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Effect of Vitamin D Supplementation with Pegylated Interferon-α and Ribavirin on Erythrocyte Indices, Iron Parameters and Erythropoietin Expression in Male Wistar Rats

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Abstract

Objectives: To measure the effect of Vitamin D3 (Vit D) on liver and serum iron parameters, erythrocyte indices, serum and kidney Erythropoietin (EPO) in normal rats treated with Pegylated Interferon-α (Peg-INF-α) and Ribavirin (RBV).

Materials and Methods: Sixty four male Wistar rats were divided equally into 8 groups. 'Control'; 'P': only received Peg-INF-α; 'PD': Peg-INF-α/Vit D; 'PR': Peg-INF-α/RBV; 'PRD': Peg-INF-α/RBV/Vit D; 'R': only received RBV; 'RD': RBV/Vit D and 'VitD': only received vitamin D3. Peg-INF-α-2a was injected subcutaneously (12 μg/rat/week) for 4 weeks. RBV (4 mg/rat/day) and Vit D (500 IU/rat/day) were given orally for 5 weeks. Blood samples were collected to measure erythrocyte indices and serum 25 (OH) vitamin D. Iron, ferritin, Total Iron Binding Capacity (TIBC) and transferrin saturation were measure in blood and liver tissue. EPO was measured in serum samples and kidney specimens by ELISA.

Results: All groups, except 'R' group, showed significant decrease in liver iron, ferritin and transferrin saturation, and increase in TIBC ($P>0.05$). However, there was no significant difference in those parameters at the serum level. RBV ± Peg-INF-α significantly decreased the RBCs count, haemoglobin, serum and kidney EPO compared to control and 'P' groups ($P>0.05$). Vit D prevented the development of anaemia and significantly increased the concentrations of EPO at serum and kidney levels in the designated groups. Vit D also correlated negatively with liver iron and transferrin saturation and positively with serum and kidney EPO, red cell count and haemoglobin concentrations.

Conclusion: Vit D could be involved in the regulation of iron metabolism and the prevention of anaemia during the course of treatment of hepatitis C by Peg-INF-α based therapy. Further studies are needed to explore the role of Vit D during the treatment of chronic hepatitis C.

Keywords: Anaemia; Erythropoietin hormone; Iron; Pegylated interferon-α; Ribavirin; Vitamin D

Introduction

Chronic infection with Hepatitis C Virus (HCV) is a leading cause of end-stage liver diseases including liver cirrhosis and hepatocellular carcinoma. Chronic Hepatitis C (CHC) is also associated with an increase in iron overload and the deposition of iron in the liver correlates significantly with the severity and progression of the disease. CHC has been treated during the last decade by the combination of Pegylated Interferon-α (Peg-INF-α) with Ribavirin (RBV) [1,2]. Although new antiviral drugs have been recently developed for the treatment of CHC, it has been postulated that Peg-INF-α and RBV may still have a role especially in developing countries where the access to the new drugs is not definite due to its high cost [2-6].

Besides their role in the regulation of the immune system for the eradication of the virus, both Peg-INF-α and RBV have also been shown to modulate the metabolism of iron and body iron stores by regulating the expression of hepcidin *in vitro* and *in vivo* [7-9]. The success of the antiviral therapy has been reported to be associated with a decrease in hepcidin, which is the main iron regulatory hormone [7-9].

The course of CHC treatment with Peg-INF-α based therapy is dependent on the viral genotype and success rate reaches up to 60% in genotype 1 and 4 [1]. However, Peg-INF-α based therapy is associated with the development of several side effects and these adverse events could results in the termination of treatment. Haematological side effects are common during Peg-IFN-α based therapy and anaemia is the most common complication [10-14].

Peg-INF-α based therapy could lead to anaemia by haemolysis and/or suppression of erythropoiesis. Peg-INF-α have been reported to suppress progenitor cell proliferation, increase destruction of erythroid precursor cells, induce autoimmune haemolytic reactions, and reduce renal function [15-18]. RBV simultaneously has been associated with haemolytic anaemia in a dose dependent manner and it is believed that Peg-INF-α may exacerbate the haemolytic effect of RBV [19-21]. RBV may also lead to anaemia by down-regulating the expression of erythropoietin receptors [13,22,23] and both drugs were

also associated with a decrease in serum Erythropoietin hormone (EPO) [24,25].

The management of Peg-INF-α based therapy induced anaemia was initially based on decreasing the dose of RBV. Nevertheless, the correlation between response rate and higher RBV dose lead to the use of alternative methods including Erythropoiesis-stimulating agents (ESA), such as EPO, and blood transfusion to support anaemic patients during the course of treatment [12,26-29].

Vitamin D (Vit D) has recently been described in the regulation of the several systems including iron metabolism and erythrocyte production. The administration of Vit D in rat caused hypoferraemia and alteration in the tissue distribution of iron compared to controls [30]. Additionally, low serum Vit D is directly related to the extent of iron loading in patients with hemochromatosis [31] and thalassemia major [32]. Vit D depletion was also associated with an increase in liver iron accumulation in a murine model of iron overload [33].

Serum levels of Vit D also correlates negatively with the prevalence of anemia and the use of ESA regardless of kidney function in the general population [34] and the administration of Vit D has been associated with dose reductions of ESA and increased reticulocytosis in haemodialysis patients [35,36]. It has also been shown that Vit D interacts with EPO and other cytokines to increase the production of EPO receptor and erythroid progenitor cells [37].

