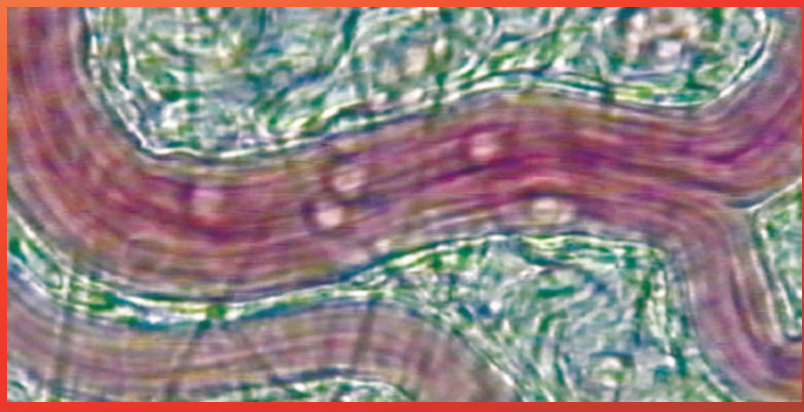
ISSN 2182-6005

publicação semestral
Julho-Dezembro
vol. 32 n.º 2 2017
BSPHM



www.hemorreologia.com

Boletim da Sociedade Portuguesa de Hemorreologia e Microcirculação

Bulletin of the Portuguese Society of Hemorheology and Microcirculation

BOLETIM

Sociedade Portuguesa de Hemorreologia e Microcirculação

Bulletin of the Portuguese Society of Hemorheology and Microcirculation

Vol. 32 n.º 2 Julho-Dezembro 2017

Sumário / Summary

Sociedade Portuguesa de Hemorreologia e Microcirculação

Presidente Honorário: Prof. Doutor João Alcindo Martins e Silva

ÓRGÃOS SOCIAIS DA SPHM / BOARDS (2017-2019)

Direção / Executive Committee

Presidente
Prof.ª Doutora Maria Carlota Saldanha Lopes

Vice-Presidente
Dr. José António Pereira Albino

Secretário-Geral
Prof. Doutor Flávio Reis

Tesoureira
Dr.ª Ana Santos Silva Herdade

Secretários-Adjuntos
Prof.ª Doutora Alice Santos Silva
Dr. Mário Manuel M. G. Marques
Dr. Luís Sargento

Assembleia Geral / General Assembly

Presidente
Prof. Doutor J. M. Braz Nogueira

1.º Secretário
Prof. Doutor Luís Mendes Pedro

2.º Secretário
Prof. Doutor Henrique Sobral do Rosário

1.º Secretário Suplente
Dr.ª Sandra Maria Maurício Hilário Pires

2.º Secretário Suplente
Dr. Paulo Ferreira da Silva

Conselho Fiscal / Finance and Audit Committee

Presidente
Dr. Carlos Manuel dos Santos Moreira

1.º Vogal
Dr.ª Maria Helena Baptista Manso Ribeiro

2.º Vogal
Dr. Paulo Farber

Comissão de Delegados / Committee of Delegates

Delegado da Região Norte
– Dr. Manuel Campos
Delegado da Região Centro
– Dr. João Morais
Delegado da Região Sul e Regiões Autónomas
– Dr. Mário Marques

MEMBROS CONSULTIVOS, HONORÁRIOS E CORRESPONDENTES / / CONSULTIVE, HONORARY AND CORRESPONDENT MEMBERSHIP

Conselho Científico / / Scientific Council

Axel Pries (Alemanha)
David Lominadze (Estados Unidos)
Friedrich Jung (Alemanha)
Gregório Caimi (Itália)
J. Braz Nogueira (Portugal)
J. Fernandes e Fernandes (Portugal)
Jean Frederic Brun (França)
Jerard Nash (Reino Unido)
João Morais (Portugal)
José M. Ferro (Portugal)
Nadia Antonova (Bulgária)
Sayon Roy (Estados Unidos)

Individualidades / / Distinguished Members

A. Diniz da Gama (Portugal)
A. M. Ehrly (Alemanha)
Carlos Ribeiro (Portugal)
Fernando Lacerda Nobre (Portugal)
Helbert J. Meiselman (Estados Unidos)
Helena Saldanha Oliveira (Portugal)
J. Esperança Pina (Portugal)
J.M.G. Toscano Rico (Portugal)
Jean François Stoltz (França)
Joaquim Silva Carvalho (Portugal)
John A. Dormandy (Grã-Bretanha)
John Edward Tooke (Grã-Bretanha)
Luís Providência (Portugal)
LuisTeixeira Diniz (Portugal)
M. Freitas e Costa (Portugal)
Manuel Carrageta (Portugal)
Mário Andreia (Portugal)
Michel Boisseau (França)
Políbio Serra e Silva (Portugal)
Rafael Ferreira (Portugal)
Ricardo Seabra Gomes (Portugal)
Sandro Forconi (Itália)
Sayon Roy (Estados Unidos)
Yukihide Isogai (Japão)

FILIAÇÃO INTERNACIONAL

EUROPEAN SOCIETY FOR CLINICAL HEMORHEOLOGY
EUROPEAN SOCIETY FOR MICROCIRCULATION

Referência da capa: Vénula pós-capilar (diâmetro aproximado: 30 mm) de rede microvascular em mesentério de rato (*Rattus norvegicus*), observada por microscopia intravital de transiluminação. No interior do vaso sanguíneo visualizam-se leucócitos a interagir com a parede vascular. Imagem obtida por Henrique Sobral do Rosário (Instituto de Biopatologia Química – Prof.ª Doutora Carlota Saldanha, Faculdade de Medicina de Lisboa; Unidade de Biopatologia Vascular, Instituto de Medicina Molecular)

Esta publicação foi subsidiada por:
FCT: Fundação para a Ciência e Tecnologia (Ministério da Educação e Ciência – Portugal),
ao abrigo do: **Apoio do Programa Operacional Ciência, Tecnologia, Inovação do Quadro Comunitário de Apoio III.**

O Boletim (ISSN 2182-6005) é publicado semestralmente pela Sociedade Portuguesa de Hemorreologia e Microcirculação. **Depósito Legal** 30 525/89. **Tiragem** 100 exemplares **Distribuição** sócios, sociedades científicas afins, entidades oficiais e privadas de âmbito médico e áreas de educação da ciência. **Preço de cada número avulso:** 5 €, a que acresce 2,5 € para portes de correio. **Editor, Proprietário, Administração e Secretariado:** Sociedade Portuguesa de Hemorreologia e Microcirculação, a/c Instituto de Bioquímica, Faculdade de Medicina da Universidade de Lisboa. **Endereço do Secretariado:** Apartado 4098, 1501-001 Lisboa, Portugal. **Telefone** 217 985 136; Fax: 217 999 447 **Execução Gráfica:** Publicações Ciência e Vida, Lda. **Telef.:** 214 787 850; **Fax:** 214 020 750. **E-mail:** pub@cienciaevida.pt

MICRO INSTRUMENTOS EM HEMORREOLOGIA

MICRO SCALE APPARATUS IN HEMORHEOLOGY

Com o início deste século XXI aumentou na literatura científica o número de estudos de bioquímica, biofísica, biomecânica, de simulação e modelação matemática aplicados às propriedades reológicas do fluxo sanguíneo. Um dos objetivos comum é amplificar o conhecimento para a criação de instrumentos de pequenas dimensões que facultem a quantificação dos parâmetros hemorreológicos. Para além da necessidade em reduzir a quantidade de volume das amostras de sangue, pretende-se que possibilitem a utilização nos laboratórios de análise clínica, nas unidades de cuidados intensivos (UCI) e nos serviços de internamento. Um dos biossensores em uso há vários anos em algumas UCI permite monitorizar a microcirculação sublingual, avaliar parâmetros hemodinâmicos e prever a evolução do prognóstico, por exemplo, de doentes com sepsis.

Também se comercializaram biossensores que permitem acompanhar o desenvolvimento da ação terapêutica em doentes ou com acidentes vasculares cerebrais, ou com anemia hemolítica originada pela drepanocitose ou em portadores de doença infeciosa pela neurotoxina Botulina.

A vantagem da realização dos testes clínicos, em locais adjacentes ao doente acamado, com instrumentação microfluídica, ou tecnologia de pequenos discos portáteis ou de leitura de sinais fornece rapidez à decisão clínica e ao início do tratamento. Acresce em sincronia, o baixo custo a menor complexidade processual e a possibilidade de se juntarem a outro equipamento portátil.

Relativamente à comercialização de instrumentos microfluídicos, para a quantificação dos parâmetros hemorreológicos, muito trabalho é necessário desenvolver, se não vejamos.

O sangue e os elementos figurados nele contidos estão em permanente contacto com a parede vascular, a qual está sujeita a mecanismos de controlo nomeadamente hemodinâmico, miogénico e metabólico. A parede vascular ao separar dois espaços de fluidos corporais, o intravascular e o extravascular, fica na dependência de sinais bioquímicos, biofísicos e biomecânicos. Estes sinais de diversa natureza interferem na adesão ou não dos eritrócitos, leucócitos, plaquetas, lipoproteínas à parede vascular, aos "choques" entre eles, por exemplo, o "empurrão" dos leucócitos para a parede vascular resultante da agregação eritrocitária ou de forças biomecânicas. Há ainda a possibilidade de ocorrer ou extravasão de plasma ou oscilações do equilíbrio da pressão oncótica. A diversidade estrutural do sistema vascular, por exemplo, comprimento, diâmetro, número de ramificações e a variedade de valores dos parâmetros hemodinâmicos influenciam o hematócrito. Este parâmetro hemorreológico é tomado em consideração no trabalho de Joseph Sherwood e colaboradores no qual explicam a influência do comportamento hemorreológico da viscosidade sanguínea na quantificação dos perfis de velocidade do fluxo nas bifurcações de pequenos vasos.

Considerando o número e a diversidade dos estudos de modelação, com múltiplas condições de fluxo sanguíneo de cisalhamento, que afetam a alteração do perfil parabólico da circulação sanguínea, por exemplo oscilações de grande amplitude, presença de bifurcações na rede vascular ou a existência de placas de ateroma, cresce o número de variáveis a dificultar a manufatura de instrumentos de micro escala para a quantificação dos parâmetros hemorreológicos.

Deixo, contrariamente ao habitual, nas referências os títulos para despertar a curiosidade de alguns dos contributos entre os quais, e passando a propaganda do nosso trabalho, por exemplo, a transducção de sinal do monóxido de azoto (NO) no eritrócito.

A complexidade de variáveis a influenciar os parâmetros hemorreológicos é extensa.

Naturalmente que não pretendo que esta pequena narrativa seja pessimista, mas antes alertar para o muito que há a fazer entre os vários centros de investigação multidisciplinar nacionais e internacionais.

Dedico este editorial a Eiichi Fukada e a todos os sócios SPHM e aos que passam pelo nosso site **www.hemorreologia.com**.

Festas felizes e Excelente 2018

Carlota Saldanha
Presidente da SPHM

REFERÊNCIAS

Saldanha C. Human Erythrocyte Acetylcholinesterase in Health and Disease. Molecules. 2017 Sep 8;22(9). pii: E1499. doi: 10.3390/molecules22091499

Saldanha C, Silva-Herdade AS. Physiological properties of human erythrocytes in inflammation. Journal of Cellular Biotech.2017; 3: 15–20.

Saldanha C., Messias A. Sepsis needs follow-up studies in intensive care units-Another avenue for translational research Nov Appro Drug Des Dev (1) NAPDD.MS.ID.555555 (2017).

Sousa P,Pinho FT, Alves MA, Oliveira MSN. A review of hemorheology: measuring techniques and recente Advances. Korea-Australia Rheology J. 1016; 28: 1-22

Saldanha C. Instrumental analysis applied to erythrocyte properties. J Cell Biotech.2015; 1: 81–93.

Sherwood JM, Kaliviotis E, Dusting J, Balabani S. Hematocrit, viscosity and velocity distributions of aggregating and non-aggregating blood in a bi-furcating microchannel. Biomech Model Mechanobiol. 2014; 13:259-73.

