Int J Clin Exp Med 2014;7(9):2667-2676
www.ijcem.com /ISSN:1940-5901/IJCEM0001650

Original Article

Ribavirin induced anaemia: the effect of vitamin D supplementation on erythropoietin and erythrocyte indices in normal Wistar rat

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Received August 1, 2014; Accepted August 26, 2014; Epub September 15, 2014; Published September 30, 2014

Abstract: Objectives: To measure the effect of vitamin D3 (VitD) supplementation on erythrocyte indices, serum and kidney erythropoietin (EPO) in normal rats treated with Pegylated interferon-α (Peg-INF-α) and ribavirin (RBV). Materials and methods: Eighty male Wistar rats were divided equally into 8 groups. 'Control'; 'P': only received Peg-INF-α; 'PD': Peg-INF-α/VitD; 'PR': Peg-INF-α/RBV; 'PRD': Peg-INF-α/RBV/VitD; 'R': only received RBV; 'RD': RBV/VitD and 'VitD': only received vitamin D3. Peg-INF-α-2a was injected subcutaneously (6 μg/rat/week) for 4 weeks. RBV (4 mg/rat/day) and VitD (500 IU/rat/day) were given orally for 5 weeks. Blood samples were collected to measure erythrocyte indices and serum 25(OH) vitamin D. EPO was measured in serum samples and kidney specimens by ELISA. Results: Peg-INF-α alone did not affect the RBCs count, haemoglobin, serum and kidney EPO compared to control ($P > 0.05$). RBV significantly decreased ($P < 0.05$) the erythrocyte count, haemoglobin and EPO levels in kidney and serum, either individually (R group) or combined with Peg-INF-α (PR group), compared to 'Control' and 'P' groups. VitD prevented the development of anaemia and significantly increased the concentrations of EPO at serum and kidney levels in the 'RD' and 'PRD' groups compared to 'R' and 'PR' groups. There was a significant positive correlation between blood levels of VitD with serum and kidney EPO, Red cell count and haemoglobin concentrations. Conclusion: VitD could have a potential beneficial role in the prevention of ribavirin-induced anaemia by promoting endogenous EPO. Further studies are needed to explore the role of vitamin D in the prevention of ribavirin associated anaemia.

Keywords: Anaemia, erythropoietin hormone, pegylated interferon-α, ribavirin and vitamin D

Introduction

Chronic hepatitis C (CHC) infection is a major health problem affecting 170 million persons worldwide with an estimated annual incidence of 3-4 million acquired new cases [1]. The current treatment of CHC consists of the combination of a weekly injection of pegylated interferon-α (Peg-INF-α) and daily oral ribavirin (RBV) and the duration of the treatment is based on the viral genotype [2, 3]. Despite the recent development of new antiviral drugs, the estimated cost is 60,000-100,000 USD and therefore, it is expected that peg-INF-α and RBV may still have a role especially for those patients living in developing countries and for whom access to the new drugs is not definite [3-7].

Almost all patients treated with peg-INF-α and RBV experience one or more adverse events during the course of therapy. Adverse events are a major reason that patients decline or stop therapy altogether. In the registration trials of peg-INF-α-2a and 2b plus RBV, 10% to 14% of patients had to discontinue therapy due to an adverse event [1, 2]. Haematological abnormalities are major side effects of Peg-IFN-α based therapy and the most common and important one is the development of anaemia [8-12].

Several mechanisms for the development of anaemia during Peg-INF-α based therapy have been suggested. Peg-INF-α may lead to anaemia by suppressing the proliferation of progenitor cell, increasing erythroid precursor cells destruction, autoimmune haemolytic reactions, and reducing renal function [13-16]. Alternatively, RBV induces haemolytic anaemia in a dose-dependent manner, which is believed to be exacerbated by Peg-INF-α [17-19]. RBV may also lead to anaemia by down-regulating the

expression of erythropoietin receptors [11, 20, 21] and both drugs were also associated with a decrease in serum erythropoietin hormone (EPO) [22, 23]. Hence, Peg-INF-α based therapy could induce anaemia by haemolysis and/or suppression of erythropoiesis.

The initial clinical management of anaemia associated with Peg-INF-α based therapy was the reduction of RBV dosage. However, several reports have demonstrated a correlation between response rate and higher RBV dose [10, 24-27]. Therefore, the use of erythropoiesis-stimulating agents (ESA), such as EPO, and blood transfusion have been introduced as alternatives for RBV dose reduction to support anaemic patients during the course of treatment [27, 28].

