

Journal of the Hellenic Veterinary Medical Society

Vol 71, No 2 (2020)



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doi: [10.12681/jhvms.23644](https://doi.org/10.12681/jhvms.23644)

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To cite this article:

SULTAN, F., GANAIE, B., MIR, D., & DAR, J. (2020). Recombinant erythropoietin treatment does not alter the blood pressure despite elevated haematological parameters in normotensive Wistar rats. *Journal of the Hellenic Veterinary Medical Society*, 71(2), 2157–2162. <https://doi.org/10.12681/jhvms.23644>

Recombinant erythropoietin treatment does not alter the blood pressure despite elevated haematological parameters in normotensive Wistar rats

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ABSTRACT: Use of erythropoietin (EPO) is believed to be associated with adverse cardiovascular events, especially high blood pressure. Also, its illegal use in blood doping is thought to result in detrimental events both in humans and equines. To test this hypothesis, normal Wistar rats were treated with recombinant erythropoietin (rEPO @ 400 i.u/kg s.c) or normal saline one day apart for one week. Heart rate, systolic, diastolic, mean arterial pressure and blood count were determined. Rats were also observed for their behaviour during the study period. rEPO significantly ($P<0.001$) increased the erythrocyte count (RBC), haemoglobin (Hb), hematocrit (HCT) and platelet count (PLT) in comparison to control animals. Despite such an increase in hematocrit which in turn increases blood viscosity, the systemic blood pressure and heart rate did not differ between the groups. rEPO treatment did not cause any untoward behavioural change in animals. In conclusion, despite the profound effect on haematological parameters (especially hematocrit), rEPO was without any effect on blood pressure and heart rate and the hypothesis of short-term erythropoietin-induced alterations in cardiac parameters was not verified.

Keywords: Rats, Blood doping, Erythropoietin, Hypertension, Hematocrit, Plethysmography.

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Date of initial submission: 01-07-2019
Date of revised submission: 12-01-2020
Date of acceptance: 19-03-2020

INTRODUCTION

Erythropoietin is a glycoprotein which is primarily secreted from the kidney. It acts on erythroid progenitor cells in the bone marrow and stimulates their proliferation and differentiation (Jelkmann, 1992). Clinical applications of erythropoietin include severe anaemic hemodialysis patients, predialysis patients and patients in which anaemia is caused by a tumour or chemotherapy. In veterinary practice, rEPO is used in canine and feline patients suffering from non-regenerative anaemia secondary to chronic renal failure (Mikiciuk *et al.*, 1990; Giger, 1992; Cowgill *et al.*, 1998). Erythropoietin is illegally used in “blood doping” which is defined as an artificial increase in Hb/hematocrit to enhance performance. Jelkmann (2000, 2001) has reviewed the use of rEPO as an anti-anaemic and performance-enhancing drug. EPO is used by top endurance athletes such as long-distance runners and cyclists on a systemic basis (Pommering, 2007). Blood doping in racehorses is done to increase red blood cell count and thus enhancing oxygen-carrying capacity (Ungemach, 1985; Toutain, 2010).

Therapy with rEPO is associated with the development of high blood pressure or else its aggravation (Eschbach *et al.*, 1989; Abraham *et al.*, 1991). EPO therapy completely prevented the chronic renal failure (CRF)-associated anaemia but led to a marked rise in arterial blood pressure which began one week after the onset of EPO administration (Ni *et al.*, 1998). It was also shown that in normotensive subjects there was a significant increase in resting mean arterial pressure (MAP) of +6mmHg (Lundby *et al.*, 2007). Even it has been shown that rEPO produces a direct pressor effect on bilateral rat renal resistance vessels (Heidenreich *et al.*, 1991) and human placental vessels (Resch *et al.*, 2003). It was previously seen that rEPO impaired nitric oxide production and eNOS mRNA expression in pulmonary vasculature (Sultan *et al.*, 2017).

