CARDIO-RENAL ANTI-APOPTOTIC AND PRO-PROLIFERATIVE EFFECT OF RECOMBINANT HUMAN ERYTHROPOIETIN IN A MODERATE STAGE OF CHRONIC RENAL FAILURE IN THE RAT

Teixeira AM[1,2], *Garrido P*[1], *Rodrigues-Santos P*[2], *Costa E*[3,4], *Parada B*[1], *Sereno J*[1], *Mascarenhas-Melo F*[1], *Alves R*[5], *Belo L*[4,6], *Santos-Silva A*[4,6], *Teixeira F*[1], *Reis F*[1]

ABSTRACT

We hypothesize that recombinant human erythropoietin (rhEPO) therapy under circumstances of moderate chronic renal failure (CRF), with lower kidney and heart lesion/impairment, may have a protective effect beyond the correction of anemia, whose mechanism deserve better elucidation by clarifying the impact on cardio-renal gene expression profile on markers of apoptosis, inflammation, proliferation, angiogenesis and lesion/stress. Four groups of rats were studied over a period of 15 weeks (n=7 each): control – without surgery and without drug treatment; rhEPO – treated with 50 IU/kg/week of rhEPO-beta; CRF – submitted to partial nephrectomy (3/4); CRF+rhEPO – CRF with rhEPO treatment after the 3rd week of surgery. The kidney and the heart were collected in order to evaluate the gene expression, by real-time qPCR, of markers of apoptotic machinery (Bax, Bcl2, Fas, Faslg and caspases 3 and 9), inflammation/immunology (TNF-α, NF-Kβ and IL-2), proliferation/angiogenesis (TGF-β, VEGF and PCNA) and lesion/stress (cytochrome c and NOS2 and NOS3). The main finding obtained were: a) – CRF rats has demonstrate overexpression of EPO-R in the heart ($P>0.005$) and a trend to higher values in the kidney, without changes on EPO expression, together with overexpression of Bax/Bcl2 ratio, PCNA and IL-2 in both tissues ($P<0.005$ vs control); b) – rhEPO therapy on the remnant kidney and on the heart of

[1] Laboratory of Pharmacology and Experimental Therapeutics, IBILI, Medicine Faculty, Coimbra University.
[2] Laboratory of Immunology and Oncology, Center for Neuroscience and Cell Biology, Coimbra.
[3] Institute of Health Sciences of University Catholic, Porto.
[4] Institute for Molecular and Cellular Biology, Porto University.
[5] Department of Nephrology, Medicine Faculty, Coimbra University.
[6] Biochemistry Department, Pharmacy Faculty, Porto University; Portugal.

Corresponding author:
Flávio Reis, PhD
Laboratory of Pharmacology and Experimental Therapeutics, IBILI, Medicine Faculty
Sub-Unit 1 (Pólo III), Coimbra University,
3000-548 Coimbra, Portugal
Tel: +351 239 480053; Fax: +351 239 480065;
E-mail: freis@fmed.uc.pt

the rats with CRF induced by partial 3/4 nephrectomy promoted non-hematopoietic protection, demonstrated by the apoptosis prevention in both tissues, viewed by the Bax/Bcl2 balance (P<0.05 vs CRF), by the promotion of proliferation, due to PCNA increment, particularly in the heart (P<0.01 vs CRF), and by the immunomodulatory action, expressed by the prevention of IL-2 increment in the remnant kidney (P>0.01 vs CRF). These effects were accompanied by a trend to higher values of EPO gene expression in the heart and of EPO-R in the remnant kidney and in the cardiac tissue. In conclusion, in this model of moderate CRF, rhEPO treatment showed important cardio-renal non-hematopoietic effects, expressed mainly by the anti-apoptotic and the pro-proliferative action. This data suggest that early rhEPO in moderate stages of CRF, before critical lesion of the tissues, might be a therapeutic measure with further benefits.

Key-words: moderate chronic renal failure, cardio-renal protection, gene expression, poptosis, inflammation, proliferation/angiogenesis, lesion/stress, animal model

INTRODUCTION

The appearance and development of chronic kidney disease (CKD) is often associated to a state of low levels of erythropoietin (EPO) production, due to loss of peritubular cells of the kidney, responsible for its formation, with consequent reduction in erythropoiesis, resulting in anemia[1]. The reduction of tissue oxygenation then causes an increase of cardiac function, and subsequent left ventricular hypertrophy, in order to reply to the oxygen demands by the peripheral tissues, which accounts for subsequent development of heart failure, a triad of dysfunctions is known as the cardio-renal anemia syndrome[1-3]. The relationship between the anemia-secondary to kidney disease and the heart failure is already well described and accounts for the high morbidity and mortality rates found in CKD patients[4,5].

The use of recombinant human erythropoietin (rhEPO) has emerged in the early 90s as the most appropriate method to control anemia in patients with CRF, and was responsible for a better and longer life in those patients, both from amelioration of anemia and beneficial cardiovascular impact[4-6]. Therefore, EPO has been recognized as a key player in a broad variety of processes in cardiovascular pathophysiology, including apoptosis, cell proliferation, ischaemia and the nitric oxide pathway, which give particular relevant to their use in non-haematological conditions[7]. However, for a significant percentage of patients, rhEPO losses efficacy, becoming resistant, recommending dose increment with further deterioration of heart function, most probably due to the expected hyperviscosity and thromboembolisms[7-10]. Therefore, although enhanced EPO synthesis is viewed as an appropriate compensatory mechanism in the cardio-renal syndrome, excessive EPO synthesis in the advanced stages of both the chronic renal failure and congestive heart failure appears to be predictive of higher mortality[10]. Thus, early rhEPO use, in stages of yet moderate renal failure and low car-

diac deterioration, might have additional benefits. However, this impact remains poorly investigated.

Considering that the use of human tissues is obviously limited by ethical reasons, our group has previously extensively characterized a model of moderate chronic renal failure, which is a good tool to assess the pathological evolution of the triad, as well as to analyze the beneficial impact of early rhEPO use[11]. The model, induced by partial (3/4) nephrectomy, was consistent with a moderate but maintained degree of chronic renal failure (CRF), together with transitory anemia and iron metabolism disturbances. The remnant kidney presented a reasonable degree of functionality, mainly due to hypertrophic compensation, and there was several important cardiovascular modifications, including hypertension, tachycardia, dyslipidemia, erythropoietic disturbances, sympathetic activation, proliferation, angiogenesis and oxidative stress, which are features seen in CKD patients[11,12].

Using our model, we found that rhEPO treatment in the moderate CRF promotes erythrocytosis and prevented tachycardia, catecholamines increment and dyslipidemia, with a rise of serum TGF-β1. Furthermore, the decreased kidney gene expression of EPO and the overexpression of Caspase 9 were prevented, demonstrating a renoprotective action on the remnant kidney[12,13]. Thus, rhEPO therapy has promoted a protective effect on the cardio-renal axis, which might be attributed to its protective actions, namely of pro-proliferative and anti-apoptotic nature, as was previously suggested in other conditions and pathologies[3-6], but that deserves a more extensive evaluation, particularly in moderate stages of CKD, before critical impairment of the kidney and heart.