Vitamin D (VitD) has been shown to play important roles in the regulation of several systems beside its role in bone and mineral metabolism [29-31]. Vitamin D regulates the process of erythropoiesis by stimulating erythroid progenitor cells in a synergistic fashion with other hormones and cytokines, including EPO, and it has been reported that vitamin D is crucial for normal red blood cell production [32]. The prevalence of anemia and the use of ESA have been found to be negatively correlated with serum VitD levels regardless of kidney function in the general population [30]. The role of vitamin D in erythropoiesis has also been suggested by several clinical observations, especially in haemodialysis patients, where administration of VitD has been has been associated with dose reductions in ESA and increased reticulocytosis [33, 34]. Furthermore, vitamin D3 (calcitriol), in synergism with EPO, increases the production of EPO receptor at the mRNA and protein levels in vitro [32].

Little is known about the role of VitD as a prophylactic/treatment for anaemia associated with Peg-INF-α based therapy. We therefore hypothesis that vitamin D3 supplementation may provide protection against anaemia associated with the current treatment of CHC. The present work is a preclinical study to measure the effect of vitamin D3 (cholecalciferol) supplementation with Peg-INF-α and RBV on erythrocyte indices and concentrations of EPO in serum and kidney of normal rat.

Materials & methods

Drugs

Pegylated interferon-α-2a (Pegasys®, HoffmannLa 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) was 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 80 male Wistar rats weighing 250-300 gm were used. All animals received humane care during the study protocol and during sacrification. The animals were divided equally into 8 groups as follow: The first group included 10 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 + VitD 'PD group', The fourth group received Peg-INF-α + ribavirin 'PR group', The fifth group received Peg-INF-α + RBV + VitD 'PRD group', the sixth group received RBV only 'R' group, the seventh group received RBV + VitD '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 9.5 ml sterile normal saline to prepare a final volume of 10 ml and the final concentration was 18 μg/ml. Each rat in the 'P', 'PD', 'PR' and 'PRD' groups received a weekly subcutaneous injection of 0.33 ml (6 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 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]) [2].

Cholecalciferol (4500 IU/mL) was prepared by adding 4 ml 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) for the full study duration. Cholecalciferol, and its dose, was chosen over calcitriol, the hormonal form of vitamin D, to avoid the risk of soft tissue calcification [35]. Following 4 injections, the rats were sacrificed in the fifth week 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 anaesthesia using diethyl ether (Fisher Scientific UK Ltd, Loughborough, UK) a week after the last injection. One ml of blood was collected on EDTA for CBC and 3ml 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 and EPO.

A specimen weighing 1gm from both kidneys (0.5 gm from each) was obtained from each animal and it was 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 candidate proteins in kidney using ELISA.

Measurement of extracted protein concentrations

The concentrations of the total proteins extracted from the kidney 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.

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

Routine biochemistry

There was no significant difference (P > 0.05) using one way ANOVA between the different study groups in body weight, 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

Table 1. Summary of liver enzymes, bilirubin (total and indirect), renal function parameters and serum 25-OH vitamin D in all study groups

	Control	P Group	PD group	PR group	PRD group	R group	RD group	VitD group
ALP (IU/L)	122.6 ± 11.2	125.7 ± 9.7	120.8 ± 12.4	127.3 ± 11.9	121.6 ± 11.1	119 ± 9.9	122.8 ± 8.7	120.7 ± 12.3
ALT (U/L)	67 ± 2.4	71.2 ± 6.7	68.7 ± 4.1	66.4 ± 5.3	67.3 ± 3.7	66.1 ± 2.6	69.6 ± 3.3	66.7 ± 4.2
AST (U/L)	77.7 ± 3.9	81.8 ± 7.3	79.4 ± 5.1	75.2 ± 4.9	77.9 ± 4.8	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)	43.19 ± 8.1	39.6 ± 6.7	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}

$^{a}p < 0.05$ 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.

Table 2. Summary of erythrocyte indices, serum and renal concentrations of EPO in all study groups

	Control	P Group	PD group	PR group	PRD group	R group	RD group	VitD group
RBCs (X10^{6}/μl)	9.14 ± 1.2	8.5 ± 0.8	8.95 ± 0.7	7.9 ± 0.6^{a,c}	9.15 ± 0.8^{b,d}	7.58 ± 0.7^{a,b,c,e}	9.3 ± 0.9^{d,f}	8.9 ± 0.5^{d,f}
Hb (g/dL)	15.5 ± 1.07	14.7 ± 0.4	15.1 ± 0.6	13.8 ± 0.7^{a,b,c}	15.54 ± 0.5^{d}	13.1 ± 1^{a,b,c,e}	15.3 ± 0.5^{d,f}	15.1 ± 0.7^{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 ± 0.9^{c,e}	35.8 ± 0.6^{a,b,d,f}	34 ± 0.8^{c,e,g}
Serum EPO (ng/mL)	2.1 ± 0.5	1.9 ± 0.2	2.8 ± 0.6^{a,b}	0.6 ± 0.13^{a,b,c}	1.8 ± 0.5^{c,d}	0.7 ± 0.3^{a,b,c,e}	2 ± 0.7^{c,d,f}	2.9 ± 0.4^{a,b,d,e,f,g}
Kidney EPO (ng/mL)	4.7 ± 0.9	4.2 ± 0.4	5.9 ± 1.2^{a,b}	2 ± 0.6^{a,b,c}	4.4 ± 0.8^{c,d}	2.3 ± 0.7^{a,b,c,e}	4 ± 1.1^{c,d,f}	5.8 ± 1^{a,b,d,e,f,g}

$^{a}p < 0.05$ 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.

cholecalciferol compared to the other study groups (**Table 1**).