Little is known about the role of Vit D in the regulation of iron and prevention/treatment of anaemia associated with Peg-INF-α based therapy. We therefore hypothesize that vitamin D3 supplementation may regulated serum and liver iron levels and could provide protection against anaemia associated with the current treatment of CHC. The current study measured the effect of vitamin D3 (cholecalciferol) supplementation with Peg-INF-α and RBV on erythrocyte indices, serum and liver iron parameters and concentrations of EPO in serum and kidney of normal rat.

Materials & Methods

Drugs

Pegylated interferon-α-2a (Pegasys®, Hoffmann-La Roche, Nutley, NJ) was used. The ready to use syringe contains 180 μg/0.5 ml. Ribavirin capsules (Viracure®, 6th October Pharm, Egypt) were used and each capsule contains 400 mg of ribavirin. Vitamin D3 (cholecalciferol 4500 IU/mL) oral drops (VitD3, Novartis International AG, Basel, Switzerland) were used in the study.

Study design

All experimental protocols were approved by the Committee for the Care and Use of Laboratory Animals at Umm Al-Qura University and were in accordance with the EU Directive 2010/63/EU for animal experiments.

A total of 64 male Wistar rats weighing 250-300 gm were used. All animals received humane care during the study protocol and during euthanasia. The animals were divided equally into 8 groups as follow: the first group included 8 rats and they served as 'Control group', the second group consisted of those that only received Peg-INF-α 'P group', the third group received Peg-INF-α+Vit D 'PD group', the fourth group received Peg-INF-α+ribavirin 'PR group', the fifth group received Peg-INF-α+RBV+Vit D 'PRD group', the sixth group received RBV only 'R' group, the seventh group received RBV+Vit D 'RD group' and the last group consisted of rats that received vitamin D3 only 'VitD group'.

Treatment protocol

The study duration was 5 weeks. Peg-INF-α-2a was prepared by diluting the content of a full syringe (180 μg/0.5 ml) in 4.5 ml sterile normal saline to prepare a final volume of 5 ml and the final concentration was 36 μg/ml. Each rat in the 'P', 'PD', 'PR' and 'PRD' groups received a weekly subcutaneous injection of 0.33 ml (12 μg/rat) for a total of 4 injections. The drug was prepared fresh on the day of use.

One capsule of ribavirin (400 mg) was dissolved in 50 ml saline every day of the experiment and each rat in the 'PR', 'PRD', 'RBV' and 'RD' received 0.5 ml (4 mg/day) orally using a feeding syringe for the whole length of the study similar to the highest dose of the drug recommended from human during CHC treatment (12 mg/kg [1200 mg for body weight ≥ 75 Kg]) [1].

Cholecalciferol (4500 IU/mL) was prepared by adding 4 ml of the oral drops (Novartis International AG, Basel, Switzerland) to 16 ml saline every morning to form a final concentration of 1000 IU/mL. Each rat in the 'PD', 'PRD', 'PR' and 'VitD' groups received 0.5 ml/day (500 IU/day) orally through a feeding syringe for the full study duration. Cholecalciferol, and its dose, was chosen over calcitriol, the hormonal form of Vit D, to avoid the risk of soft tissue calcification [38]. Following 4 injections, the rats were sacrificed at the time of the 5th injection would have been given. Ribavirin and vitamin D3 were continued till the day before sacrification.

Types of sample

All rats were sacrificed on the same day under diethyl ether anaesthesia (Fisher Scientific UK Ltd, Loughborough, UK) a week after the last injection. One ml of blood was collected on EDTA for CBC and 3 ml of blood were collected in plain tube immediately after cutting the vena cava. Blood samples in plain tubes were centrifuged and the serum was stored in -20°C for routine biochemistry and to measure serum concentrations of 25-OH vitamin D, EPO and iron parameters.

Specimens weighing 1 gm from both kidneys (0.5 gm from each) and 1 gm from the middle lobe of liver was obtained from each animal and they were used immediately for protein extraction using 3 ml of RIPA lysis buffer containing protease inhibitors (Santa Cruz Biotech, USA) and electrical homogeniser. All samples were centrifuged at 14000 rpm for 30 minutes and small aliquots (0.5 ml) of the resultant supernatant were placed in Eppindorff tubes and stored in -20°C till processed to measure the levels of EPO hormone in kidney using ELISA and iron, total iron binding capacity and ferritin in liver using Electro-chemiluminescence immunoassay (ECLIA).

Measurement of extracted protein concentrations

The concentrations of the total proteins extracted from the kidney and liver specimens were measured using the BioSpec-nano (Shimadzu Corporation, Japan) at 280 OD. All protein samples were diluted using normal sterile saline to make a final concentration of 500 μg/ml of total protein.

Determination of haematological profile

Whole blood samples (1 ml) collected on EDTA were processed on Sysmex XS 500 (Sysmex, IL, USA) for the measurement of haemoglobin concentrations, RBCs count, Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC).

Liver and renal function parameters

The quantitative measurement of serum liver enzymes (ALP, ALT and AST), total and indirect bilirubin, creatinine and urea was done using Cobas e411 (Roche Diagnostics International Ltd, Switzerland) according to the manufacturer protocol.

Enzyme Linked Immunosorbant Assay (ELISA)

ELISA was used for quantitative measurement of serum total 25-OH Vitamin D (Dialab, Objekt, Austria) and, serum and kidney EPO concentrations (Cusabio, Hubei, China). All samples were processed in duplicate on a fully automated ELISA system (Human Diagnostics, Germany) and according the manufacturers' instructions. The optical density of the plates was measured within 5 minutes at 450 nm as recommended by the manufacturers.

