Erythropoietin treatment elevates haemoglobin concentration by increasing red cell volume and depressing plasma volume

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Erythropoietin (Epo) has been suggested to affect plasma volume, and would thereby possess a mechanism apart from erythropoiesis to increase arterial oxygen content. This, and potential underlying mechanisms, were tested in eight healthy subjects receiving 5000 IU recombinant human Epo (rHuEpo) for 15 weeks at a dose frequency aimed to increase and maintain haematocrit at approximately 50%. Red blood cell volume was increased from 2933 ± 402 ml before rHuEpo treatment to 3210 ± 356 ($P < 0.01$), 3117 ± 554 ($P < 0.05$), and 3172 ± 561 ml ($P < 0.01$) after 5, 11 and 13 weeks, respectively. This was accompanied by a decrease in plasma volume from 3645 ± 538 ml before rHuEpo treatment to 3267 ± 333 ($P < 0.01$), 3119 ± 499 ($P < 0.05$), and 3323 ± 521 ml ($P < 0.01$) after 5, 11 and 13 weeks, respectively. Concomitantly, plasma renin activity and aldosterone concentration were reduced. This maintained blood volume relatively unchanged, with a slight transient decrease at week 11, such that blood volume was 6578 ± 839 ml before rHuEpo treatment, and 6477 ± 573 (NS), 6236 ± 908 ($P < 0.05$), and 6495 ± 935 ml (NS), after 5, 11 and 13 weeks of treatment. We conclude that Epo treatment in healthy humans induces an elevation in haemoglobin concentration by two mechanisms: (i) an increase in red cell volume; and (ii) a decrease in plasma volume, which is probably mediated by a downregulation of the rennin–angiotensin–aldosterone axis. Since the relative contribution of plasma volume changes to the increments in arterial oxygen content was between 37.9 and 53.9% during the study period, this mechanism seems as important for increasing arterial oxygen content as the well-known erythropoietic effect of Epo.

Erythropoietin (Epo) is a glycoprotein growth factor produced by the kidney to regulate red blood cell volume mass primarily by stimulating proliferation, differentiation and maturation of erythroid progenitor cells. Epo synthesis is mainly governed by hypoxia sensitive cells within the kidney. However, factors such as blood volume (Ehmke *et al.* 1995) and the rennin–angiotensin system (Freudenthaler *et al.* 1999, 2000; Donnelly & Miller, 2001) have also been shown to affect the production of Epo. In situations with reduced oxygen availability, such as at high altitude, Epo synthesis is increased leading to a progressive elevation in haemoglobin (Hb) mass.

Concomitantly, however, plasma volume is depressed within days, resulting in a reduced blood volume, but also in a marked haemoconcentration, thereby ensuring an increase in arterial oxygen content to levels not much different from those at sea level (Lundby *et al.* 2004). This could suggest that arterial oxygen content, rather than blood volume, is the crucial factor to be controlled in hypoxia. In agreement with this, it has recently been shown in four chronic heart failure patients that treatment with recombinant human Epo (rHuEpo) increased red blood cell volume by 250 ml and simultaneously decreased plasma volume by more than

1 l (but this was not commented on) (Mancini *et al.* 2003). Thus, administrating rHuEpo in these patients induced haemoconcentration by inducing erythropoiesis and at the same time depressing plasma volume. Chronic hypoxia elicits similar changes in healthy subjects, i.e. stimulation of erythropoiesis and reduction of plasma volume (Pugh, 1964; Wolfel *et al.* 1991). Interestingly, when calculating plasma volumes from Hb mass or blood volume and haematocrits reported in studies where rHuEpo has been injected in healthy subjects, plasma volumes are indeed also decreased in these studies (but not reported) (Ekblom & Berglund, 1991; Parisotto *et al.* 2001). Since only reported sparsely, no potential mechanisms have been proposed for this effect. If in fact Epo, or rHuEpo injections, regulate arterial oxygen content not only by increasing erythropoiesis, but also by downregulating plasma volume, then this would be of major importance considering the global use of rHuEpo in numerous diseases.