Erythrocyte indices

The administration of pegylated interferon-α-2a alone (P group) did not affect any of the erythrocyte parameters compared to 'Control' group' ($p > 0.05$). The addition of RBV to Peg-INF-α (PR group), significantly decreased the RBCs count ($7.9 \pm 0.6 \times 10^{-6}$/μl; $p = 0.002$) and haemoglobin concentration (13.8 ± 0.7 g/dL; $p = 0.0001$) compared to control (**Table 2**). Additionally, the combination of RBV with Peg-INF-α (PR group) significantly decreased the haemoglobin compared to the Peg-INF-α only (P) group. The lowest RBCs count ($7.58 \pm 0.7 \times 10^{-6}$/μl) and haemoglobin concentration (13.1 ± 1 gm/dL) was detected with ribavirin only (R group) and it was significantly lower compared to the control group ($p = 0.0004$) and 'P group' ($p = 0.003$). 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**), suggesting that the induced anaemia by the drugs is normocytic normochromic.

The addition of cholecalciferol prevented the development of anaemia in the designated groups as the erythrocyte indices in the rats supplemented with vitamin D3 (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**). Additionally, supplementation with cholecalciferol significantly increased the MCHC in all the treated groups, except for 'VitD group', compared to the control and the non-treated groups. However, there was no significant difference in PCV, MCH and MCV between the different study groups (**Table 2**).

Serum and kidney concentrations of EPO

Treatment with RBV, either individually or combined with Peg-INF-α, significantly decreased the levels of EPO at the serum and kidney levels compared to the 'control' and 'P' groups (**Table 2**). On the other hand, the addition of cholecalciferol, either alone or in combination with the other drugs, significantly augmented the kidney ($p < 0.05$) and serum ($p < 0.05$) concentrations

