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ORIGINAL ARTICLE

Response to erythropoietin in pediatric patients with chronic kidney disease: insights from an in vitro bioassay

Rachel Gavish[1] · Salmas Watad[1] · Nathalie Ben-Califa[2] · Ori Jacob Goldberg[1] · Orly Haskin[3] · Miriam Davidovits[3] · Gili Koren[3] · Yafa Falush[3] · Drorit Neumann[2] · Irit Krause[1]

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Abstract

Background Decreased production of erythropoietin (EPO) is a major cause of anemia associated with chronic kidney disease (CKD). Treatment with recombinant human EPO (rHuEPO) improves patients' quality of life and survival; however, there is a marked variability in response to rHuEPO. At present, no available laboratory test is capable of evaluating responsiveness to EPO treatment. The aim of the present study was to use an in vitro bioassay to estimate the effect of uremic environment on EPO-dependent erythroid cell proliferation.

Methods EPO-dependent human erythroleukemia cells (UT-7) were incubated with exogenous EPO (2 u/ml) and sera obtained from 60 pediatric patients (aged 1–23 years). Three groups were studied: (1) 12 children on dialysis (4 peritoneal, 8 hemodialysis); (2) 28 patients with CKD 1–5 (not on dialysis), and (3) 20 healthy children.

Results Sera from dialysis patients inhibited UT-7 cell growth compared to the CKD group and healthy controls at 48 h ($p = 0.003$ and $p = 0.04$, respectively) and 72 h of culture ($p = 0.02$ and $p = 0.07$, respectively). In 18 patients treated with rHuEPO, a significant inverse correlation was found between the EPO resistance index and cell proliferation at 48 h ($p = 0.007$, $r = -0.63$) and 72 h ($p = 0.03$, $r = -0.52$).

Conclusions Our findings support the presence of erythropoiesis inhibitory substances in uremic sera. EPO/EPO-R-dependent mechanisms may play a role in inhibiting erythropoiesis. The in vitro bioassay described herein may serve as an indicator of rHuEPO responsiveness which may encourage further investigation of underlying mechanisms of EPO resistance.

Keywords Dialysis · Pediatric · Anemia · Erythropoietin resistance index · UT-7 cells

Introduction

Anemia is a common complication of chronic kidney disease (CKD) affecting approximately 33% of pediatric and adolescent patients [1]. Possible adverse effects of anemia in CKD include growth failure, cardiovascular morbidity and mortality, lower quality of life, and cognitive disabilities [2–4].

Hypoxia induces an increase in production of the hormone erythropoietin (EPO) in the kidney, which subsequently circulates in the plasma and binds to EPO receptors (EPO-R). These receptors, which are abundantly expressed on erythroid progenitor cells in the bone marrow, promote the viability, proliferation, and terminal differentiation of erythroid precursors and cause an increase in red blood cell mass. The oxygen-carrying capacity of the blood is thereby enhanced, increasing tissue oxygen tension, thus completing the feedback loop and suppressing further elevation of EPO [5]. Decreased synthesis of EPO by the kidneys is a major cause of anemia associated with CKD [4]. The use of recombinant human EPO (rHuEPO) in the treatment of anemia in CKD patients reduces iron load, avoids multiple blood transfusions, enhances quality of life, and prolongs survival [6].

Rachel Gavish, Salmas Watad, Drorit Neumann and Irit Krause contributed equally to this work.