EPO gene activation, mainly by hypoxic states, is linked with several important pathways, with unequivocal impact on renal and cardiac tissues function and structure. The 3' end of the EPO gene contains an element of response to situations of hypoxia (HRE), which interacts with multiple transcription factors, including hypoxia-induced factor (HIF), a regulator of the genes that are involved in adaptation to alterations in oxygen levels. The HIF will act on several genes, including vascular endothelial growing factor (VEGF) and the glucose transporter GLUT-1[14]. Although the regulation of erythropoiesis occurs mainly in the peritubular cells of the kidneys, 10% of EPO is expressed in extrarenal tissues such as in liver, brain, spleen and lungs[15-17]. Binding of EPO to its receptor (EPO-R) causes a change in intracellular calcium levels, including the formation of IP3 that is able to inhibit the occurrence of apoptosis due to activation of its effector, Akt, an apoptotic blocker involved in proliferation and cell survival[16]. The interaction between the EPO and its receptor also leads to activation of ras/MAPK signaling pathways, by activating nuclear factor kB (NF-kB), with consequent strengthening of cell proliferation[15-17].

Our previous findings concerning a protective role of rhEPO, together with the unequivocal impact of EPO on pivotal signalling pathways for apoptosis, proliferation, angioge-

nesis and inflammation, was the basis for the present study, which was designed to assess the effects of rhEPO on gene expression profile on the kidney and heart in a moderate stage of moderate CRF. We hypothesize that rhEPO under that circumstances of lower tissue lesion/impairment may have a protective effect beyond the correction of anemia, whose mechanism deserve better elucidation by clarifying the impact on gene expression profile of several markers of apoptosis, inflammation, proliferation, angiogenesis and lesion/stress.

MATERIALS AND METHODS

Animals and diets

Male Wistar rats (Charles River Lab. Inc., Barcelona, Spain), weighing ± 275g, were maintained in an air conditioned room, subjected to 12-h dark/light cycles and given standard rat chow (IPM-R20, Letica, Barcelona, Spain) and free access to tap water. Animal experiments were conducted according to the European Communities Council Directives on Animal Care. The rats were divided into 4 groups (7 rats each), during a 15-week protocol: control – without drugs and surgery; rhEPO (beta) – 50 IU/Kg/week s.c. Recormon® (Roche Pharmaceuticals), without surgery; CRF – induced by a two-stage (3/4) nephrectomy: firstly, about half of the left kidney was removed and, one week later, the entire right kidney was removed; CRF+rhEPO – treated with rhEPO after the 3rd week of surgery. All the animals have completed the protocol.

Kidney and heart collection and preparation

The rats were sacrificed by cervical dislocation, after intraperitoneal anesthesia with a 2 mg/kg BW of a 2:1 (v:v) 50 mg/mL ketamine (Ketalar®, Parke-Davis, Lab. Pfeizer Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil®, Rhône-Poulenc Rorer, Lab. Vitória, Amadora, Portugal). The heart and the kidneys were immediately removed, placed in ice-cold Krebs' buffer and carefully cleaned of adherent fat and connective tissue, freezing therefore in RNAlater tubes at –80ºC.

Kidney and heart gene expression analysis

Total RNA isolation: Kidneys and heart were isolated in autopsy and stored in RNA later™ solution (Ambion, Austin, USA). Samples were removed from preservation solution and 1200 µl of RLT Lysis Buffer were added to proceed with disruption and homogenization for 2 minutes at 30Hz using TissueLyser (Qiagen, Hilden, Germany). Tissue lysate were processed according to the protocol from RNeasy® Mini Kit (Qiagen, Hilden, Germany). Total RNA was eluted in 50 µl of RNase-free water (without optional treatment with DNAse). In order to quantify the amount of total RNA extracted and verify RNA integrity (RIN, RNA Integrity Number), samples were analyzed using 6000 Nano Chip® kit, in Agilent 2100 bioanalyzer (Agilent Technologies, Walbronn, Germany) and 2100 expert software, following manufacturer instructions. The yield

from isolation was from 0.5 to 3 µg; RIN values were 6.0-9.0 and purity (A_{260}/A_{280}) was 1.8-2.0.

Reverse Transcription: RNA was reverse transcribed with Super-Script™ III First-Strand Synthesis System for RT-PCR (Invitrogen, California, USA). One microgram of total RNA was mixed with a 2X First--Strand Reaction Mix and a Super-Script™ III Enzyme Mix (Oligo(dT) plus Random hexamers). Reactions were carried out in a thermocycler Gene Amp PCR System 9600 (Perkin Elmer, Norwalk, USA), 10 min at 25ºC, 50 min at 50ºC and 5 min at 85ºC . Reaction products were then digested with 1 µl RNase H for 20 min at 37ºC and, finally, cDNA eluted to a final volume of 100 µl and stored at –20ºC.

Relative quantification of gene expression: Performed using 7900 HT Sequence Detection System (Applied Biosystems, Foster City, USA). A normalization step preceded the gene expression quantification, using geNorm Housekeeping Gene Selection kit for *Rattus norvegicus* (Primer Design, Southampton, UK) and geNorm software (Ghent University Hospital, Center for Medical Genetics, Ghent, Belgium) to select optimal housekeeping genes to this study[18]. Real-time PCR reactions used specific QuantiTect Primer Assays (Qiagen, Hilden, Germany) with optimized primers for TGF-β1 (QT00190953) and PCNA (QT00178647), as a proliferative markers; vascular endothelial growing factor (QT00198954) as a angiogenesis marker; a synthase 2 (inducible) and 3 (constitutive, endothelial) from nitric oxide, NOS2 (QT00186340) and NOS3 (QT01570618) as indicators of endothelial and constitutive enzyme activity; Cytochrome C (QT00366205) as a vascular damage factor; IL-2 (QT00185360), NF-kB (QT01573334) and TNF-α (QT00178717) as inflammatory markers; and at least apoptotic indicators such as caspase 9 (QT00188734), caspase 3 (QT01794429), Bax (QT01081752), Bcl2 (QT00184863), Fas (QT00196595) and Fas ligand (QT00178171). Endogenous controls were also used: GAPDH (QT00199633), ACTB (QT00193473), TOP1 (QT01820861) and RPL13 (QT00178675) together with QuantiTect SYBR Green PCR Kit Gene expression (Qiagen, Hilden, Germany) according to manufacturer's instructions. RT-qPCR reactions were carried out with: 100ng cDNA sample, primers (50-200 nM) and 1X QuantiTect SYBR Green PCR Master Mix. Non template control reactions were performed for each gene, in order to assure no unspecific amplification. Reactions were performed with the following thermal profile: 10 min. at 95ºC plus 40 cycles of 15 seconds at 95ºC and 1 min. at 60ºC. Real-time PCR results were analyzed with SDS 2.1 software (Applied Biosystems, Foster City, USA) and quantification used the $2^{-\Delta\Delta Ct}$ method[19].