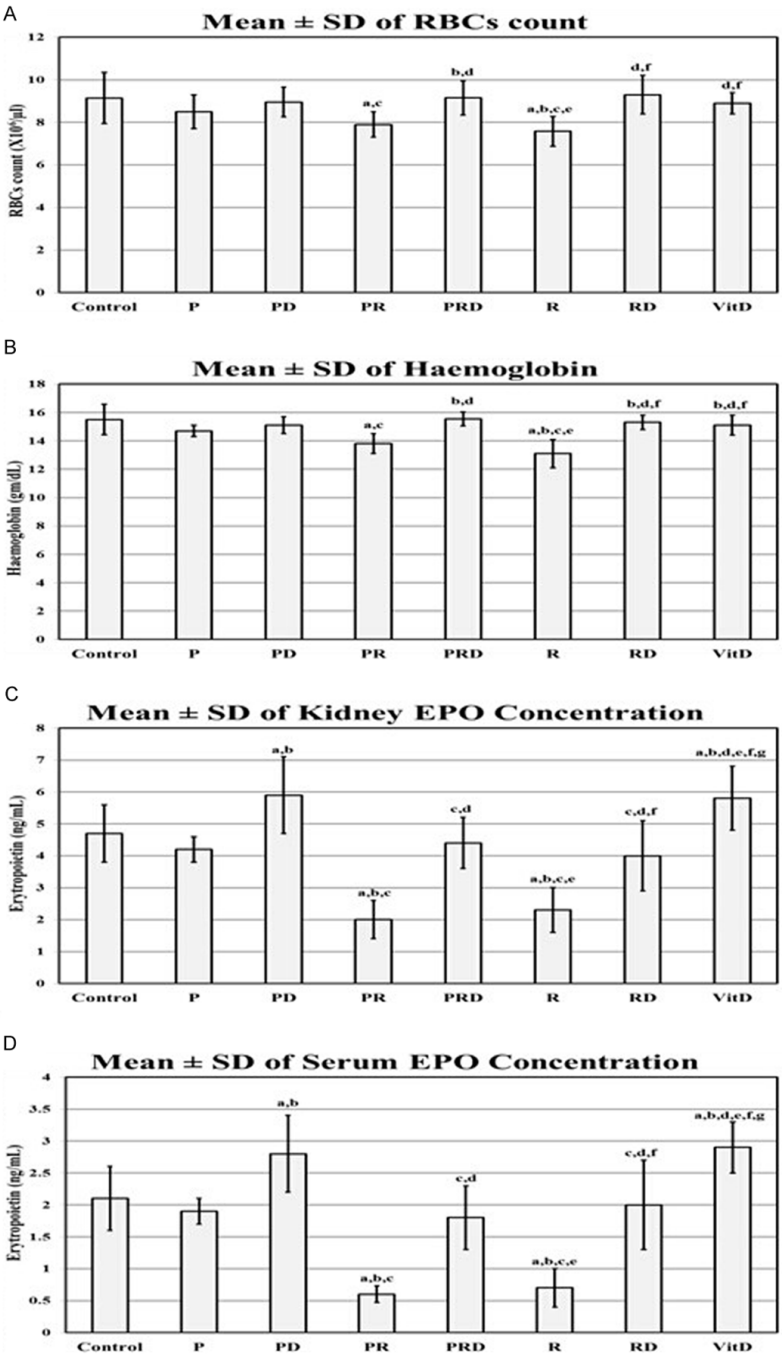

Figure 1. Mean ± SD of (A) erythrocyte count, (B) haemoglobin concentration, (C) kidney erythropoietin and (D) serum erythropoietin in the different study groups (a = p 0.05 compared to control; b = $p < 0.05$ compared to 'P group', c = $p < 0.05$ compared to 'PD' group, d = $p < 0.05$ compared to 'PR' 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).

of the hormone compared to the control and the non-vitamin D (P, PR and R) groups (**Figure 1**).

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

Serum EPO correlated positively and significantly with kidney EPO levels ($r = 0.874$; $p = 0.2 \times 10^{-7}$). Additionally, there was a significant positive correlation between serum levels of 25-OH vitamin D with serum EPO ($r = 0.644$; $p = 0.1 \times 10^{-6}$) and kidney EPO concentrations ($r = 0.736$; $p = 0.2 \times 10^{-9}$) (**Figure 2**).

Both renal and serum EPO correlated significantly ($p < 0.01$) with the RBCs count ($r = 0.33$ and 0.335, respectively) and with the haemoglobin concentration ($r = 0.577$ and 0.455, respectively) (**Table 3**). The RBCs count and haemoglobin also correlated significantly with the serum levels of 25-OH vitamin D ($r = 0.244$ and 0.326, respectively). There was no correlation between serum EPO, kidney EPO and serum 25-OH vitamin D with PCV, MCV and MCH (**Table 3**).

Discussion

The current study is the first to report a protective role for vitamin D3 supplementation against the development of ribavirin-induced anaemia by promoting the kidney and serum EPO concentrations in experimental animal model. Our results demonstrated a significant decrease in RBCs count and haemoglobin concentration following the use of RBV either individually or in combination with Peg-INF-α. However, there was no significant difference in the values of indirect bilirubin,