To further test the hypothesis that rHuEpo treatment in healthy subjects increases arterial oxygen content by augmenting erythropoiesis, but also by depressing plasma volume, we determined red blood cell volume and plasma volume repeatedly during 14 weeks of rHuEpo treatment in healthy subjects, and also investigated potential regulating mechanisms.

Methods

Eight healthy male volunteers (age 27 ± 7 years, height 180 ± 4 cm, weight 83 ± 7 kg) participated in the study, which was approved by the local ethical committee of the communities of Copenhagen and Frederiksberg and conformed to the Declaration of Helsinki. All subjects gave written informed consent to participate. The presented data are part of a large study, and this report includes factors related to blood volume changes.

Experimental setup

On the experimental day, the subjects reported to the laboratory at 08.00 h, and catheters were placed under local anaesthesia (2% lidocaine (lignocaine)). A 20 gauge catheter (Ref. ES-14150; Arrow, Reading, PA, USA) was inserted percutaneously using the Seldinger technique into the right femoral artery, 2 cm below the inguinal ligament, and advanced 5–10 cm in the proximal direction. This catheter was connected to a blood pressure transducer positioned at the height of the fourth intercostal space (T100209A; Baxter, Unterschleissheim, Germany). An additional catheter was placed in the femoral vein to study the vasodilatory response to the infusion of ATP and adenosine; those results will be reported in another paper. An antecubital vein was also catheterized to inject indocyanine green (ICG; Akorn, Inc, IL, USA)

for measuring cardiac output, as explained below. Blood pressure was therefore measured before and after the treatment period through a femoral arterial indwelling catheter in the supine position as reported elsewhere (Calbet *et al.* 2006).

Resting venous blood samples for haematological parameters were assessed on four occasions, separated by 1 week, before rHuEpo treatment, and blood volume was determined twice, also separated by 1 week, before the rHuEpo treatment started. The treatment aimed to increase and maintain the haematocrit to around 50% throughout the study period. Two weeks prior to rHuEpo treatment, all subjects received iron at 100 mg day^{-1} orally, and this was maintained throughout the entire study period. Following baseline measurements, 5000 IU rHuEpo (NeoRecormon; Roche, Mannheim, Germany) was injected as follows. Weeks 1 and 2: one injection every second day; week 3: three injections on three consecutive days; weeks 4–15: one injection every week. All injections occurred between 08.00 and 10.00 h, and were preceded by 30 min of supine rest and a venous blood sample (6 ml) for analysis. In the hours preceding the first one and two rHuEpo injections, four of the subjects reported general malaise.

Venous blood samples were analysed for haematocrit, haemoglobin, white blood cell count, middle cell volume, and middle cell haemoglobin concentration, using a Sysmex R-3000 (Sysmex Europe, Norderstedt, Germany). Elongation measurements of the red blood cells were done by laser diffractometry at shear stresses between 0.11 and 22.5 Pa (Rheodyn-SSD; Myrenne, Röttgen, Germany). Plasma renin activity (PRA) was measured by radioimmunoassay of generated and antibody trapped angiotensin I and aldosterone were quantified by a solid-phase radioimmunoassay kit (Coat-A-Count; Diagnostic Products Corporation, USA). Total haemoglobin mass, red blood cell volume, plasma volume and blood volume were determined by a carbon monoxide rebreathing method (Burge & Skinner, 1995) before rHuEpo treatment, and after 5, 11 and 13 weeks of treatment. To access the variability of the method, all subjects were investigated twice (separated by 1 week) before rHuEpo treatment, and the coefficients of variation (CVs) for red cell volume, plasma volume, and blood volume were 2.7, 3.7 and 2.6%, respectively. The reported pretreatment value is the average of these two measurements. Carboxyhaemoglobin, haematocrit and arterial oxygen content were analysed in triplicate for each measurement on an automated system (Radiometer ABL 700; Radiometer, Copenhagen, Denmark).

Calculations and statistical analysis

Statistical differences over time were assessed by the non-parametric Friedman test, and the non-parametric

Wilcoxon test was used as a *post hoc* test. Statistical difference was set to $P < 0.05$. All values reported are means $\pm$ s.d.