Statistical analysis

For statistical analysis, we used the GraphPad Prism, Version 5.0. Results are presented as means ± standard error of means (SEM). Comparisons between groups and between different times of evaluation were performed using two-way ANOVA and the Post´hoc Bonferroni test. Significance was accepted at p less than 0.05.

RESULTS

Effect of rhEPO on kidney and heart EPO and EPO-R mRNA gene expression

We found that rhEPO treatment, per se (rhEPO group), promoted a trend to overexpression of EPO gene in the kidney (Fig. 1A1), with a statistically significant (P<0.05) reduction of EPO-R gene expression (Fig. 1A2). In the cardiac tissue, rhEPO was able to significantly (P<0.05) increase EPO gene expression (Fig. 1B1), together with a trend to higher values of EPO-R (Fig. 1B2), when compared with the control animals (Fig. 1). In the CRF animals, no significant changes were encountered in EPO gene expression in both the kidney and heart tissues (Fig. 1A1 and 1B1, respectively), but a trend to higher values of EPO-R was found in the kidney and statistically significant elevated in the heart (Fig. 1A2 and 1B2), when compared with the control rats. rhEPO treatment in the CRF animals (CRF+rhEPO group) showed a trend to higher values of EPO gene expression in the heart (Fig. 1B1), without further changes when compared with the CRF rats without rhEPO therapy (Fig. 1).

Effect of rhEPO on kidney and heart mRNA gene expression of apoptotic markers

The following proteins were evaluated as markers of the apoptotic machinery: Bax, Bcl2, Fas, FasLg and caspases 3 and 9. Concerning the rhEPO treatment, we found in the kidney tissue a significant (P<0.001) increment in Bax gene expression and a trend to higher expression of

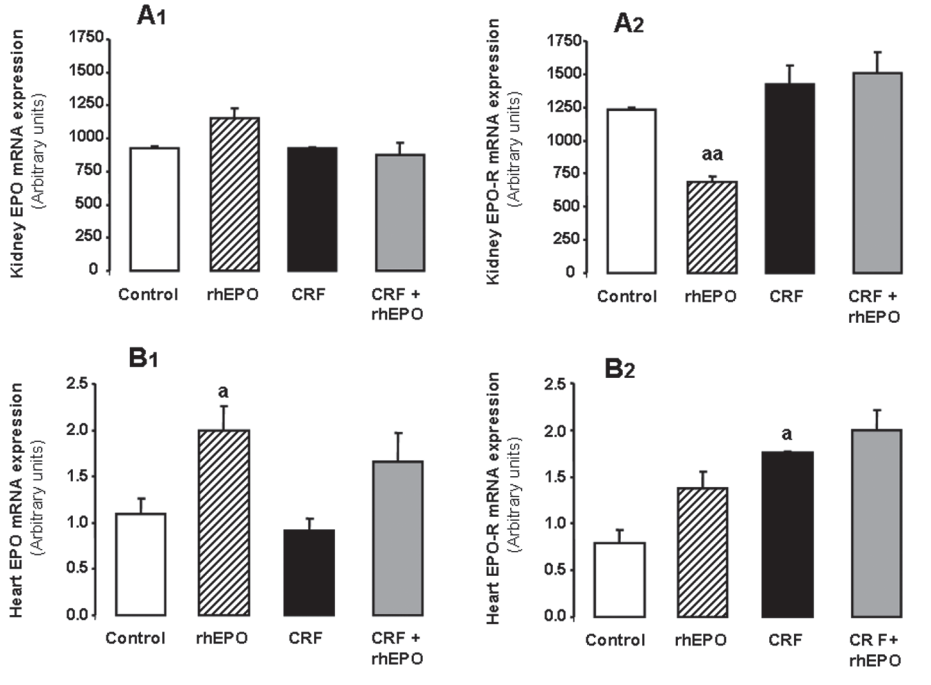

Figure 1 – Kidney (A) and heart (B) mRNA erythropoietin (1) and erythropoietin receptor (2) gene expression for the groups under study, at the final time. Results are means ± SEM (7 rats/group): $^{a}p<0.05$ and $^{aa}p<0.01$ *vs* the control group.

