The effect of erythropoietin on calcium levels during hypoxia reoxygenation injury in rats

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Abstract

This experimental study examined the effect of erythropoietin (Epo) on rat model and particularly in a hypoxia-reoxygenation protocol. The effect of that molecule was studied biochemically using blood mean calcium levels (Ca^{++}). Forty rats of mean weight 247.7 g were used in the study. Ca^{++} levels were measured at 60 min (groups A and C) and at 120 min (groups B and D) of reoxygenation. Erythropoietin was administered only in groups C and D. Epo administration non-significantly decreased the Ca^{++} levels by 0.56%±1.13% (P=0.5761). Reoxygenation time non-significantly increased the Ca^{++} levels by 0.65%±1.12% (P=0.5281). However, Epo administration and reoxygenation time together non-significantly decreased the Ca^{++} levels by 0.34%±0.68% (P=0.6095). Epo administration whether it interacted or not with reoxygenation time had non-significant decreasing short-term effects on calcium levels. Perhaps, a longer study time than 2 h or a higher Epo dose may reveal more significant effects.

Introduction

Permanent or transient damage with serious implications on adjacent organs and certainly on patients' health may be due to tissue hypoxia and reoxygenation (HR). Although important progress has been made regarding the usage of erythropoietin (Epo) in managing this kind of damages, satisfactory answers have not been given yet to fundamental questions, as, by what velocity this factor acts, when should it be administered and at what dosage. The particularly satisfactory action of Epo in stem blood cells recovery has been noted in several performed experiments. However, just few relative reports were found concerning Epo trial in ischemia reperfusion (IR) experiments, not covering completely this particular matter. A meta-analysis of 23 published seric variables,[1-4] coming from the same experimental setting, tried to provide a numeric evaluation of the Epo efficacy at the same endpoints (Table 1). Furthermore, several publications addressed trials of other similar molecules of growth factors, which the studied molecule also belongs to.

The aim of this experimental study was to examine the effect of Epo on rat model and particularly in an HR protocol. The effect of that molecule was studied by measuring the blood mean calcium (Ca^{++}) levels. Hypocalcemia although seriously underdiagnosed[5] in geriatric units may be deteriorated by Epo administration.

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Materials and Methods

Animal preparation

This experimental study was licensed by Veterinary Address of East Attiki Prefecture under 3693/12-11-2010 and 14/10-1-2012 decisions. All consumables, equipment and substances, were a courtesy of Experimental Research Centre of ELPEN Pharmaceuticals Co. Inc. S.A. at Pikermi, Attiki. Accepted standards of humane animal care were adopted for Albino female Wistar rats. Pre-experimental normal housing in laboratory for 7 days included *ad libitum* diet. Post-experimental awakening and preservation of the rodents was not permitted, even if euthanasia was needed. They were randomly delivered to four experimental groups by 10 animals in each one. Hypoxia for 45 min followed by reoxygenation for 60 min (group A). Hypoxia for 45 min followed by reoxygenation for 120 min (group B). Hypoxia for 45 min followed by immediate Epo intravenous (IV) administration and reoxygenation for 60 min (group C). Hypoxia for 45 min followed by immediate Epo IV administration and reoxygenation for 120 min (group D). The molecule Epo dosage was 10 mg/kg body weight of animals.

Prenarcosis preceded of continuous intra-experimental general anesthesia, oxygen supply, electrocardiogram and acidometry of animals.[1-4]

The protocol of HR was followed. Hypoxia was caused by laparotomic forceps clamping inferior aorta over renal arteries for 45 min. Reoxygenation was induced by removing the clamp and reestablishment of inferior aorta patency. The molecules were administered at the time of reoxygenation, through catheterized inferior vena cava. The Ca^{++} levels measurements were performed at 60 min of reoxygenation (for groups A and C) and at 120 min of reoxygenation (for groups B and D). The mean weight of the forty (40) female Wistar albino rats used was 247.7 g [standard deviation (Std. Dev): 34.99172 g], with min weight ≥165 g and max weight ≤320 g. Rats' weight could be potentially a confusing factor, *e.g.*, the more obese rats to have higher Ca^{++} levels. This assumption was investigated.

Model of hypoxia-reoxygenation injury

Control groups

Twenty control rats [mean mass 252.5 g (Std. Dev: 39.31988 g)] experienced hypoxia for 45 min followed by reoxygenation.

Group A

Reoxygenation lasted for 60 min (n=10 controls rats) mean mass 243 g (Std. Dev: 45.77724 g), mean Ca^{++} levels 10.53 mg/dL (Std. Dev: 0.3465705 mg/dL) (Table 2).

Group B

Reoxygenation lasted for 120 min (n=10 controls rats) mean mass 262 g (Std. Dev:

31.10913 g), mean Ca^{++} levels 10.69 mg/dL (Std. Dev: 0.3984694 mg/dL) (Table 2).

Erythropoietin group

Twenty Epo rats [mean mass 242.9 g (Std. Dev: 30.3105 g)] experienced hypoxia for 45 min followed by reoxygenation in the beginning of which 10 mg Epo/kg body weight were IV administered.