In order to investigate the effect of recombinant erythropoietin on cardiovascular function and haematology, normal rats were treated with rEPO or saline for one week. Heart rate, systolic blood pressure, diastolic blood pressure, mean arterial blood pressure and haematological parameters were determined. We hypothesised that erythropoietin induces excessive erythrocytosis which can cause increased blood viscosity and increased systemic blood pressure. If this hypothesis was verified, more cautious use of erythropoietin is warranted.

MATERIALS AND METHODS

Animals

Male Wistar rats (150-250g, 6-9 weeks of age) were acclimated for ten days before the experiments, placed in transparent polycarbonate cages and housed in an environmentally controlled room. They were kept under 12-h dark/light cycle and allowed free access to feed and water. A diet of following composition was provided to the rats: crude wheat 61 %, crushed maize 30 %, wheat bran 7 %, common salt 1 % and mineral mixture 1 %. The rats were divided into two groups of eight animals each. During the acclimatization, we measured the blood pressure of rats at least three times using the tail-cuff method (NIBP system, CODA, Kent Scientific Corporation, Torrington, CT, USA). We didn't get any animal that was hypertensive thus ensuring that we are using normotensive animals. According to “resource equation method” eight animals per group in our study is an acceptable sample size. The protocol was approved by the Institutional Animal Ethics Committee of Indian Veterinary Research Institute, Izzatnagar (Approval No. F1-53/2012-13/JDR).

Experimental design

Animals were divided into two groups i.e. Control group and Test group. The test group was administered recombinant erythropoietin subcutaneously at 400 IU/kg (Sultan *et al.*, 2017) on every other day (i.e. day 1, 3, 5 and 7) for one week. In a similar fashion, the control group received normal saline. On the 8th day, the blood pressure was taken by tail-cuff plethysmography. After measuring the blood pressure rats were anaesthetized with urethane (1.2 g/kg body weight i.p). Blood was collected from the retro-orbital plexus with the help of a capillary tube.

Blood pressure and heart rate

Initially, the rats were trained for ten days to make them friendly while taking blood pressure and heart rate. Systolic, diastolic and mean arterial pressures (MAP) were measured at the end of the treatment by tail-cuff plethysmography (IITC, mode 31, Woodland Hills, CA, USA) following established procedures. This method is analogous to sphygmomanometry in humans. Animals were adjusted to the experimental cages by bringing them into measuring chamber 3-4 times before the start of the experiment for a period of 30-60 min. To measure blood pressure, a tubular inflatable cuff was placed around the base of the tail and a piezoelectric pulse detector was positioned dis-

tal to the cuff. The systolic pressure was detected and subsequently recorded on a polygraph. Each measurement was repeated at least three times and the mean of these values was used for further calculations. All the recordings and data analysis was done using a computerized data acquisition system (BPMon, version 1.32).

Haematological parameters

On the 8th day, blood was collected using EDTA containing vacutainers from retro-orbital plexus after proper anaesthesia. The collected blood was used for haematological assessment viz. haemoglobin (Hb), red blood cells (RBC), platelets (PLT), white blood cells (WBC), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). These haematological parameters were determined by using automated haematology analyser MEK-6450 (Nihon Kohden, Japan).

Statistical analysis

GraphPad Prism version 4.00 (San Diego, California, USA) software was used for statistical analysis. Student's *t*-test was used in comparing data from two groups. For statistical comparisons, a *P*-value <0.01

was considered significant. All data given are as means \pm standard error of the mean (SEM).

RESULTS

Systolic (SAP), diastolic (DAP), mean arterial pressure (MAP) and heart rate were measured at the end of the treatment by tail cuff-plethysmography (IITC, mode 31, Woodland Hills, CA, USA). No significant differences were observed in these parameters as shown in Table 1. Animals were also observed for any abnormal behaviour during the whole study and were found active and normal.