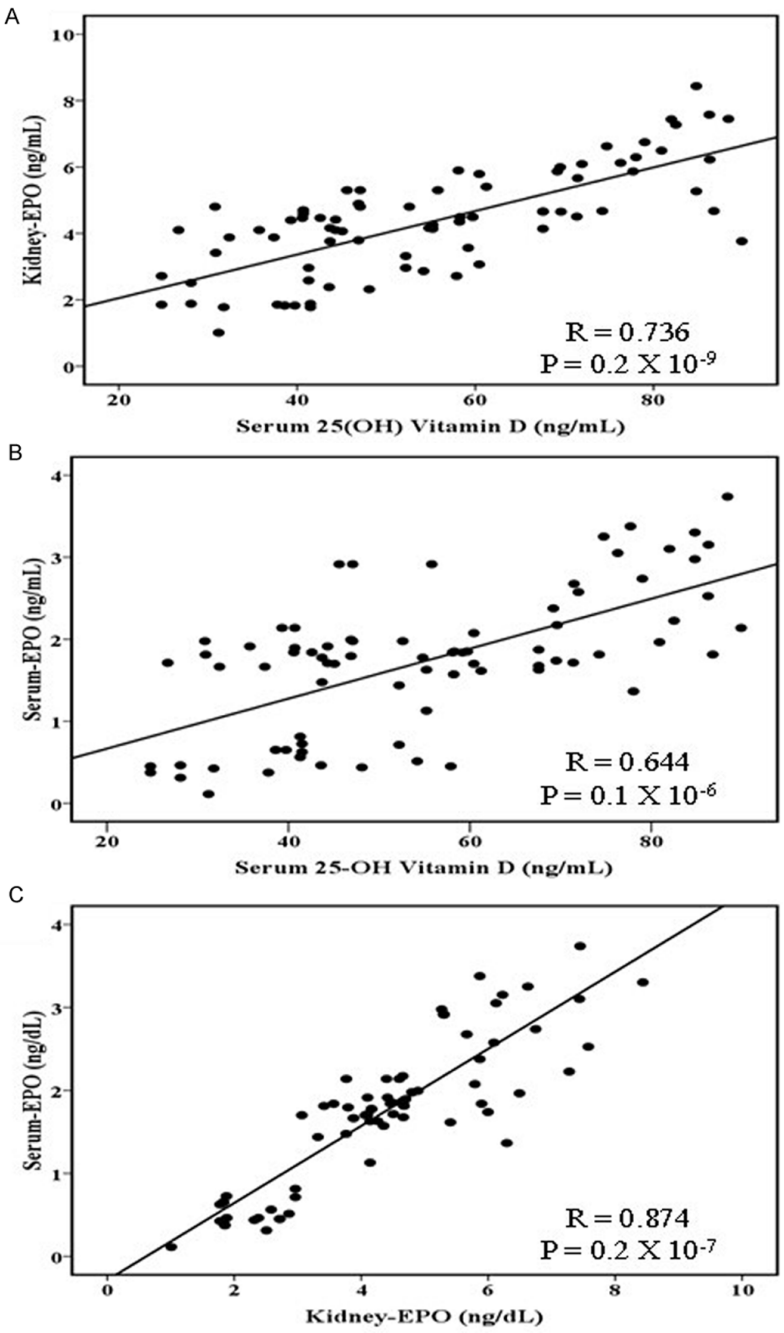

Figure 2. Correlation of (A) serum 25-(OH) vitamin D with kidney EPO, (B) serum 25-(OH) vitamin D with serum EPO and (C) Kidney EPO with serum EPO by Pearson's correlation test.

MCV and MCH between the treated groups and control, suggesting that the treatment lead to the development of normocytic normochromic anaemia by suppressing erythropoiesis. This is supported by the observation that ribavirin ± Peg-INF-α significantly decreased the concentrations of EPO at the kidney and serum levels. These observations were detected in the 'R' and 'PR' groups.

The addition of cholecalciferol (500 IU/day) during the course of the treatment rescued the drugs-induced anaemia in all groups (PRD and RD groups) and the erythrocytes parameters were similar to those of the control group. Furthermore, a significant increase in the concentrations of EPO was detected at the serum and kidney levels in all groups treated with vitamin D3. There was also a significant positive correlation between 25-OH vitamin D serum levels with both kidney and serum EPO, RBCs count and haemoglobin concentrations.

Our results suggest that, besides its recently reported potential role in increasing the response rate to Peg-INF-α based therapy [36-38], vitamin D could have a potential beneficial role in the prevention of anaemia during the treatment of chronic hepatitis C by promoting serum and renal EPO.

The combination of Peg-INF-α and ribavirin is the currently used therapy for CHC [1, 2]. However, the current treatment protocol is associated with several adverse effects, including anaemia, that could result in the termination of therapy [9, 11, 12, 21-23, 29]. The incidence of anaemia in the registered trials using the combination of Peg-INF-α and RBV was about 12% with 2.5-3 mg/dL decrease in haemoglobin during the first 4 weeks of treatment and the severity of anaemia was dependent on the dose of RBV [10, 11, 27, 28, 39,

Table 3. 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

		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	0.1×10^{-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	0.1×10^{-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

*$P < 0.05$.

40]. The majority of treatment-induced anaemia is due to RBCs intoxication with RBV resulting in haemolysis [17-20, 28]. Peg-INF-α, which was reported to produce anaemia by several mechanisms including bone marrow suppression and autoimmune haemolysis, exaggerates RBV-induced anaemia in the currently applied treatment protocol [12-16]. However, clinical trials have shown that the prevalence of anaemia was significantly lower in Peg-INF-α monotherapy compared to Peg-INF-α and RBV dual therapy [41].

The current study correlates with the previous reports as there was no significant change in erythrocyte count and haemoglobin in the 'P' group compared to control. Moreover, a significant decrease in the number of RBCs and haemoglobin concentration was observed after 5 weeks of treatment with RBV either individually or in combination with Peg-INF-α. However, there was no significant changes in indirect bilirubin, MCV and MCH between the RBV ± PEG-INF-α treated groups and control, suggesting that the treated rats developed normocytic normochromic anaemia. The observed significant decrease in serum and kidney EPO concentrations suggests that the observed anaemia with ribavirin could be due to bone marrow suppression rather than haemolysis.

Ribavirin-induced anaemia is primarily dependent on the plasma concentration of the drug rather than the dose/Kg body weight [27, 28]. Ribavirin and its metabolites accumulate in human RBCs, leading to oxidative stress and mitochondrial toxicity and subsequently results in RBCs haemolysis [17-21]. The uptake rate of RBV by erythrocytes showed dose and species dependency [42]. Canonico et al. reported that the largest accumulation of the drug was observed in monkey erythrocyte, followed by human and the lowest accumulation was detected in rat cells [42]. Furthermore, the authors reported that in vitro incubation of erythrocytes from the 3 species with RBV revealed that monkey, human and rat red cells retained 77, 45 and 20% of their initial drug content, respectively [42]. Neither osmotic fragility nor deformability was altered by exposure of red cells to RBV in vitro [42-44].