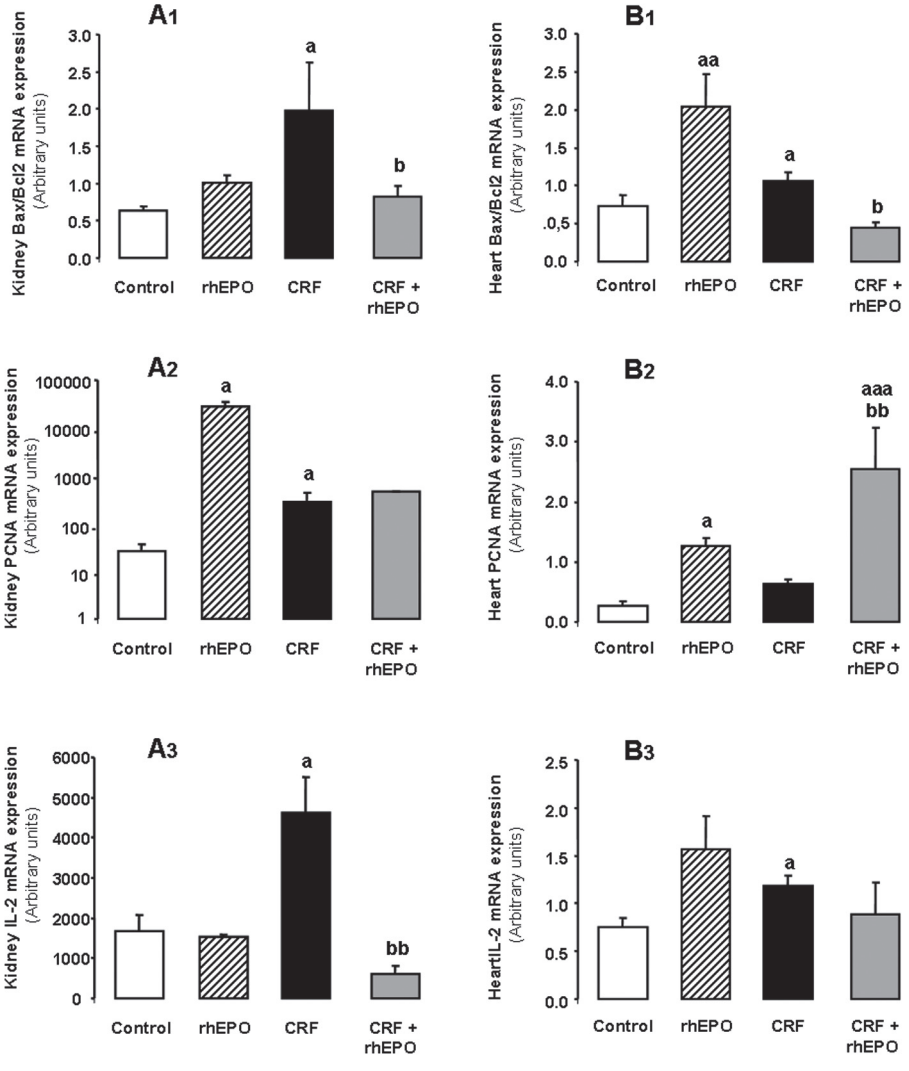

Figure 2 – Kidney (A) and heart (B) mRNA Bax/Bcl2 (1), PCNA (2) and IL-2 (3) gene expression for the groups under study, at the final time. Results are means ± SEM (7 rats/group): $^{a}p<0.05$, $^{aa}p<0.01$ and $^{aaa}p<0.001$ *vs* the control group; $^{b}p<0.05$ and $^{bb}p<0.01$ *vs* the CRF group.

Bcl2; Bax/Bcl2 ratio showed no significant changes when compared with the control (Fig. 2A1). No further changes were encountered in the other markers, including Fas, FasLg and caspases 3 and 9, despite a trend to higher expression of Fas, caspase 3 and 9 and lower of FasLg (Table I). In the heart tissue, however, rhEPO was able to promote a statistically significant increase (P<0.01) in Bax/Bcl2 ratio (Fig. 2B1), as a result of a trend to higher values of the pro-apoptotic protein Bax and lower of the anti-apoptotic Bcl2 (Table II). The other markers of apoptosis in the heart tissue were also unchanged, with again a trend to higher values of caspase 3 and 9 gene expression (Table II).

In the CRF animals, and concerning the kidney tissue, a statistically significant increment (P<0.05) in Bax/Bcl2 ratio (Fig. 2A1) was found, together with no further changes on the other proteins, Fas, FasLg and

Table 1 – Effects of rhEPO treatment on kidney mRNA gene expression of markers of apoptosis, proliferation, angiogenesis, inflammation and lesion/oxidative stress in a rat model of moderate CRF, at the final time (15 weeks)

Renal Parameters	Control (No surgery)		CRF (¾ Nephrectomy)	
Arbitrary units (AU)	**Vehicle**	**rhEPO**	**Vehicle**	**rhEPO**
Apoptosis				
Bax	567.45 ± 85.42	1,481.67 ± 104.93^{aaa}	679.40 ± 64.43	809.88 ± 119.40
Bcl2	897.15 ± 55.45	1.446,70 ± 176.75	400.05 ± 88.59	1,053.43 ± 194.95
Bax/Bcl2	0.63 ± 0.06	1.01 ± 0.11	1.98 ± 0.65^{a}	0.82 ± 0.15
Fas	1,007.55 ± 507,75	1,822.20 ± 171.83	1,626.45 ± 416.01	656.78 ± 329.77
Faslg	2,426.51 ± 707.54^{a}	938.30 ± 148.56	993.45 ± 351.92	1,133.08 ± 288.64
Caspase 3	956.35 ± 213.99	1,194.70 ± 145.64	656.65 ± 273.87	867.23 ± 69.87
Caspase 9	952.25 ± 84.15	1,582.51 ± 171.11	1,131.20 ± 271.82	739.08 ± 75.19
Proliferation and angiogenesis				
TGF-1β	1,130.10 ±102.13	1,335.02 ± 85.42a	790.60 ± 198.84	709.05 ± 86.21
Citocrom c	591.45 ±180.34	2,330.71 ± 177.98^{aaa}	550.95 ± 19.08	473.40 ± 100.32
VEGF	1,025.25 ± 231.95	991.35 ± 135.56	1,108.55 ± 294.71	934.43 ± 155.11
Inflammation/Stress				
TNF-α	1,273.01 ± 19.05	1,247.44 ± 85.94	1,152.05 ± 105.63	656.85 ±166.88^{a}
NF-Kβ	1,066.55 ± 272.19	1,442.20 ± 187.02	882.65 ± 107.59	751.83 ± 117.24
NOS2	696.75 ± 265.73	2,821.01 ± 384.21^{aa}	566.80 ± 99.77	371.18 ± 88.25
NOS3	2,210.50 ± 417.71	456.89 ± 76.88^{aaa}	3,159.01 ± 387.98	1,804.25 ± 183.21^{bb}

Results are means ± SEM (7 rats/group): ^{a} $p<0.05$, ^{aa} $p<0.01$ and ^{aaa} $p<0.001$ *vs* the control group; ^{bb} $p<0.01$ *vs* the CRF group.

caspases 3 and 9 (Table I). In the heart, the same pro-apoptotic profile concerning Bax/Bcl2 was found, with a significant increase (P<0.05) versus the control group (Fig. 2B1), without any other significant effect on Fas, FasLg and caspases mRNA gene expression (Table II).

Concerning the rats under rhEPO treatment with CRF (CRF+rhEPO group), in the kidney tissue there was a prevention of the pro-apoptotic effect promoted by the renal failure, with a statistically significant (P<0.05) reduction of Bax/Bcl2 ratio when compared with the animals without rhEPO therapy (Fig. 2A1). Once again, no further changes were found for the other parameters (Table I). In the heart tissue, a similar anti-apoptotic protection was encountered in Bax/Bcl2 ratio (P<0.05) (Fig. 2B1), due to a significantly increment (P<0.01) on Bcl2 gene expression (Table II).

Effect of rhEPO on kidney and heart mRNA gene expression of proliferative and angiogenic markers

To assess the influence of rhEPO treatment on kidney and heart proliferation and angiogenesis, we evaluated the following proteins: PCNA, TGF-β1, citocrom c and VEGF. Concerning the rhEPO treatment, per se (rhEPO group), we found a remarkable increment in PCNA mRNA

Table 2 – Effects of rhEPO treatment on heart mRNA gene expression of markers of apoptosis, proliferation, angiogenesis, inflammation and lesion/oxidative stress in a rat model of moderate CRF, at the final time (15 weeks)