✉ Irit Krause
Iritk3@clalit.org.il

[1] Department of Pediatrics C, Schneider Children's Medical Center of Israel, Sackler Faculty of Medicine, Tel-Aviv University, 14 Kaplan St., Petah Tikva, Israel

[2] Department of Cell and Developmental Biology, Sackler Faculty of Medicine, Tel-Aviv University, Tel Aviv, Israel

[3] Institute of Pediatric Nephrology, Schneider Children's Medical Center of Israel, Sackler Faculty of Medicine, Tel-Aviv University, Tel Aviv, Israel

However, there is a marked variability in the response to rHuEPO; > 20% of pediatric patients with CKD stage 4 have low hemoglobin (Hgb) levels even when treated with rHuEPO [4, 6]. Underlying mechanisms of this phenomenon include inflammation, iron deficiency, malnutrition, inadequate dialysis, and metabolic bone disease [7–9]. The presence of pro-inflammatory cytokines in a uremic milieu is a factor in reducing red blood cell production [9–11]; however, the precise mechanism is still unclear. EPO-R-associated mechanisms may also play a role in EPO resistance [12].

The EPO resistance index (ERI) is a tool commonly used in clinical research to estimate the responsiveness of rHuEPO therapy. There is a paucity of studies relating to the estimation of EPO resistance in pediatric and adolescent populations. Adjustment of EPO dosages in clinical practice are based on serial Hgb measurements, thereby often delaying attainment of the optimal dose. At present, there is no available laboratory test that can estimate patient responsiveness to EPO treatment. UT-7 cells are EPO-dependent human leukemic erythroid progenitors with a high expression of EPO-R [13]; hence, these cells can be utilized in examining the inhibitory effect of uremic serum on EPO-dependent cell growth.

The aim of the current study was to examine the hypothesis that sera of CKD patients inhibit erythropoiesis, by utilizing the UT-7 in vitro model. This in vitro model may also serve as an experimental model for investigating underlying mechanisms of EPO resistance.

Subjects and methods

Our study cohort was comprised of three groups of pediatric patients: (1) children on dialysis; (2) patients with CKD1-5 (not on dialysis) [14], and (3) those with neither CKD nor anemia, serving as controls. Patients with CKD were recruited from the Nephrology Institute, Schneider Children's Medical Center, Israel. Children in the control group were recruited either from the Day Care Unit or the Department of Pediatrics of our institution. Calculation of estimated GFR (eGFR) was based on the new Schwartz formula [15]. Underlying diseases of CKD patients included dysplastic kidneys (25), Alport syndrome (2), infantile nephrotic syndrome (1), focal segmental glomerulosclerosis (1), autosomal dominant polycystic kidney disease (1), lupus nephritis (1), atypical hemolytic uremic syndrome (1), prune belly syndrome (1), nephronophthisis (1), and Denys-Drash syndrome (1). Demographic and clinical data were collected from the patients' computerized medical charts. Informed consent was obtained from all patients.

Blood sample analysis

Blood samples were processed for complete blood cell count and serum levels of urea, creatinine, electrolytes, parathyroid hormone (PTH), iron, transferrin, ferritin, vitamin B_{12}, folic acid, and C-reactive protein (CRP). The ERI in the subgroup of patients treated with rHuEPO was calculated according to the following equation: ERI = rHuEPO dose (units/week/kg)/Hgb (g/dl) [16]. Transferrin saturation was calculated as Fe/Transferrin × 70.9 [17].

Cell proliferation assay

EPO-dependent UT-7 cells [13] were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FCS, 2 mM L-glutamine, 10 u/ml penicillin, 10 μg/ml streptomycin, and 2 u/ml rHuEPO. Cells were seeded in triplicates in 96-well, flat-bottom culture plates (5×10^3 cells per well) and incubated at 37 °C with 2 u/ml rHuEPO (EPREX®-Epoetin Alfa) and sera (diluted 1:10) obtained from patients or control subjects for 24, 48, and 72 h. Cell viability was determined by sodium 39-(phenylaminocarbonyl)-3,4-tetrazolium-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT). After incubation, 50 μl of XTT was added to the cells and they were further incubated for 4 h at 37 °C. Plates were read by the ELISA SpectraMAX 190 at 492 nm. Proliferation of cells at 48 and 72 h was normalized to the values at 24 h for each tested serum sample.

