
First group	8.000-22.000	13.600±3.851	-2.282	0.030
Second group	10.000-21.000	16.467±2.973		

* Unpaired student t-test

Table (4): Comparison of first group and second group as regards ALT level in serum

	ALT Iu/L		T-test*	
	Range	Mean±SD	t	*p*-value
First group	5.000-15.000	8.533±2.800	-1.707	0.099
Second group	6.000-15.000	10.200±2.541		

* Unpaired student t-test

Table (5): Comparison of first group and second group as regards serum albumin

	S. albumin (g/L)		T-test*	
	Range	Mean±SD	t	*p*-value
First group	3.100-4.200	3.573±0.371	-0.141	0.889
Second group	3.000-4.300	3.593±0.404		

* Unpaired student t-test

Table (6): Comparison of first group and second group as regards hemoglobin (Hgb) level

	Hgb (g/dL)		T-test*	
	Range	Mean±SD	t	*p*-value
First group	7.800-14.500	10.113±2.144	1.992	0.056
Second group	5.700-12.400	8.600±2.015		

* Unpaired student t-test

Table (7): Comparison of first group and second group as regards hematocrit (Hct) level

	Hct (%)		T-test*	
	Range	Mean±SD	t	*p*-value
First group	20.800-41.700	31.080±6.487	1.990	0.056
Second group	17.300-37.000	26.687±5.568		

* Unpaired student t-test

Table (8): Comparison of first group and second group as regards white blood cells

	WBC (x 10^9/L)		T-test*	
	Range	Mean±SD	t	*p*-value
First group	3000.000-87.00	5373.333±1810.472	0.430	0.670
Second group	2100.000-8000.00	5073.333±2005.516		

* Unpaired student t-test

Table (9): Comparison of first group and second group as regards serum IgM level

	IgM (ug/mL)		T-test*	
	Range	Mean±SD	t	*p*-value
First group	40.300-213.700	119.467±61.781	0.238	0.814
Second group	43.000-214.500	114.647±48.536		

* Unpaired student t-test

Table (10): Comparison of IgM level in serum in males and females in both first and second groups together

	IgM (ug/mL)		T-test*	
	Range	Mean±SD	t	*p*-value
Female	40.300-213.700	125.025±52.345	0.850	0.403
Male	43.000-214.500	107.950±57.722		

* Unpaired student t-test

Table (11): Correlation of serum level of IgM and different parameters of the study in first group

First group	IgM	
	R*	*P*-value
Age	-0.119	0.674
S. creatinine	-0.374	0.170
BUN	-0.470	0.077
SGOT (AST)	-0.467	0.079
SGPT	-0.247	0.375
S. albumin	0.035	0.901
EPO dose	0.242	0.384
Hgb	0.005	0.987
Hct	0.122	0.665
WBC	-0.104	0.712

* Linear correlation coefficient (r)

Table (12): Correlation of serum level of IgM and different parameters of the study in second group

Second group	IgM	
	R*	*P*-value
Age	-0.147	0.600
S. creatinine	-0.064	0.821
BUN	0.300	0.277
SGOT (AST)	0.338	0.218
SGPT (ALT)	0.467	0.079
S. albumin	-0.085	0.762
Hgb	-0.305	0.270
Hct	-0.223	0.425
WBC	0.172	0.540

* Linear correlation coefficient (r)

Table (13): Correlation of serum level of IgM and different parameters of the study in both first and second group

1st &2nd group	IgM	
	R*	*P*-value
Age	-0.131	0.491
S. creatinine	-0.227	0.229
BUN	-0.057	0.766
SGOT (AST)	-0.167	0.377
SGPT (ALT)	0.034	0.858
S. albumin	-0.021	0.912
EPO dose	0.242	0.384
Hgb	-0.102	0.591
Hct	0.000	1.000
WBC	0.028	0.885

* Linear correlation coefficient (r)

4. Discussion

The depression of the immune response in the uremic patient is global and concerns both humoral and cellular sectors(Foley and Collins , 2007).

Disorders of both innate and adaptive immune systems and functional abnormalities of monocytes, neutrophils and dendritic cells, are directly linked with infection risk in this patient population (Lim *et al.*, 2007).

Death from sepsis is 50 times higher in hemodialysis patients than in the general population even after accounting for other comorbidities. One of the most difficult causes to treat is the development of an acquired immune dysfunction associated with chronic kidney disease (CKD) and dialysis therapy (Geara *et al.*, 2010).

Hepatitis C virus (HCV) is commonly associated with autoimmune disease as extra-hepatic manifestations (EHM).

The most important auto-immune diseases associated with HCV are mixed essential cryoglobulinemia (MEC) and Sjogren syndrome (SS) (Awad *et al.*, 2011). Increasing evidence suggests that HCV can interfere with innate immune activation at multiple levels (Jang and Chung, 2010).