Results

Blood compounds

Haemoglobin concentration was 14.2 ± 0.6 before treatment and reached a peak of $17.1 \pm 0.5\ \mathrm{g\ dl^{-1}}$ after 12 weeks of rHuEpo administration ($P < 0.05$, from weeks 7–15) (Fig. 1). The haematocrit followed a similar pattern, being increased from 0.42 ± 0.03 to $0.49 \pm 0.03\%$ at week 12 ($P < 0.05$, from weeks 6–15). Reticulocytes were increased by approximately 134% ($P < 0.05$) in weeks 6–7, as compared with pretreatment values. rHuEpo treatment had no significant effect on either the size (mean cell volume) or the amount of Hb within the erythrocyte (mean cell Hb concentration). Also of note, is that rHuEpo had no effect on the number of white blood cells (Fig. 1*D*).

Blood volume, plasma volume and red cell blood volume

Red blood cell volume was increased from 2933 ± 402 ml before rHuEpo treatment to 3210 ± 356 ($9.8 \pm 1.3\%$, $P < 0.01$), 3117 ± 554 ($5.9 \pm 2.0\%$, $P < 0.05$), and 3172 ± 561 ml ($7.8 \pm 2.0\%$, $P < 0.01$) after 5, 11 and 13 weeks, respectively (Fig. 2). However, the increase in red blood cell volume was accompanied by an almost similar plasma volume decrease from 3645 ± 538 ml before rHuEpo treatment to 3267 ± 333 ($-9.8 \pm 2.0\%$, $P < 0.01$), 3119 ± 499 ($-14.3 \pm 2.0\%$, $P < 0.05$), and 3323 ± 521 ml ($-8.8 \pm 1.5\%$, $P < 0.01$) after 5, 11 and 13 weeks, respectively. This kept blood volume relatively unchanged throughout the study period, the initial value being 6578 ± 839 ml, and then 6477 ± 537 ($-1.1 \pm 1.5\%$, NS), 6236 ± 908 ($-5.5 \pm 1.5\%$, $P < 0.05$), and 6495 ± 935 ml ($-1.3 \pm 1.3\%$, NS) at weeks 5, 11 and 13, respectively. The relative contribution of erythropoiesis and plasma volume changes to the augmentation of arterial oxygen content are shown in Table 1, and they indicate approximately equal importance of both factors.

Cardiovascular parameters

Resting heart rate, cardiac output and systemic vascular conductance were not significantly affected by the rHuEpo treatment. However, mean arterial pressure showed a trend toward a higher value following the rHuEpo treatment period (96.7 ± 3.8 and 102.0 ± 6.0 mmHg, $P = 0.05$) (Table 2).

Plasma renin and aldosterone

Plasma renin activity was $1.29 \pm 0.5\ \mathrm{ng\ ml^{-1}\ h^{-1}}$ before treatment, and decreased to $0.75 \pm 0.3\ \mathrm{ng\ ml^{-1}\ h^{-1}}$ ($P < 0.05$) after 96 h (two injections) of rHuEpo treatment, and to $0.51 \pm 0.4\ \mathrm{ng\ ml^{-1}\ h^{-1}}$ ($P < 0.01$) after 13 weeks. The response of aldosterone was similar, decreasing from 150.8 ± 83 to 77.3 ± 50.8 ($P < 0.05$) and $54.0 \pm 52.7\ \mathrm{pg\ ml^{-1}}$ ($P < 0.01$) after 96 h and 13 weeks of treatment, respectively.

Elongation

We found no differences in erythrocyte elongation at shear rates within the physiological range, or at shear forces that can be applied without damaging the cells.

Discussion

The main finding of the present study is that rHuEpo treatment in humans increases Hb concentration, and thus arterial oxygen content, by simultaneously increasing Hb mass and depressing plasma volume. These counteracting volume changes kept total blood volume relatively unchanged. Possibly the rHuEpo-induced decrease in plasma volume serves as a fast responding mechanism to increase arterial oxygen content in critical oxygen-limited situations, whereas the well-known effects of rHuEpo on erythropoiesis may restore oxygen homeostasis at a slower pace.

