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EFFECTS OF ERYTHROPOIETIN ALPHA ON CIRCULATING CELL POPULATIONS IN AN EXPERIMENTAL MODEL

Original Article

DENEYSEL HAYVAN MODELİNDE ERİTROPOETİN ALFA'NIN DOLAŞAN ENDOTEL HÜCRE POPÜLASYONU ÜZERİNE ETKİLERİ

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ABSTRACT

Objective: In this study we aimed to investigate the effects of erythropoietin alpha on the number of circulating mature endothelial cells (MEC) and circulating endothelial progenitor cells (EPC) in an experimental model.

Methods: Twenty-four male wistar albino rats were used for the study. Rats were divided into two groups, 12 of each. Group 1 received vehicle only (0,5 cc serum physiologic/twice a week by intraperitoneal injection for 60 days), while intraperitoneal erythropoietin enjection (1000unit/kg/twice a week week) was administered to group 2. At the end of the study period rats were sacrified and circulating cell populations were measured by flow-cytometric analysis. Mann-Whitney U test and analysis of variance were used for statistical purposes.

Results: At the end of the study erythropetin treatment resulted with higher haemoglobin levels as expected. The body weights at the end of the study were also significantly higher in erythropoietin group. Circulating MEC numbers were significantly decreased with erythropoietin administration while circulating EPC s were significantly increased compared to control group.

Conclusion: The study showed that treatment with erythropoietin alpha is associated with an increase in EPC number. These findings may suggest that erythropoietin alpha mediated increase in EPC number could be associated in part with the beneficial effects observed in

treated patients, irrespective of the rise in hemoglobin levels.

Key words: *Erythropoietin alpha; Mature endothelial cells; Endothelial progenitor cells.*

ÖZET

Amaç: : Bu çalışmada deneysel hayvan modelinde eritropetin alfa (Epo) uygulamasının endotel hasarının bir göstergesi olan dolaşan matür endotel hücresi (MEC) ve endotel progenitör hücre (EPC) sayıları üzerine olan etkileri değerlendirilmiştir.

Gereç ve yöntemler: Çalışmamızda 16 adet, ortalama ağırlıkları 300–350 gram olan wistar albino cinsi erkek sıçanlar kullanılmıştır ve her grupta 8 adet sıçan olacak şekilde iki gruba ayrılmışlardır. Grup 1 kontrol grubu olup, çalışma boyunca sadece intraperitoneal enjeksiyon yoluyla 0,5cc. serum fizyolojik haftada 2 gün uygulanmıştır. Grup 2' ye ise çalışma süresince eritropoetin alfa 1000ünite/kg/hafta dozu iki güne bölünmüş olarak intraperitoneal enjeksiyon yoluyla uygulanmıştır. 60 günlük çalışmanın sonunda hayvanlar sakrifiye edilerek alınan kan örneklerinde flow-sitometrik olarak EPC ve MEC sayıları değerlendirilmiştir.

Sonuç: Çalışmanın sonunda, eritropetin alfa alan grupta hemoglobin seviyeleri dbeklendiği üzere yüksek çıkmıştır. Eritropetin alfa uygulaması sonucunda dolaşan MEC sayılarında anlamlı azalma saptanırken, EPC sayılarında ise anlamlı artış saptanmıştır.

Özet: Eritropetin alfa, çalışmamızda kullanılan doz ve sürede EPC sayısında artışa sebep olmuştur. Eritropetin alfa uygulaması sonucunda EPC sayılarında artış olması, bu molekülün hemoglobin düzeylerini arttırıcı etkisinden bağımsız olarak saptanan yararlı etkilerini açıklayabilir.

Key words: *Eritropetin alfa; Matür endotel hücreleri; Endotel progenitör hücreleri.*

INTRODUCTION

Currently,coronary artery disease (CAD) is one of the most important causes of mortality and morbidity in patients with renal disease. (1-3) Endothelial dysfunction plays an important role in the pathogenesis of cardiovascular diseases. Among the potential causative factors involved in uraemic endothelial dysfunction, the role of endothelial progenitor cells (EPCs) has recently been a subject of intensive research. EPCs are bonemarrow derived, monocyte-like circulating mononuclear cells with the ability to adhere to the damaged vessel wall and replace/shed endothelial cells.[4] It is known that EPCs circulate in very low numbers in the peripheral blood and respond to local and systemic stimuli to be recruited at the site of the vascular damage. There is evidence that EPCs may be involved in the process of endothelial maintenance and neovascularisation.(4-7) Functional early EPC is charactarised by three markers: CD133, CD34 and vascular endothelial growth factor receptor-2 (VEGFR-2) which is also called kinase insert domain receptor (KDR) or Flk-1. In the peripheral circulation of adults, more mature EPCs are found that obviously have lostCD133, but are positive for CD34 and VEGFR-2.(8)