The detection range of the EPO kit according to the manufacturer was 0.156 to 10 ng/mL, sensitivity was 0.039 ng/mL and the intra-assay and inter-assay precision was <8% and 10%, respectively. For total vitamin d kit, the detection range was 2.9 to 130 ng/mL, sensitivity was 2.89 ng/mL and the intra-assay and inter-assay precision was 4.7% and 9.7%, respectively.

Iron parameters

The quantitative measurement of serum and liver iron, ferritin and Total Iron Binding Capacity (TIBC) were performed on Cobas e411 (Roche Diagnostics International Ltd, Switzerland) according to the manufacturer protocol. Transferrin Saturation (TfSat) was calculated as follow: (Serum iron/TIBC) X100.

Iron kit sensitivity of 5 µg/dL and the intra- and interassay variation was <4% and 6.2%, respectively. The TIBC sensitivity was 16.8 µg/dL and the intra- and interassay was 1.6% and 2.4%, respectively. The sensitivity of the ferritin kit was 0.5 ng/mL and the intra- and interassay was 5.5% and 7.1%, respectively.

Statistical analysis

Statistical analysis of the results was performed using SPSS version 16. Normality and homogeneity of data were assessed with the Kolmogorov and Smirnoff test and Levene test, respectively. One way ANOVA followed by LSD post hoc test were used to compare between the different groups. Correlations were determined using Pearson's test. P value<0.05 was considered significant.

Results

Biochemical findings

There was no significant difference (P>0.05) between the different study groups in liver enzymes, total bilirubin, indirect bilirubin and renal function parameters (Table 1). However, serum concentrations of total 25-OH Vitamin D were significantly higher in the groups that received cholecalciferol compared to the other study groups (Table 1).

	Control	P group	PD group	PR group	PRD group	R group	RD group	VitD group
ALP (IU/L)	121.2 ± 11.5	126.1 ± 8.9	120.1 ± 11.8	126.2 ± 12.3	121.5 ± 11.6	118 ± 9.5	123.1 ± 8.3	119.2 ± 13.1
ALT (U/L)	68 ± 3.1	70.1 ± 6.2	67.7 ± 5.1	66.2 ± 5.4	68.1 ± 3.9	67.3 ± 3.1	68.7 ± 4.1	67.1 ± 3.9
AST (U/L)	77.5 ± 3.4	80.1 ± 6.8	79.1 ± 5	76.5 ± 5.1	80.1 ± 5.2	78.4 ± 3.2	76.8 ± 6.1	75.8 ± 4.2
Total Bilirubin (mg/dL)	0.5 ± 0.18	0.48 ± 0.16	0.49 ± 0.11	0.51 ± 0.21	0.48 ± 0.13	0.49 ± 0.14	0.47 ± 0.2	0.51 ± 0.23
Indirect Bilirubin (mg/dL)	0.15 ± 0.05	0.16 ± 0.07	0.17 ± 0.05	0.19 ± 0.06	0.18 ± 0.08	0.17 ± 0.07	0.16 ± 0.03	0.15 ± 0.08
Creatinine (mg/dL)	0.22 ± 0.06	0.2 ± 0.08	0.21 ± 0.02	0.2 ± 0.03	0.21 ± 0.05	0.22 ± 0.03	0.23 ± 0.07	0.2 ± 0.06
Urea (mg/dL)	47.6 ± 5.1	52.3 ± 4	47.2 ± 3.8	54.6 ± 6.5	52.4 ± 7	47.3 ± 5.8	53.9 ± 6.4	48.1 ± 5.2
25-OH Vitamin D (ng/mL)	42.3 ± 7.8	39.8 ± 6.1	68.5 ± 9.1^{a,b}	37.7 ± 9.5^{c}	65.8 ± 9.1^{a,b,d}	42 ± 10^{c,e}	67.9 ± 10.4^{a,b,d,f}	69.9 ± 9.8^{a,b,d,f}

Table 1: Mean ± SD of liver enzymes, bilirubin (total and indirect), renal function parameters and serum 25-OH vitamin D in all study groups. (a=p<0.05 compared to control group; b=p<0.05 compared to P group; c=p<0.05 compared to PD group; d=p<0.05 compared to PR group; e=p<0.05 compared to PRD group and f=p<0.05 compared to R group).

Erythrocyte indices

The administration of pegylated interferon-α-2a alone (P group) did not affect any of the erythrocyte parameters compare to 'Control' group' (p>0.05). The addition of RBV to Peg-INF-α (PR group), significantly decreased the RBCs count (7.25 ± 0.45x10^{-6}/µl; p=0.0003) and haemoglobin concentration (13.1 ± 0.5 gm/dL; p=0.000001) compared to control. Additionally, the combination of RBV with Peg-INF-α (PR group) significantly decreased the haemoglobin compared to the Peg-INF-α only (P) group (Table 2). The lowest RBCs count (6.97 ± 0.9x10^{-6}/µl) and haemoglobin concentration (12.6 ± 1 gm/dL) was detected with ribavirin only (R group) and it was significantly lower compare to the control group (p=0.00002) and 'P group' (p=0.0004). However, there was no significant difference between the 'PR' and 'R' groups. Additionally, there was no significant difference between all study groups in PCV, MCV and MCH (Table 2).