The effect of one-week EPO-treatment on various haematological parameters is presented in Table 2 and Figure 1. Erythropoietin significantly ($p < 0.001$) increased the erythrocyte count (RBC) $8.83 \pm 0.27 \times 10^6/\mu\text{l}$ in comparison to control ($7.44 \pm 0.13 \times 10^6/\mu\text{l}$); hemoglobin (Hb) $16.3 \pm 0.38 \text{ g/dL}$ in comparison to control ($13.3 \pm 0.20 \text{ g/dL}$); hematocrit (PCV/HCT) $51.2 \pm 1.09 \%$ in comparison to control ($40.1 \pm 0.56 \%$) and platelet count (PLT) $903 \pm 54 \times 10^3/\mu\text{l}$ in comparison to control $669 \pm 46 \times 10^3/\mu\text{l}$. There was no significant effect on leukocyte count (WBC), mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH). There was a significant decrease in mean corpuscular haemoglobin concentration (MCHC).

Table 1. Effect of EPO-treatment on hemodynamic parameters. Data are presented as mean \pm SEM; Pressure is in mmHg and Heart rate in Beats/min

Group	Systolic pressure (mmHg)	Diastolic pressure (mmHg)	MAP (mmHg)	Heart rate (BPM)
Control	146 ± 1.77	112 ± 1.82	122.8 ± 1.67	485.6 ± 10.68
rEPO-treated	146 ± 1.73	112 ± 2.13	122.9 ± 1.92	468.6 ± 9.46

Table 2. Effect of one-week rEPO-treatment on various haematological parameters. Data are presented as mean \pm SEM, ** $P < 0.001$ and *** $P < 0.0001$ in comparison to control animals.

Parameter	Control	rEPO-treated
WBC ($10^3/\mu\text{l}$)	13 ± 1.2	15 ± 1.4
RBC ($10^6/\mu\text{l}$)	7.44 ± 0.13	$8.83 \pm 0.27^{***}$
Hb (g/dL)	13.3 ± 0.20	$16.3 \pm 0.38^{***}$
HCT (%)	40.1 ± 0.56	$51.2 \pm 1.09^{***}$
PLT ($10^3/\mu\text{l}$)	669 ± 46	$903 \pm 54^{**}$
MCV (fL)	53.9 ± 0.51	58.1 ± 0.83
MCH (pg)	17.9 ± 0.19	18.6 ± 0.26
MCHC (g/dl)	33.1 ± 0.22	$31.9 \pm 0.15^{***}$