HYPOXIA-INDUCIBLE FACTORS PLAY A MAJOR ROLE IN RENAL AND HEPATIC ERYTHROPOIETIN REGULATION IN AN ANIMAL MODEL OF ANEMIA ASSOCIATED WITH CHRONIC KIDNEY DISEASE

João Fernandes[1,2], Sandra Ribeiro[1,2], Patrícia Garrido[2], José Sereno[4], Sara Nunes[2], Elísio Costa[1], Flávio Reis[2,3], Alice Santos-Silva[1*]*

ABSTRACT

Anemia of chronic kidney disease (CKD) is a common clinical problem, primarily due to decreased erythropoietin (EPO) production and/or iron deficiency. Regulation of EPO production is mediated by the hypoxia-inducible factor (HIF) system. Although the pivotal role of HIF-1α and HIF-2α in renal EPO synthesis is documented, the expression patterns and functional properties of these molecules, as well of HIF-3α and aryl hydrocarbon receptor nuclear translocator (ARNT) subunits, remain to be elucidated in CKD. The present study aims to address in vivo changes of mRNA and protein expression of HIF-1α, HIF-2α, HIF-3α, ARNT1 and ARNT2 in CKD, and their correlation with EPO production at both renal and hepatic level, using the remnant kidney rat model of CKD-associated anemia, with and without recombinant human erythropoietin (rHuEPO) treatment. Distinct mRNA levels and protein concentration of all HIF subunits were detectable in liver and kidney, appearing to differ in an organ-specific fashion, as well to depend on rHuEPO treatment. Our data suggest that HIF-2α/ARNT1 dimer play a key role in hepatic EPO production, while in the remnant kidney the HIF-2α/ARNT2 combination seems to be the main responsible. In addition, HIF-3α appears to be positively associated with EPO synthesis in both organs, while HIF-1α overproduction in CKD is most probably related with fibrosis and immune response mechanisms. In this condition of CKD-associated anemia there is an increase expression of EPO mRNA in renal and hepatic tissue, which was further translated in an EPO synthesis rate increase by both organs. Moreover, our results suggest that the mechanisms behind EPO synthesis in CKD differ in an organ-specific fashion, with the distinct isoforms having different contribution. These new insights on the mechanisms responsible by tissue-specific regulation of EPO in CKD may be clinically useful to identify new targets and therapies to stimulate higher levels of EPO, otherwise not achievable by physiological means.

Key words: CKD; anemia; hypoxia-inducible factor isoforms; extra-renal erythropoietin

[1] Research Unit on Applied Molecular Biosciences (UCIBIO), REQUIMTE, Department of Biological Sciences, Laboratory of Biochemistry, Faculty of Pharmacy, University of Porto, Portugal;
[2] Laboratory of Pharmacology & Experimental Therapeutics, Institute for Biomedical Imaging and Life Sciences (IBILI), Faculty of Medicine, University of Coimbra, Portugal;
[3] Center for Neuroscience and Cell Biology – Institute for Biomedical Imaging and Life Sciences (CNC.IBILI) Research Consortium, University of Coimbra, Portugal;
[4] Institute for Nuclear Sciences Applied to Health (ICNAS), University of Coimbra, Portugal.
[*] Equally contributed

Correspondence to:
Flávio Reis
Laboratory of Pharmacology & Experimental Therapeutics,
Institute for Biomedical Imaging and Life Sciences (IBILI),
Faculty of Medicine, University of Coimbra,
3000-548 Coimbra, Portugal.
Tel: +351 239 480 053; Fax: +351 239 480 065
E-mail: freis@fmed.uc.pt

INTRODUCTION

Erythropoietin (EPO), a 30.4-kDa glycoprotein, is a key hormone in the regulation of red blood cell production, in response to changes in tissue oxygenation. EPO induces erythropoiesis through the stimulation of viability, proliferation, and terminal differentiation of erythroid progenitor cells that reside in the bone marrow, thus enhancing the oxygen-carrying capacity of blood. EPO expression is tightly regulated by developmental, tissue-specific, and physiological cues[1,2].

The dominant role of kidneys and liver in EPO production was first reported after a serie of experiments involving organ ablation[3,4]. On those experiments with adult animals, the increase in serum EPO levels, in response to stimulation, was severely attenuated after bilateral nephrectomy. In addition, the remaining EPO production was completely hampered when nephrectomy was combined with subtotal hepatectomy[5]. Although primary EPO production switches from liver to kidney after the birth, the liver maintains the capacity to express EPO. Hepatocytes are the primary cell type responsible for EPO expression in the liver[6]. During normal erythropoiesis, the ratio between kidney and liver EPO levels in the adult is 9:1[7]. However, under conditions of severe reduced oxygen concentrations (hypoxia), it has been estimated that liver EPO production may account for more than 33% of total EPO[8].

On the molecular level, adaptation to hypoxia depends on the activation of the hypoxia-inducible factors (HIFs), which enables critical processes such as glycolysis, angiogenesis and erythropoiesis[9]. HIFs are heterodimeric transcription factors consisting of an oxygen-sensitive α-subunit and a constitutive β-subunit, also known as ARNT (aryl-hydrocarbon-nuclear-translocator). Upon hypoxia, the α-subunit becomes stabilized, translocates to the nucleus where it dimerizes with the β-subunit and stimulates HIF target genes expression. Under normoxia, HIF is hydroxylated by specific prolyl hydroxylases (PHDs) within its α subunits. Prolyl hydroxylation promotes interaction with the von Hippel–Lindau protein E3 ubiquitin ligase complex and proteolytic inactivation by proteasomal degradation, while asparaginyl hydroxylation by factor inhibiting HIF (FIH) blocks co-activator recruitment, preventing transcription[10-13].

To date, in mammals, three genes have been shown to encode HIF-α subunits – HIF-1α, which seems to be expressed in almost all cell types; HIF-2α (also known as HIF-1-like factor or endothelial PAS domain protein), which displays a more tissue- and cell-specific expression pattern; and HIF-3α with a less clear role with respect to oxygen-regulated gene expression[11,14]. The role of each HIF isomer in the hypoxic induction of *EPO* remains controversial. Although HIF-1α is ubiquitously expressed, the expression pattern of HIF-1α and HIF-2α in the kidney appears to be specific[15]. Studies using murine models with inducible knockouts of either HIF-1α or HIF-2α and PHD inhibition studies in rats have underscored the importance of HIF-2α for renal and hepatic EPO expression in rodents[16,17]. In human hepatoma and neuroblastoma cell lines (Hep3B and Kelly, respectively), a predominant role of HIF-2α in the regulation of EPO was proposed using siRNA approaches[15]. Furthermore, using a Cre-Lox recombination to ablate renal HIF-2α, Kapitsinou *et al.* showed that HIF-2α takes over as the main regulator of the serum EPO level[18]. In contrast, in human renal allograft biopsies and in the developing kidney, HIF-1α was predominantly detected by immunohistochemistry[19,20].

Although major advances have been achieved during the last years regarding HIF system physiology and pathophysiology, in distinct conditions[21-24], some of the molecular details and the regulatory mechanisms remain almost completely unknown in some conditions. Knowledge on how HIF and ARNT different isoforms shape EPO production in different renal pathologies may be clinically useful to identify new targets and therapies to stimulate higher levels of EPO, otherwise not achievable by physiological means. Chronic kidney disease (CKD) is a slowly progressive decline in the kidneys' ability to filter metabolic waste from the blood[25]. In addition, kidneys play a major role in multiple metabolic and endocrine functions, such as controlling erythropoiesis, which may also become seriously impaired. Therefore, anemia usually develops during the course of CKD and is probably the source of several of its adverse outcomes[26]. This may be the result of a number of factors

affecting CKD patients – functional or absolute iron deficiency, blood loss, presence of uremic inhibitors, reduced half-life of circulating blood cells, deficiencies of folate or vitamin B12 and endogenous EPO resistance[27-30]; however, inappropriate synthesis of EPO, with serum levels of EPO being disproportionately low for the degree of anemia seems to be the primary cause of anemia in CKD[31,32]. Numerous studies reported the association of anemia on CKD patients with several changes in the structure and function of the kidney; yet, until now, there has been no unequivocal information on the contribution of HIF different subunits on CKD.

To specifically dissect the role of the three HIF-α subunits on renal and hepatic EPO expression in CKD, we have used the remnant kidney rat model of CKD-associated anemia induced by a two stage 5/6 nephrectomy, and focus on the evaluation of gene and protein expression of different HIF isoforms upon kidney and liver tissue. We demonstrate that distinct mRNA and protein levels of all HIF subunits are detectable in the liver and kidney, appearing to differ in an organ-specific fashion, as well to depend on rHuEPO treatment.

MATERIALS AND METHODS

Animals and experimental protocol

Male Wistar rats (Charles River Lab. Inc., Barcelona, Spain), 280-320g, were maintained in an air conditioned room, subjected to 12-h dark/light cycles and given standard laboratory rat diet (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 and to the National Authorities, including the Local Ethics Committee.

The rats were divided into 3 groups (8 rats each): a Sham group (rats submitted to surgery without kidney mass reduction), treated with saline solution; a severe CKD group (submitted to a 5/6 nephrectomy), treated with saline solution; a severe CKD group, under rHuEPO (400 IU/Kg/week, s.c, Recormon®, Roche Pharmaceuticals) treatment. All the animals have completed the 6-week protocol.

Sample collection and preparation

Blood: blood samples were taken from all rats at the beginning of the experimental protocol (T0), and at the end of the 4th and 6th weeks (T1 and T2, respectively). First, rats were subjected to 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), afterwards blood samples were collected by venipuncture from the jugular vein into vacutainer tubes without anticoagulant (to obtain serum) or with EDTA as anticoagulant (for hematological studies).

Tissues: the rats were sacrificed by cervical dislocation and the liver and kidneys (the remnant kidney in the case of nephrectomized animals) were immediately removed, placed in ice-cold Krebs' buffer and carefully cleaned.

Biochemical and hematological assays

Renal function: serum creatinine and urea levels were assessed using the Roche/Hitachi 717 analyzer (Roche Diagnostics Inc., MA, USA).

Hematological data: red blood cell (RBC), hematocrit (HTC) and hemoglobin (Hb) concentration were assessed in whole blood EDTA by using an automatic Coulter Counter® (Beckman Coulter Inc., USA, CA). Reticulocytes were evaluated via light microscope evaluation of blood smears stained with New methylene blue.

Iron metabolism: serum iron concentration was determined using a colorimetric method (Iron, Randox Laboratories Ltd., North Ireland, UK), whereas serum ferritin and transferrin were measured by immunoturbidimetry (Laboratories Ltd., North Ireland, UK).

Inflammatory and angiogenesis markers: serum levels of high-sensitive C-reactive protein (hs-CRP) were determined by using a rat-specific Elisa kit from Alpha Diagonostic International (San Antonio, USA). Vascular endothelial growth factor (VEGF) levels were measured by using a rat-specific Quantikine ELISA kit from R&D Systems (Minneapolis, USA).

Quantitative real-time PCR (qRT-PCR)

In order to isolate total RNA, 0.2 g of liver and kidney samples, from each rat, were immersed in RNA laterTM (Ambion, Austin, USA) upon collection and stored at 4 °C, for 24h; afterwards, samples were frozen at -80.ºC. Subsequently, tissue samples weighing 50±10 mg were homogenized in a total volume of 1 ml TRI® Reagent using a homogenizer, and total RNA was isolated according to manufacturer instructions (Sigma, Sintra, Portugal). To ensure inactivation of contaminating RNAses, metal objects and glassware were cleaned with detergent, immersed in RNAse-free water (0.2% diethyl pyrocarbonate) for 2 h and finally heated at 120.ºC for 1 h. RNA integrity (RIN, RNA Integrity Number) was analyzed using 6000 Nano ChipW 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 1.5 µg; RIN values were 7.8–9.0 and purity (A260/A280) was 1.8–2.0. The concentration of the RNA preparations was confirmed with NanoDrop1000 (ThermoScientific,Wilmington, DE, USA). Possible contaminating remnants of genomic DNA were eliminated by treating these preparations with deoxyribonuclease I (amplification grade) prior to qPCR amplification. Reverse transcription and relative quantification of gene expression were performed as previously described[33]. qPCR reactions were performed for the following genes: *EPO*, *HIF-1α, HIF-2α, HIF-3α, ARNT1* and *ARNT2*, which were normalized in relation to the expression of beta-actin (*Actb*), and 18S ribosomal subunit (*18S*). Results were analyzed with SDS 2.1 software (Applied Biosystems, Foster City, CA, USA) and relative quantification calculated using the $2^{\Delta\Delta Ct}$ method[34].

Hypoxia markers

Enzyme-linked immunosorbent assay: serum, renal and hepatic levels of HIF-1α, HIF-2α, HIF-3α, ARNT1, ARNT2 and EPO were measured by rat-specific Quantikine ELISA kits from MyBioSource (San Diego, USA).