HCV itself seems to be able to stimulate B cells through different pathways and mechanisms (Bokle and Sepp, 2010).

The persistent of stimulation of B cells by viral antigen could be responsible for leading to polyclonal and later to monoclonal expansion of B cells (Ito *et al.*, 2011).

The highest level of B-lymphocyte stimulator have been found in chronic HCV-infected subjects with clinical and laboratory features of autoimmunity (Bokle and Sepp, 2010).

EPO structure presents elements of cytokines composition and that is why, it is considered that this hormone, a part from its influence on red blood cells

system, can regulate immunological responses (Liwoska *et al.,* 2011 b).

Studies over the last 12 years demonstrated that erythropoietin is probably able to modulate or amplify some signaling pathways important for human lymphocytes and monocyte functions. There are also many studies demonstrating the role of rHu EPO in improving immune responses in CRF patients and at the same time suggesting that rHu EPO may act as an immunomodulating cytokine in the human organism (Liwoska *et al.,* 2011 a).

In our study, there was no statistical significant difference as regards age (P = 0.834) and sex distribution (p = 0.140) between the first group with EPO therapy and the second group without EPO therapy. Serum immunoglobulin concentrations tend to increase with age (Gonzalez *et al.,* 2008).

On comparing first and second groups, there was no statistical significant difference as regards serum creatinine levels (p = 0.774) and blood urea nitrogen levels (p = 0.871), which means that HCV infection together with concomitant EPO therapy didn't influence these two parameters in ESRD patients on regular HD.

In our study, second group showed higher serum AST levels than first group on EPO therapy (p = 0.03). Also, second group showed a borderline significantly higher serum ALT levels than first group (p = 0.099). In our study, EPO seems to have an anti-inflammatory response influencing our markers of hepatic inflammation or may be it may have a liver supporting effect. Further studies are needed to elucidate this role using liver biopsy findings.

Compared to non-uremic HCV patients, ESRD patients with chronic hepatitis C have milder hepatic necroinflammation and fibrosis (Trevizoli *et al.,* 2008).

Patients with ESRD and HCV infection displayed normal ALT levels. Indeed ALT levels in these patients were significantly lower than those found in patients infected with HCV without renal damage but with similar grades and stages of liver alterations. It has been proposed that the increase in hepatocytes of HCV-infected patients with ESRD who are an chronic dialysis produces a hepatoprotective effect (Contreras *et al.*, 2007).

Causes of reduction in ALT activity in these patients are only partially known, such as a reduction in pyridoxal – 5' – phosphate, vitamin B_{12}, coenzymes of ALT, suppression of AST and ALT synthesis in hepatocytes and an inhibition of AST and ALT released from hepatocytes into the blood stream, as well as the possibility of liver protection by the hepatocyte growth factor, which is higher in patients with chronic renal failure (Lin *et al.,* 2008).

Among HD patients, serum ALT levels are elevated in 4-67% patients with positive anti-HCV antibodies, 12-31% of patients with positive HCV-RNA and one third of patients with biopsy proven hepatitis(Perira and Levey , 1997).

Shin *et al.* (2006) reported on two cases after accidental ten times overdose administration of recombinant human erythropoietin (rHu EPO) up to 318.000 units a day in acute myocardial infarction, that the only side effects they found were elevated liver enzymes and hemoglobin levels. These patients were followed up as out patients and elevated enzymes soon normalized.

In Berglund and Ekblom study (1991), that aimed to evaluate the effect of treatment with subcutaneous recombinant human erythropoietin (rHuEPO), 20-40 Iu/kg body weight, 3 times a week, Serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT) were unchanged after rHu EPO treatment.

We didn't find a statistically significant difference in serum albumin between first and second group (p = 0.889), which means that EPO had no effect on serum albumin level.

Rhee and Erickson (2012) reported that protein energy malnutrition (PEM) diminishes immunoglobulin (IgA, IgM and IgG) concentrations and cytokine production.

This is not the case in our study as serum albumin is within normal range.

In our study, hemoglobin (Hgb) levels were borderline higher in first group than second group (p = 0.056). Also hematocrit (Hct) levels were borderline higher in first group than second group (p = 0.056) and this was expected due to administration of EPO in first group.

Khurana *et al.* (2008) hypothized that the chronic inflammation as a result of HCV infection or the increased production from the regenerating liver cells causes increased circulating EPO causing improved Hct in these patients. Also, he reported that hepatitis C patients tend to have higher baseline hemoglobin and decreased need for EPO therapy on dialysis.