	Control	P group	PD group	PR group	PRD group	R group	RD group	VitD group
RBCs (x10^6/µl)	9.3 ± 2.1	8.7 ± 1.1	9 ± 0.9	7.9 ± 0.6^{a,c}	9.15 ± 0.8^{b,d}	7.25 ± 0.45a,b,c,e	9.3 ± 0.9^{d,f}	8.9 ± 0.5^{d,f}
Hb (g/dL)	15.5 ± 1.07	14.9 ± 0.7	15.2 ± 0.8	13.1 ± 0.5^{a,b,c}	15.54 ± 0.5^{d}	12.6 ± 1a,b,c,e	14.9 ± 0.6^{d,f}	15.1 ± 0.8^{d,f}
PCV (%)	46.4 ± 7.1	42.3 ± 3.1	43.1 ± 1.4	41.9 ± 1.3	45.1 ± 0.6	42.1 ± 2.7	42.9 ± 1.3	44.9 ± 1.8
MCV (fL)	61 ± 3.5	62.7 ± 3.3	62.1 ± 0.8	61.1 ± 2.9	59.2 ± 3.6	64.3 ± 4.2	59.5 ± 1.6	61.3 ± 1.8
MCH (pg)	17.6 ± 0.9	17.8 ± 0.9	17.7 ± 0.4	17.5 ± 0.7	17.1 ± 0.6	17.6 ± 0.7	17.4 ± 0.5	17.4 ± 0.7
MCHC (pg/dL)	34.7 ± 1	33.8 ± 0.5	35.9 ± 0.6^{a,b}	34.5 ± 1^{c}	36.8 ± 1^{a,b,d}	34.4 ± 0.9c,e	35.8 ± 0.6^{a,b,d,f}	34 ± 0.8^{c,e,g}
Serum EPO (ng/mL)	2.3 ± 0.8	1.92 ± 0.3	2.9 ± 0.8^{a,b}	0.63 ± 0.15^{a,b,c}	1.9 ± 0.6^{c,d}	0.7 ± 0.4a,b,c,e	2.1 ± 0.9^{c,d,f}	2.9 ± 0.4^{a,b,d,e,f,g}
Kidney EPO (ng/mL)	4.9 ± 0.7	4.2 ± 0.4	6.1 ± 1.1^{a,b}	2 ± 0.6^{a,b,c}	4.4 ± 0.8^{c,d}	2.3 ± 0.7a,b,c,e	4 ± 1.1^{c,d,f}	5.8 ± 1^{a,b,d,e,f,g}

Table 2: Mean ± SD of erythrocyte indices, serum and renal concentrations of EPO in all study groups. (a=p<0.05 compared to control group; b=p<0.05 compared to P group; c=p<0.05 compared to PD group; d=p<0.05 compared to PR group; e=p<0.05 compared to PRD group; f=p<0.05 compared to R group and g=p<0.05 compared to RD group).

Supplementation with cholecalciferol rescued the observed decrease in RBCs count and haemoglobin content in the designated groups and the erythrocyte indices in the supplemented rats with VitD3 (PD, PRD, RD and VitD groups) were similar to the control group (p>0.05) and significantly higher (p<0.05) compared to the corresponding non-vitamin D groups (P, PR and R groups) (Figure 1). However, there was no significant difference in PCV, MCH and MCV between the different study groups (Table 2).

Serum and kidney concentrations of EPO

RBV significantly decreased the levels of EPO at the serum and kidney levels compared to the 'control' an 'P' groups either individually or combined with Peg-INF-α, (Table 2). Coherently, supplementation with VitD3 significantly increased the concentrations of the hormone in kidney (p<0.05) and serum (p<0.05) samples compared to the control and the non-vitamin D (P, PR and R) groups (Figure 1).

Serum and liver iron parameters

A significant decrease in the median of liver iron, ferritin and transferrin saturation, and a significant increase in TIBC was observed in all groups, except for RBV only group, when compared to control (Figure 2). However there was no significant change (p>0.05) between the different study groups in serum iron parameters (Table 3).

Correlations between erythrocyte indices, liver & serum iron parameters, serum & kidney EPO and serum vitamin D

Liver concentrations of iron correlated significantly and negatively with serum (r=-0.345; p=0.005) and kidney (r=-0.350; p=0.004) EPO levels, haemoglobin levels (r=-0.254; p=0.04) and serum vitamin D concentrations (r=-0.300; p=0.01). Same results were also observed for liver transferrin saturation with serum (r=-0.420; p=0.001) and kidney (r=-0.380; p=0.002) EPO and serum vitamin D concentrations (r=-0.421; p=0.0004) (Figure 3).There was no significant correlation detected either for the other liver iron parameters (TIBC and ferritin) or for serum iron parameters with serum and kidney EPO and serum vitamin D (p>0.05).

Serum EPO correlated positively and significantly with kidney EPO levels (r=0.896; p=0.6x10^{-18}). Additionally, there was a significant positive correlation between serum levels of 25-OH vitamin D with serum EPO (r=0.644; p=0.1x10^{-6}) and kidney EPO concentrations (r=0.711; p=0.3x10^{-9}) (Figure 4).

Both renal and serum EPO correlated significantly (p<0.01) with the RBCs count (r=0.36 and 0.38, respectively) and with the haemoglobin concentration (r=0.557 and 0.481, respectively) (Table 4). The RBCs count and haemoglobin also correlated significantly with the serum levels of 25-OH vitamin D (r=0.244 and 0.323, respectively). There was no correlation between serum EPO, kidney EPO and serum 25-OH vitamin D with PCV, MCV and MCH (Table 4).