Circulating mature endothelial cells (MECs) may appear in the circulation by detaching from activated or damaged vessels and express CD34 and Flk-1, but, unlike the EPCs, they are negative for the haematopoietic marker CD45.(9)MECs also express endothelial markers such as CD146 and CD31. An increase of MECs has been described in several pathological conditions that involve vascular injury or instability. The enumeration of MECs released in peripheral blood usually represents a direct exploration of the endothelium.

Erythropoietin (Epo) is an endogenous protein that controls production of red blood cells produced principally by the adult kidney.(10) Epo is induced by hypoxia via the hypoxia-inducible factor (HIF) family of transcription factors, which mediate a series of events culminating in general adaptation to tissue hypoxia. It appears that Epo possesses several properties that are associated with its tissue protective effects, when applied as a therapeutic protein in vivo.[11,12] A recently reported mechanism of action of Epo relates to its promoting effect on angiogenesis that led to a considerable interest in the possible application of this protein for cardiovascular protection.(13-14) Interestingly, several studies have demonstrated that Epo is a powerful mobilizer of bone marrow cells to the peripheral circulation, partially accounting for its pro-angiogenic properties.(14-16)
Treatment of congestive heart failure (CHF) with Epo has been found to be of clinical benefit and although anemia correction has been suggested as the mediating mechanism, it is probable that other mechanisms are also operable.(17)
In the current study, we aimed to evaluate the impact of Epo on endothelial functions in an experimental rat model. Endothelial effects were determined by measuring EPC and MEC numbers.

MATERIAL AND METHODS

The study comprised 24 male wistar albino rats each weighing 300-350 grams. They were housed in cages in a temperature and light-controlled environment and were allowed free access to water. The rats were divided, into 2 groups of 12 each. The animals in Group 1, which served as the control, were treated with vehicle alone (0.09% serum physiologic 0.5cc by intraperitoneal administration).Rats in Group 2 received erythropoietin alpha (1000 Unit/kg/twice a week) by intraperitoneal enjection . After 60 days, each rat was anaesthesised by intraperitoneal pentobarbital (50 mg/kg). The abdomen was opened up through a midline incision; the inferior vena cava was catheterized and a 4 cc. blood sample was obtained from each animal for the flow-cytometric analyses of EPCs and MECs. After obtaining blood from each animal, polymorphonuclear cells (PMN) were isolated by density gradient centrifugation. EPCs and MECs were identified from PMN cells by flow-cytometry. Flow-cytometric analyses were performed on a FACS Calibur flow-cytometer. For the flowcytometric analyses, 100 µl of isolated cells were stained with one of the following antibody panels for each rat: (1) FITC conjugated rabbit anti-rat Flk-1(Santa Cruz biotechnology), PeCy7 conjugated mouse monoclonal CD34 (Santa Cruz biotechnology) and PE-conjugated mouse monoclonal anti-rat CD146 (R&D Systems). (2) PeCy7 conjugated Mouse monoclonal CD34 (Santa Cruz biotechnology), seconder PE antibody conjugated goat polyclonal anti-rat CD133 (Santa Cruz biotechnology) and FITC conjugated mouse anti-rat CD45 (Bioscience). To confirm the phenotype, the expression of surface proteins was measured by flow-cytometric analysis. Cells expressing CD34/CD133/CD45 were identified and quantified as progenitor population. Cells staining positive for CD34/Flk-1/CD146 were judged to be MECs. In each analysis, 500000 events (cells) were counted. Absolute numbers ofEPCs and MECs (cells/mm3) of each subject were quantified. Statistical analyses were performed using one-way analysis of variance (ANOVA) and results were expressed as mean±SD. Differences in variables between the two groups were compared using the non-parametric Mann-Whitney U test. Differences between variables were considered statistically significant if the p-value was <0.05.

RESULTS

At the end of the study, Epo administration was associated with an increase of hemoglobin and leukocyte levels compared to the control group as expected. The body weights of the rats treated with Epo were significantly higher

than the rats in the control group (**table 1**) .

***Table 1**: Comparison of weight profiles and*

	Group 1	Group 2	p value
Weight (gram)	326±16	368±15	<0,05
Leucocyte counts (cell/mm3)	6780±2174	7730±2194	>0,05
Haemoglobin counts (mg/dl)	14.1±2.16	18.2±1.63	<0,05

haematologic parameters at the end of the study.