Recently, some studies and case reports indicated attenuated anemia in HD patients with HCV infection, and they previously considered this to be related to increased erythropoietin production after hepatic stimulation by chronic infection with hepatitis virus (Alasran *et al.,* 2009).

In Lin *et al.,* study (2008), there were increased Hb levels in chronic HCV infected patients with ESRD.

In contrast, Abdalla *et al.,* (2000), reported a higher EPO requirement in HCV positive versus HCV negative patients that was a result of altered iron metabolism induced by chronic infection.

In our study, there was no statistically significant difference in white blood cells in blood (p = 0.670) between the two groups, which means that erythropoietin didn't increase white blood cells count above normal, but it only normalized it.

Different circumstances such as chronic renal failure, hemodialysis process, chronic hepatitis C virus infection and various dietary restrictions that we practice with those patients influenced immune system and immunoglobulin production. We didn't find a statistically significant difference in IgM level in serum between first group and second group, which means that EPO didn't influence much IgM level production by stimulated B-lymphocytes by either EPO or HCV infection.

Little is known about the effect of ESRD on B-cell sub-populations (Pahl *et al.,* 2010).

The increase in PMNL counts in CKD has been suggested to be a sign of pre-activation. The number of PMNL increasers in relation to the GFR decrease ($P <$ 0.0001) PMNL decreases with increasing serum C-reactive protein and IL-6 and decreased albumin, all associated with declining GFR (Sela *et al.,* 2005).

In our study this was not the case, as we had lymphopenia but no undernourishment. Sardenberg *et al.* (2006) findings suggest that uremic toxicity plays an essential role in PMN apoptosis and that dialysis may correct or normalize apoptotic rates.

ESRD and especially HD, is associated with B-cell lymphopenia(Kato *et al.,* 2008).

HCV infection is associated with leucopenia in HD patients, is as common as in non-HD patients with liver cirrhosis (Ng *et al.,* 2008).

Chronic hepatitis C virus (HCV) infection is associated with B cell activation, although underlying mechanisms are unclear (Sugalski *et al.,* 2010).

This is evidenced by an elevation in serum immunoglobulin isotypes; IgG and its subclasses IgG, and IgG_2 and IgM. Mean serum IgM was increased in patients with HCV infection compared with healthy controls(Lotfy *et al.,* 2006).

However, it has been documented that Ig levels, serum IgG isotypes and both IgM and IgA production are normal in dialysis patients (Hauser *et al.,* 2008).

Starzyk *et al.* (1993) in their study on 10 patients with chronic renal failure treated with hemodialysis (HD) T and B cell populations were determined in peripheral blood, together with immunoglobulin concentration. There was no significant change in the concentration of IgA and IgM.

We didn't find in our study a significant difference in IgM level between males and females. This was not the case in Gonzalez *et al.* (2008)study who reported that IgM levels are higher in females than in males. Sex differences in immunoglobulins concentrations specifically high IgM levels in females, have been attributed to hormonal effects on B lymphocytes.

IgM didn't show in our study, any significant correlation to any of the measured parameters of the study including erythropoietin dose. To our knowledge, we are the first to study the effect of EPO on IgM level in HCV positive patients on regular hemodialysis.

In a previous study by Debska-Slizien *et al.* (2003), in order to find the influence of erythropoietin on immunological system of patients with chornic renal failure, it was found that treatment with EPO did not alter plasma immunoglobulin (IgG, IgM and IgA), as well as total count of lymphocytes. In a previous study, by Costa et al. (2008)[44], 50 HD patients, 25 responders and 25 non responders to rHuEPO, were compared to each other and to 25healthy controls. No statistically significant differences were found between the three groups of individuals concerning immunoglobulin serum levels (IgG, IgM and IgA).

In a previous study by Schaefer *et al.* (1992), who studied whether erythropoietin interferes with B cell function and the mechanisms of this effect,

IgM production, which appeared to be normal in uremia, remained unchanged.

A retrospective study was done to determine whether rHu EPO treatment modulates the humoral arm of the immune system in MM patients. There was a significant increase in the levels of normal Ig (IgG, IgA or IgM) in response to rHu EPO, during the 3-9 months from treatment initiation Gadassi et al.,(2007) and Prutchi-Sagiv et al. , (2006) Data indicate a direct stimulant effect of erythropoietin on B- lymphocytes in end-stage renal failure. Production of IgM was enhanced(Kimata *et al.,* 1991).

These findings also show that the pharmacologic response to rHuEPO is a function of the dose.

Moreover, these effects were seen in concentrations much higher than that used in our study.

Conclusion:

ESRD with all its restrictions seems to protect patients from increased level of serum IgM due to HCV infection and erythropoietin therapy and subsequent cryoglobulinemia. Further studies at molecular level of B-cell functions are still needed to elucidate the causes of this protection.

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