RBV could also induce anaemia by inhibiting the process of erythropoiesis through the suppression of bone marrow and decreasing the expression of both EPO and its receptor [22, 23]. 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 [42-46]. However, RBV had no effect on erythrocyte MCV, MCH and MCHC in both species [42-44]. The administration of Peg-INF-α and RBV in human was also associated with a decrease in serum EPO concentrations [22]. Therefore, we suggest that RBV, with or without PEG-INF-α, produces normocytic normochromic anaemia in rat by suppressing the bone marrow through decreasing the production of EPO from the kidney.

The management of Peg-INF-α and RBV induced anaemia during the treatment of CHC was the reduction of RBV dose [27, 28]. However, several published studies have shown that the response rate is associated with higher dose of the drug [10, 24-27]. As a result, the use of EPO as an erythropoiesis-stimulating agent has been proposed as an alternative for RBV dose reduction to maintain the treatment success rate [27, 28].

Vitamin D plays an important role in the process of erythropoiesis and data generated from haemodialysis patients have demonstrated the clinical usefulness of vitamin D supplementation in the treatment of anaemia associated with chronic renal failure [29, 31, 33, 34]. It has been demonstrated that vitamin D3 stimulates

the proliferation of erythroid progenitor cells independently from EPO [29]. It has also been shown that the vitamin D-responsive elements is located on the promoter region of the EPO receptor gene and that vitamin D3 synergises with EPO to increases the production of EPO receptor at the mRNA and protein levels in vitro [32]. 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 aim of the current study was to investigate whether vitamin D has a protective effect against the development of ribavirin-induced anaemia. Nevertheless, measuring the effect of vitamin D supplementation on the cellular expression of EPO protein and EPO receptors, at the protein level using immunohistochemistry or at the gene level using quantitative RT-PCR, in future studies is mandatory to explore the mechanism(s) by which it could promote the action(s) of endogenous EPO during the treatment of CHC.

In conclusion, we suggest that RBV could induce normocytic normochromic anaemia by decreasing endogenous EPO at the kidney and serum levels and subsequently suppressing erythropoiesis. Supplementation with vitamin D3 could protect against the associated anaemia with ribavirin 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 and the prevention of the associated anaemia.

Disclosure of conflict of interest

None.

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References

[1] Averhoff FM, Glass N and Holtzman D. Global burden of hepatitis C: considerations for healthcare providers in the United States. Clin Infect Dis 2012; 55 Suppl 1: S10-5.

[2] Ghany MG, Strader DB, Thomas DL and Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. Hepatology 2009; 49: 1335-74.

[3] Chan K, Lai MN, Groessl EJ, Hanchate AD, Wong JB, Clark JA, Asch SM, Gifford AL, Ho SB. Cost effectiveness of direct-acting antiviral therapy for treatment-naive patients with chronic HCV genotype 1 infection in the veterans health administration. Clin Gastroenterol Hepatol 2013; 11: 1503-10.

[4] Lange CM and Zeuzem S. Perspectives and challenges of interferon-free therapy for chronic hepatitis C. J Hepatol 2013; 58: 583-92.

[5] Younossi ZM, Singer ME, Mir HM, Henry L and Hunt S. Impact of Interferon Free Regimens on Clinical and Cost Outcomes for Chronic Hepatitis C Genotype 1 Patients. J Hepatol 2014; 60: 530-7.

[6] Ferrante SA, Chhatwal J, Brass CA, El Khoury AC, Poordad F, Bronowicki JP, Elbasha EH. Boceprevir for previously untreated patients with chronic hepatitis C Genotype 1 infection: a US-based cost-effectiveness modeling study. BMC Infect Dis 2013; 13: 190.

[7] Cure S, Bianic F, Gavart S, Curtis S, Lee S and Dusheiko G. Cost-effectiveness of telaprevir in combination with pegylated interferon alpha and ribavirin in previously untreated chronic hepatitis C genotype 1 patients. J Med Econ 2014; 17: 65-76.

[8] Sandokji AM, Sanai FM, Al-Ajlan AA and Al-Karawi MA. Interferon-ribavirin therapy for chronic hepatitis C: efficacy in Saudi patients. Saudi J Gastroenterol 2003; 9: 129-34.

[9] Garcia TJ, Lara PH, Morimoto TP, Higasiaraguti M, Perejao AM and Ayub MA. Side effects of the hepatitis C treatment at the ABC application center. Rev Assoc Med Bras 2012; 58: 543-9.

[10] McHutchison JG, Manns MP, Brown RS Jr, Reddy KR, Shiffman ML and Wong JB. Strategies for managing anemia in hepatitis C patients undergoing antiviral therapy. Am J Gastroenterol 2007; 102: 880-9.

[11] Kowdley KV. Hematologic side effects of interferon and ribavirin therapy. J Clin Gastroenterol 2005; 39: S3-8.

[12] Keeffe EB and Kowdley KV. Hematologic side effects of PEG interferon and ribavirin. Management with growth factors. J Clin Gastroenterol 2005; 39: S1-2.

