Down-Regulation of Cardiac Erythropoietin Receptor and its Downstream Activated Signal Transducer Phospho-STAT-5 in a Rat Model of Chronic Kidney Disease

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ABSTRACT: **Background:** Chronic kidney disease (CKD) is often accompanied by impairment of cardiac function that may lead to major cardiac events. Erythropoietin (EPO), a kidney-produced protein, was shown to be beneficial to heart function. It was suggested that reduced EPO secretion in CKD may play a role in the initiation of heart damage.

Objectives: To investigate molecular changes in the EPO/erythropoietin receptor (EPO-R) axis in rat cardiomyocytes using a rat model for CKD.

Methods: We established a rat model for CKD by kidney resection. Cardiac tissue sections were stained with Masson's trichrome to assess interstitial fibrosis indicating cardiac damage. To evaluate changes in the EPO/EPO-R signaling cascade in the myocardium we measured cardiac EPO and EPO-R as well as the phosphorylation levels of STAT-5, a downstream element in this cascade.

Results: At 11 weeks after resection, animals presented severe renal failure reflected by reduced creatinine clearance, elevated blood urea nitrogen and presence of anemia. Histological analysis revealed enhanced fibrosis in cardiac sections of CKD animals compared to the sham controls. Parallel to these changes, we found that although cardiac EPO levels were similar in both groups, the expression of EPO-R and the activated form of its downstream protein STAT-5 were significantly lower in CKD animals.

Conclusions: CKD results in molecular changes in the EPO/EPO-R axis. These changes may play a role in early cardiac damage observed in the cardiorenal syndrome.

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KEY WORDS: erythropoietin (EPO), erythropoietin receptor (EPO-R), phospho-STAT-5, chronic kidney disease (CKD), cardiac interstitial fibrosis

*The first two authors contributed equally to this study
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The clinical presentation of cardiac and renal insufficiency has been referred to as the "cardiorenal syndrome" (CRS), whereby acute or chronic dysfunction of one organ induces acute or chronic dysfunction of the other [1]. Chronic kidney disease (CKD) leading to an impairment of cardiac function – characterized by primary left ventricular hypertrophy (LVH), diastolic dysfunction, and/or increased risk of adverse cardiovascular events – is defined as type IV CRS [2]. It is currently established that cardiovascular involvement occurs in most cases of CKD and that major cardiac events account for almost 50% of CKD-related deaths [3].

Using an animal model of type IV CRS we previously showed that evoking kidney failure can induce early changes in the myocardium structure and alter gene expression. These effects can, in time, evolve into severe myocardial damage and heart failure [4].

The exact mechanism connecting renal and heart failure (HF) is unknown. However, some possible mechanisms involved in this co-morbidity have been suggested. Indeed, one of the main side effects of CKD, anemia, is proposed to play a role in both renal failure and heart disease. The development of anemia in CKD patients is mainly attributed to insufficient renal erythropoietin (EPO) production [5,6], which, in turn, might result in cardiac damage due to the known contribution of anemia to LVH and cardiac impairment [7,8]. Furthermore, several studies showed that EPO treatment of anemic patients with CKD, who often also develop congestive heart failure (CHF), resulted in a reduction in cardiac size, regression of LVH, and improvement of LV ejection fraction and myocardial contractility [9].

Recent experimental studies on the role and function of EPO revealed that apart from its direct action on erythropoiesis, EPO also exerts numerous tissue protective effects by preventing vascular and tissue damage caused by acute ischemia in the heart, brain and kidneys [1,8]. Specifically to CRS, EPO treatment was repeatedly shown to be beneficial to heart function in several distinct experimental models. In experimental