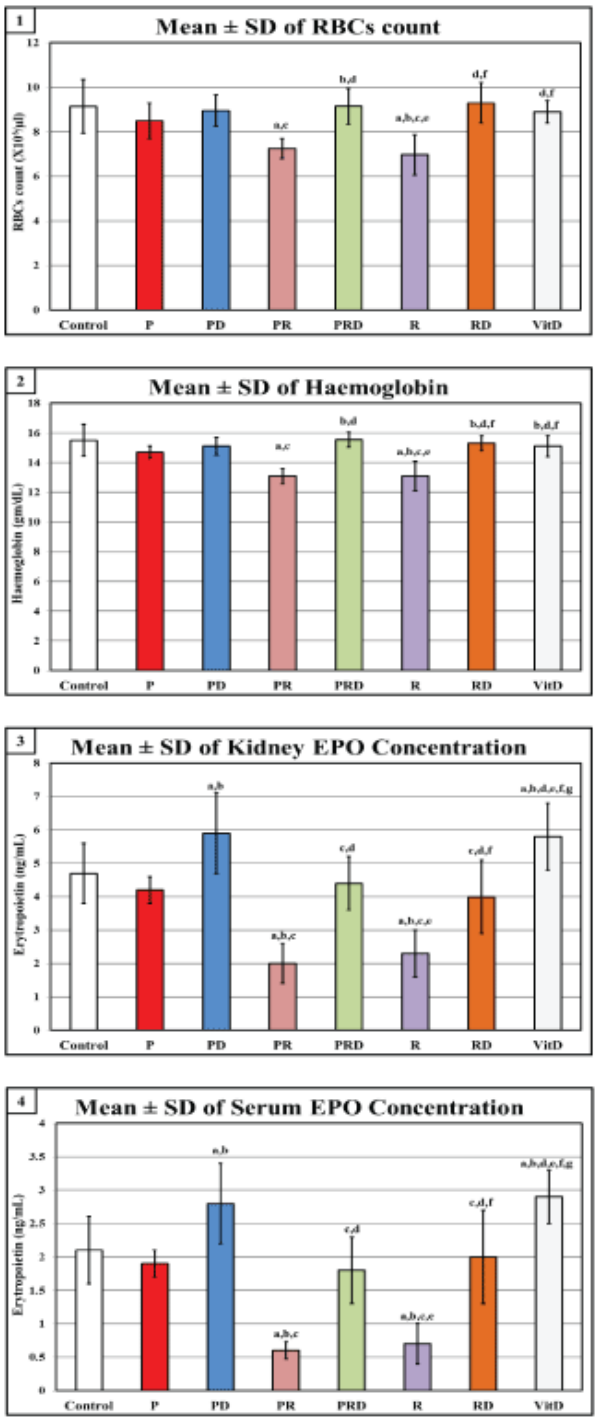

Figure 1: Mean ± SD of (1) erythrocyte count, (2) haemoglobin concentration, (3) kidney erythropoietin and (4) serum erythropoietin in the different study groups (a=p<0.05 compared to control; b=p<0.05 compared to 'P group', c=p<0.05 compared to 'PD' group, d=p<0.05 compared to 'PR' group, e=p<0.05 compared to 'PRD' group; f=p<0.05 compare to 'R' group; and g=p<0.05 compared to 'RD' group).

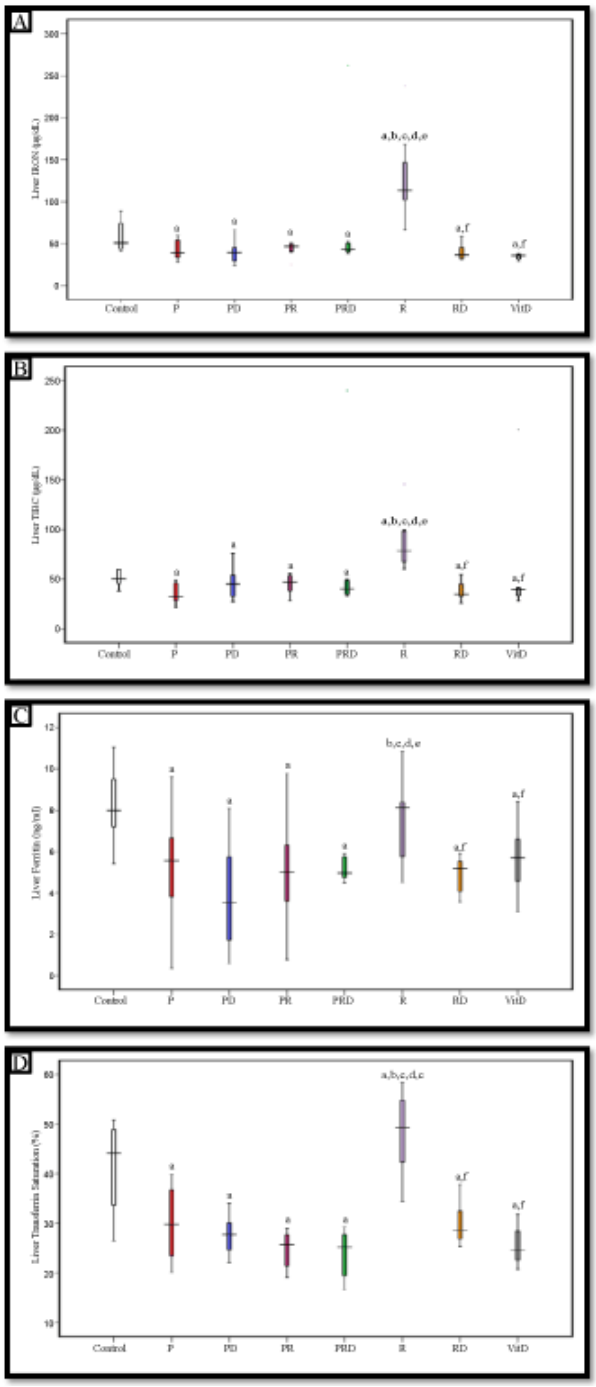

Figure 2: Correlation of liver iron (left column) with (1) serum EPO, (3) kidney EPO, (5) serum 25(OH) vitamin D, and liver transferrin saturation (right column) with (2) serum EPO, (4) kidney EPO, (6) serum 25(OH) vitamin D by Pearson's correlation test.