[13] Kurschel E, Metz-Kurschel U, Niederle N and Aulbert E. Investigations on the subclinical and clinical nephrotoxicity of interferon alpha-2B in patients with myeloproliferative syndromes. Ren Fail 1991; 13: 87-93.

[14] Sacchi S, Kantarjian H, O'Brien S, Cohen PR, Pierce S and Talpaz M. Immune-mediated and unusual complications during interferon alfa therapy in chronic myelogenous leukemia. J Clin Oncol 1995; 13: 2401-7.
[15] Tarumi T, Sawada K, Sato N, Kobayashi S, Takano H, Yasukouchi T, Takashashi T, Sekiguchi S, Koike T. Interferon-alpha-induced apoptosis in human erythroid progenitors. Exp Hematol 1995; 23: 1310-8.
[16] Kato K, Kamezaki K, Shimoda K, Numata A, Haro T, Aoki K, Ishikawa F, Takase K, Ariyama H, Matsuda T, Miyamoto T, Nagafuji K, Gondo H, Nakayama K, Harada M. Intracellular signal transduction of interferon on the suppression of haematopoietic progenitor cell growth. Br J Haematol 2003; 123: 528-35.
[17] Sulkowski MS, Wasserman R, Brooks L, Ball L and Gish R. Changes in haemoglobin during interferon alpha-2b plus ribavirin combination therapy for chronic hepatitis C virus infection. J Viral Hepat 2004; 11: 243-50.
[18] Tanaka H, Miyano M, Ueda H, Fukui K and Ichinose M. Changes in serum and red blood cell membrane lipids in patients treated with interferon ribavirin for chronic hepatitis C. Clin Exp Med 2005; 5: 190-5.
[19] Morello J, Rodriguez-Novoa S, Jimenez-Nacher I and Soriano V. Usefulness of monitoring ribavirin plasma concentrations to improve treatment response in patients with chronic hepatitis C. J Antimicrob Chemother 2008; 62: 1174-80.
[20] Van Vlierbergh H, Delanghe JR, De Vos M and Leroux-Roel G, Factors influencing ribavirin-induced hemolysis. J Hepatol 2001; 34: 911-6.
[21] De Franceschi L, Fattovich G, Turrini F, Ayi K, Brugnara C, Manzato F, Noventa F, Stanzial AM, Solero P, Corrocher R. Hemolytic anemia induced by ribavirin therapy in patients with chronic hepatitis C virus infection: role of membrane oxidative damage. Hepatology 2000; 31: 997-1004.
[22] Balan V, Schwartz D, Wu GY, Muir AJ, Ghalib R, Jackson J, Keeffe EB, Rossaro L, Burnett A, Goon BL, Bowers PJ, Leitz GJ; HCV Natural History Study Group. Erythropoietic response to anemia in chronic hepatitis C patients receiving combination pegylated interferon/ribavirin. Am J Gastroenterol 2005; 100: 299-307.
[23] Martin P and Jensen DM. Ribavirin in the treatment of chronic hepatitis C. J Gastroenterol Hepatol 2008; 23: 844-55.
[24] McHutchison JG, Manns M, Patel K, Poynard T, Lindsay KL, Trepo C, Dienstag J, Lee WM, Mak C, Garaud JJ, Albrecht JK; International Hepatitis Interventional Therapy Group. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. Gastroenterology 2002; 123: 1061-9.
[25] Shiffman ML, Salvatore J, Hubbard S, Price A, Sterling RK, Stravitz RT, Luketic VA, Sanyal AJ. Treatment of chronic hepatitis C virus genotype 1 with peginterferon, ribavirin, and epoetin alpha. Hepatology 2007; 46: 371-9.
[26] Shiffman ML, Ghany MG, Morgan TR, Wright EC, Everson GT, Lindsay KL, Lok AS, Bonkovsky HL, Di Bisceglie AM, Lee WM, Dienstag JL, Gretch DR. Impact of reducing peginterferon alfa-2a and ribavirin dose during retreatment in patients with chronic hepatitis C. Gastroenterology 2007; 132: 103-12.
[27] Poordad F, Lawitz E, Reddy KR, Afdhal NH, Hezode C, Zeuzem S, Lee SS, Calleja JL, Brown RS Jr, Craxi A, Wedemeyer H, Nyberg L, Nelson DR, Rossaro L, Balart L, Morgan TR, Bacon BR, Flamm SL, Kowdley KV, Deng W, Koury KJ, Pedicone LD, Dutko FJ, Burroughs MH, Alves K, Wahl J, Brass CA, Albrecht JK, Sulkowski MS; Protocol 6086 Investigators. Effects of ribavirin dose reduction vs erythropoietin for boceprevir-related anemia in patients with chronic hepatitis C virus genotype 1 infection--a randomized trial. Gastroenterology 2013; 145: 1035-1044, e5.
[28] Sulkowski MS, Poordad F, Manns MP, Bronowicki JP, Rajender Reddy K, Harrison SA, Afdhal NH, Sings HL, Pedicone LD, Koury KJ, Sniukiene V, Burroughs MH, Albrecht JK, Brass CA, Jacobson IM; SPRINT-2 Trial Investigators. Anemia during treatment with peginterferon Alfa-2b/ribavirin and boceprevir: Analysis from the serine protease inhibitor therapy 2 (SPRINT-2) trial. Hepatology 2013; 57: 974-84.
[29] Deicher R and Horl WH. Hormonal adjuvants for the treatment of renal anaemia. Eur J Clin Invest 2005; 35 Suppl 3: 75-84.
[30] Sim JJ, Lac PT, Liu IL, Meguerditchian SO, Kumar VA, Kujubu DA, Rasgon SA. Vitamin D deficiency and anemia: a cross-sectional study. Ann Hematol 2010; 89: 447-52.
[31] Rianthavorn P and Boonyapapong P. Ergocalciferol decreases erythropoietin resistance in children with chronic kidney disease stage 5. Pediatr Nephrol 2013; 28: 1261-6.
[32] Alon DB, Chaimovitz C, Dvilansky A, Lugassy G, Douvdevani A, Shany S, Nathan I. Novel role of 1,25(OH)(2)D(3) in induction of erythroid progenitor cell proliferation. Exp Hematol 2002; 30: 403-9.
[33] Albitar S, Genin R, Fen-Chong M, Serveaux MO, Schohn D and Chuet C. High-dose alfacalcidol improves anaemia in patients on haemodialysis. Nephrol Dial Transplant 1997; 12: 514-8.
[34] Saab G, Young DO, Gincherman Y, Giles K, Norwood K and Coyne DW. Prevalence of vitamin D deficiency and the safety and effectiveness