models of CHF, EPO was shown to enhance cardiac function [10-14]. Furthermore, it was established that treatment with EPO improves cardiac remodeling associated with LV pressure overload by decreasing myocardial interstitial fibrosis [15]. In addition, treatment with recombinant human EPO (rhEPO) was reported to significantly reduce infarct size by inhibiting apoptotic cell death, thereby preserving ventricular function [16-19]. It was proposed that the protective effects of EPO and its receptor (EPO-R) may be mediated by multiple signaling pathways, such as JAK/STAT, PI3/AKT, MAPKs and eNOS, which can influence cell survival and death and be critical for tissue repair and remodeling [18,20]. In the heart, Jing Lu et al. [21] focused on the EPO/EPO-R system in a model of diabetes-induced heart injury and found that the protective properties of this pathway are mediated specifically by the regulation of cardiac EPO-R expression levels. Moreover, it was also shown that post-infarct local treatment with EPO improved LV remodeling and function by activating pro-survival, anti-fibrosis, and angiogenesis signaling induced by the up-regulation of cardiac EPO-R and its downstream proteins [22]. The dual effect of EPO on cardiac function, both directly on the heart muscle through its signaling cascade and also indirectly through induction of anemia, suggests that EPO might play a role in CRS progression. Thus, EPO is thought to represent one of the connecting factors that mediate kidney disease and heart injury.

The regulation of EPO and EPO-R in CRS is poorly understood. In the current study we aimed to evaluate the effects of CKD on the expression of endogenous EPO/EPO-R and the downstream signal transducer molecule phospho-STAT (p-STAT) of this cascade in the heart.

MATERIALS AND METHODS

All experimental protocols involving animals were approved by the Tel Aviv Sourasky Medical Center Institutional Review Board (Helsinki Committee) which constitutes our Institutional Animal Care and Use Committee (IACUC).

IN VIVO MODEL

We employed a rat model for cardiorenal syndrome, as previously described by Van Dokkum et al. [23] and our laboratory [4,24]. Briefly, Lewis rats (300–350 g body weight, 7–10 animals/group) underwent subtotal nephrectomy (STN) in two subsequent surgeries under anesthesia with ketamine (50 mg/kg) and xylazine (10 mg/ml): two-thirds of the left kidney were removed initially followed by removal of the right kidney one week later. This phase of the experiment was associated with 70% survival of the animals that underwent STN. The study included two experimental arms with five animals per group:

- CKD only: the animals were terminated 11 weeks after completion of 5/6 STN
- Sham-operated control animals: abdominal opening only.

Prior to termination, the animals were weighed. Renal function was evaluated by measurements of blood urea nitrogen (BUN) and creatinine clearance (Cct), calculated as follows:

$$Cct\ (ml/min) = [Ucr\ (mg/dl) \times V\ (ml/min)]/\ Pcr\ (mg/dl)$$

where Ucr = urine levels of creatinine, V = urine flow rate, and Pcr = plasma levels of creatinine.

Following termination, sera were collected and aliquoted. In parallel the hearts were removed and weighed. Anemia was evaluated by measuring blood hemoglobin concentration.

WESTERN BLOT ANALYSES

LV sections from CKD or sham-operated control animals were extracted using a commercial lysis buffer (Sigma-Aldrich Israel Ltd.). Equal protein amounts (80 µg) were loaded on a 4%–20% acrylamide gel followed by electric transfer to nitrocellulose membranes. Following overnight blocking with 5% low fat milk diluted in TBS-tween, the membranes were incubated with STAT-5 or p-STAT-5 (Cell Signaling Technology, USA), anti-EPO-R (Santa Cruz Biotechnology, Inc, USA), or with an anti-GAPDH antibody used for validating equal loading (Abcam, UK, clone 6C5). The primary antibodies were followed by blotting with horseradish peroxidase-conjugated secondary antibodies. After rapid incubation with an ECL substrate (Biological Industries, Israel) the membranes were exposed to an imaging film.

EPO ELISA

EPO levels were determined using a rat erythropoietin ELISA kit according to the manufacturer's instructions (Wuhan EIAAB science Co, Ltd, China).

HEART MUSCLE STAINING FOR FIBROSIS

Heart sections from the sham and CKD groups were fixed with 4% paraformaldehyde, sliced into transverse sections and paraffinized. The blocks were then sectioned in 5 µm slices. The slides were stained with Masson's trichrome to assess interstitial fibrosis in the LV sections.

STATISTICAL ANALYSIS

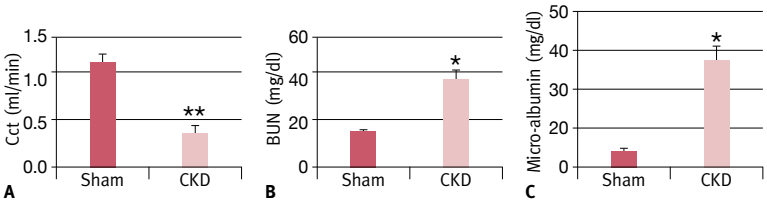
Results are expressed as mean ± SE. Comparisons between each experimental arm to sham-operated controls were performed using the two-tailed Student's *t*-test (IBM SPSS statistics 22 software). $P < 0.05$ was accepted as statistically significant.