The present effects of rHuEpo treatment on red cell mass are in line with previous reports (Ekblom & Berglund, 1991; Parisotto *et al.* 2001), and decreases in plasma volume with rHuEpo administration have been observed in four chronic heart failure patients (Mancini *et al.* 2003). Interestingly, when calculating plasma volumes from Hb mass or blood volume and haematocrits reported in studies where rHuEpo has been injected in healthy subjects, plasma volumes are also decreased in these studies, but not reported (Ekblom & Berglund, 1991; Parisotto *et al.* 2001), and thus the later findings on plasma volume did not receive any attention. The present study shows that the relative changes in plasma volume account for almost 50% of the increase in arterial oxygen content with rHuEpo treatment (Table 1), i.e. the reduction in plasma volume is a relevant physiological mechanism by which erythropoietin contributes to enhanced arterial oxygen content.

Plasma volume could be decreased by at least two mechanisms. First, recent studies have confirmed the existence of a link between volume-regulating hormones and the release of Epo. It has, for example, been shown that the rennin–angiotensin system is involved in the production of Epo in a dose-dependent manner (Freudenthaler *et al.* 1999, 2000; Donnelly
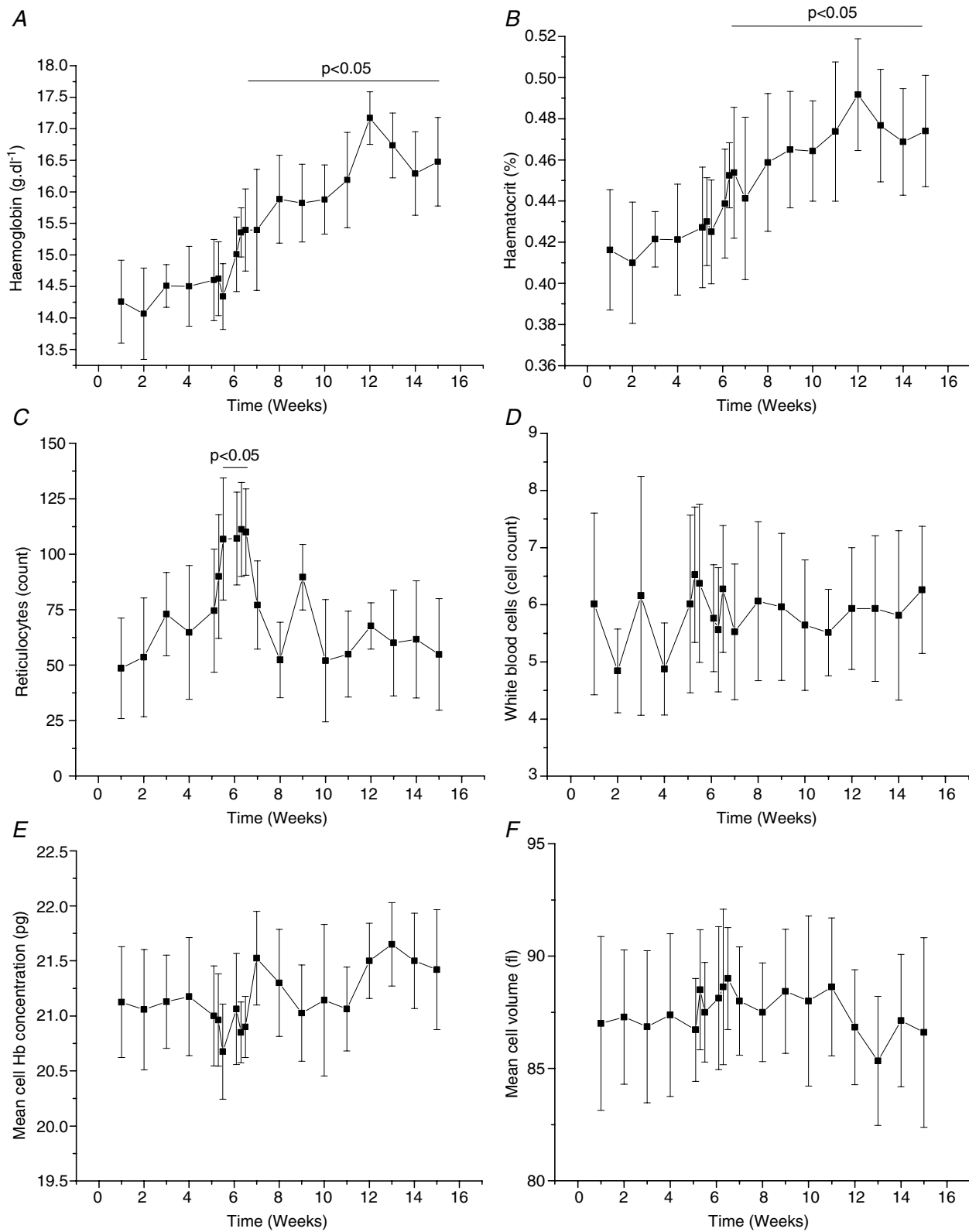
Figure 1. Blood variables throughout the study period
A, haemoglobin concentration (g dl^{-1}), *B*, haematocrit (%), *C*, reticulocytes (count), *D*, white blood cells (count), *E*, mean cell haemoglobin concentration (pg), and *F*, mean cell volume (fl). The first 4 week points are before recombinant human erythropoietin (rHuEpo) treatment. Values are means ± s.d. Horizontal bar, $P < 0.05$ compared with average pretreatment value.