Immunohistochemistry: sections of paraformaldehyde fixed kidney and liver tissues (3 to 4 µm thick) were processed by indirect immune detection technique with mouse and rabbit specific horseradish peroxidase/3,3-diaminobenzidine (HRP/DAB) detection immunohistochemistry (IHC) kit (Abcam, Cambridge, UK) using primary antibodies specific to HIF-1α (1:200), HIF-2α (1:500), HIF-3α (1:250), ARNT1 (1:250), ARNT2 (1:250) and EPO (1:250) from Santa Cruz Biotechnology (exception for HIF-3α, which was purchased from Abcam). The protocol was executed according to the manufacturer's instructions. The negative controls were obtained by omitting the primary antibody. Slides were partially counterstained with hematoxylin. Signals were analyzed with a Nikon Eclipse Ci microscope (Melville, NY, USA). Photographs were digitally recorded by means of a Nikon digital Sight DS-Fi1 (Melville, NY, USA). A staining score (Quick Score) was then calculated according to previously describe[35], using the formula: Quick Score = intensity (1, 2 or 3) multiplied by area (percentage). The final score (out of maximum of 300) for each group was obtained averaging the individual scores of each animal. All slides were reviewed independently by 2 investigators blinded to the data.

Statistical analysis

For statistical analysis, we used the Statistical Package for Social Sciences (SPSS), version 22.0. Results are presented as mean ± standard deviation. Multiple comparisons between groups were performed by one-way ANOVA supplemented with Turkey's HSD post-hoc test. For single comparisons, we used the Mann–Whitney U-test. Significance was accepted for a p minor than 0.05.

RESULTS

Renal function

Serum creatinine and urea levels observed in the Sham group at T2 (0.45±0.11 and 22.64±2.58 mg/dL, respectively) were significantly lower (p<0.005) than those found in the CKD (1.00±0.19 and 54.10±9.59

mg/dL) and CKD+rHuEPO (1.01±0.18 and 56.89±6.98 mg/dL) groups (Fig. 1A and 1B). These results are indicative of under-excretion, clearly showing kidney impairment in both nephrectomized groups.

Anemic status

In a CKD patient the chance of developing anemia increases as kidney function gets worse. The anemia becomes more severe as the glomerular filtration rate progressively decreases. Criteria used to assess anemia and its causes include Hb, HTC and RET levels assessment, as well iron stores (as measured directly by bone marrow biopsy, or indirectly as measured by serum ferritin, transferrin saturation levels). Alterations along the protocol in RBC counts, Hb concentration, HTC and in RET numbers are depicted in Fig. 1. The nephrectomy led to a significant decrease in RBCs (7.40±0.20 vs 6.10±0.31 10^{12}/L), HTC (41.10±1.58 vs. 32.10±1.56 %) and Hb (13.60±0.44 vs. 12.20±0.57 g/dL) levels (T0 *vs*. T1, $p<0.05$), which maintained nearly constant until the end of the protocol (T2) (Fig. 1C, 1D and 1E). Such decline in these erythrocyte markers is consistent with an ane-

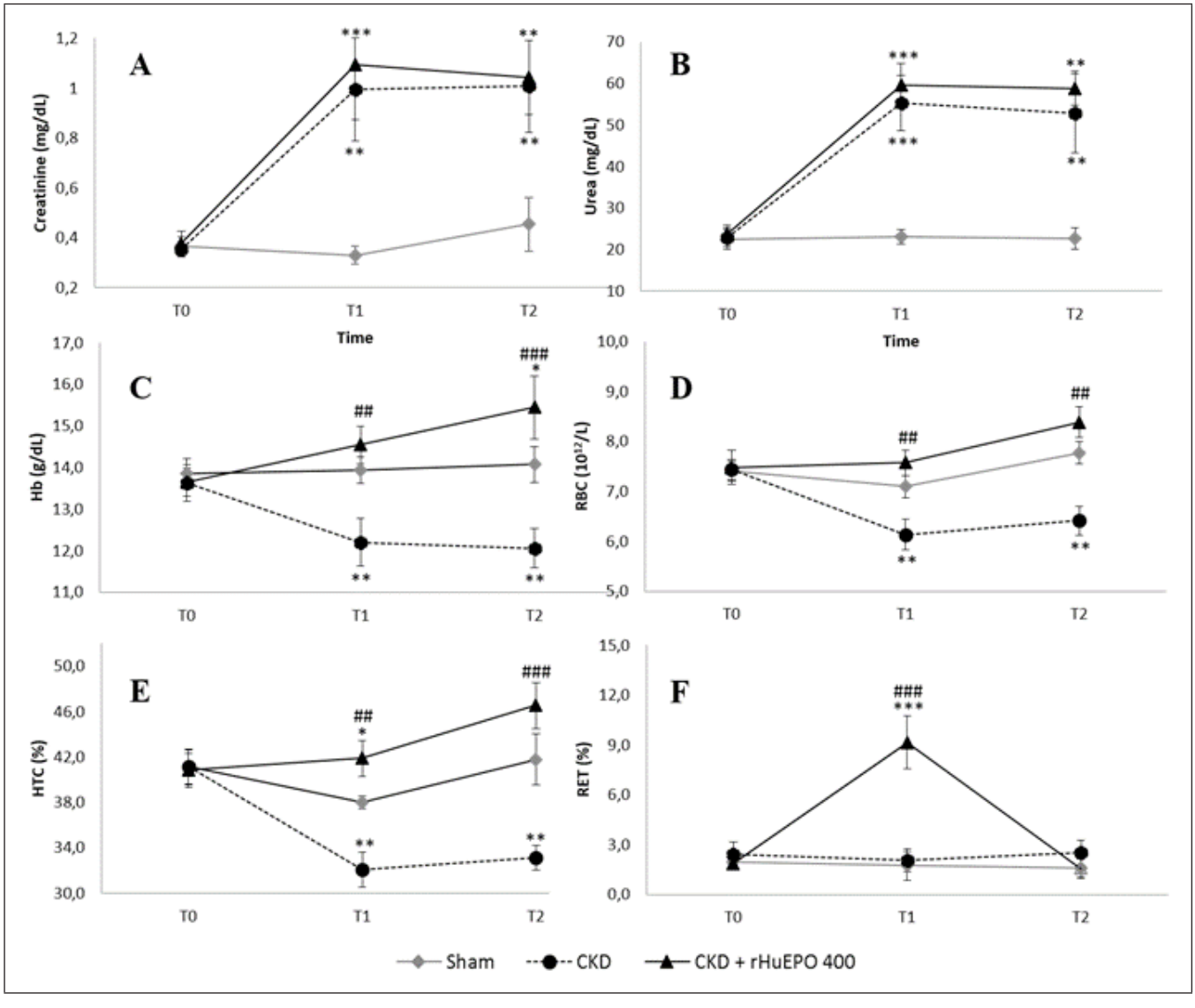


Figura 2. Renal and hematological data. Evolution of creatinine (A), urea (B), hemoglobin (C), red blood cells (D), hematocrit (E) and reticulocytes (F) values throughout the experimental protocol. Results are presented as mean ± SD: *- $p < 0.05$, **- $p < 0.01$, and ***- $p < 0.001$ *versus* Sham group; #- $p < 0.05$, ##- $p < 0.01$, and ###- $p < 0.001$ *versus* CKD group

Table 1. Iron metabolism status markers from the three groups under study

	Iron (µg/dL)	Ferritin (ng/mL)	Transferrin (mg/dL)
Sham	147.50 ± 18.21	156.70 ± 33.28	389.96 ± 41.70
CKD	151.86 ± 18.34	156.08 ± 28.06	375.47 ± 47.78
CKD+rHuEPO	142.75 ± 27.71	158.49 ± 31.08	274.82 ± 45.68

Results are means ± SD.

mic status. On the contrary, the CKD+rHuEPO group at T1 showed a slight increase in the same parameters – RBCs (7.50±0.34 vs. 7.60±0.25 10^{12}/L), HTC (40.80±1.51 vs. 45.58±1.61 %) and Hb (13.70±0.34 vs. 14.60±0.45 g/dL), accompanied by a boost in RET levels (1.70±0.42 vs. 9.20±1.60 %) (p<0.001) (Fig. 1C-1F). This boost in RET levels was completely abrogated at T2 (1.50±0.74 %), falling to values si-

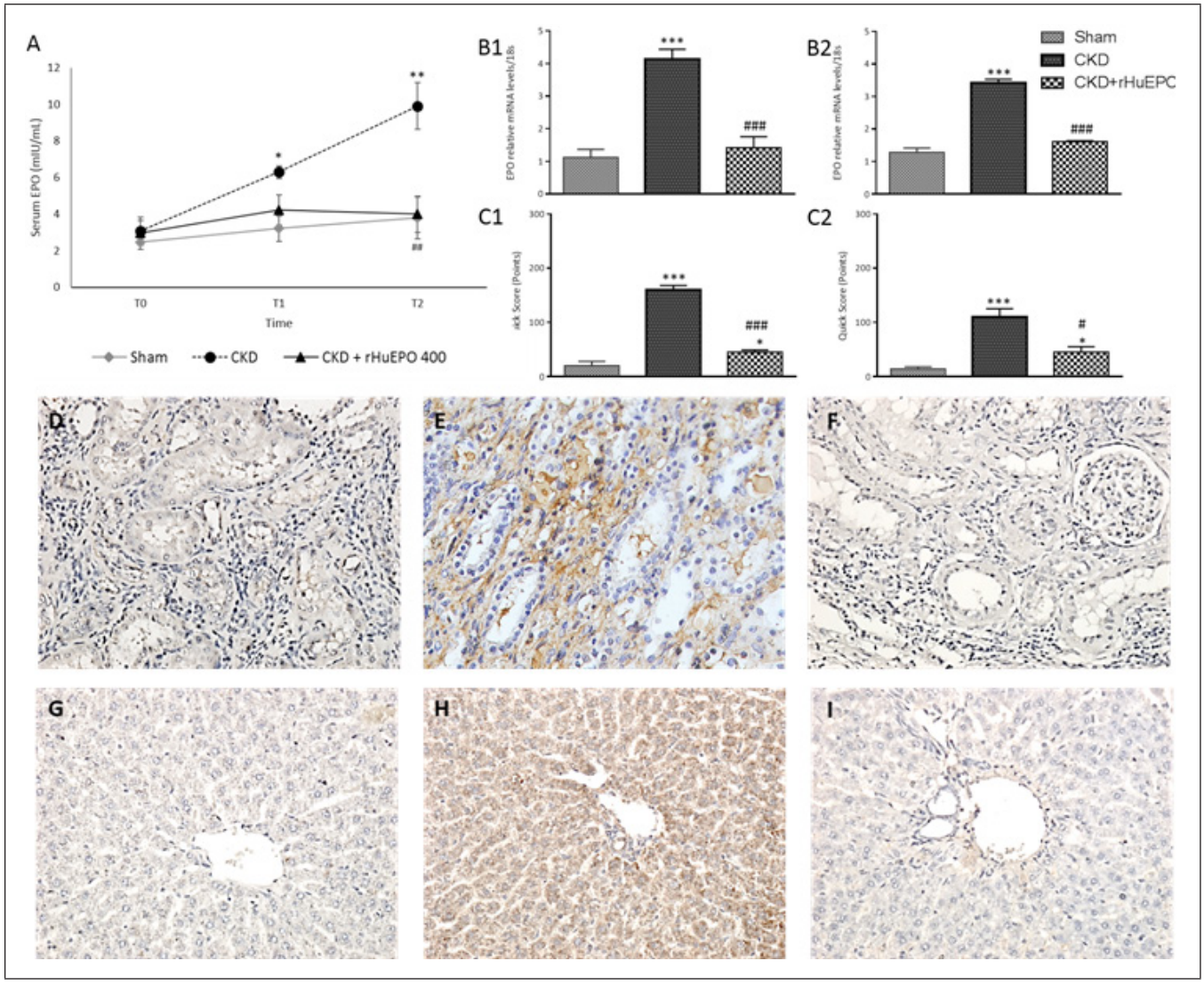


Figura 2. EPO analysis in serum and renal and hepatic tissue**.** Serum EPO (A), *EPO* mRNA levels in kidney (B1) and liver (B2) tissues; kidney (C1) and liver (C2) EPO immunohistochemical quick scores and corresponding images of EPO expression in the kidney (D – sham, E – CKD, and F – CKD+rHuEPO groups) and in the liver (G – sham, H – CKD, and I –CKD+rHuEPO groups). Results are presented as mean ± SD: *- $p < 0.05$, **- $p < 0.01$, and ***- $p < 0.001$ *versus* Sham group; #- $p < 0.05$, ##- $p < 0.01$, and ###- $p < 0.001$ *versus* CKD group.

milar to those detected in the Sham group (1.60±0.54). On the other hand, Hb, HTC and RBCs values continued to increase throughout the protocol. Iron metabolism markers did not differ significantly between the three groups under test (Table 1), suggesting that a deficient EPO synthesis may be the main responsible by CKD group anemic condition. In addition to hematological and iron metabolism data, hs-CRP analysis revealed that the inflammation status remained almost unchanged in all the groups under test, while VEGF levels disclosed a trend, yet not statistically significant, to increase upon the nephrectomized groups (Sham = 274.60±42.99 pg/mL; CKD = 337.93±38.92 pg/mL; CKD+rHuEPO = 303.17±15.51 pg/mL).