Cardiac Parameters	Control (No surgery)		CRF (¾ Nephrectomy)	
Arbitrary units (AU)	**Vehicle**	**rhEPO**	**Vehicle**	**rhEPO**
Apoptosis				
Bax	0.89 ± 0.10	1.07 ± 0.08	1.11 ± 0.00	1.26 ± 0.17
Bcl2	1.47 ± 0.25	0.63 ± 0.16	1.09 ± 0.12	2.95 ± 0.39[aa, bb]
Bax/Bcl2	0.73 ± 0.15	2.04 ± 0.43[aa]	1.06 ± 0.12	0.45 ± 0.07
Fas	1.55 ± 0.39	0.96 ± 0.62	2.48 ± 0.11	3.91 ± 0.51
Faslg	0.53 ± 0.11	0.57 ± 0.18	0.73 ± 0.02	1.13 ± 0.42
Caspase 3	0.85 ± 0.07	1.12 ± 0.12	1.23 ± 0.15	1.20 ± 0.08
Caspase 9	1.28 ± 0.26	1.14 ± 0.21	0.66 ± 0.24	0.54 ± 0.04
Proliferation and angiogenesis				
TGF-1β	1.28 ± 0.23	1.43 ± 0.37	0.98 ± 0.31	0.60 ± 0.06
Cytochrome c	1.54 ± 0.29	0.82 ± 0.16	1.24 ± 0.22	0.70 ± 0.05
VEGF	1.15 ± 0.16	1.84 ± 0.67	2.08 ± 0.39	2.39 ± 0.10
Inflammation/Stress				
TNF-α	1.23 ± 0.19	1.46 ± 0.15	1.05 ± 0.31	1.25 ± 0.18
NF-Kβ	0.61 ± 0.09	1.17 ± 0.26	1.16 ± 0.01	1.29 ± 0.15
NOS2	0.62 ± 0.10	1.01 ± 0.19	0.51 ± 0.17	0.83 ± 0.03
NOS3	0.67 ± 0.09	1.12 ± 0.19	1.10 ± 0.19	1.45 ± 0.17

Results are means ± SEM (7 rats/group): [aa] $p<0.01$ *vs* the control group; [bb] $p<0.05$ *vs* the CRF group.

gene expression in both the kidney and the heart ($P<0.05$) (Figs. 2A2 and 2B2, respectively). We should mention that the values in the kidney changed from the control group of 25.91 ± 10.18 AU to 27,933.45 ± 7,251.23 in the rhEPO animals, whcih is an unequivocal increment. rhEPO treatment also promoted a trend to higher values of TGF--β1 gene expression in the kidney, together with statistically significant increment in citocrome c gene expression ($P<0.001$), without unchanges values of VEGF (Table I). In the heart, rhEPO treatment promoted no changes on TGF-β1, citocrome c and VEGF gene expression, despite a trend to higher values of TGF-β1 and lower of citocrome (Table II).

In the CRF animals, and concerning the kidney tissue, we found a notoriously higher value of PCNA (287.22 ±154.27 AU; $P>0.05$) when compared with the control animals (25.91 ± 10.18 AU) (Fig. 2A2). Similar patern was found for the cardiac tissue, with a tend to higher values (0.63 ±0.10 AU) versus the control rats (0.27 ± 0.07 AU) (Fig. 2B2). Concerning the other markers, no changes were encountered in the kidney and in the heart for TGF-β1, citocrome c and VEGF gene expression levels (Table I and II, respectivelly).

In the rats under rhEPO treatment with CRF (CRF+rhEPO group), PCNA gene expression was identical to that found in the kidney of CRF rats

and was even higher (P<0.01) in the heart (Fig. 2A2 and 2B2, respectivelly). No other significant chnages were encountered concerning the other proteins, both in the kidney and in the heart (Table I and II, respectivelly).

Effect of rhEPO on kidney and heart mRNA gene expression of markers of inflammation and lesion/stress

We also assess the influence of rhEPO treatment on kidney and heart IL-2, TNF-α, NF-KB and NOS2 and NOS3 gene expression. Concerning the rhEPO treatment, per se (rhEPO group), we found no significant changes for all the parameters analyzed in the heart tissue, despite a trend to higher expression in all of them (Fig. 2B3 and Table II). Concerning the kidney, rhEPO also promoted no changes on IL-2, TNF-α and NF-KB gene expression, but NOS2 was significantly higher (P<0.01) and NOS3 lower (P<0.001), versus the control group (Table I).

In the CRF animals, in both the kidney and the heart, IL-2 gene expression was higher (P<0.05) that in the control rats (Fig. 2A3 and 2B3, respectively), without no further significant changes on all the other markers (Table I and II, respectively).

In the rats under rhEPO treatment with CRF (CRF+rhEPO group), IL-2 gene overexpression found in the CRF animals was prevented in the kidney (P<0.01) and a trend to identical variation was found in the heart (Fig. 2A3 and 2B3, respectively). Apart from those changes, a trend to lower values was found for the kidney TNF-α gene expression and higher for heart NF-KB, together with a reduction of NOS3 in the kidney (P<0.01) versus the CRF group (Table I and II).

DISCUSSION

The introduction of recombinant human erythropoietin (rhEPO) therapy allowed a significant reduction of anemia-associated adverse effects, allowing for a prolonged life expectance in end-stage renal disease stages[4]. Apart the anemia correction, rhEPO therapy has been associated with positive beneficial effects on non-hematopoietic cells[7-9], which have been attributed to its anti-apoptotic, anti-inflammatory and antioxidant actions, that underlies the cardio and neuro protection in other conditions[20,21]. Functional EPO receptors have been found to be expressed in several tissues, included the cardiovascular system, namely in endothelial cells and cardiomyocytes[22,23] and EPO is a key player in a broad variety of processes in cardiovascular pathophysiology, including apoptosis, cell proliferation, ischaemia and the nitric oxide pathway[7]. An increase in cardiac systolic function has been observed in patients with chronic heart failure treated with EPO. Other beneficial effects appear to be related to the pro-angiogenic properties on endothelial cells and could be useful for treatment of ischemic heart disease[24]. These findings suggest that rhEPO could provide potential therapeutic benefits in the management of cardiovascular diseases beyond anaemia correction. Nevertheless, although enhanced EPO synthesis is viewed as an appropriate compensatory mecha-

nism in the cardio-renal syndrome, excessive EPO synthesis in the advanced stages of both the CRF and CHF appears to be predictive of higher mortality[10]. In this context, earlier rhEPO therapy in anaemic CKD patients might have a positive cardio--renal impact, such as previously reported in ischemic injury, contributing to organ protection/ regeneration[23-26]. However, the impact of early rhEPO use, in stages of yet moderate renal failure and low cardiac deterioration, remains poorly investigated.

In order to further evaluate the effect of rhEPO on the cardio-renal axis, our group has previously characterized a rat model of moderate chronic renal failure induced by partial (3/4) nephrectomy, which demonstrated a moderate but maintained degree of CRF, together with transitory anemia and iron metabolism disturbances[11-13]. The remnant kidney presented a reasonable degree of functionality, mainly due to hypertrophic compensation, and there was several important cardiovascular modifications, including hypertension, tachycardia, dyslipidemia, erythropoietic disturbances, sympathetic activation, proliferation, angiogenesis and oxidative stress, which are features seen in CKD patients[11-13]. The use of rhEPO in that model showed important renal and cardio effects, including prevention of tachycardia, catecholamines increment and dyslipidemia, together with a notorious pro-proliferative action on the remnant kidney and on the heart tissue[12,13]. Considering that EPO interaction with its receptor leads to activation of several important pathways related with apoptosis, proliferation, angiogenesis, inflammation and lesion/stress[15-17,21], we hypothesize that the putative protective effect of early rhEPO use might be linked with adaptations on kidney and heart gene expression.