Table 1. Relative contributions of haemoglobin and plasma volume changes in arterial oxygen content (C_{aO_2}) after 5, 11 and 13 weeks of rHuEpo treatment

	Baseline	5 weeks	11 weeks	13 weeks
C_{aO_2} (ml l^{-1})	191.1 ± 13.0	222.1 ± 13.0*	223.9 ± 18.8*	218.8 ± 18.4*
Contribution Hb (%)	—	61.6 ± 2.1	46.1 ± 1.8	62.1 ± 1.3
Contribution PV (%)	—	38.4 ± 1.2	53.9 ± 1.8	37.9 ± 1.4

rHuEpo, recombinant human erythropoietin; Hb, haemoglobin; PV, plasma volume; C_{aO_2}, arterial oxygen content. Values are means $\pm$ s.d. *Significant difference ($P < 0.05$) from baseline values.

Table 2. Resting heart rate (HR), stroke volume (SV), cardiac output (Q), mean arterial pressure (MAP), and systemic vascular conductance (Sys VC), after 14 weeks treatment

Parameter	Baseline	14 W
HR (beats min^{-1})	62.7 ± 8.7	61.6 ± 7.9
SV (ml min^{-1})	79.7 ± 51.9	89.8 ± 57.5
$\dot{Q}$ (l min^{-1})	6.8 ± 0.5	7.7 ± 1.1
MAP (mmHg)	96.7 ± 3.8	102.0 ± 6.0*
Sys VC (ml min^{-1} mmHg^{-1})	69.4 ± 6.3	74.5 ± 11.9

HR, heart rate; SV, stroke volume; $\dot{Q}$, cardiac output; MAP, mean arterial pressure; Sys VC, systemic vascular conductance.