To discover whether the inhibitory effect of uremic sera was mediated via the EPO/EPO-R axis, we added sera of the dialysis patients and controls to the human erythroleukemia K562 cell line [18]. Although these cells express low levels of EPO-Rs on their surface, they are not dependent on the presence of the hormone for their proliferation [19]. The cell proliferation assay was carried out according to the method described above (without an addition of EPO). The effect of the sera on the EPO-dependent proliferation of the UT-7 cells was correlated with ERI and eGFR. By utilizing both UT-7 and K562 cell lines, we were able to compare the effect of the patients' sera on EPO-dependent and EPO-independent cell proliferation.

Statistical analysis

Variables with non-normal distributions were described as median ± range; qualitative variables were expressed as number and percentage. The effect of the sera on EPO-driven proliferation of UT-7 cells at 48 and 72 h was examined in three study groups (controls, non-dialysis CKD patients, and dialysis patients). A similar analysis (the Kruskal-Wallis H test) was performed to determine the effect of sera on the proliferation of the K562 cells. The Mann-Whitney *U* test was performed for non-parametric variables and the chi-squared test for binary variables.

The correlation between the ERI, cell proliferation, PTH, transferrin saturation, vitamin B_{12}, folic acid, and CRP in a subgroup of patients treated with rHuEPO was performed by

the Spearman correlation test. A log transformation was performed to achieve a linear distribution. A two-tailed p value of < 0.05 was considered statistically significant. All analyses were conducted using SPSS software version 17. The study was approved by the Ethics Committee of the Rabin Medical Center, Israel. Informed consent was obtained from the study participants who were > 18 years old or from the legal guardians of those who were < 18 years old.

Results

Sixty children, 40 boys and 20 girls (aged 1–23 years), were enrolled in the study. Twelve were on dialysis (4 peritoneal and 8 hemodialysis), 28 were classified with stages 1–5 CKD (1 with CKD1, 7 with CKD2, 9 with CKD 3, 7 with CKD 4, and 4 with CKD 5), and 20 controls. Length of time spent on dialysis was 0.25–9.25 years in the hemodialysis group and 1.3–2.0 years in the peritoneal dialysis group. There was no significant difference between age, gender distribution, and length of time spent on dialysis between the hemodialysis patients and those on peritoneal dialysis. All dialysis patients and six of the CKD patients were treated with rHuEPO; 16 received darbopoietin (Aranesp) and two epoietin beta injections (Recormon). Conversion of the dosages of the two medications was performed according to the manufacturer's instructions: 1 mcg darbopoietin alfa = 200 u epoietin beta (Aranesp (darbopoietin-alfa) product monograph, Mississauga (ON): Amgen Canada Inc., 2011). The three study groups were age-matched with a male predominance observed in the CKD group. Lower Hgb levels were found in the dialysis patients compared with the non-dialysis CKD patients and controls; serum intact PTH levels were significantly higher in the dialysis group. Serum folic acid levels were lower in the CKD group compared to the dialysis patients. Serum vitamin B_{12} levels and transferrin saturation were similar in all groups. Serum CRP was higher in the dialysis patient group (Table 1).

A significant positive correlation was found between the ERI and PTH ($p = 0.003$; $r = 0.52$) and the ERI and CRP ($p = 0.05$; $r = 0.48$). An inverse correlation was found between the ERI and transferrin saturation ($p = 0.03$; $r = -0.52$) (Fig. 1). No correlation was found between the ERI and serum folic acid levels. UT-7 EPO-dependent cell proliferation was significantly decreased in the presence of the dialysis patients' sera compared to the proliferation in the presence of sera from the CKD and control groups at 48 h ($p = 0.003$ and $p = 0.04$, respectively) and 72 h ($p = 0.02$ and $p = 0.07$, respectively) (Fig. 2). A significant inverse correlation between the ERI and cell proliferation was found at 48 and 72 h ($p = 0.007$; $r = -0.63$ and $p = 0.03$; $r = -0.52$, respectively) (Fig. 3). There was no significant difference in UT-7 cell proliferation between the sera from the CKD and control groups at 48 and 72 h ($p = 0.36$ and $p = 0.9$, respectively) (Fig. 2). No correlation was found between cell proliferation rates and eGFR in the CKD group. After incubation of K562 cells with sera from the dialysis group and controls, a decrease in cell proliferation was observed in the dialysis group compared with the controls at 48 h ($p = 0.002$). There was no significant difference between the groups at 72 h ($p = 0.55$).