RESULTS

VALIDATION OF CKD

To test the relationship between chronic kidney disease and cardiac damage we employed an established protocol of kid-

Figure 1. Validation of the chronic kidney disease (CKD) model. **[A]** Bar graphs of creatinine clearance (Cct, ml/min). **[B]** Serum blood urea nitrogen (BUN) levels (mg/dl). **[C]** Urine excretion of micro-albumin levels (mg/dl). Data are represented as mean ± SEM



**P < 0.01,*P < 0.05

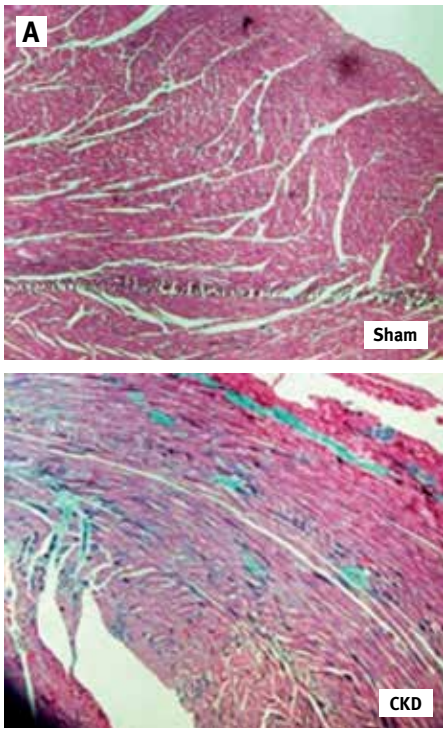
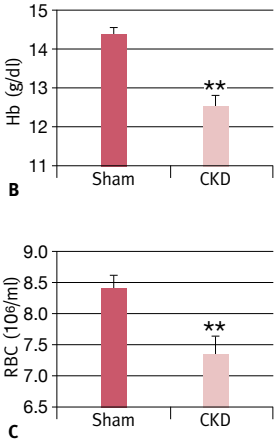


Figure 2. Cardiac and systemic pathology induced by CKD. **[A]** Histological staining of cardiac sections for Masson's trichrome showing interstitial fibrosis. **[B]** Blood levels of hemoglobin (Hb, g/dl), and **[C]** red blood cell count (RBC, x 10^6/ml)



**P < 0.01

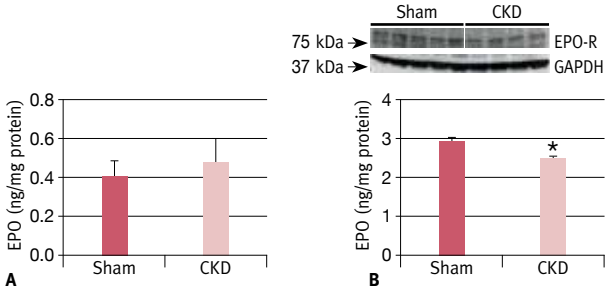
ney injury [24]. To evaluate the induction of a chronic state of renal failure we measured blood levels of both creatinine and BUN. We observed a significant decline in Cct (0.4 ± 0.08 versus 1.2 ± 0.10 mg/dl, $P < 0.001$) [Figure 1A] accompanied by a significant elevation in BUN levels (40.50 ± 4.30 vs. 16.75 ± 0.63, $P < 0.002$) [Figure 1B] in CKD rats compared to sham-operated controls respectively. Urine micro-albumin secretion was also markedly reduced, confirming the renal injury and damage (34.50 ± 1.48 mg/dl vs. 5.50 ± 0.78 in CKD vs. sham, respectively, $P < 0.01$) [Figure 1C].