& Miller, 2001). The present data indicate that a negative-feedback mechanism elicited by an excess of rHuEpo results in an attenuation of the activity of the rennin–angiotensin–aldosterone axis, avoiding the development of excessive hypervolaemia, and thereby maintaining total blood volume within a narrow range. Such a mechanism would have the advantage of increasing [Hb] faster than it would be possible by simply increasing erythropoiesis. In situations with acute reductions in oxygen content, such as with exposure to high altitude, it may take weeks or months to restore oxygen homeostasis if depending entirely on erythropoiesis (Reynafarje *et al.* 1959). Therefore the augmented Epo concentration with altitude exposure, and the concomitant decrease in plasma volume, may serve as a fast responding mechanism restoring oxygen content independently of Hb mass. The same mechanism could be valid for hypoxic patients receiving rHuEpo, such as chronic heart failure and kidney failure patients. Besides effectively increasing arterial oxygen content, the proposed mechanism also keeps total blood volume within a normal range, thereby reducing the load on the heart, which should be of advantage especially for chronic heart failure patients, particularly and particularly those who are prone to retain fluids. Since plasma renin activity and aldosterone were depressed after 96 h of rHuEpo treatment, i.e. when no enhanced release of erythrocytes from the bone marrow into the circulation is assumed to have occurred yet, it seems reasonable to suggest that the reduction in plasma renin and aldosterone was mediated directly by rHuEpo, and not by an elevation of Hb and blood volume, therefore supporting a direct effect of Epo on this volume regulating system.

Theoretically a second mechanism by which rHuEpo may decrease plasma volume could be through its effects on vascular tone and mean arterial pressure. Epo itself is reported to induce vasoconstriction (Heidenreich *et al.* 1991), which may be associated with a loss of plasma volume (Nette *et al.* 2006). Thus, vasoconstriction could be part of the plasma lowering mechanism. However, in our subjects, mean arterial pressure was minimally affected by rHuEpo treatment, while cardiac output and systemic vascular conductance remained close to their pre-Epo respective values. This corroborates previous studies showing that in the range of haematocrits observed in this study (from 42 to 49%), a small increase of haematocrit has no major impact on resting mean arterial pressure and hence on systemic vascular conductance (Berglund & Ekblom, 1991). In agreement, this study shows that the flexibility of the red blood cells remained unchanged. Based on this, blood viscosity may be assumed to be relatively unchanged. Although systemic vascular conductance was not negatively affected by rHuEpo, this does not rule out the possibility for a specific regulation of vascular tone in some territories, as for example the skeletal muscle when blood Hb concentration is increased (Calbet *et al.* 2003).
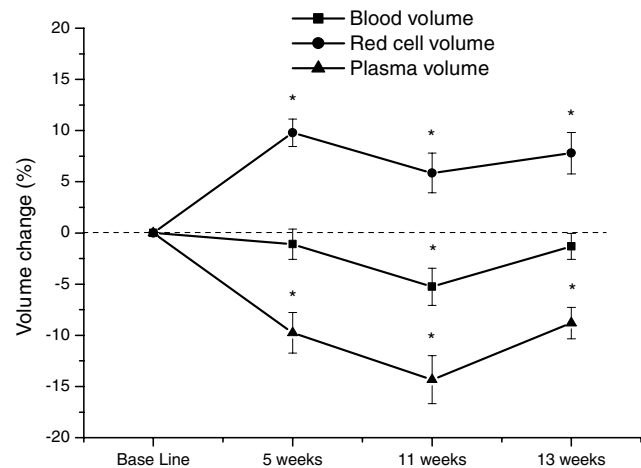
Figure 2. Blood, red cell, and plasma volume changes (%) after 5, 11 and 13 weeks of rHuEpo treatment
Values are means $\pm$ s.d., *$P < 0.05$ compared with baseline values.

In summary, this study clearly shows that rHuEpo is not only an erythropoeitic hormone, it also has an effect of lowering plasma volume, which is regulated by reducing the activity of rennin–angiotensin–aldosterone axis. With acute induction of systemic hypoxia, the blood volume reducing effect of rHuEpo appears critical in order to promptly increase the oxygen-carrying capacity of blood before new red cells reach the vascular stream. Because millions of chronic renal and cancer patients worldwide are treated with rHuEpo, an understanding of the effects of rHuEpo on the human body is crucial and should be included in the focus of future studies. Such studies could address acute measurements of haematocrit upon rHuEpo injection to determine changes in plasma volume, blockade of sympathetic receptors in combination with rHuEpo injections, and prolonged rHuEpo treatment in combination with blockade of volume-regulating hormones. This would provide better selection procedures for patients for whom rHuEpo treatment could prove beneficial.

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