of monthly ergocalciferol in hemodialysis patients. Nephron Clin Pract 2007; 105: c132-8.
[35] Salum E, Kampus P, Zilmer M, Eha J, Butlin M, Avolio AP, Põdramägi T, Arend A, Aunapuu M, Kals J. Effect of vitamin D on aortic remodeling in streptozotocin-induced diabetes. Cardiovasc Diabetol 2012; 11: 58.
[36] Bitetto D, Fabris C, Falleti E, Fornasiere E, Fumolo E, Fontanini E, Cussigh A, Occhino G, Baccarani U, Pirisi M, Toniutto P. Vitamin D and the risk of acute allograft rejection following human liver transplantation. Liver Int 2010; 30: 417-44.
[37] Abu-Mouch S, Fireman Z, Jarchovsky J, Zeina AR and Assy N. Vitamin D supplementation improves sustained virologic response in chronic hepatitis C (genotype 1)-naive patients. World J Gastroenterol 2011; 17: 5184-90.
[38] Nimer A and Mouch A. Vitamin D improves viral response in hepatitis C genotype 2-3 naive patients. World J Gastroenterol 2012; 18: 800-5.
[39] Maddrey WC. Safety of combination interferon alfa-2b/ribavirin therapy in chronic hepatitis C-relapsed and treatment-naive patients. Semin Liver Dis 1999; 19 Suppl 1: 67-75.
[40] Jacobson IM, Gonzalez SA, Ahmed F, Lebovics E, Min AD, Bodenheimer HC Jr, Esposito SP, Brown RS Jr, Bräu N, Klion FM, Tobias H, Bini EJ, Brodsky N, Cerulli MA, Aytaman A, Gardner PW, Geders JM, Spivack JE, Rahmin MG, Berman DH, Ehrlich J, Russo MW, Chait M, Rovner D, Edlin BR. A randomized trial of pegylated interferon alpha-2b plus ribavirin in the retreatment of chronic hepatitis C. Am J Gastroenterol 2005; 100: 2453-62.
[41] Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002; 347: 975-82.
[42] Canonico PG, Kastello MD, Spears CT, Brown JR, Jackson EA and Jenkins DE. Effects of ribavirin on red blood cells. Toxicol Appl Pharmacol 1984; 74: 155-62.
[43] Canonico PG, Kastello MD, Cosgriff TM, Donovan JC, Ross PE, Spears CT, Stephen EL. Hematological and bone marrow effects of ribavirin in rhesus monkeys. Toxicol Appl Pharmacol 1984; 74: 163-72.
[44] Cosgriff TM, Hodgson LA, Canonico PG, White JD, Kastello MD, Donovan JC, Ross PE. Morphological alterations in blood and bone marrow of ribavirin-treated monkeys. Acta Haematol 1984; 72: 195-200.
[45] D'Souza UJ and Narayana K. Mechanism of cytotoxicity of ribavirin in the rat bone marrow and testis. Indian J Physiol Pharmacol 2002; 46: 468-74.
[46] Narayana K, D'Souza UJ and Seetharama Rao KP. The genotoxic and cytotoxic effects of ribavirin in rat bone marrow. Mutat Res 2002; 521: 179-85.