		Control	P group	PD group	PR group	PRD group	R group	RD group	VitD group
Iron (µg/dL)	Serum	187 (110-347)	220 (157-278)	188 (166-263)	204 (134-248)	178 (171-276)	176 (167-247)	223 (172-329)	248 (133-297)
	Liver	51 (41-137)	39 (28-60)[a]	39.5 (24-67)[a]	42 (25-51)[a]	33.5 (24-62)[a]	113 (66-238)[a,b,c,d,e]	36.5 (31-59)[a,f]	36 (27-67)[a,f]
TIBC (µg/dL)	Serum	536 (483-668)	505 (492-606)	591 (538-689)	545 (465-637)	560 (494-614)	556 (501-585)	576 (513-675)	570 (529-599)
	Liver	50.2 (38-135)	73.7 (51-169)[a]	75.1 (50-181)[a]	67.6 (48-142)[a]	70.2 (53-130)[a]	35 (25-554)[a,b,c,d,e]	72.3 (45-161)[a,f]	68.4 (42-130)[a,f]
Ferritin (ng/mL)	Serum	2.9 (2.3-7.2)	2.2 (1.9-6.1)	2.6 (1.8-6.3)	2.6 (1.17-5.7)	2.8 (2.1-7)	2.4 (1.8-6.8)	2.5 (2-7.3)	2.3 (1.7-6)
	Liver	8.4 (4.4-11)	5.5 (1.3-9)[a]	3.5 (1.5-8)[a]	5 (1.7-9.7)[a]	4.9 (4.4-8)[a]	8.8 (5-13.2)[b,c,d,e]	5 (2.5-5.8)[a,f]	5.7 (3-8.4)[a,f]
Transferrin Saturation (%)	Serum	33.6 (20.7-51)	39.3 (25-55)	32.1 (26.2-44)	35.3 (26.3-46)	34.1 (29-47)	34.6 (29-46)	36.7 (29-55)	35 (25-49)
	Liver	42.9 (29-59)	35.1 (21-46)[a]	27.7 (22-34)[a]	34.8 (26-53)[a]	32.1 (20-55)[a]	51 (44-58)[a,b,c,d,e]	33 (25-31)[a,f]	31.8 (24-47)[a,f]

Table 3: Median of iron parameters in liver and serum in all study groups (a=p<0.05 compared to control group; b=p<0.05 compared to P group; c=p<0.05 compared to PD group; d=p<0.05 compared to PR group; e=p<0.05 compared to PRD group; f=p<0.05 compared to R group and g=p<0.05 compared to RD group).

		RBCs	Hb	MCV	MCH	MCHC	PCV
Serum EPO	R Value	0.335*	0.557*	-0.135	-0.159	-0.022	0.152
	P Value	0.002	$0.1x10^{-5}$	0.2	0.15	0.8	0.17
Kidney EPO	R Value	0.330*	0.455*	-0.174	-0.321*	-0.163	0.158
	P Value	0.003	$0.1x10^{-6}$	0.12	0.004	0.14	0.16
25-OH vitamin D	R Value	0.244*	0.326*	-0.205	-0.322*	-0.116	0.121
	P Value	0.02	0.003	0.069	0.004	0.3	0.2

Table 4: Results of correlation analysis using Pearson's test for serum EPO, kidney EPO an serum 25-OH vitamin D with RBCs count, haemoglobin concentration, MCV, MCV, MCH, MCHC and PCV (*=P<0.05).

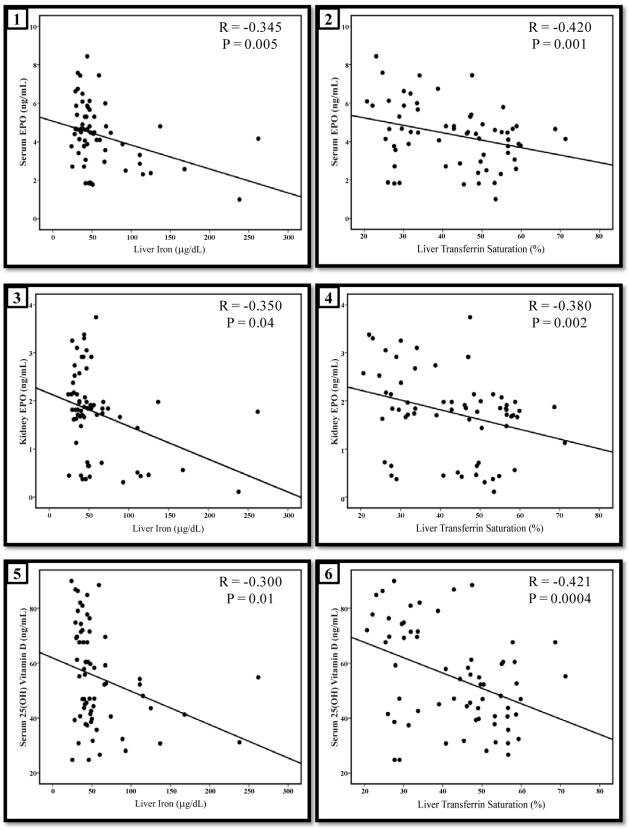

Figure 3: Median of liver (A) iron levels, (B) TIBC, (C) ferritin and (D) transferrin saturation in the different study groups (a=p<0.05 compared to control; b=p<0.05 compared to 'P group', c=p<0.05 compared to 'PD' group, d=p<0.05 compared to 'PR' group, e=p<0.05 compared to 'PRD' group; f=p<0.05 compare to 'R' group; and g=p<0.05 compared to 'RD' group).