Discussion

The current study presents a novel application of an in vitro bioassay designed to examine the effect of uremic environment on EPO-dependent cell proliferation by utilizing a human erythroleukemia (UT-7) cell line.

Resistance to EPO in CKD patients is multifactorial. Various mechanisms have been proposed such as iron deficiency, B_{12} and folate deficiency, and metabolic bone disease [7–9, 20]. In concordance with previous reports [21], we found a significant positive correlation between the ERI and PTH and an inverse correlation between the ERI and transferrin saturation. Folic acid deficiency is an important factor in EPO resistance [22]. No correlation was found between folic acid levels and the ERI. There is a limitation of reliance on serum levels of folic acid in the diagnosis of folate deficiency, since serum folate undergoes significant changes related to recent food intake. Therefore, serum folate levels do not accurately reflect long-term body folate status in comparison to red blood cell folate concentration [23].The measurement of red blood cell folate concentration was unattainable; therefore, the exact folate status could not be determined. Although numerous nutritional and metabolic factors are involved in EPO resistance, hyporesponsiveness to rHuEPO occurs in CKD patients with adequate iron stores and with no hyperparathyroidism or vitamin deficiency; therefore, other factors may play a role in EPO resistance. EPO-R function, as well as interaction between EPO-R and EPO, is crucial in the process of erythropoiesis. There is some evidence that a uremic environment may affect the EPO-R/EPO axis [24–26].

Our study was designed to examine the direct effect of the uremic environment on EPO-dependent erythropoiesis by using the UT-7 cell line as a surrogate for erythroid progenitor cells. Saturation of EPO-R in UT-7 cells is attained in the presence of rHuEPO at a concentration of 2 u/ml [13, 27]. We found that sera from pediatric dialysis patients inhibited UT-7 cell growth even when the EPO receptors were saturated. Since there were no significant differences between demographic characteristics and duration on dialysis between hemodialysis and peritoneal dialysis patients, and because the total number of patients on dialysis was relatively small, we analyzed these patients as one group. Interestingly, the inhibitory effect was not observed in CKD patients who were not on dialysis. Moreover, no correlation between eGFR and cell

Table 1 Demographic and laboratory parameters of the study groups

	CKD[1] ($n = 28$)	Dialysis ($n = 12$)	Control ($n = 20$)	p value
Age (years)[a]	14.5(19.58)	12.7 (16.75)	10.2 (22)	0.22
Sex (% male)[b, c]	24 (85.7)	7 (58.3)	9 (45)	0.01
Hgb[2] (g/dl)[a, d]	12.5 (5.4)	11.75 (3)	12.85 (4.6)	0.03
PTH[3] (pg/ml)[a, e]	53.8(359.3)	252.25 (721.6)	21.25 (50.2)	< 0.001
Folic acid (nmol/l)[a, f]	22.4 (39.4)	42.8 (38.6)	16.1 (22)	0.08
B_{12} (pmol/l)[a]	389 (1326)	515.5 (1205)	335.5 (504)	0.11
Transferrin saturation (%)[a]	23.5 (64)	23.7 (34.5)		0.54
CRP[4] (mg/dl)[a, g]	0.07 (0.83)	0.3 (2.65)	0.05 (0.59)	0.003

[1] *CKD* chronic kidney disease

[2] *Hgb* hemoglobin

[3] *PTH* parathyroid hormone

[4] *CRP* C-reactive protein

[a] Variables described as median and range

[b] Variables described as number and percentage

[c] CKD group included a higher percentage of males compared with the dialysis group and controls