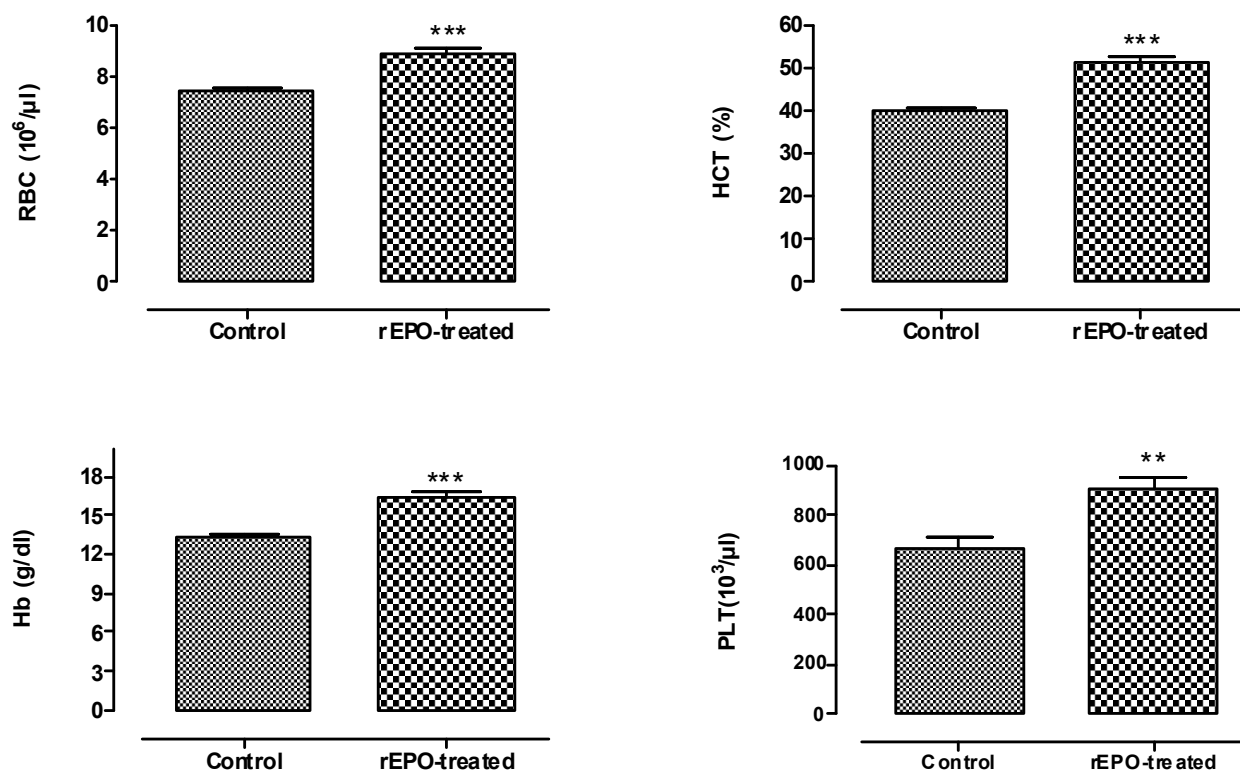


Figure 1: Bar diagrams showing the effect of EPO-treatment on various haematological parameters. ** $P < 0.001$ and *** $P < 0.0001$ in comparison to control animals

DISCUSSION

An expanding increase in the clinical use of erythropoietin is witnessed. Apart from the clinical use, its illegitimate use has reached the point where various athletes have relied on its systemic use (Pommering, 2007). The annual prescription sales of EPO and erythropoiesis-stimulating agents (ESA) are expanding (Steinbrook, 2007). The EPO doping is dangerous because of excessive erythropoiesis which can result in high blood viscosity, thromboembolism, and death. A meta-analysis showed an increased risk of venous thromboembolism and increased mortality rates after EPO administration (Bennet, 2008). The mechanisms of such events are not fully elucidated but EPO-induced arterial hypertension is a leading candidate (Fishbane, 2007). A cause-effect relationship was hypothesized in the present study.

We got profound changes in some haematological parameters in short term rEPO use in otherwise normal Wistar rats. Hematocrit was highly elevated from 40% to 51% which is a profound increase. Haemoglobin level jumped from 13g/dl to 16 g/dl within a week. We assumed that an increase in the hematocrit

will result in increased blood viscosity and thus may result in hypertension since peripheral resistance is governed by blood viscosity apart from blood vessel diameter and total vessel length. Blood viscosity is a major determinant of cardiac work and tissue perfusion (Levy et al., 2008). The more viscous the blood, the greater the resistance it encounters and the higher the blood pressure. A direct correlation between blood pressure and viscosity has been shown in both normotensive and hypertensive subjects (Letcher et al., 1981).

However, despite the increase in HCT the normal rats did not develop hypertension. No significant difference in blood pressure was found between the rEPO treated and control rats. It is unlikely that the dose of EPO in our study was too low to have any effect as we used a high dose over one week. Athletes associated with endurance sports possibly use EPO for short durations as in our study. EPO treatment was without effect on heart rate, blood pressure and behaviour except haematology variables in normal rats. The short-term changes described for these parameters in normal animals are therefore unlikely to

cause changes in cardiovascular function. Some authors found no alteration of blood pressure, heart rate, or cardiac output, in transgenic mice over-expressing EPO (Vogel et al., 2003). They explain this by discussing an adaptive mechanism that involves the increased activity of endothelial nitric oxide synthase and increased flexibility of transgenic erythrocytes.

CONCLUSION

Acute and high-dose administration of rEPO in-

stigated a profound effect on haematological parameters (especially hematocrit) but it was without any effect on heart rate or blood pressure. Our study does not provide evidence for pronounced cardiovascular side-effects of rEPO treatment over a period of one week.

CONFLICT OF INTEREST

None declared.

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