Effect of CKD-associated anemia on EPO levels (protein and mRNA levels)

When anemia and other complications associated to low oxygen levels occur, the healthy kidney cells are able to increase the EPO synthesis rate so that hypoxia can be remitted spontaneously. However, once the kidneys are severely damaged, EPO production becomes insufficient allowing anemia to easily progress. Serum EPO levels along the entire protocol are depicted in Fig. 2A. EPO levels were maintained relatively stable within the Sham group, while in the CKD group we verified a continuous increase of EPO concentration with time (T0 = 3.08±0.55; T1 = 6.29±0.35; and, T2 = 9.91±0.45 mIU/mL). CKD+rHuEPO group showed a small, yet not significant, increase in EPO levels after

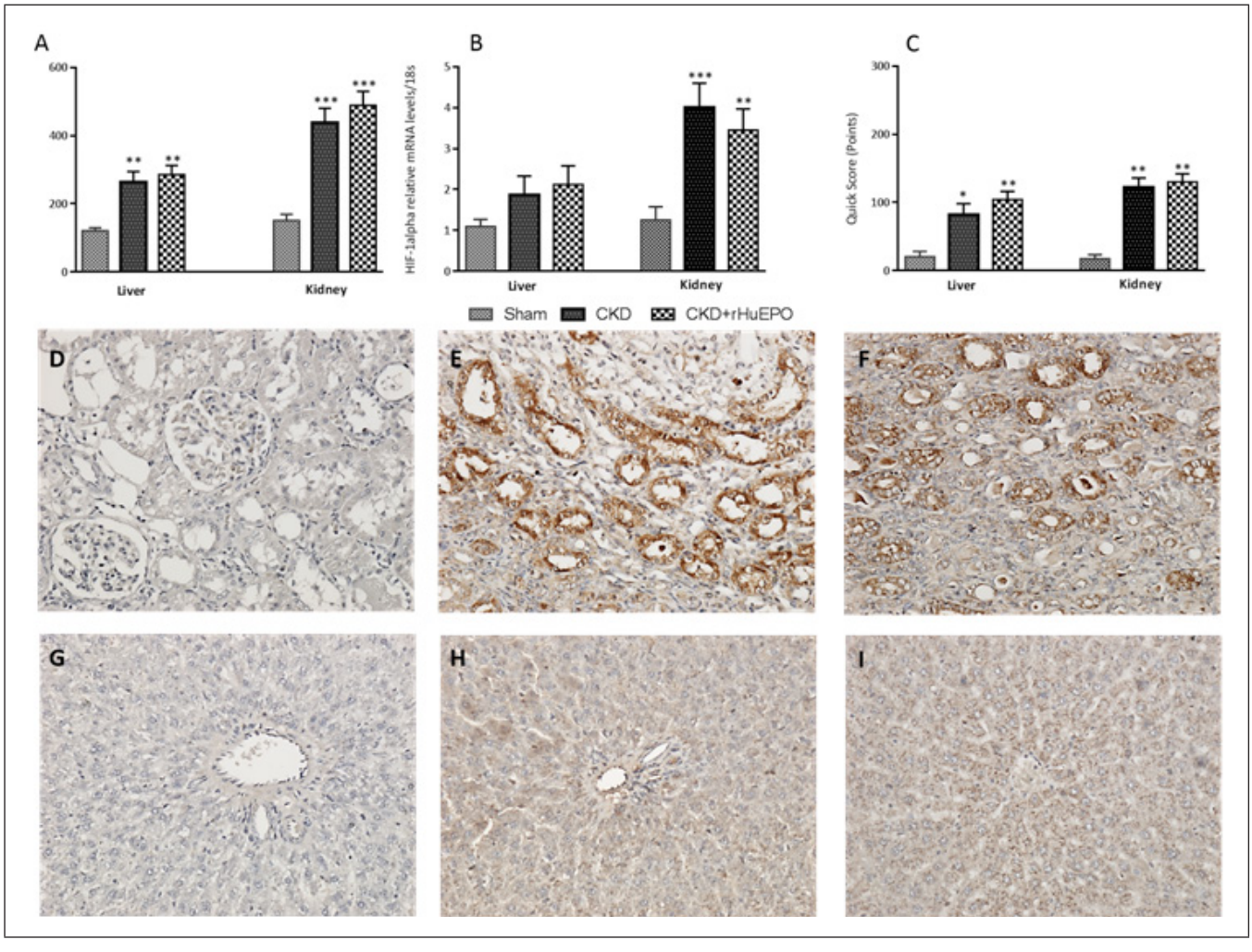


Figura 3. HIF-1α analysis in renal and hepatic tissue. Hepatic and renal HIF-1α concentration (A) and mRNA levels (B); kidney and liver (C) HIF-1α immunohistochemical quick scores and corresponding images of HIF-1α expression in the kidney (D – sham, E – CKD, and F – CKD+rHuEPO groups) and in the liver (G – sham, H – CKD, and I –CKD+rHuEPO groups). Results are presented as mean ± SD: *- $p < 0.05$, **- $p < 0.01$, and ***- $p < 0.001$ *versus* Sham group; #- $p < 0.05$, ##- $p < 0.01$, and ###- $p < 0.001$ *versus* CKD group.

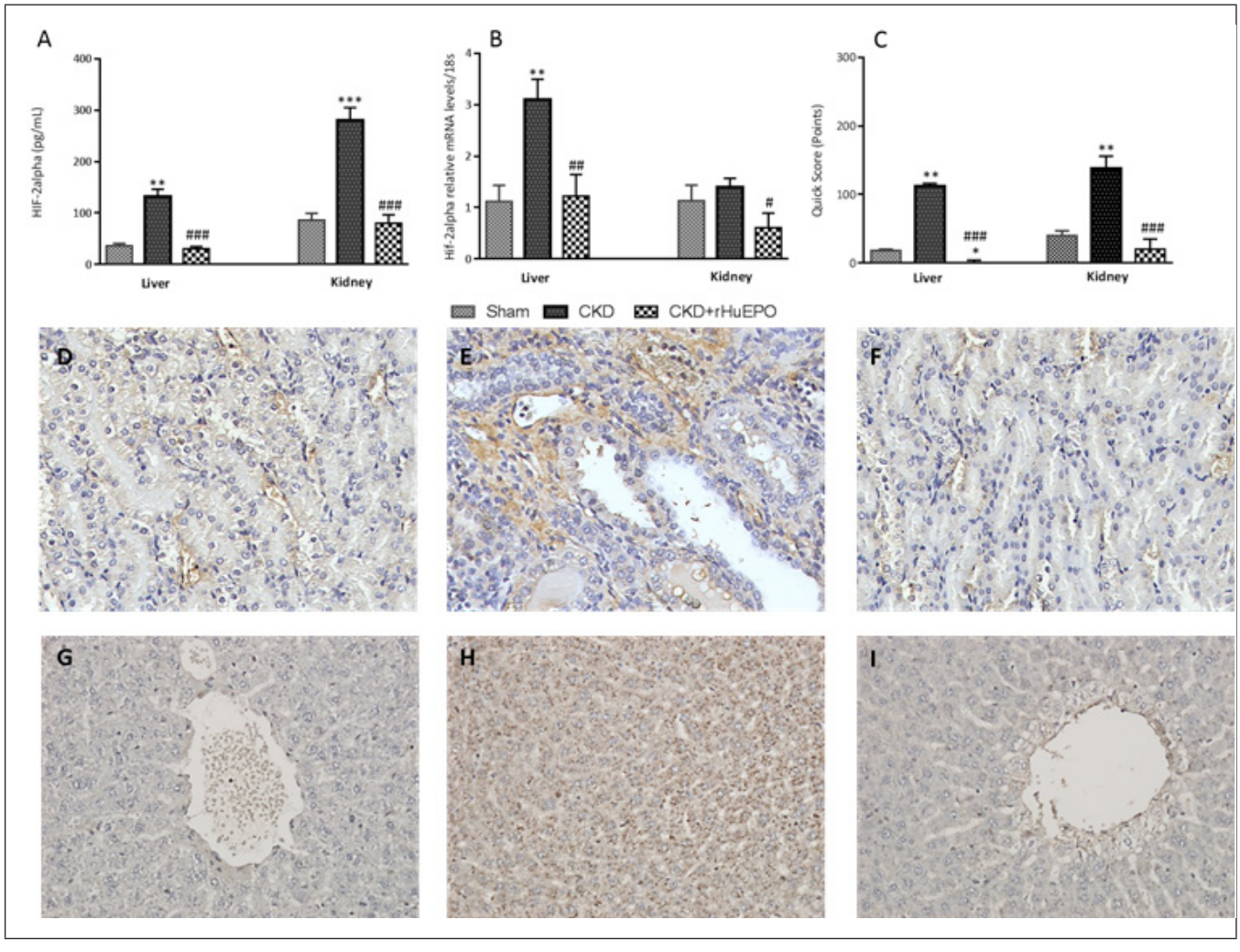


Figura 4. HIF-2α analysis in renal and hepatic tissue. Hepatic and renal HIF-2α concentration (A) and mRNA levels (B); kidney and liver (C) HIF-2α immunohistochemical quick scores and corresponding images of HIF-2α expression in the kidney (D – sham, E – CKD, and F – CKD+rHuEPO groups) and in the liver (G – sham, H – CKD, and I –CKD+rHuEPO groups). Results are presented as mean ± SD: *- $p < 0.05$, **- $p < 0.01$, and ***- $p < 0.001$ *versus* Sham group; #- $p < 0.05$, ##- $p < 0.01$, and ###- $p < 0.001$ *versus* CKD group.

the nephrectomy procedure – T0 (2.96±0.89) and T1 (4.23±0.81). Nevertheless, at T2, EPO levels returned to values similar to those from the Sham group (3.99±0.98), thus significantly lower than those observed in the CKD group. In agreement with the reported circulating levels of EPO, we found that renal and hepatic EPO mRNA abundance significantly increased in CKD group (4.14±0.29 and 3.42±0.11, respectively), while in the CKD+rHuEPO group mRNA levels remained almost unchanged (1.41±0.33 and 1.51±0.03, respectively) (Fig. 2B). These results were confirmed by immunohistochemistry, which revealed the presence of higher levels of this hormone in CKD hepatic (in the hepatocytes) and renal (in the peritubular interstitial fibroblast-like cells, as well in the peritubular endothelial cells, from the deep cortex and in the outer medulla) tissues than those seen in the Sham and CKD+rHuEPO groups (Fig. 2C-I).

Expression of HIF subunits and related transcription factors genes

We speculated that the changes observed in hepatic and renal EPO synthesis might be mediated via different HIF subunits, which may correspond to different adaptive mechanisms by these organs against hypoxia. To determine which HIF subunits were stimulated in CKD, we performed qPCR in renal and hepatic tissues from the three groups (Fig. 3 to 5).