[d] Dialysis patients had lower Hgb levels compared with the CKD group and controls

[e] Dialysis patients had higher PTH levels compared with the CKD group and controls

[f] CKD patients had a lower level of folic acid compared with dialysis group, $p = 0.03$ (no significant difference was found when the three study groups were compared)

[g] Dialysis patients had higher CRP levels compared with the CKD group and controls

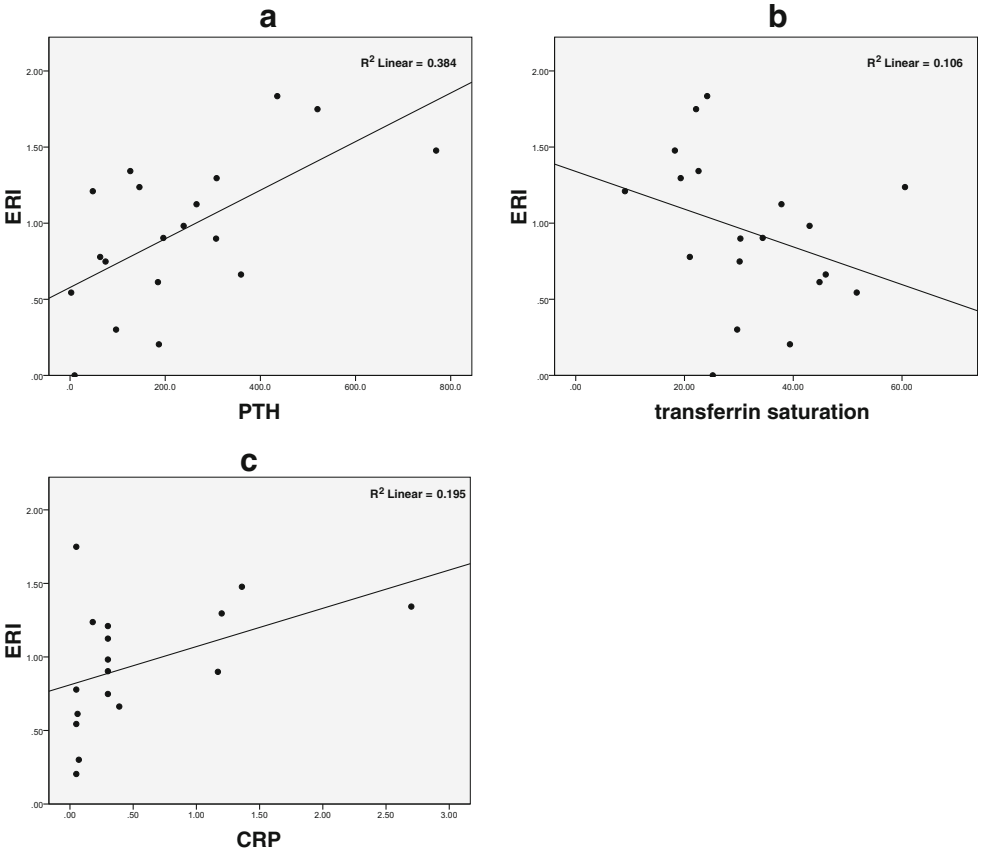

Fig. 1 Correlation between ERI and PTH, transferrin, and CRP. **a** Correlation between ERI and PTH, $p = 0.003$; $r = 0.52$. **b** Correlation between ERI and transferrin, $p = 0.03$; $r = -0.52$. **c** Correlation between ERI and CRP, $p = 0.05$; $r = 0.48$. A log transformation was performed for ERI. *ERI* erythropoietin resistance index, *PTH* parathyroid hormone, *CRP* C-reactive protein