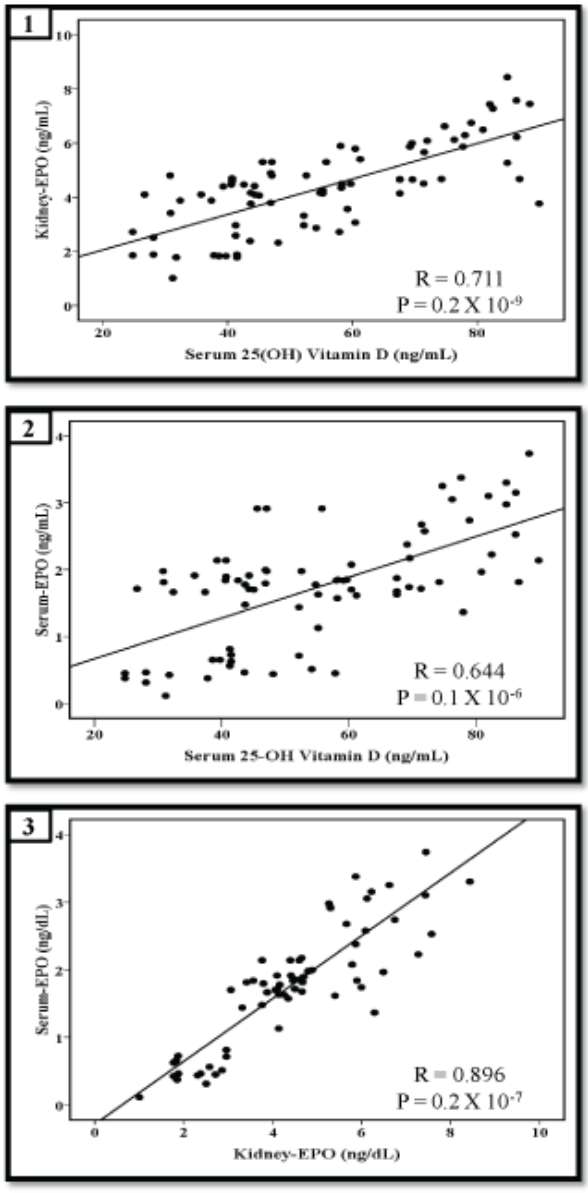

Figure 4: Correlation of (1) serum 25-(OH) vitamin D with kidney EPO, (2) serum 25-(OH) vitamin D with serum EPO and (3) Kidney EPO with serum EPO by Pearson's correlation test.

Discussion

CHC is a major health problem worldwide and it is associated with iron accumulation in the liver, which correlates with the severity and progression of the disease [9]. Additionally, the use of Peg-INF-α based therapy for the treatment of CHC is associated with several side effects, including anaemia, which could lead to the termination of treatment [10-14]. Recently, the addition of vitamin D to Peg-INF-α therapy during the treatment of CHC has shown a potential role in increasing the response rate [39-41].

The current study investigated the effect(s) of Peg-INF-α based therapy on serum and liver iron parameters, serum and kidney concentrations of EPO, erythrocyte indices and the effect of vitamin D3 supplementation on the aforementioned parameters in experimental animal model. The results showed that RBV, either alone or in combination with Peg-INF-α, significantly decreased RBCs count and haemoglobin concentration. Additionally, RBV increased iron, ferritin and transferrin saturation, and significantly decreased TIBC in liver. Furthermore, ribavirin ± Peg-INF-α significantly decreased the concentrations of EPO at the kidney and serum levels.

However, there was no significant difference observed between the study groups in the values of serum iron parameters. Additionally, there was no significant difference in serum levels of indirect and total bilirubin or in MCV, MCH and MCHC between the treated groups and control, suggesting that RBV resulted in the development of normocytic normochromic anaemia by suppressing erythropoiesis.

Both Peg-INF-α and/or vitamin D3 significantly decreased liver iron, ferritin and transferrin saturation. Nevertheless, both drugs had no effect on the iron parameters in serum. Correspondingly, there was a significant negative correlation between serum vitamin D and liver iron concentrations and transferrin saturation. Moreover, supplementation with vitamin D3 prevented the development of anaemia and the erythrocytes indices were not different from the control group. Coherently, a significant increase in the concentrations of EPO was also detected at the serum and kidney levels in all groups treated with vitamin D3 and a significant positive correlation was observed for serum 25-OH vitamin D with both kidney and serum EPO, RBCs count and haemoglobin concentrations.

The current results suggest that vitamin D could have a potential beneficial role in decreasing the accumulation of iron in the liver and the prevention/treatment of anaemia during the treatment of CHC by promoting serum and renal EPO.

The combination of Peg-INF-α and ribavirin is still currently used for the treatment of CHC [1,42]. In addition to its role in the modulation of immune system, Peg-INF-α based therapy has been shown to regulate iron metabolism and decrease the liver iron content, probably by modulating the expression of hepcidin [7-9]. Iron overload and increase in liver iron content has been reported to correlate with the severity of liver damage and the progression of the disease [43,44]. Additionally, high serum levels of ferritin have been associated with decrease response to Peg-INF-α based therapy [45,46]. Therefore, it has been postulated that the success of the antiviral treatment could be dependent on decreasing iron overload and ferritin levels by modulating the expression of hepcidin, which is also known to have anti-inflammatory properties, by the liver [7-9].