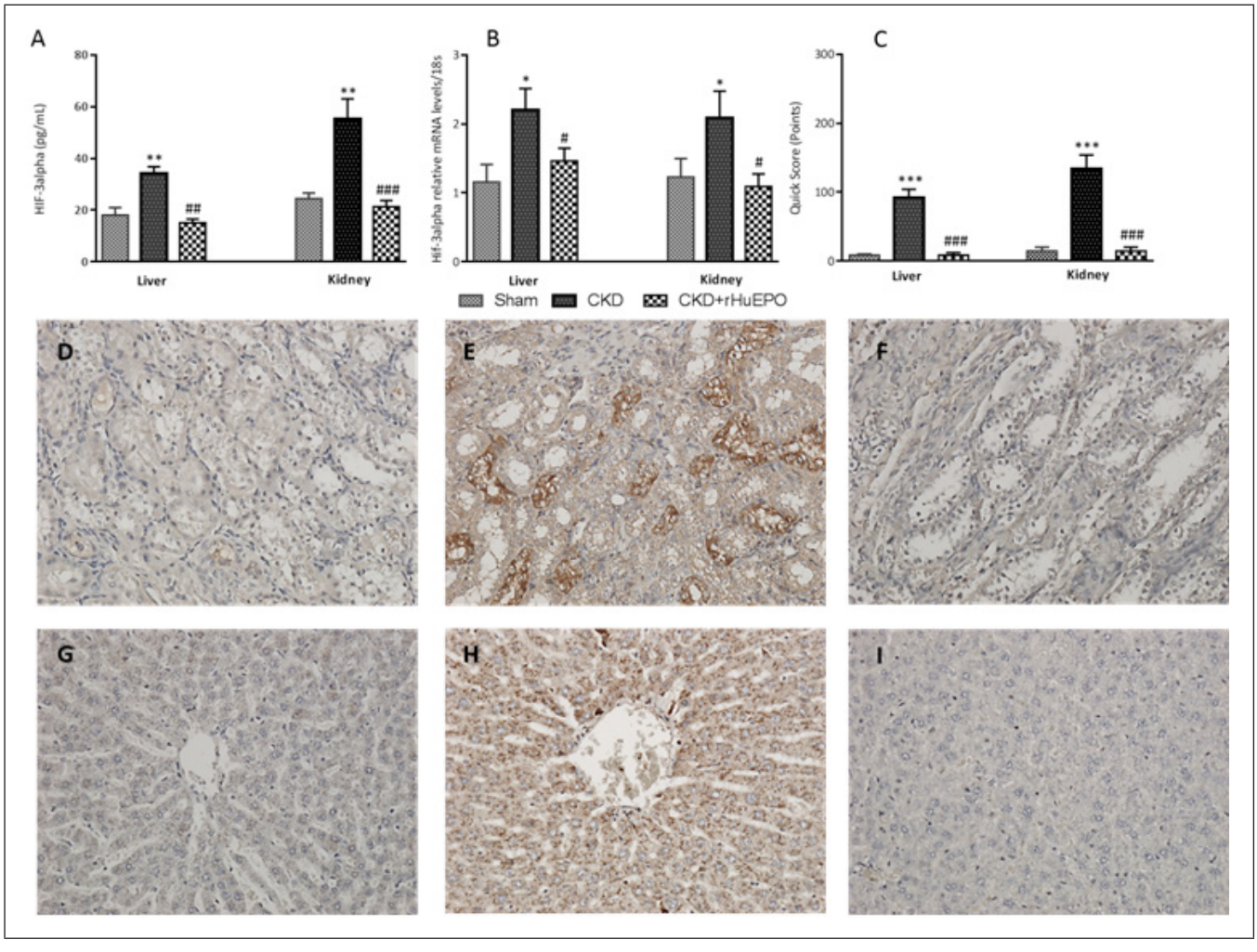


Figura 5. HIF-3α analysis in renal and hepatic tissue. Hepatic and renal HIF-3α concentration (A) and mRNA levels (B); kidney and liver (C) HIF-3α immunohistochemical quick scores and corresponding images of HIF-3α expression in the kidney (D – sham, E – CKD, and F – CKD+rHuEPO groups) and in the liver (G – sham, H – CKD, and I –CKD+rHuEPO groups). Results are presented as mean ± SD: *- $p < 0.05$, **- $p < 0.01$, and ***- $p < 0.001$ *versus* Sham group; #- $p < 0.05$, ##- $p < 0.01$, and ###- $p < 0.001$ *versus* CKD group.

The results reveal major differences among the three groups under test, as well as between the tissues. Regarding the kidney, the CKD group showed an increased expression of genes responsible by all the three HIF subunits – 1α (4.01±0.59), 2α (1.39±0.17, not significant) and 3α (2.09±0.38), while only 1α subunit was increased on CKD+rHuEPO group (3.45±0.51). The expression of ARNT1 gene was clearly up-regulated in the kidneys from both nephrectomized groups, whereas ARNT2 mRNA levels significantly increased exclusively in the CKD group (2.95±0.42) (Fig. 6 and 7).

Hepatic gene expression analysis showed an increased expression on CKD group of both HIF-2α (3.11±0.21) and HIF-3α (2.20±0.31); HIF-1α expression slightly increased in both CKD groups. Regarding ARNT subunits, ARNT1 mRNA levels significantly increased in CKD group (2.82±0.13), while in the CKD+rHuEPO group barely increased (1.41±0.35); in its turn, ARNT2 mRNA were not detected in the liver for any group.

Following the gene expression analysis, we sought to confirm the data obtained by immunohistochemistry and ELISA methods. In agreement with the qPCR results, HIF-1α protein levels in the CKD group were significantly higher than those detected in the Sham group, both in renal and hepatic tissues (3- and 2-fold higher, respectively). Similarly, CKD+rHuEPO renal and hepatic HIF-1α concentration increased 3-fold when compared with control values from the Sham

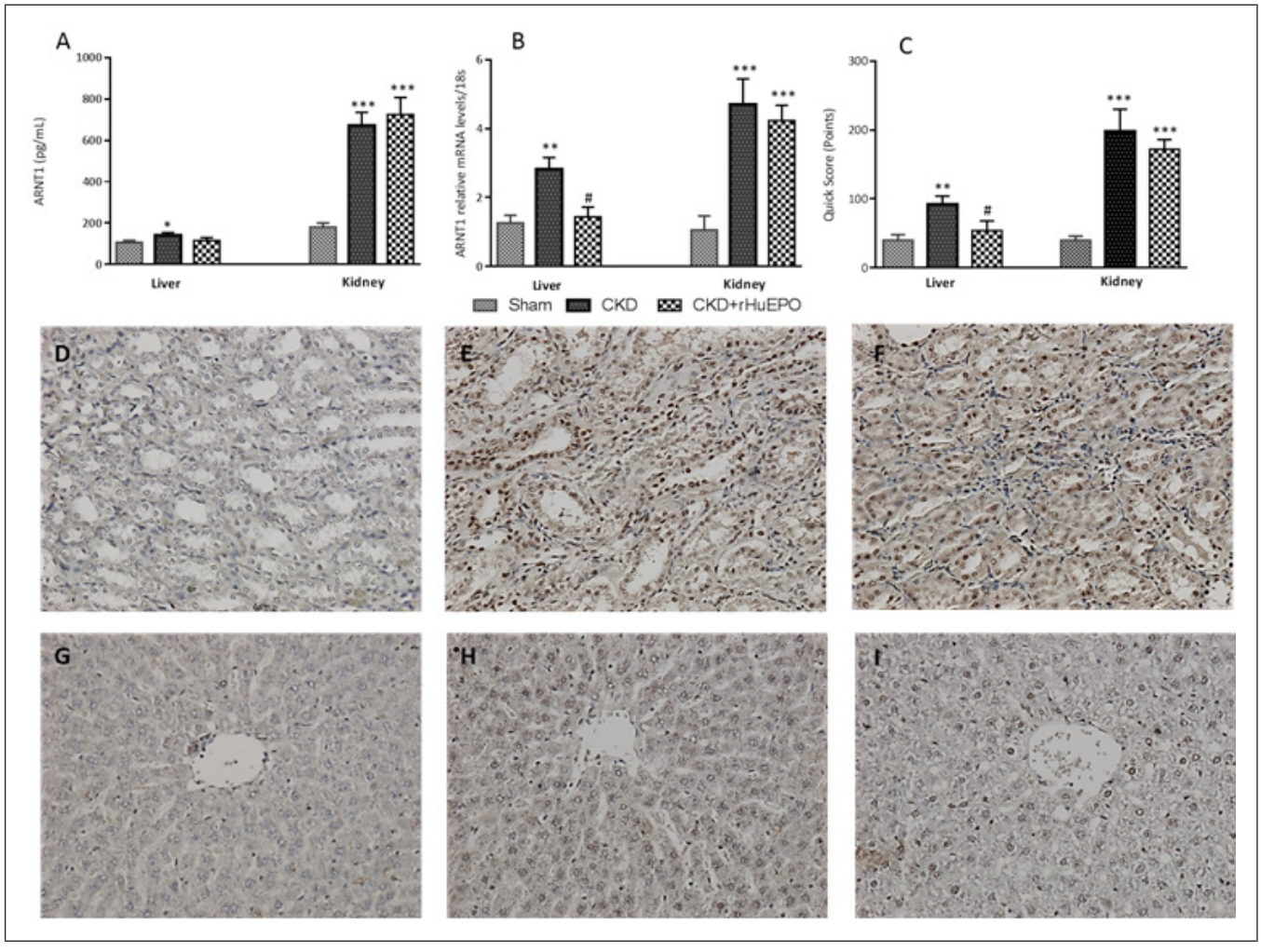


Figura 6. ARNT1 analysis in renal and hepatic tissue. Hepatic and renal ARNT1 concentration (A) and mRNA levels (B); kidney and liver (C) ARNT1 immunohistochemical quick scores and corresponding images of ARNT1 expression in the kidney (D – sham, E – CKD, and F – CKD+rHuEPO groups) and in the liver (G – sham, H – CKD, and I –CKD+rHuEPO groups). Results are presented as mean ± SD: *- $p < 0.05$, **- $p < 0.01$, and ***- $p < 0.001$ *versus* Sham group; #- $p < 0.05$, ##- $p < 0.01$, and ###- $p < 0.001$ *versus* CKD group.

group. Immunohistochemistry revealed high expression upon renal and hepatic tissue of such molecule, mainly in the renal tubular epithelial cells and hepatocytes. HIF-2α renal concentration was statistically augmented in the CKD group ($p<0.001$), even though mRNA levels were almost unchanged. Moreover, a statistically significant raise of this protein was observed at hepatic level ($p<0.005$). On the contrary, CKD+rHuEPO group exhibited a slight reduction compared to Sham results of about 10-20%, on renal and hepatic HIF-2α concentration. The presence of HIF-2α was mainly detected in the peritubular area (i.e. endothelial and interstitial cells) at kidney level, whereas in the liver appeared widely spread. The analysis of the third HIF isoform – HIF-3α, revealed a significant increase on this molecule (about twice the concentration detected in Sham rats), both in renal and hepatic tissues from CKD group ($p<0.005$). Results also showed a reduction on HIF-3α levels in CKD+rHuEPO hepatic tissue, while at renal level there were no significant changes on its concentration. HIF-3α presence in the kidney was detected in the same cells than HIF-1α. Regarding the two ARNT subunits, results showed an increase in ARNT1 levels in both renal and hepatic tissues from CKD group, while in CKD+rHuEPO ARNT1 levels only increased in renal tissue. Although ARNT2 could not be detected in the liver from any of the three groups under test, results showed a great increase (ca. 7-fold higher than Sham) of this molecule in renal tissue from

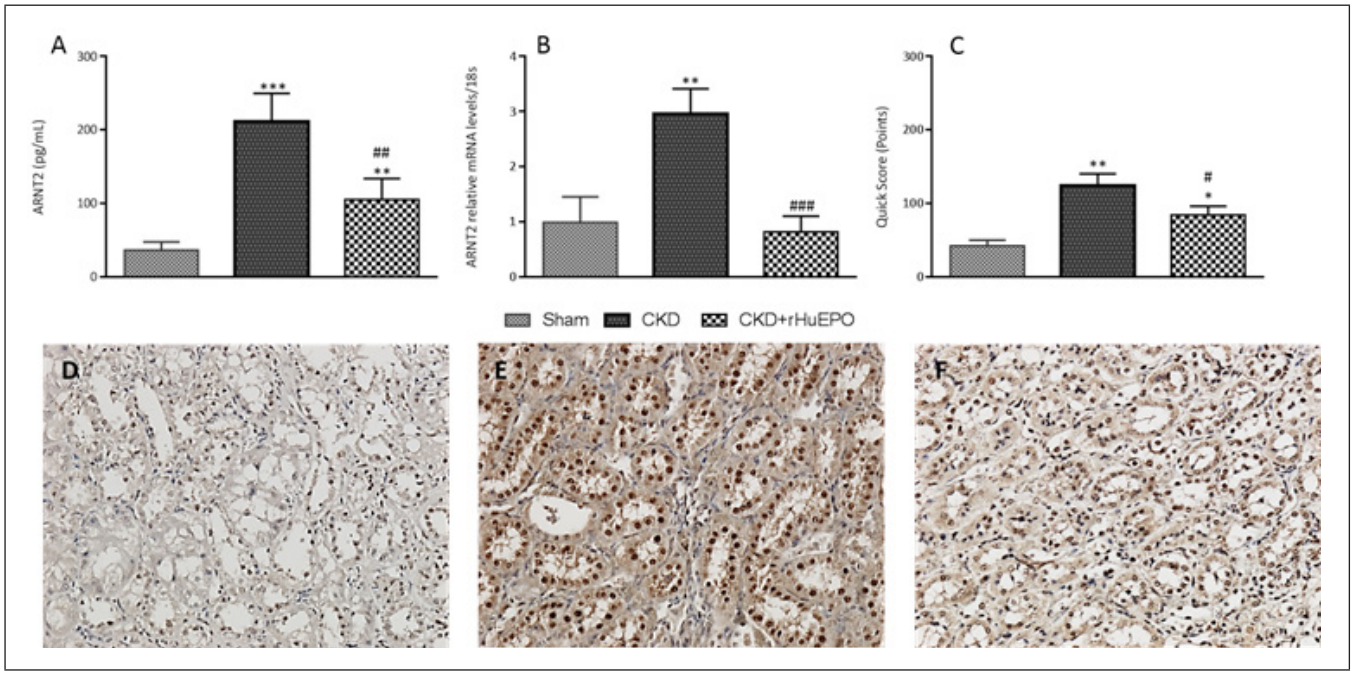


Figura 7. ARNT2 analysis in renal tissue. Renal ARNT2 concentration (A) and mRNA levels (B); kidney and liver (C) ARNT2 immunohistochemical quick scores and corresponding images of ARNT2 expression in the kidney (D – sham, E – CKD, and F – CKD+rHuEPO groups). Results are presented as mean ± SD: *- $p < 0.05$, **- $p < 0.01$, and ***- $p < 0.001$ *versus* Sham group; #- $p < 0.05$, ##- $p < 0.01$, and ###- $p < 0.001$ *versus* CKD group.