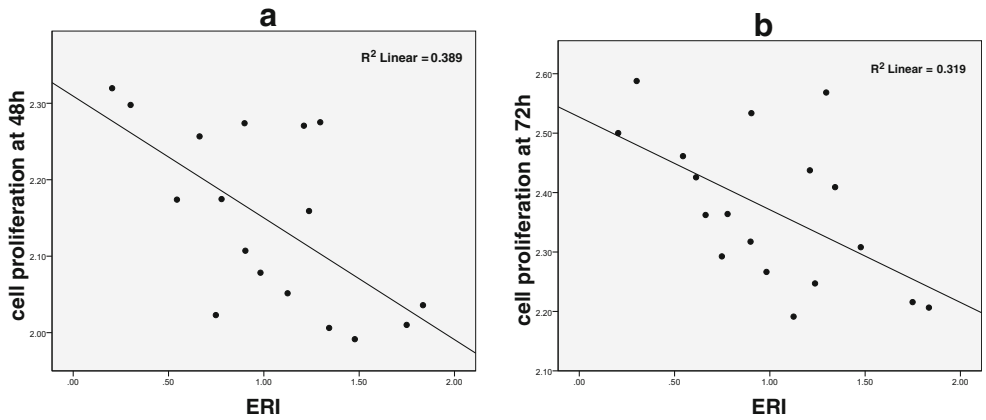

Fig. 3 Correlation between ERI and cell proliferation at 48 h (**a**) and 72 h (**b**). **a** Correlation between cell proliferation at 48 h and ERI, $p = 0.007$, $r = -0.63$. **b** Correlation between cell proliferation at 72 h and ERI, $p = 0.03$, $r = -0.52$. A log transformation was performed for ERI and cell proliferation. *ERI* erythropoietin resistance index, *CKD* chronic kidney disease

proliferation was noted. Only six of the CKD patients were treated with rHuEPO (Table 1). Hemoglobin levels of the CKD group did not significantly differ from the control group. We, therefore, presume that sera from CKD patients who were not on dialysis did not contain sufficient amounts of inhibitory substances for UT-7 cell growth.

The ERI is widely accepted as an indicator of responsiveness to rHuEPO treatment. Although correlation between the ERI, cardiovascular morbidity, and overall mortality has been reported in the adult population [28, 29], several reports have challenged its relevance [16]. Since no other parameters were reported superior to the ERI, we used it as a clinical estimator of EPO resistance.

Analysis of 18 patients treated with rHuEPO showed a significant inverse correlation between the ERI and erythroid cell proliferation.

To assess whether the inhibitory effect of uremic sera was specific to EPO-driven proliferation, we examined the growth of K562 cells (EPO-independent) in the presence of sera obtained from the dialysis patients and controls. Uremic sera inhibited K562 cell growth at 48 h but not at 72 h. These findings may suggest multiple mechanisms interfering with erythropoiesis in CKD patients. Some of these may act on the EPO/EPO-R axis while other factors act by EPO-R-independent pathways. One of the proposed mechanisms is high levels of plasma-soluble EPO-R which may impede the binding of EPO to the transmembrane receptor and is also found to correlate with EPO resistance in CKD patients [24, 25]. Other factors may be involved in the inhibition of EPO. The accumulation of binding proteins plays an important role in resisting the action of the growth hormone and insulin growth factor 1 (IGF-1) in patients with CKD [30]. Although, to the best of our knowledge, EPO binding proteins have not, as yet, been identified in patients with CKD, further studies should focus on the recognition of possible inhibitors. Several additional factors such as fibroblast growth factor 23 (FGF23) may affect erythropoiesis in CKD patients. FGF23 is a pro-phosphaturic hormone secreted by osteocytes and its