The present results correlate with the previous reports as they showed that Peg-INF-α, but not RBV, significantly decreased liver iron, ferritin and transferrin saturation and significantly increased TIBC in liver. Moreover, the addition of vitamin D also demonstrated a significant decrease in these parameters in the liver either solely or in combination with the other drugs. Vitamin D has been shown to decrease iron accumulation in liver tissue in patients suffering from thalassemia major and hemochromatosis [31,32], possibly by regulating the expression of hepcidin [47]. Furthermore, the administration of Vit D in rat resulted in a decrease in the levels of iron in blood and altered the distribution of iron in different tissue, including liver, compared to controls [30]. Hypovitaminosis Dwasalso associated with an increase in liver iron accumulation in a murine model of iron overload [33].

Findings of the present study suggest that supplementation with vitamin D with Peg-INF-α during the treatment of CHC could have a beneficial role in modulating the liver content of iron, especially in those patients suffering from haemolytic anaemia. Further studies are needed to illustrate the role of vitamin D supplementation in the regulation of iron metabolism during the treatment of CHC.

Several side effects, including anaemia, develop during the course of CHC treatment with Peg-INF-α based therapy and could result in the termination of treatment [11,13,14,23-25,48]. In the registered trials using the combination of Peg-INF-α and RBV, anaemia affected 12% of the patients with 3 mg/dL decrease in haemoglobin during the first 4 weeks of treatment and the severity of anaemia was dependent on the dose of RBV [12,13,29,49-51]. It is believed that the majority of anaemia during the course of therapy are haemolytic in nature due to the intoxication of RBCs with RBV [19-22,51]. Peg-INF-α could also exaggerate the haemolytic effect of RBV in the currently applied treatment protocol [14-18]. However, the prevalence of anaemia was significantly lower in Peg-INF-α monotherapy compared to Peg-INF-α and RBV dual therapy [52].

The current study agrees with the aforementioned observations as there was no significant change in RBCs count and haemoglobin concentration in the 'P' group compared to control. Moreover, a significant decrease in the number of erythrocytes and haemoglobin was observed with RBV either individually or in combination with Peg-INF-α compared to the 'Control' and 'P' groups. However, there were no significant changes in total and indirect bilirubin, MCV and MCH between the RBV ± PEG-INF-α treated groups and control, suggesting that the treated groups developed normocytic normochromic anaemia. Hence, the observed significant decrease in serum and kidney EPO concentrations could suggest that the RBV associated anaemia could be due to bone marrow suppression rather than haemolysis.

Anaemia associated with RBV is believed to be dependent on the plasma concentration of the drug rather than the dose/Kg body weight [29,51]. The accumulation of RBV and its metabolites in human RBCs causes oxidative stress, mitochondrial toxicity and RBCs haemolysis [19-23]. However, the uptake rate of RBV by erythrocytes has been reported to differ according to dose and species [53]. The largest accumulation of RBV was observed in monkey, followed by human and the lowest accumulation was detected in rat erythrocyte [53]. Moreover, *in vitro* incubation of erythrocytes from the 3 species with RBV showed that the retention rate of the drug was 77% in monkey, 45% in human and 20%, in rat red cells [53]. Exposure of red cells to RBV *in vitro* did not alter the osmotic fragility and deformability of the cells [53-55].

RBV induced anaemia could also be due the inhibiting effect of RBV on the process of erythropoiesis through the suppression of bone marrow and decreasing the expression of both EPO and its receptor [24,25]. RBV was also shown to decrease red cell survival as well as inhibit the release of red cell from the bone marrow in monkey and rat [53-57]. However, RBV had no effect on erythrocyte MCV, MCH and MCHC in both species [53-55]. The administration of Peg-INF-α and RBV in human was also associated with a decrease in serum EPO concentrations [24]. Therefore, it could be postulated that RBV produces normocytic normochromic anaemia in rat by suppressing

the bone marrow through decreasing the production of EPO from the kidney.

Gathered data from clinical studies on haemodialysis patients have proven that vitamin D supplementation is clinically useful in the management of chronic renal failure induced anaemia [35,36,48,58]. Vitamin D3 has been shown to stimulate the proliferation of erythroid progenitor cells independently from EPO [48]. Additionally, vitamin D3 has been reported to synergise with EPO to increases the production of EPO receptor at the mRNA and protein levels *in vitro* [37]. The present study agrees with the previous data as supplementation with cholecalciferol prevented the development of anaemia with both Peg-INF-α and RBV and significantly increased endogenous EPO concentrations at the kidney and serum levels. The current results suggest that vitamin D supplementation with Peg-INF-α therapy could have potential clinical usefulness in the prevention of RBV induced anaemia, especially in patients with chronic renal failure. More studies are needed to explore the potential clinical role of vitamin D supplementation in the prevention/treatment of anaemia associated with CHC treatment.

A limitation of the present study is that it did not measure the effect of vitamin D supplementation on the cellular expression of EPO protein, EPO receptors, ferritin, hepcidin and transferrin at the protein level using immunohistochemistry or at the gene level using quantitative RT-PCR. Studying the expression of these molecules at the tissue level could reveal the mechanism(s) by which vitamin D regulates the metabolism of iron and the action(s) of endogenous EPO during the treatment of CHC.

In conclusion, supplementation with vitamin D3 could prevent the accumulation of iron in hepatic tissue. Furthermore, VitD may provide protection against ribavirin induced anaemia by stimulating the production of endogenous EPO. Further studies are needed to illustrate the clinical value of vitamin D supplementation in the treatment of hepatitis C virus, especially in those patients suffering from chronic haemolytic anemia and/or chronic renal failure.

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Conflict of Interest

The author has nothing to declare.

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