CARDIAC INTERSTITIAL FIBROSIS AND ANEMIA IN THE CKD MODEL

We have previously shown that 4 week CKD results in gene expression coding for pro-apoptotic protein fibrosis, cell hypertrophy, cell irregularity and disarrangement [4]. In the current study, the representative micrograph of heart sections revealed enhanced fibrosis in cardiac sections of CKD relative to the sham, pointing to the detrimental effect of CKD on the heart [Figure 2A].

As mentioned above, anemia is known to be relevant to both CKD and cardiac damage. Induction of anemia observed in this model could play a role in exacerbating the injury of the cardiac muscle. Indeed, we found that kidney insult led to a decrease in blood hemoglobin concentration (12.57 ± 0.96 vs. 14.40 ± 0.89 mg/dl in the CKD versus sham, respectively, $P < 0.05$) [Figure 2B] and to red blood cell count (RBC) count decrement (7.36 ± 0.49 x 10^6/ml vs. 8.43 ± 0.43 x 10^6/ml in the CKD versus sham, respectively, $P < 0.04$) [Figure 2C].

Figure 3. The cardiac axis of EPO/EPO-R. **[A]** Cardiac EPO normalized to total protein levels (ng/mg). **[B]** Cardiac EPO-R normalized to GAPDH level



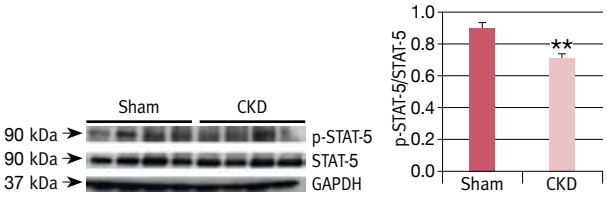
*P < 0.05

CKD INDUCES CHANGES IN THE EPO/EPO-R AXIS IN THE HEART

To better understand the connection between CKD and heart injury we investigated whether CKD could influence the EPO/EPO-R axis in the heart. EPO expression levels were measured in heart lysates derived from both CKD and sham animals by EPO ELISA. We did not observe any significant changes in EPO expression in LV sections of the two groups (0.48 ± 0.04 vs. 0.40 ± 0.06 ng/mg in the CKD versus sham, respectively, $P = 0.39$) [Figure 3A]. Since activation of the signaling pathway of this axis might not be regulated through EPO but rather by regulation of its receptor levels in the heart [21], we evaluated the expression levels of cardiac EPO-R by Western blot. Interestingly, we found that the protein levels of EPO-R were significantly reduced in the CKD compared to the sham operated group by 14% (2.5 ± 0.13 vs. 2.9 ± 0.21 respectively, $P = 0.013$) [Figure 3B]. This result implies that the erythropoietin receptor might act as a key component in CRS.

To further substantiate this result and explore the involvement of the EPO/EPO-R cascade in mitigating cardiac injury induced by CKD, we sought to assess the expression levels of downstream elements of this signaling pathway. Receptor-associated Janus family tyrosine kinase (JAK)/STAT are known

Figure 4. Cardiac levels of p-STAT-5 are attenuated in kidney disease. The cardiac protein expression ratio of p-STAT-5/STAT-5 quantitated by Western analysis.



**$P < 0.01$

to be downstream mediators of EPO-R signaling in cardiac cells, both in vitro and in vivo [22]. Indeed, in accordance with reduced EPO-R cardiac expression, we observed a significant reduction in STAT-5 activation, as indicated by 22% attenuated levels of the phosphorylated form of STAT-5 (p-STAT-5) in the heart of the CKD group compared with the sham operated group (0.70 ± 0.03 vs. 0.90 ± 0.11, respectively, $P = 0.022$) [Figure 4].

DISCUSSION

In this work we aimed to study the potential effects of CKD on cardiac expression of the erythropoietin receptor and its signaling pathway. We found that CKD resulted in down-regulation of EPO-R accompanied by reduced phosphorylation of STAT-5 in the myocardium of the LV, despite unchanged cardiac EPO levels. The exact mechanism of cardiac injury induced by chronic kidney damage is not yet clear. However, it was previously found that several cardiac changes occur against a background of renal injury leading to cardiac remodeling that includes hypertrophy, fibrosis and late-stage apoptosis, resulting in loss of cardiac mass [2,9].