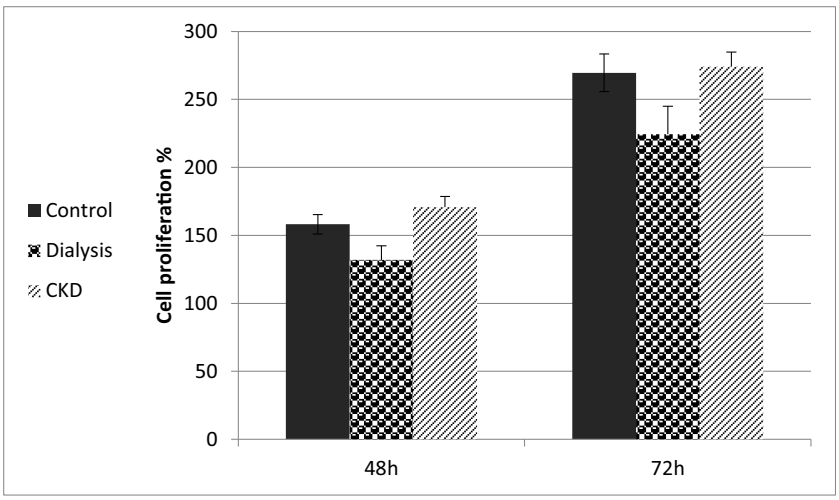

Fig. 2 Effect of sera on EPO-dependent UT-7 proliferation. Cell proliferation was significantly decreased in the dialysis group compared to the CKD[5] group and controls at 48 h ($p = 0.003$ and $p = 0.04$, respectively) and at 72 h ($p = 0.02$ and $p = 0.07$, respectively). There is no statistical difference between cell proliferation of control and CKD groups at 48 and 72 h ($p = 0.36$ and $p = 0.9$, respectively)

levels increase in CKD [31]. A direct inhibitory effect of FGF23 on prenatal and postnatal erythropoiesis has been reported in an animal model [32]. Moreover, high FGF23 levels were associated with low Hgb levels in CKD patients [33].

Chronic kidney disease is associated with persistent inflammation [34, 35]. As previously reported, a significant positive correlation was found between CRP levels and the ERI in our study group. Uremic toxins have been implicated in the propagation of pro-inflammatory cytokines such as IL-1, IL-6, interferon gamma, and tumor necrosis factor which may interfere with the EPO/EPO-R axis via several mechanisms [35] including downregulating the expression of EPO-R on erythroid progenitor cells [25]. Furthermore, pro-inflammatory cytokines promote increased production of cytokine-inducible SH2-containing proteins which bind directly to the cytoplasmic domain of EPO-R as well as decreasing EPO-R activation [36]. Other potential uremic toxins such as growth arrest-specific protein 6 (Gas6) were reported to inhibit erythropoiesis in CKD patients [37]. Hepcidin, an iron-regulatory hormone, plays an important role in a feedback-regulated mechanism that maintain adequate plasma concentrations of iron-transferrin for erythropoiesis [38]. In CKD, inflammation and impaired renal clearance increase plasma hepcidin levels, thus inhibiting duodenal iron absorption and sequestering iron in macrophages. These effects cause functional iron deficiency and resistance to EPO [39]; hence, the uremic environment contains multiple substances which may inhibit erythropoiesis.

Adjustment of rHuEPO dosage in CKD patients is based on serial measurements of Hgb levels; therefore, attaining an optimal dose may be delayed. Although the in vitro bioassay presented herein cannot replace multiple clinical parameters based on which treatment decisions are made, it may provide an additional tool for estimating responsiveness to EPO and facilitating the adjustment of rHuEPO dosage.

In conclusion, to the best of our knowledge, our study is the first to examine the direct effect of sera obtained from CKD patients on erythroid cell proliferation in vitro. Our findings support the presence of inhibitory factors in the sera of these patients. We present the data of our relatively small cohort in anticipation that it will stimulate further research in this area with larger cohorts and expect that additional studies employing similar experimental systems will identify relevant inhibitory substances and mechanisms contributing to EPO resistance.

Dedication This work is dedicated to our dearest friend, the late Professor Miriam Souroujon, with deep appreciation and love.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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