In our study, rhEPO treatment, per se, without CRF, showed a trend to higher EPO gene expression and a significant downregulation of EPO-R in the kidney, which was accompanied by overexpression of EPO and EPO-R gene in the heart. In the group with CRF, rhEPO treatment did not change EPO and EPO-R expression in the kidney, but a trend to higher values of both genes in the heart was found. This data suggest that rhEPO use, in this circumstances of moderate CRF, might produce a relevant extra--renal impact, namely at the level of cardiac tissue, as previously suggested by us using this model, as well as by others in other conditions[6,27].

Our study also showed that both the kidney and the heart from the rats with CRF presented a pro-apoptotic profile, viewed by the significantly increased ratio of Bax/Bcl2 expression in both tissues. This pattern was accompanied by the absence of other significant changes, including on gene expression of the par Fas-Faslg and on caspases 3 and 9. Recombinant erythropoietin use in these animals promoted prevention of apoptosis in both the remnant kidney and the heart, due to the significant prevention of Bax/Bcl2 increment induced by renal failure. This effect was previously documented for rhEPO use in other situations, in both the kidney[28-31] and in the heart, due to effect on cardiac myocytes[32-34], but was not previously evaluated on a moderate stage of chronic renal failure. A

similar preventive use, by the pleiotropic action, including mechanisms of anti-apoptotic action, was recommended by Bernhardt and Eckardt (2008) as protective against development of acute kidney injury in critically ill patients at higher risk of acute nephropathy[35].

Chronic kidney disease is associated with inflammatory mechanisms and is present in CKD patients, viewed by increment in the synthesis of acute phase proteins, such as C-reactive protein (CRP), a prominent marker of inflammatory response in the general population and in chronic kidney disease (CKD) patients, which was reported elevated in hemodialyzed patients[36-38]. Chronic inflammation is also associated with atherosclerotic cardiovascular disease (CVD) and the high rate of morbidity and mortality observed in haemodialyzed patients[39,40] could reflect the inflammatory process in CKD patients favouring CVD events. rHEPO has been shown a pleiotropic action[41-43], which includes an anti-inflammatory activity already reported on other conditions, including in cardiomyocytes with hypoxia/reoxygenation injury, in the brain, as well as in the kidney[44-48]. In our study, CRF was not associated with significant changes on the inflammatory markers, viewed by normal gene profile of both TNF-α and NF-kβ in both the remnant kidney and in the heart, which is in agreement with our previous assays in serum markers of inflammation[11-13]. However, the IL-2 gene overexpression in both tissues was prevented with rhEPO, suggesting an inhibitory effect on T-cell induced IL-2 production. Previous studies on CKD patients have shown serum IL-2 increment when compared with controls[49,50], an effect which was reduced by rhEPO treatment, demonstrating that rhEPO has an immunomodulatory action. Our data reinforces this notion, demonstrating that the rhEPO effect is due to downregulation of CRF induced IL-2 gene overexpression in the remnant kidney and in the heart on a moderate model of CRF.

Erythropoietin (EPO) is a hormone regulating the proliferation and differentiation of erythroid precursor cells, which have been demonstrating pro-proliferative and pro-angiogenic activity[24,51]. In our model of moderate CRF, kidney and heart gene expression of TGF-β1 and VEGF, which are well-recognized markers of proliferation and angiogenesis, respectively, was unchanged, thus demonstrating that other pathways should play a role on the pro-proliferative profile encountered in this model, previously demonstrated by us in his model by the increment in the trophism of both tissues, as well as by the increased levels of serum TGF-β1[13]. rhEPO treatment, accordingly, did not modify the expression of both genes in those tissues of the CRF rats. However, rhEPO was able to exacerbate the effect of chronic renal failure on PCNA expression in the remnant kidney and in the heart, thus demonstrating a proliferative action associated with DNA synthesis and repair, which are known functions of PCNA[52,53], thus suggesting a regenerative function of rhEPO on both tissues. Similar effect was reported in a model of acute renal failure induced by cisplatin in the rat[54], as well in the liver after resection in the rat[55], but was not previously documented nei-

ther on remnant kidney of moderate chronic renal failure nor on the corresponding heart.

Our study also assessed the influence of rhEPO treatment on kidney and heart gene expression of cytochrome c, viewed as a marker of mitochondrial injury, as well as on the expression of NO synthase 2 and 3, which are the key enzymes in inducible and constitutive NO synthesis and which participate in oxidative stress under unimpaired circumstances. Erythropoietin as been indicated as an antioxidant tissue-protective cytokine[56,57]. In our model of moderate CRF, both cytochrome c and NO synthases gene expression were unchanged in the remnant kidney and in the heart, suggesting that both tissues were in an earlier stage of oxidative lesion. rhEPO treatment, per se, without CRF, showed interesting effects on the kidney, including an increment of NOS2 gene expression, a complementary reduction of NOS3 and a significant augment of cytochrome c. Previous studies showed the influence of rhEPO on vascular nitric oxide synthase expression and activity[58,59], as well as in cytochrome c synthesis[60]. However, rhEPO use in the CRF animals has prevented the trend to increased kidney NOS3 gene expression in the CRF group, without any further effects in the CRF rats for NOS2 or NOS3 in the kidney or the heart, which is in agreement with the absence of effect found by other group in rats with chronic renal failure[61].

In conclusion, in this model of moderate chronic renal failure, rhEPO treatment showed important non-hematopoietic effects, expressed mainly by the anti-apoptotic and the pro-proliferative action on the kidney and on the heart. This data suggest that early rhEPO in moderate stages of CRF, before critical lesion of the tissues, might have further benefits.

Declaration of interest: The authors report no conflict of interest.