CKD group. In addition, immunohistochemistry revealed a subcellular distribution of ARNT1 limited to hepatocytes and renal cell's nucleus, while ARNT2 was solely observed in renal cell's nucleus.

DISCUSSION

In this research work, we first confirmed the impairment of the renal function on our rat CKD model. The observed levels of urea and creatinine indicate that we are in fact in the presence of a moderate CKD animal model[36]. The loss of renal function was followed by a generalized decrease in erythrocyte parameters, confirming that our CKD model developed anemia. Anemia is commonly associated complication of CKD, affecting over 27% of moderate CKD patients and 76% of severe CKD patients[37]. The correlation between impaired renal function and low levels of Hb or RBCs has been reported in diverse animal and clinical studies, and can be easily found in literature[26,38]. The detected anemic condition was further hold up by an absence of a reticulocytes raise, which is indicative that the bone marrow in not providing an efficient response to such stimuli. On the contrary, in the group treated with rHuEPO, a boost in circulating reticulocytes was detected, as well as a concomitant increase in all erythrocyte parameters. This generalized increase of the erythrocyte parameters is directly correlated with the EPO administration, which strongly suggested that the anemic status observed in the CKD group was mostly due to a deficiency in EPO. Therefore, we measured serum EPO levels and noted its increase with the time in CKD group. In fact, at the end of the protocol (T2), serum EPO levels were three times higher than the concentration detected in the Sham group. Even though, serum EPO levels were unable to correct anemia, suggesting that these were disproportionately low for the anemia degree.

Diverse clinical studies have demonstrated that serum EPO levels in CKD patients are within the normal range and may even increase, but to levels inappropriately low for the severity of anaemia. Koch & Radtke firstly reported that the initial phase of renal anemia is accompanied by a compensatory increase of serum EPO concentration, which was lately termed as relative erythropoietin deficiency[39]. Fehr *et al.* assessed serum EPO level in a large number of CKD patients previously to dialysis therapy, and detected

a gradual increase in serum EPO level with plasma creatinine level in patients with mild to moderate CKD [32]. Sanada *et al*. reported the conservation of the capacity to synthesize EPO in rats with a mild CKD condition (creatinine under 1.2 mg/dL and an average Hb level of 12.1 g/dL)[40]. Moreover, Sanada *et al*. reported increasing levels of serum EPO in these rats until a maximum of 25 mIU/mL, without detecting any positive impact upon the anemic condition of the rats. As suggested both in Sanada *et al.* and Fehr *et al.* works, the problem may not be an insufficient EPO production, but instead an altered set point for EPO production. The assumption that EPO production by these animals is non-proportional to the anemia degree was further supported by the response given by the CKD+rHuEPO group to the administration of a high dose of rHuEPO (400 IU/Kg/week); such dose was more than enough to boost up reticulocyte numbers (in T1) and to increase all erythrocyte parameters, thus completely eliminating any sign of anemia in the CKD+rHuEPO rats.

As an attempt to disclose the underlying mechanism of EPO production in a case of moderate CKD, we decided to assess the contribution of renal and extra-renal EPO production (i.e. in liver), in addition to the transcription factors responsible by its regulation. Induction of CKD with an anemic status associated, led to the appearance of EPO-encoding mRNA in liver and kidney of CKD animals group. This increase was translated into EPO production in both organs. The majority of EPO production seen in our model is responsibility of the remnant kidney, however a part of the circulating EPO is produced by the liver. It is irrefutable that the kidney is not the only EPO-producing tissue in adults[41]; in fact, early estimates indicated that around 10% of the circulating EPO originates from non-renal tissue. This minor role, yet essential, played by the liver has been previously identified and may increase in specific circumstances. Querbes *et al*. reported that it is feasible to reactivate hepatic EPO synthesis using systemically delivered siRNAs targeting cellular oxygen sensors specifically in the liver, leading to increased RBC numbers in models of anemia caused by either acute renal insufficiency or chronic inflammation with enhanced hepcidin production[42]. Bernhardt *et al*, showed in anephric patients that prolyl hydroxylases inhibition was capable of generating an erythropoietic response owing to increased HIF-α levels and *EPO* expression in the liver[43]. In addition, Tan *et al*. efforts to quantify the contribution of hepatic EPO upon rats submitted to subtotal nephrectomy revealed that hepatic *EPO* mRNA was greatly increased and accounted for almost 40% of the total; however, this increase in EPO mRNA did not generate a significant impact upon EPO circulating levels[44].

Our data shows that in a CKD situation all the three HIF isoforms appear to be active at renal level. HIF-2α has been described in multiple reports as the main responsible by hypoxic induction of renal EPO[45,46]. Such statement stems from several studies, including those showing that EPO-producing renal cells coincided with the location of HIF-2α–expressing renal cells[47]. Moreover, this theory has been further cemented by diverse studies with adult mice that are either germline deficient for HIF-2α or made HIF-2α deficient during postnatal life with the use of a ubiquitously expressed Cre-recombinase. Our results suggest the presence of HIF-2α in renal interstitial fibroblast-like cells in the CKD as well in endothelial peritubular cells, in spite of the modest levels of HIF-2α mRNA in this tissue. The reduced presence of this protein in the CKD+rHuEPO renal tissue led us to hypothesise that HIF-2α abundance in the remnant kidney may be essentially associated with hypoxia-induced EPO production. On the contrary, HIF-1α concentration and mRNA levels were increased in both nephrectomized groups – with and without anemia. Such results suggest that this HIF subunit may be increased in CKD renal tissue for other purposes rather than being involve in EPO regulation. Actually, HIF-1α has been implicated in the regulation of several other genes in circumstances requiring cell and organ adaptation to changes beyond oxygen supply, such as in redox balance, inflammation status or in wound healing situations. Therefore, the presence of high concentrations of HIF-1α in both CKD groups is most probably an outcome of the nephrectomy procedure itself. Among the possible physiologic adaptations to a nephrectomy procedure found in the literature we highlight the fibrosis (large deposits of fibrous tissue were seen in the remnant kidneys of both CKD groups, at T3), immune reactions (HIF-1α is activated by cytokines as IL-1β or TNF-α), presence of nitric oxide (responsible by

improving renal blood flow in the remnant kidney) and reactive oxygen species, apoptosis, as well other hypoxic related responses like angiogenesis, glucose metabolism (switch from the oxygen-dependent tricarboxylic acid cycle to the oxygen-independent glycolysis) and cell proliferation[48,49].

HIF-3α is the least well understood member of HIF isomers, considered by several authors as a piece of a multipart system of feedback mechanisms that attenuate the hypoxic response thus preventing detrimental effects of an unopposed, prolonged HIF activation. In addition, this HIF subunit has been reported to be regulated by HIF-1α, however, most studies on this molecule are based predominantly on *in vitro* over-expression experiments[50,51]. We observed an increase in HIF-3α total mRNA and protein concentration in renal tissue, only in the absence of EPO treatment (CKD group). Under rHuEPO administration, HIF-3α levels significantly decreased, even in the presence of elevated levels of HIF-1α. These results suggest a possible contribution of HIF-3α upon EPO synthesis in CKD situations. Fang Li *et al*. reported a strong increase on HIF-3α (protein and mRNA levels) in lung A549 cells, under hypoxic conditions[50]. In addition, Tanaka *et al*. reported that hypoxia (1% CO2) stimulates *HIF-3*α mRNA (3-fold) in Caki-1 renal carcinoma cells[51], while Heidbreder *et al.* described HIF-3α as a rapidly reacting component of the HIF system in protection against hypoxic damage[52] Nonetheless, none of the mentioned studies demonstrated a possible relation between HIF-3α and EPO levels.

Although, as mentioned previously, the synthesis of HIF-2α and EPO in the same region could lead us to designate this HIF subunit as the one responsible by EPO synthesis in CKD, recent works have been showing that EPO synthesis is more widespread along the kidney than first believed. Nagai *et al*. showed that hypoxia induces EPO mRNA expression in peritubular cells (not seen in normoxia), proximal convoluted tubules, distal convoluted tubules and cortical collecting ducts[53]. Mujais *et al*. reported EPO gene expression in the transporting tubular epithelial cells[54]. Furthermore, Beirão *et al*. showed by means of *in situ* hybridization on kidney biopsies that EPO is mainly expressed by epithelial distal tubular cells and collecting tubules, as well in some situation by glomerular cells[55]. Accordingly, we speculated that the increased expression and synthesis of HIF-3α in peritubular endothelial cells may play, at least, a small role in EPO up-regulation in CKD.

It has been widely reported that in response to hypoxia, ARNT1 and ARNT2 form heterodimers with HIF-α subunits, which will then bind to hypoxia response element of genes responsible for the adaptation to oxygen deprivation. Numerous mouse tissues and various cell culture lines showed that endogenous ARNT1 is ubiquitously expressed, while its analogue ARNT2 expression is limited to kidney, central nervous system, and retinal epithelium[56,57]. In this study we report the expression of ARNT1 and ARNT2 mRNA's, as well as of both proteins in CKD rat's kidney. However, the administration of rHuEPO led to a decline, both in mRNA and protein levels of ARNT2, whereas ARNT1 reduction was quite insignificant. Such results led us to link the presence of ARNT2 in kidney to hypoxia-induced EPO production, contrasting with ARNT1, which presence in renal tissue seems to be related with other processes related with the nephrectomy procedure or with the rHuEPO therapy, rather than with hypoxia-induced EPO production. Actually, the dimerization between HIF-alpha subunits with ARNT1 has been associated with transcription activation of several other genes than EPO[58]. Such genes are involved in various processes crucial to sustain the remnant kidney function from both CKD groups (e.g. vascular endothelial growth factor, vascular endothelial growth factor receptor-1 and -2 or Wilms tumor protein).

Most of the studies regarding EPO production regulation mechanisms in CKD are concentrated in the kidney. However, as also showed in this report, part of the EPO synthesized in those cases has its origin in extra-renal sources, namely in the liver. As our results demonstrate, the mechanisms behind hepatic *EPO* expression seem to mainly depend on HIF--2α and ARNT1. Both molecules were significantly increased in hepatic tissue derived from CKD animals, when compared to Sham or CKD+rHuEPO groups. In the last years, the importance attributed to HIF-2α role on extra-renal EPO regulation has been increasing [2]. Several studies, mainly based on mice with liver-specific or global gene inactivation and total kidney ablation, have been showing that in the

absence of renal HIF-2α, hepatic HIF-2α takes over as the main regulator of the serum EPO pool[18,2,59]. Our results support the significance of such molecule to generate EPO in a CKD situation, where the amount of EPO produced by the damage kidneys is not enough to fight back anemia. The dimerization of HIF-2α with ARNT1 and not with ARNT2 was somehow expected, owing to the described lack of capacity of hepatic tissue to produce ARNT2[56,60]. On the contrary, ARNT1 has been described to be synthesized in liver, both in embryonic and adult life[56,61]. Yim *et al*. reported the pivotal role of ARNT1 in liver as a heterodimerization partner of Hif-1α and Hif-2α[62]. Heidbreder *et al* revealed that parallel to Hif-1α, ARNT1 levels are required to be maintained at a certain level to sustain target gene transcription for physiological basal energy homeostasis[52].