Using animal models of CKD-induced cardiac injury it was already demonstrated that treatment with EPO can inhibit cardiac remodeling, improve general heart function and induce an anti-fibrotic effect [7,8,10-14]. Taking into consideration that EPO-R is expressed on cardiomyocytes, it is possible that the overall beneficial protective effect of EPO treatment was mediated by regulation of the expression levels of this receptor in the heart. The current data re-substantiate that EPO-R is expressed in the myocardium and indicate that under CKD conditions the expression levels of this receptor in the myocardium are reduced. Furthermore, we have shown that phosphorylation of cardiac STAT-5, which is suggested to be induced by activation of the EPO/EPO-R signaling pathway thus promoting cell survival [20,25], is also significantly reduced in CKD. These results support the hypothesis that CKD can affect pivotal signaling pathways within cardiomyocytes and that EPO-R may play a critical role in this process. Indeed, recently, several lines of evidence suggested that both EPO and EPO-R have an important role in mediating cardiac changes in different heart injury models. Wang et al. [15] showed in a model of LV pressure overload that EPO/EPO-R signaling induces phosphorylation of STAT-5, AKT and e-NOS and improves cardiac remodeling. Moreover, in a different model of myocardial infarction-induced damage, Kobayashi et al. [22] found that systemic administration of slow-release EPO treatment is sufficient to induce a significant increase in EPO-R levels in the heart. The elevation of EPO-R levels in response to EPO treatment was also shown in a diabetes-induced heart damage study. The study demonstrated that this up-regulation of cardiac EPO-R can suppress the deleterious effects of diabetes on the heart [21]. These findings suggest a crucial role for the EPO/EPO-R axis in cardiac homeostasis that is relevant in many direct and indirect cardiac injuries. In line with these data, our results also suggest that the EPO/EPO-R axis may also play a role in cardiac damage induced by CKD.

Many different mechanisms may take part in the cardiac damage induced by CKD, including anemia, inflammation, renal hormone secretion, and changes in the renin-angiotensin axis. The complex and diverse systemic changes that occur in the presence of chronic kidney disease make it extremely difficult to identify a single target that can explain the resulting heart injury. The evident reduction in both EPO-R and p-STAT-5 implies that the EPO/EPO-R axis is a good candidate that mediates the cardiac damage and in the future might even serve as a valid target for therapy. Future studies in this model will yield more information and better understanding regarding the exact mechanisms that control EPO receptor levels in cardiomyocytes and the influence it potentially has on cell survival and tissue repair.

CONCLUSIONS

Additional experiments to treat CKD rats with recombinant EPO injections are required. These experiments could further elucidate the role of EPO in controlling cardiac EPO-R levels and thus directly support the hypothesis that connects renal failure to heart damage via changes in the EPO/EPO-R pathway. This will be the basis for our impending thorough in vivo study.

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References

1. Ben-Shoshan J, Entin-Meer M, Guzner-Gur H, Keren G. The cardiorenal syndrome: a mutual approach to concomitant cardiac and renal failure. *IMAJ* 2012; 14 (9): 570-6.
2. Clementi A, Virzi GM, Goh CY, et al. Cardiorenal syndrome type 4: a review. *Cardiorenal Med* 2013; 3 (1): 63-70.