REFERENCES

1. Deicher R, Hörl WH. Anaemia as a risk factor for the progression of chronic kidney disease. Curr Opin Nephrol Hypertens. 2003; 12: 139-143.
2. Miranda S, Macedo R, Junior G, *et al.* Cardio-renal syndrome: pathophisiology and treatment. Rev Assoc Med Bras 2009; 55(1): 89-94.
3. van der Meer P, Voors A, Lipsic E, et al. Erythropoietin in cardiovascular disease. Eur Heart J 2004; 25: 285-291.
4. Foley RN, Parfrey PS, Harnett JD, Kent GM, Murray DC, Barre PE. The impact of anaemia on cardiomyopathy morbidity and mortality in end-stage renal disease. Am J Kidney Dis. 1996; 28: 53-61.
5. Locatelli F, Conte F, Marcelli D. The impact of hematocrit levels and erythropoietin treatment on overall and cardiovascular mortality and morbidity – the experience of the Lombardy dialysis registry. Nephrol Dial Transplant 1998; 13: 1642-1644.
6. Latini R, Brines M, Fiordaliso F. Do non-hemopoietic effects of erythropoietin play a beneficial role in heart failure? Heart Fail Rev. 2008; 13: 415-423.
7. Mastromarino V, Volpe M, Musumeci MB, Autore C, Conti E. Erythropoietin and the heart: facts and perspectives. Clin Sci (Lond). 2011; 120(2): 51-63.
8. van der Putten K, Braam B, Jie KE, Gaillard CA. Mechanisms of Disease: erythropoietin resistance in patients with both heart and kidney failure. Nat Clin Pract Nephrol. 2008; 4(1): 47-57.
9. Besarab A, Bolton WK, Browne JK, *et al.* The effects of normal as compared with low hematocrit values in patients with cardiac disease who are receiving hemodialysis and epoetin. N Engl J Med 1998; 339: 584-590.
10. 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): 2161-2167.
11. Garrido P, Reis F, Costa E, Teixeira-Lemos E, Parada B, Alves R, Piloto N, Sereno J, Figueiredo

A, Pinto R, Carvalho L, Rocha-Pereira P, Belo L, Santos-Silva A, Teixeira F. Characterization of a rat model of moderate chronic renal failure--focus on hematological, biochemical, and cardio-renal profiles. Ren Fail. 2009; 31(9): 833-842.
12. Garrido P, Reis F, Costa E, Almeida A, Parada B, Teixeira-Lemos E, Santos P, Alves R, Sereno J, Pinto R, Tavares CA, Figueiredo A, Rocha-Pereira P, Belo L, Santos-Silva A, Teixeira F. Effect of recombinant human erythropoietin in a rat model of moderate chronic renal failure – focus on inflammation, oxidative stress and function/renoprotection. The Open Drug Discovery Journal 2010; 2: 25-32.
13. Teixeira AM, Garrido P, Santos P, Alves R, Parada B, Costa E, Almeida A, Teixeira-Lemos E, Sereno J, Pinto R, Belo L, Santos-Silva A, Teixeira F, Reis F. Recombinant human erythropoietin treatment protects the cardio-renal axis in a model of moderate chronic renal failure. Ren Fail. 2010; 32(9): 1073-1080.
14. Fandrey J. Oxygen-dependent and tissue-specific regulation of erythropoietin gene expression. Am J Physiol Regul Integr Comp Physiol 2004; 286: R977-R988.
15. Jelkmann W. Control of erythropoietin gene expression and its use in medicine. Methods Enzymol. 2007; 435: 179-197.
16. Lappin T. The cellular biology of erythropoietin receptors. Oncologist. 2003; 8 Suppl 1: 15-18.
17. Weidemann A, Johnson RS. Nonrenal regulation of EPO synthesis. Kidney Int. 2009; 75: 682-688.
18. Vandesompele J, De Preter K, Pattyn F, *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002; 3: RESEARCH0034.
19. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- ΔΔCT. Method Methods 2001; 25: 402–408.
20. Bogoyevitch MA. An update on the cardiac effects of erythopoietin cardioprotetion by erythropoietin and the lessons learnt from studies in neuroprotection. Cardiovasc Res. 2004, 63: 208-216.
21. Manolis AS, Tzeis S, Triantafyllou K, *et al.* Erythropoietin in heart failure and other cardiovascular diseases: hematopoietic and pleiotropic effects. Curr Drug Targets Cardiovasc Haematol Disord. 2005; 5: 355-375.
22. Anagnostou A. Erythropoietin receptor mRNA expression in human endothelial cells. Proc Natl Acad Sci USA 1994; 91: 3974-3978.
23. Tramontano AF, Muniyappa R, Black AD, *et al.* Erythropoietin protects cardiac myocytes from hypoxia-induced apoptosis through an Akt-dependent pathway. Biochem Biophys Res Commun 2003; 308: 990-994.
24. Raddino R, Robba D, Caretta G, Bonadei I, Teli M, Zanini G, Madureri A,Vizzardi E, Dei Cas L. [Erythropoietin: a new perspective in cardiovascular therapy]. Monaldi Arch Chest Dis. 2008; 70(4): 206-213.
25. Abdelrahman M, Sharples EJ, McDonald MC, *et al.* Erythropoietin attenuates the tissue injury associated with hemorrhagic shock and myocardial ischemia. Shock 2004; 22: 63-69.
26. Ma R, Xiong N, Huang C, *et al.* Erythropoietin protects PC12 cells from beta-amyloid(25-35)-induced apoptosis via PI3K/Akt signalling pathway. Neuropharmacology 2009; 56: 1027-1034.
27. van der Meer P, van Veldhuisen DJ. [New applications of erythropoietin in cardiovascular disease: from haematopoiesis to cardiac protection]. Ned Tijdschr Geneeskd 2008; 152(16): 923-927.
28. Moriyama MT, Tanaka T, Morita N, Ishii T, Chikazawa I, Suga K, Miyazawa K, Suzuki K. Renal protective effects of erythropoietin on ischemic reperfusion injury. Cell Transplant 2010; 19(6): 713-721.
29. Pallet N, Bouvier N, Legendre C, Beaune P, Thervet E, Choukroun G, Martinez F. Antiapoptotic properties of recombinant human erythropoietin protects against tubular cyclosporine toxicity. Pharmacol Res. 2010; 61(1):71-75.
30. Toba H, Sawai N, Morishita M, Murata S, Yoshida M, Nakashima K, Morita Y, Kobara M, Nakata T. Chronic treatment with recombinant human erythropoietin exerts renoprotective effects beyond hematopoiesis in 2-induced diabetic rat. Eur J Pharmacol 2009; 612(1-3):106-114.
31. Wang W, Zhang J. Protective effect of erythropoietin against aristolochic acid-induced apoptosis in renal tubular epithelial cells. Eur J Pharmacol 2008; 588(2-3):135-140.
32. Timmer SA, De Boer K, Knaapen P, Götte MJ, Van Rossum AC. The potential role of erythropoietin in chronic heart failure: from the correction of anemia to improved perfusion and reduced apoptosis? J Card Fail 2009; 15(4):353-361.
33. Fu P, Arcasoy MO. Erythropoietin protects cardiac myocytes against anthracycline-induced apoptosis. Biochem Biophys Res Commun 2007; 354(2):372-378.
34. Tramontano AF, Muniyappa R, Black AD, Blendea MC, Cohen I, Deng L, Sowers JR, Cutaia MV, El-Sherif N. Erythropoietin protects cardiac myocytes from hypoxia-induced apoptosis through an Akt-dependent pathway. Biochem Biophys Res Commun 2003; 308(4):990-994.
35. Bernhardt WM, Eckardt KU. Physiological basis for the use of erythropoietin in critically ill patients at risk for acute kidney injury. Curr Opin Crit Care 2008; 14(6):621-626.
36. Schindler R, Senf R, Frei U. Influencing the inflammatory response of haemodialysis patients by cytokine elimination using large-pore membranes. Nephrol Dial Transplant 2002; 17:17-19.
37. Caglar K, Peng Y, Pupim LB, *et al.* Inflammatory signals associated with hemodialysis. Kidney Int 2002; 62:1408-1416.
38. Costa E, Rocha S, Rocha-Pereira P, Castro E, Reis F, Teixeira F, Miranda V, Do Sameiro Faria M, Loureiro A, Quintanilha A, Belo L, Santos-Silva A. Cross-talk between inflammation,coagulation/fibrinolysis and vascular access in hemodialysis patients. J Vasc Access 2008; 9(4):248-253.
39. Hung C, Chen Y, Chou C, Yang C. Nutritional and inflammatory markers in the prediction of mortality in Chinese hemodialysis patients. Nephron Clin Pract 2005; 100:c20-6.
40. Wanner C, Zimmermann J, Schwedler S, Metzger T. Inflammation and cardiovascular risk in dialysis patients. Kidney Int 2002; 61 (suppl): S99-102.
41. Chatterjee PK. Pleiotropic renal actions of erythropoietin. Lancet 2005; 365(9474):1890-1892.