HIF-3α presence in CKD animals' livers leads us to claim that the relation of this molecule to EPO production is not limited to the kidney. The elevated levels of HIF-3α during hypoxia have already been associated with early phases of tissue adaptation to low O_2 tensions[52]. Furthermore, it was previously suggested HIF-3α capacity to induce target genes like EPO and GLUT-1 in such situation[63]. The precise role of HIF-3α in EPO regulation in a CKD condition cannot be described in detail with the results obtained during this work. However, our results strongly suggest that in addition to HIF-2α, HIF-3α is also an important piece in the EPO regulation mechanism in CKD patients.

In conclusion, our results suggest that the mechanisms behind EPO synthesis in CKD differ in an organ-specific fashion. The dimer constituted by HIF-2α/ARNT1 appears to play a key role in EPO production at hepatic level, while in the remnant kidney the HIF-2α/ARNT2 combination looks to be the main responsible. In addition, our results suggest that HIF-3α is also positively associated with EPO synthesis in both organs, while HIF-1α overproduction in CKD is probably related with fibrosis, redox imbalance and immune responsive mechanisms. Therefore, these new insights on the mechanisms responsible by tissue-specific regulation of EPO in CKD may be clinically useful to identify new targets and therapies to stimulate higher levels of EPO, otherwise not achievable by physiological means.

Acknowledgements: This work was financed by the European Regional Development Fund (ERDF), through Centro 2020 Regional Operational Programme: project CENTRO-01-0145-FEDER-000012-HealthyAging2020, the COMPETE 2020-Operational Programme for Competitiveness and Internationalisation and Portuguese national funds via FCT (Fundação para a Ciência e a Tecnologia, I.P.) through projects POCI-01-0145-FEDER-007440 and FCOMP-01-0124-FEDER-028417, as well as by FCT/MEC through national funds and cofinanced by FEDER, under the Partnership Agreement PT2020 (UID/MULTI/04378/2013-POCI/01/0145/FEDER/007728). Authors also acknowledge FCT for the Sara Nunes PhD scholarship (SFRH/BD/109017/2015) and UID/NEU/04539/2013 funds (CNC.IBILI Consortium).

Conflicts of interest: The authors declare that they have no competing interests.

REFERENCES

1. Lombardero M, Kovacs K, Scheithauer BW. Erythropoietin: A Hormone with Multiple Functions. *Pathobiol* 2011;78:41–53.
2. Rankin EB, Biju MP, Liu Q, et al. Hypoxia-inducible factor–2 (HIF-2) regulates hepatic erythropoietin *in vivo*. *J Clin Invest* 2007;117:1068–77.
3. Jacobson LO, Goldwasser E, Fried W, Plzak L. Role of the kidney in erythropoiesis. *Nature* 1957;179:633–4.
4. Eckardt KU, LeHir M, Tan CC, Ratcliffe PJ, Kaissling B, Kurtz A. Renal innervationplays no role in oxygen-dependent control of erythropoietin mRNA levels. *Am J Physiol Renal Physiol* 1992;263:F925–30.
5. Fried W. The liver as a source of extrarenal erythropoietin production. *Blood* 1972;40:671–77.
6. Koury ST, Bondurant, MC, Koury MJ, Semenza GL. Localization of cells producing erythropoietin in murine liver by in situ hybridization. *Blood* 1991;77:2497–503.
7. Fandrey J. Oxygen-dependent and tissue-specific regulation of erythropoietin gene expression. *Am J Physiol Regul Integr Comp Physiol* 2004;286:R977–88.
8. Eckardt KU, Kurtz A. Regulation of erythropoietin production. *Eur J Clin Invest* 2005;35:13–9.
9. Semenza GL. Regulation of oxygen homeostasis by hypoxia-inducible factor 1. *Physiology* 2009;24:97–106.
10. Rankin EB, Giaccia AJ. The role of hypoxia-inducible factors in tumorigenesis. *Cell Death Differ* 2008;15:678–85.
11. Fernandes JC, Reis F, Costa E, Santos-Silva A. Modulation of Erythropoietin Gene Expression. In: Costa E, Reis F, Santos-Silva A, editors. Frontiers in Drug Discovery Erythropoietic Stimulating Agents. Benthan Editions; 2013. p. 27–42.
12. Bruick RK. Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. *Genes & Dev* 2003;17:2614–23.
13. Yan Q, Bartz S, Mao M, Li L, Kaelin WG. The hypoxia-inducible factor 2α N-terminal and C-terminal transactivation domains cooperate to promote renal tumorigenesis in vivo. *Mol Cell Biol* 2007; 27:2092–102.
14. Hu C-J, Wang L-Y, Chodosh LA, Keith B, Simon MC. Differential Roles of Hypoxia-Inducible Factor 1α (HIF-1α) and HIF-2α in Hypoxic Gene Regulation. *Mol Cell Biol* 2003;23:9361–74.

15. Warnecke C, Zaborowska Z, Kurreck J, et al. Differentiating the functional role of hypoxia-inducible factor (HIF)-1α and HIF-2α (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2α target gene in Hep3B and Kelly cells. *Physiol Rev* 1992;72:449–89.
16. Scortegagna M, Ding K, Zhang Q, et al. HIF-2alpha regulates murine hematopoietic development in an erythropoietin-dependent manner. *Blood* 2005;105:3133–40.
17. Gruber M, Hu CJ, Johnson RS, Brown EJ, Keith B, Simon MC. Acute postnatal ablation of Hif-2alpha results in anemia. *Proc Natl Acad Sci USA* 2007;104:2301–6.
18. Kapitsinou PP, Liu Q, Unger TL, et al. Hepatic HIF-2 regulates erythropoietic responses to hypoxia in renal anemia. *Blood* 2010;116:3039–48.
19. Tung-Wei H, Jia-Hung L, Kun-Tu Y, et al. Renal expression of hypoxia inducible factor-1α in patients with chronic kidney disease: a clinicopathologic study from nephrectomized kidneys. *Indian J Med Res* 2013;137:102–10.
20. Bernhardt WM, Schmitt R, Rosenberger C, et al. Expression of hypoxia--inducible transcription factors in developing human and rat kidneys. *Kidney Int* 2006;69:114–122.
21. Yim HE, Kim JH, Yoo KH, et al. Spironolactone, but not enalapril, potentiates hypoxia-inducible factor-1 alpha and Ets-1 expression in newborn rat kidney. *J Physiol Pharmacol* 2010;61:73-81.
22. Garrido P, Ribeiro S, Fernandes J, et al. Iron-hepcidin dysmetabolism, anemia and renal hypoxia, inflammation and fibrosis in the remnant kidney rat model. *PLoS One* 2015;10(4):e0124048.
23. Pawlik MW, Kwiecien S, Pajdo R, et al. Esophagoprotective activity of angiotensin-(1-7) in experimental model of acute reflux esophagitis. Evidence for the role of nitric oxide, sensory nerves, hypoxia-inducible factor-1alpha and proinflammatory cytokines. *J Physiol Pharmacol* 2014;65:809-22.
24. Zubieta-Castillo G Sr, Zubieta-Calleja GR Jr, Zubieta-Calleja L. Chronic mountain sickness: the reaction of physical disorders to chronic hypoxia. *J Physiol Pharmacol* 2006;57 Suppl 4:431-42.
25. O'Mara NB. Anemia in patients with chronic kidney disease. *Diabetes Spectrum* 2008;21:12–9.
26. Kalantar-Zadeh K, Aronoff GR. Hemoglobin variability in anemia of chronic kidney disease. *J Am Soc Nephrol* 2009;20:479–87.
27. Lankhorst CE, Wish JB. Anemia in renal disease: Diagnosis and management. *Blood Rev*. 2010;24:39–47.
28. Ribeiro S, Belo L, Reis F, Santos-Silva A. Iron therapy in chronic kidney disease: Recent changes, benefits and risks. *Blood Rev* 2015. pii: S0268--960X(15)00062-4.
29. Costa E, Pereira BJ, Rocha-Pereira P, et al. Role of prohepcidin, inflammatory markers and iron status in resistance to rhEPO therapy in hemodialysis patients. *Am J Nephrol* 2008;28(4):677-83.
30. Costa E, Swinkels DW, Laarakkers CM, et al. Hepcidin serum levels and resistance to recombinant human erythropoietin therapy in haemodialysis patients. *Acta Haematol* 2009;122(4):226-9.
31. van der Putten K, Braam B, Jie KE, Gaillard CAMJ. Mechanisms of disease: erythropoietin resistance in patients with both heart and kidney failure. *Nat Clin Pract Nephrol* 2008;4:47–57.
32. Fehr T, Ammann P, Garzoni D, et al. Interpretation of erythropoietin levels in patients with various degrees of renal insufficiency and anemia. *Kidney Int* 2004;66:1206–11.
33. Teixeira M, Rodrigues-Santos P, Garrido P, et al. Cardiac antiapoptotic and proproliferative effect of recombinant human erythropoietin in a moderate stage of chronic renal failure in the rat. *J Pharm Bioallied Sci* 2012;4:76–83.
34. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$. *Method Methods* 2001;25:402–8.
35. Acs G, Zhang PJ, McGrath CM, Acs P, McBroom J, Mohyeldin A, et al. Hypoxia-inducible erythropoietin signaling in squamous dysplasia and squamous cell carcinoma of the uterine cervix and its potential role in cervical carcinogenesis and tumor progression. *Am J Pathol* 2003;162:1789–806.
36. Velenosi TJ, Fu AYN, Luo S, Wang H, Urquhart BL. Down-regulation of hepatic cyp3a and cyp2c mediated metabolism in rats with moderate chronic kidney disease. *Drug Metab Dispos* 2012;40:1508–14.
37. Schmidt RJ, Dalton CL. Treating anemia of chronic kidney disease in the primary care setting: cardiovascular outcomes and management recommendations. *Osteopath Med Prim Care* 2007;1:1–11.
38. Hörl WH. Clinical Aspects of Iron Use in the Anemia of Kidney Disease. *J Am Soc Nephrol* 2007;18:382–93.
39. Koch KM, Radtke HW. Role of erythropoietin deficiency in the pathogenesis of renal anemia. *Klin Wochenschr* 1979;57:1031–6.
40. Sanada S, Toyama H, Ejima Y, Matsubara M. Potential for erythropoietin synthesis in kidney of uraemic rat alters depending on severity of renal failure. *Nephrol* 2009;14:735–42.
41. Weidemann A, Johnson RS. Nonrenal regulation of EPO synthesis. *Kidney Int* 2009;75:682–8.
42. Querbes W, Bogorad RL, Moslehi J, et al. Treatment of erythropoietin deficiency in mice with systemically administered siRNA. *Blood* 2012;120:1916–22.
43. Bernhardt WM, Wiesener MS, Scigalla P, et al. Inhibition of Prolyl Hydroxylases Increases Erythropoietin Production in ESRD. *J Am Soc Nephrol* 2010;21:2151–6.
44. Tan CC, Eckardt KU, Ratcliffe PJ. Organ distribution of erythropoietin messenger RNA in normal and uremic rats. *Kidney Int* 1991;40:69–76.
45. Paliege A, Rosenberger C, Bondke A, et al. Hypoxia-inducible factor-2alpha--expressing interstitial fibroblasts are the only renal cells that express erythropoietin under hypoxia-inducible factor stabilization. *Kidney Int* 2010;77:312–8.
46. Wenger RH, Hoogewijs D. Regulated oxygen sensing by protein hydroxylation in renal erythropoietin-producing cells. *Am J Physiol Renal Physiol* 2010;298:F1287–96.
47. Rosenberger C, Mandriota S, Jurgensen JS, et al. Expression of hypoxia--inducible factor-1alpha and -2alpha in hypoxic and ischemic rat kidneys. *J Am Soc Nephrol* 2002;13:1721–32.
48. Ke Q, Costa M. Hypoxia-Inducible Factor-1 (HIF-1). *Mol Pharmacol* 2006;70:1469–80.
49. Gunaratnam L, Bonventre JV. HIF in Kidney Disease and Development. *J Am Soc Nephrol* 2009;20:1877–87.
50. Li QF, Wang XR, Yang YW, Lin H. Hypoxia upregulates hypoxia inducible factor (HIF)-3alpha expression in lung epithelial cells: characterization and comparison with HIF-1alpha. *Cell Res* 2006;16:548–58.
51. Tanaka T, Wiesener M, Bernhardt W, Eckardt KU, Warnecke C. The human HIF (hypoxia-inducible factor)-3alpha gene is a HIF-1 target gene and may modulate hypoxic gene induction. *Biochem J* 2009;424:143–51.
52. Heidbreder M, Fröhlich F, Jöhren O, Dendorfer A, Qadri F, Dominiak P. Hypoxia rapidly activates HIF-3α mRNA expression. FASEB J 2003;17:1541–3.
53. Nagai T, Yasuoka Y, Izumi Y, et al. Reevaluation of erythropoietin production by the nephron. *Biochem Biophys Res Commun* 2014;449:222–8.
54. Mujais SK, Beru N, Pullman TN, Goldwasser E. Erythropoietin is produced by tubular cells of the rat kidney. *Cell Biochem Biophys* 1999; 30:153–66.
55. Beirão I, Moreira L, Barandela T, et al. Erythropoietin production by distal nephron in normal and familial amyloidotic adult human kidneys. *Clin Nephrol* 2010;74:327–35.
56. Aitola MH, Pelto-Huikko MT. Expression of Arnt and Arnt2 mRNA in developing murine tissues. *J Histochem Cytochem* 2003;51:41–54.
57. Dougherty EJ, Pollenz RS. Analysis of Ah Receptor-ARNT and Ah Receptor-ARNT2 Complexes in Vitro and in Cell Culture. *Toxicol Sci* 2008;103:191–206.
58. Mandl M, Kapeller B, Lieber R, Macfelda K. Hypoxia-inducible factor-1β (HIF-1β) is upregulated in a HIF-1α-dependent manner in 518A2 human melanoma cells under hypoxic conditions. *Biochem Biophys Res Commun* 2013;434:166–72.
59. Kim WY, Safran M, Buckley MR, et al. Failure to prolyl hydroxylate hypoxia-inducible factor alpha phenocopies VHL inactivation in vivo. *EMBO J* 2006;25:4650–62.
60. Hirose K, Morita M, Ema M, et al. cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS factor (Arnt2) with close sequence similarity to the aryl hydrocarbon receptor nuclear translocator (Arnt). *Mol Cell Biol* 1996;16:1706–13.
61. Jain S, Maltepe E, Lu MM, Simon C, Bradfield CA. Expression of ARNT, ARNT2, HIF1α, HIF2α and Ah receptor mRNAs in the developing mouse. *Mech Develop* 1998;73:117–23.
62. Yim SH, Shah Y, Tomita S, et al. Disruption of the Arnt gene in endothelial cells causes hepatic vascular defects and partial embryonic lethality. *Hepatol* 2006;44:550–60.
63. Gu YZ, Moran SM, Hogenesch JB, Wartman L, Bradfield CA. Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha.. 1998;7:205–13.