We have tested populations of circulating EPC s and MEC s by FACS ***(**Figure 1**).***

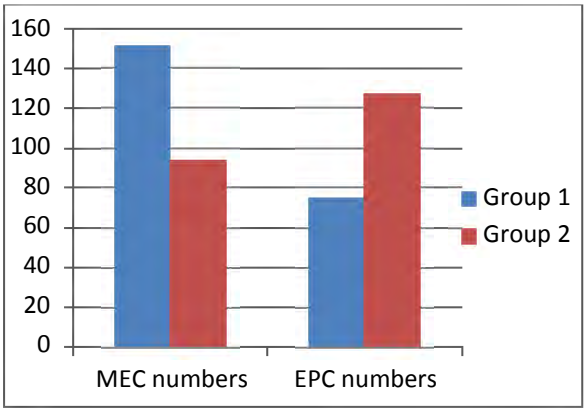

***Figure 1.**Number of circulating mature endothelial cells and endothelial progenitorcells.*
a: MEC number was significantly decresed in Group 2 (p<0,005).
b:EPC number was significantly increased in Group 2 (p<0,005).

The flow-cytometric quantification of MECs showed significantly decreased number MECs in the Epo treated group compared to the control group (94.36 ± 4.66 vs 151.04±11.44, p<0,05). The flow-cytometric quantification of EPC s also showed significantly (p<0.05) elevated amounts of EPC in the Epo-treated group compared to the control group (127±8.64 vs 75±4.11 respectively, p<0.05).

DISCUSSION

To our knowledge, this is the first experimental study assessing the effects of erythropoietin alpha on MECs and EPCs in vivo. The main finding of this study was that erytropoietin treatment resulted in increased EPC number and decreased MEC number which could partially explain the pleiotropic effects of Epo irrespective of its hemoglobin elevating properties.

Endothelial progenitor cells (EPC) are present in the circulation and have the capacity to transform to mature endothelial cells thereby promoting postnatal angiogenesis and vasculogenesis. (18-19) Circulating numbers of EPC has been shown to negatively correlate with risk factors for atherosclerosis (20-21) and with disorders associated with vascular dysfunction (22,23), whereas a positive association was found in the presence of myocardial ischemia. (24,25) These initial observations were soon followed by cell transfer studies showing promising data with regard to the ability to attenuate myocardial dysfunction upon intracoronary provision of progenitor cells.[7,26] Interestingly, several studies have demonstrated that Epo is a powerful mobilizer of bone marrow cells to the peripheral circulation, partially accounting for its pro-angiogenic properties.(14-16)

Epo exerts a variety of pleiotropic effects irrespective of its hemoglobin elevating properties. (26) Accordingly, Epo has been shown to protect cardiomyocytes from ischemic reperfusion damage and to reduce the size of experimental myocardial infarction.(27-29) Recently, several studies have shown that Epo mobilizes endothelial progenitor cells from the bone marrow to the periphery and enhances progenitor cell differentiation and proliferation after a short period of administration. (14-16) These properties could potentially be associated with the clinical benefit observed in patients treated with Epo. Animal models of ischemia/reperfusion have demonstrated

that single-dose erythropoietin may reduce infarct size, decrease apoptosis, and increase neovascularization, possibly through mobilization of endothelial progenitor cells. In the present study, we assessed the effects of Epo on circulating MEC and CEC number in vivo on an experimental model. Epo administration for 60 days resulted in decreased number of MEC s. Circulating MEC s may appear in the circulation by detaching from damaged vessels. These decreased MEC number observed in our study may be due to vasculoprotective effects of Epo. Moreover , circulating EPC number was also increased by Epo administration which were correlated with the current literature.

It appears from recent literature, that not only the absolute number of EPC is of importance in assessment of patients with vascular disorders, but also their functional properties. (18) Among these, migration of EPC (7), resistance to apoptotic cell death (30), secretion of angiogenic cytokines, capacity to support tube formation(15-16), proliferation(15) and adhesion(7,25) .Unfortunately we were unable to evaluate the function of circulating progenitor cells. This is the major limitation of our study.

In conclusion, we have found that EPC numbers treated with Epo are increased, yet their adhesive and proliferative properties are not known. These findings suggest that Epo-mediated increase in EPC number could be associated in part with the beneficial effects observed in treated patients, irrespective of the rise in hemoglobin levels.

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