3. Di Lullo L, House A, Gorini A, Santoboni A, Russo D, Ronco C. Chronic kidney disease and cardiovascular complications. *Heart Fail Rev* 2015; 20 (3): 259-72.
4. Entin-Meer M, Pasmanik-Chor M, Ben-Shoshan J, et al. Renal failure is associated with driving of gene expression towards cardiac hypertrophy and reduced mitochondrial activity. *Clin Exp Cardiol* 2012; 3: 2-9.
5. Deicher R, Horl WH. Anaemia as a risk factor for the progression of chronic kidney disease. *Curr Opin Rephrol Hypertens* 2003; 12 (2): 139-43.
6. Opasich C, Cazzola M, Scelsi L, et al. Blunted erythropoietin production and defective iron supply for erythropoiesis as major causes of anaemia in patients with chronic heart failure. *Eur Heart J* 2005; 26 (21): 2232-7.
7. van der Meer P, Voors AA, Lipsic E, van Gilst WH, van Veldhuisen DJ. Erythropoietin in cardiovascular diseases. *Eur Heart J* 2004; 25 (4): 285-91.
8. Lipsic E, Schoemaker RG, van der Meer P, Voors AA, van Veldhuisen DJ, van Gilst WH. Protective effects of erythropoietin in cardiac ischemia: from bench to bedside. *J Am Coll Cardiol* 2006; 48 (11): 2161-7.
9. Silberberg J, Racine N, Barre P, Sniderman AD. Regression of left ventricular hypertrophy in dialysis patients following correction of anemia with recombinant human erythropoietin. *Can J Cardiol* 1990; 6 (1): 1-4.
10. Hamed S, Barshack I, Luboshits G, et al. Erythropoietin improves myocardial performance in doxorubicin-induced cardiomyopathy. *Eur Heart J* 2006; 27 (15): 1876-83.
11. Hirata A, Minamino T, Asanuma H, et al. Erythropoietin enhances neovascularization of ischemic myocardium and improves left ventricular dysfunction after myocardial infarction in dogs. *J Am Coll Cardiol* 2006; 48 (1): 176-84.
12. Li L, Takemura G, Li Y, et al. Preventive effect of erythropoietin on cardiac dysfunction in doxorubicin-induced cardiomyopathy. *Circulation* 2006; 113 (4): 535-43.
13. van der Meer P, Lipsic E, Henning RH, et al. Erythropoietin induces neovascularization and improves cardiac function in rats with heart failure after myocardial infarction. *J Am Coll Cardiol* 2005; 46 (1): 125-33.
14. Teixeira AM, Garrido P, Santos P, et al. Recombinant human erythropoietin treatment protects the cardio-renal axis in a model of moderate chronic renal failure. *Renal Fail* 2010; 32 (9): 1073-80.
15. Wang W, Kagaya Y, Asaumi Y, Fukui S, Takeda M, Shimokawa H. Protective effects of recombinant human erythropoietin against pressure overload-induced left ventricular remodeling and premature death in mice. *Tohoku J Exp Med* 2011; 225 (2): 131-43.
16. Cai Z, Manalo DJ, Wei G, et al. Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. *Circulation* 2003; 108 (1): 79-85.
17. Moon C, Krawczyk M, Ahn D, et al. Erythropoietin reduces myocardial infarction and left ventricular functional decline after coronary artery ligation in rats. *Proc Natl Acad Sci USA* 2003; 100 (20): 11612-17.
18. 18.Parsa CJ, Matsumoto A, Kim J, et al. A novel protective effect of erythropoietin in the infarcted heart. *J Clin Invest* 2003; 112 (7): 999-1007.
19. Wright GL, Hanlon P, Amin K, Steenbergen C, Murphy E, Arcasoy MO. Erythropoietin receptor expression in adult rat cardiomyocytes is associated with an acute cardioprotective effect for recombinant erythropoietin during ischemia-reperfusion injury. *FASEB J* 2004; 18 (9): 1031-3.
20. Burger D, Xenocostas A, Feng QP. Molecular basis of cardioprotection by erythropoietin. *Curr Mol Pharmacol* 2009; 2 (1): 56-69.
21. Lu J, Yao YY, Dai QM, et al. Erythropoietin attenuates cardiac dysfunction by increasing myocardial angiogenesis and inhibiting interstitial fibrosis in diabetic rats. *Cardiovasc Diabetol* 2012; 11: 105.
22. Kobayashi H, Minatoguchi S, Yasuda S, et al. Post-infarct treatment with an erythropoietin-gelatin hydrogel drug delivery system for cardiac repair. *Cardiovasc Res* 2008; 79 (4): 611-20.
23. van Dokkum RP, Eijkelkamp WB, Kluppel AC, et al. Myocardial infarction enhances progressive renal damage in an experimental model for cardio-renal interaction. *J Am Soc Nephrol* 2004; 15 (12): 3103-10.
24. Entin-Meer M, Ben-Shoshan J, Maysel-Auslender S, et al. Accelerated renal fibrosis in cardiorenal syndrome is associated with long-term increase in urine neutrophil gelatinase-associated lipocalin levels. *Am J Nephrol* 2012; 36 (2): 190-200.
25. Ratajczak J, Majka M, Kijowski J, et al. Biological significance of MAPK, AKT and JAK-STAT protein activation by various erythropoietic factors in normal human early erythroid cells. *Br J Haematol* 2001; 115 (1): 195-204.