42. Gluhovschi G, Gluhovschi C, Bob F, Velciov S, Trandafirescu V, Petrica L, Bozdog G. Multiorgan--protective actions of blockers of the renin--angiotensin system, statins and erythropoietin: common pleiotropic effects in reno-, cardio- and neuroprotection. Acta Clin Belg 2008; 63(3): 152--169.
43. Buemi M, Donato V, Bolignano D. [Erythropoietin: pleiotropic actions]. Recenti Prog Med 2010; 101(6):253-267.
44. Chang YK, Choi DE, Na KR, Lee SJ, Suh KS, Kim SY, Shin YT, Lee KW. Erythropoietin attenuates renal injury in an experimental model of rat unilateral ureteral obstruction via anti--inflammatory and anti-apoptotic effects. J Urol 2009; 181(3):1434-1443.
45. Qin C, Xiao YB, Zhong QJ, Chen L, Wang XF. Anti-inflammatory effect of erythropoietin pretreatment on cardiomyocytes with hypoxia/reoxygenation injury and the possible mechanism. Chin J Traumatol 2008; 11(6):352-358.
46. Yatsiv I, Grigoriadis N, Simeonidou C, Stahel PF, Schmidt OI, Alexandrovitch AG, Tsenter J, Shohami E. Erythropoietin is neuroprotective, improves functional recovery, and reduces neuronal apoptosis and inflammation in a rodent model of experimental closed head injury. FASEB J 2005; 19(12):1701-1703.
47. Aguilera A, Selgas R. Effect of recombinant human erythropoietin on inflammatory status in dialysis patients. Nephrol Dial Transplant 2004; 19(Suppl 5):V46-53.
48. Agnello D, Bigini P, Villa P, Mennini T, Cerami A, Brines ML, Ghezzi P. Erythropoietin exerts an anti-inflammatory effect on the CNS in a model of experimental autoimmune encephalomyelitis. Brain Res 2002; 952(1):128-134.
49. Costa E, Lima M, Alves JM, Rocha S, Rocha--Pereira P, Castro E, Miranda V, do SF, Loureiro A, Quintanilha A, Belo L, Santos-Silva A. Inflammation, T-cell phenotype, and inflammatory cytokines in chronic kidney disease patients under hemodialysis and its relationship to resistance to recombinant human erythropoietin therapy. J Clin Immunol 2008; 28(3):268-275.
50. Debska-Slizień A, Rutkowski B, Manitius J, Zdrojewski Z, Szołkiewicz M, Bułło B, Lizakowski S, Myśliwska J, Myśliwski A, Bryl E, Trzonkowski P, Bakowska A, Rachoń D. Influence of erythropoietin on immunological system of patients with chronic renal failure. Pol Merkur Lekarski 2003; 15(88):326-327.
51. Efthimiadou A, Pagonopoulou O, Lambropoulou M, Papadopoulos N, Nikolettos NK. Erythropoietin enhances angiogenesis in an experimental cyclosporine A-induced nephrotoxicity model in the rat. Clin Exp Pharmacol Physiol 2007; 34(9):866-869.
52. Shivji KK, Kenny MK, Wood RD. Proliferating cell nuclear antigen is required for DNA excision repair. Cell 1992; 69(2):367-374.
53. Essers J, Theil AF, Baldeyron C, van Cappellen WA, Houtsmuller AB, Kanaar R, Vermeulen W (2005). "Nuclear dynamics of PCNA in DNA replication and repair". Mol Cell Biol 25(21):9350--9359.
54. Bagnis C, Beaufils H, Jacquiaud C, Adabra Y, Jouanneau C, Le Nahour G, Jaudon MC, Bourbouze R, Jacobs C, Deray G. Erythropoietin enhances recovery after cisplatin-induced acute renal failure in the rat. Nephrol Dial Transplant 2001; 16(5):932-938.
55. Schmeding M, Boas-Knoop S, Lippert S, Ruehl M, Somasundaram R, Dagdelen T, Neuhaus P, Neumann UP. Erythropoietin promotes hepatic regeneration after extended liver resection in rats. J Gastroenterol Hepatol 2008; 23(7 Pt 1):1125-1131.
56. Johnson DW, Forman C, Vesey DA. Novel renoprotective actions of erythropoietin: new uses for an old hormone. Nephrology (Carlton) 2006; 11(4):306-312.
57. Katavetin P, Tungsanga K, Eiam-Ong S, Nangaku M. Antioxidative effects of erythropoietin. Kidney Int Suppl 2007; 107: S10-15.
58. Kanagy NL, Perrine MF, Cheung DK, Walker BR. Erythropoietin administration in vivo increases vascular nitric oxide synthase expression. J Cardiovasc Pharmacol 2003; 42(4):527-533.
59. Banerjee D, Rodriguez M, Nag M, Adamson JW. Exposure of endothelial cells to recombinant human erythropoietin induces nitric oxide synthase activity. Kidney Int 2000; 57(5):1895-1904.
60. Chong ZZ, Kang JQ, Maiese K. Apaf-1, Bcl-xL, cytochrome c, and caspase-9 form the critical elements for cerebral vascular protection by erythropoietin. J Cereb Blood Flow Metab 2003; 23(3): 320-330.
61. Kim SW, Lee J, Kang DG, Jung K, Kim NH, Suh SP, Choi KC, Kang YJ. Erythropoietin does not affect nitric oxide system in rats with chronic renal failure. J Korean Med Sci 2000; 15(2):183-188.