ASSOCIATION OF ALTERED HEMORHEOLOGY WITH OXIDATIVE STRESS AND INFLAMMATION IN METABOLIC SYNDROME.

Gyawali P, Richards RS[1]

Abstract

Objective: We have shown increased whole blood viscosity (WBV), decreased erythrocyte deformability, and increased erythrocyte aggregation in metabolic syndrome (MetS) in our previous study. The objective of the study was to find out if the altered hemorheology shown in MetS in our previous study is associated with chronic inflammation and oxidative stress in the same subjects.

Methods: One hundred recruited participants were classified into three groups based on the number of the MetS components present following National Cholesterol Education Program, Adult Treatment Panel III definitions. WBV, erythrocyte aggregation, erythrocyte deformability, oxidative stress markers (erythrocyte reduced glutathione (GSH), superoxide dismutase (SOD), and urinary isoprostanes), inflammatory markers high-sensitivity C-reactive protein (hsCRP), and thrombotic marker D-dimer were measured. Data were analyzed by IBM SPSS 20 software.

Results: We found a significant association of altered hemorheology with chronic inflammation and oxidative stress in MetS. There was a linear increase in the level of hsCRP and a linear decrease in the level of SOD and GSH across the quartiles of erythrocyte aggregation. Similarly, the thrombotic marker D-dimer showed a linear increase and oxidative stress marker GSH showed a linear decrease trend across the quartiles of WBV.

Discussion: Alterations of hemorheology in MetS are probably due to the effect of chronic inflammation and oxidative stress. The negative effects of chronic inflammation and oxidative stress on the cardiovascular system could be due to the resulting altered hemorheology. **[Redox Rep. 2015 May;20(3):139-44.] PMID: 25494675**

[1] School of Community Health, Charles Sturt University, Australia

CLINICAL DISORDERS RESPONSIBLE FOR PLASMA HYPERVISCOSITY AND SKIN COMPLICATIONS

Namdee K, Carrasco-Teja M, Fish MB, Charoenphol P, Eniola-Adefeso O[1]

Abstract

Animal models are extensively used to evaluate the in vivo functionality of novel drug delivery systems (DDS). However, many variations likely exist in vivo between the animals and human physiological environment that significantly alter results obtained with animal models relative to human system. To date, it is not clear if the variation in hemorheology and hemodynamics between common animal and human models affect the functionality of DDS. This study investigates the role of hemorheology of humans and various animal models in dictating the binding efficiency of model vascular-targeted carriers (VTCs) to the wall in physiological blood flows. Specifically, the adhesion of sLe(A)-coated nano- and micro-spheres to inflamed endothelial cells monolayers were conducted via a parallel plate flow chamber assay with steady and disturbed red blood cells (RBCs)-in-buffer and whole blood flows of common animal models. Our results suggest that the ratio of carrier size to RBC size dictate particle binding in blood flow. Additionally, the presence of white blood cells affects the trend of particle adhesion depending on the animal species. Overall, this work sheds light on some deviation in VTC vascular wall interaction results obtained with in vivo animal experimentation from expected outcome and efficiency in vivo in human. **[Sci Rep. 2015;5:11631.] PMID: 26113000**

[1] Department of Chemical Engineering, University of Michigan, Ann Arbor, MI 48109

EFFECT OF VASCULAR BRADYKININ ON PANCREATIC MICROCIRCULATION AND HEMORHEOLOGY IN RATS WITH SEVERE ACUTE PANCREATITIS

Liu LT[1], Li Y, Fan LQ, Zhao Q, Wang D, Cheng SJ, Zhang AM, Qin Y, Zhang B.

Abstract

Objective: To investigate the effect of vascular bradykinin on pancreatic microcirculation and hemorheology in rats with severe acute pancreatitis (SAP).

Materials and methods: Ninety male Wistar rats were randomly divided into a blank control group, an SAP group and a vascular bradykinin treatment group. The SAP model was induced by the retrograde injection of 5% sodium taurocholate in the pancreaticobiliary duct. The vascular bradykinin treatment group underwent gastrostomy, with a fine plastic tube placed in the stomach that led out of body through the abdominal wall.Vascular bradykinin was fully dissolved and administered at a dose of 20 U/kg once every 8 h. The pancreatic microcirculatory blood flow volume and velocity, microvascular permeability, hemorheology were evaluated respectively by double-channel laser Doppler flowmetry, the Evans blue leakage test, a blood rheology test instrument.

Results: The pancreatic microcirculatory blood flow volume and velocity in the vascular bradykinin treatment group increased gradually after 48 h compared with the SAP group, and the changes were significantly different ($p < 0.05$). The pancreatic microvascular permeability of the vascular bradykinin treatment group was significantly reduced after 48 h compared with the SAP group ($p < 0.05$). The low shear rate blood viscosity, hematocrit and erythrocyte aggregation index of the vascular bradykinin treatment group were significantly decreased after 48 h compared with the SAP group ($p < 0.05$).

Conclusions: Vascular bradykinin can improve pancreatic microcirculation and hemorheology in rats with severe acute pancreatitis. **[Eur Rev Med Pharmacol Sci. 2015;19(14):2646-50.] PMID:26221896**

[1] Department of General Surgery, The No. 4 Affiliated Hospital of Hebei Medical University, Shijiazhuang, China. yonglicn@yeah.net.

ELEIÇÕES NA SPHM PARA ÓRGÃOS SOCIAIS

No dia 15 de dezembro de 2017, decorreram em Assembleia Geral, as eleições para os Órgãos Sociais da SPHM para o biénio 2017-2019.

Após se ter procedido à contagem dos votos, comprovou-se que a única lista concorrente foi aprovada por unanimidade, tendo sido apurados os seguintes resultados: 13 votos a favor, zero votos brancos e zero votos nulos.

A lista eleita é constituída pelos seguintes membros:

Direcção

Presidente – Prof.ª Doutora Maria Carlota Saldanha Lopes
Vice-Presidente – Dr. José António Pereira Albino
Secretário-Geral – Prof. Doutor Flávio Reis
Tesoureira – Dr.ª Ana Santos Silva Herdade
Secretários-Adjuntos – Prof.ª Doutora Alice Santos Silva, Dr. Mário Manuel M. G. Marques e Dr. Luís Sargento

Assembleia Geral

Presidente – Prof. Doutor J. M. Braz Nogueira
1.º Secretário – Prof. Doutor Luís Mendes Pedro
2.º Secretário – Prof. Doutor Henrique Sobral do Rosário
1.º Secretário Suplente – Dr.ª Sandra Maria Maurício Hilário Pires
2.º Secretário Suplente – Dr. Paulo Ferreira da Silva

Conselho Fiscal

Presidente – Dr. Carlos Manuel dos Santos Moreira
1.º Vogal – Dr.ª Maria Helena Baptista Manso Ribeiro
2.º Vogal – Dr. Paulo Farber

Comissão de Delegados

Delegado da Região Norte – Dr. Manuel Campos
Delegado da Região Centro – Dr. João Morais
Delegado da Região Sul e Regiões Autónomas – Dr. Mário Marques

REGRAS DE PUBLICAÇÃO / INSTRUCTIONS TO AUTHORS

CONVITE

A Sociedade Portuguesa de Hemorreologia e Microcirculação (SPHM) aceita para publicação no seu BOLETIM artigos de curta extensão. O Boletim é editado duas vezes por ano em formato electrónico (www.hemorreologia.com).

INSTRUÇÕES

1. Todos os textos enviados para publicação estão sujeitos a apreciação editorial e aprovação. A decisão é baseada no mérito científico e cultural dos trabalhos.

2. São aceites somente os trabalhos preparados em versão *PDF* ou *Microsoft Word*.

3. Os textos devem ser redigidos em Português ou Inglês.

4. Os manuscritos com o pedido de publicação devem ser enviados por *e-mail* ao Editor (carlotasaldanha@fm.ul.pt).

– Comunicações Originais (artigos curtos) – Os textos serão considerado para publicação rápida, com a seguinte estrutura: Sumário (50-70 palavras), Introdução, Material e Métodos, Resultados, Discussão e Conclusões. O(s) autor(es) são estimulados a englobar em conjunto os resultados, discussão e conclusões.
(Extensão máxima do texto: 5 a 6 páginas a um espaço (letra de corpo 11), incluindo figuras tabelas e quadros(e respetivas legendas),agradecimentos e até 30 referências bibliográficas).

– Artigos de Revisão – O BOLETIM terá a maior satisfação em acolher curtas revisões sobre assuntos de particular interesse, no âmbito da Hemorreologia, Microcirculação ou assuntos de âmbito médico ou de outras áreas científicas afins, que sejam submetidos diretamente para publicação ou mediante convite especial do Editor.
(Extensão máxima do texto:8 a 10 páginas (letra de corpo 11) incluindo figuras, tabelas, quadros, fotos (e respetivas legendas), agradecimentos e até 60 referências bibliográficas).

INVITATION

The Portuguese Society on Hemorheology and Microcirculation (Sociedade Portuguesa de Hemorreologia e Microcirculação, SPHM) is pleased to welcome short papers for publication in its BOLETIM. This online publication (www.hemorreologia.com), is distributed two times a year.

INSTRUCTIONS

1. All submitted manuscripts are subjected to editorial review and approval. The decision to publish is dependent on the scientific and cultural merit of the papers.

2. Only contributions prepared and submitted as *PDF* or *Microsoft Word* will be accepted.

3. Texts must be written in Portuguese or in English.

4. All scientific contributions, including manuscript submission and further correspondence should be addressed by *email* to the Editor (carlotasaldanha@fm.ul.pt)

– Original Communications – Manuscripts may be considered for rapid processing as short communications. All manuscripts should be arranged in the following sections: Abstract (50-70 words), Introduction, Material and Methods, Results, Discussion, Acknowledgements and References. The author(s) may combine some of the sections normally included in a full paper, namely the results, discussion and conclusions.
(Maximum communication length – 5-6 single spaced typed pages, including figures, tables, legends, acknowledgments and up to 30 references).

– Short Reviews – The BOLETIM will publish reviews on subjects of particular interest in its field, either following a special invitation or a submission by the author, and in the latter case only after approval by an Editorial Board member. Further information can be obtained from the editor.
(Maximum review length – 8-10 full pages, including figures, tables, photos, legends, acknowledgments and up to 60 references)