Group C

Reoxygenation lasted for 60 min (n=10 Epo rats) mean mass 242.8 g (Std. Dev: 29.33636 g), mean Ca^{++} levels 10.56 mg/dL (Std. Dev: 0.1349897 mg/dL) (Table 2).

Group D

Reoxygenation lasted for 120 min (n=10 Epo rats) mean mass 243 g (Std. Dev: 32.84644 g), mean Ca^{++} levels 10.54 mg/dL (Std. Dev: 0.516828 mg/dL) (Table 2).

Statistical analysis

Every weight and Ca^{++} levels group was compared with each other from 4 remained groups applying statistical standard t-test (Table 3). Any emerging significant difference among Ca^{++} levels, was investigated whether owed in the above-mentioned probable significant weight ones. The application of generalized linear models (glm) with dependent variable the Ca^{++} levels was followed. The 3 independent variables were the Epo administration or no, the reoxygenation time and their interaction. Inserting the rats' weight as independent variable at glm, a non-significant relation turned on with Ca^{++} levels (P=0.1279), so as to further investigation was not needed.

Results

The glm resulted in: Epo administration non-significantly decreased the Ca^{++} levels by 0.06 mg/dL (–0.2969947 mg/dL-0.1769949 mg/dL) (P=0.6113). This finding was in accordance with the results of standard t-test (P=0.5409). Reoxygenation time non-significantly increased the Ca^{++} levels by 0.07 mg/dL (–0.1666988 md/dL-0.3066988 mg/dL) (P=0.5529), in accordance also with standard t-test (P=0.5034). However, Epo administration and reoxygenation time together non-significantly decreased the Ca^{++} levels by 0.0363636 mg/dL (–0.1792719 mg/dL-0.1065446 mg/dL) (P= 0.6095). Reviewing the above and Table 3, the Tables 4 and 5 sum up concerning the alteration influence of Epo in connection with reoxygenation time.

Discussion

There are not described situations concerning whether ischemia can influence the Ca^{++} levels in bibliography. On the contrary, there are a lot of cases reporting how the Ca^{++} levels fluctuations affect the function of various organs. Such examples are described herein. Isolated Ca^{++} administration is impossible. It is means that, Ca^{++} is administered by means of a Ca^{++} salt; this is, Ca^{++} conjugated with another drug or ion. The conjugate part may also influence the Ca^{++} levels. Assayag *et al.* found mitochondrial function significantly down-regulated by HR or Ca^{++} overload insults[6] in isolated IR rat heart mitochondria. Gonçalves *et al.* noted non significantly increased[7] thiobarbituric acid

Table 1. The erythropoietin influence (±standard deviation) on the levels of some seric[1] variables concerning reperfusion time.

Variable	1 h rep (%)	P-value	1.5 h rep (%)	P-value	2 h rep (%)	P-value	Interaction of Epo and rep (%)	P-value
White BCC	+24.01±13.38	0.1012	+22.09±9.11	0.0351	+20.17±12.94	0.0902	+14.63±5.40	0.0080
Red BCC	+1.45±3.31	0.6589	+0.37±3.02	0.9048	–0.70±4.68	0.8844	+0.81±1.79	0.6446
Hematocrit	+0.14±2.89	0.9626	–0.61±2.37	0.8072	–1.37±4.05	0.7485	+0.24±1.38	0.8586
MCH	+0.01±1.29	0.9904	+0.67±0.80	0.3549	+1.34±1.08	0.1509	–0.36±0.47	0.4430
RbcDW[2]	–1.85±4.24	0.6703	–1.64±2.53	0.5159	–1.43±3.34	0.6078	–1.06±1.43	0.4733
Platelet DW	+1.60±0.80	0.0765	+1.36±0.58	0.0205	+1.13±0.74	0.1152	+0.37±0.37	0.0615
Platelet-crit	–16.47±10.40	0.0921	–13.74±7.01	0.0158	–11.01±7.34	0.0882	–6.88±3.69	0.0615
Urea	+21.42±7.84	0.0115	+20.11±7.25	0.0059	+18.80±9.44	0.0709	+15.64±4.04	0.0003
Creatinine	–0.10±9.78	0.9904	–4.84±5.78	0.3721	–9.59±7.74	0.1509	–2.62±3.49	0.4430
Uric acid	+10.13±15.10	0.4917	+15.86±10.21	0.1408	+21.59±15.45	0.1940	+9.33±6.16	0.1264
Total protein	–0.02±2.47	0.9904	–1.27±1.51	0.3721	–2.52±2.03	0.1509	–0.68±2.48	0.4430
ALT	+18.89±12.42	0.1372	+7.63±18.94	0.6396	–3.63±25.19	0.8617	+8.03±11.36	0.4698
γGT	–19.35±18.58	0.2362	–12.70±13.11	0.3541	–6.06±19.96	0.7800	–4.62±7.97	0.5534
ALP	+0.20±18.57	0.9904	+10.70±12.78	0.3549	+21.20±17.11	0.1509	+5.79±7.72	0.4430
ACP	+0.06±5.79	0.9904	+3.11±3.71	0.3172	+6.16±4.97	0.1509	+1.68±2.23	0.4430
CPK	+0.15±14.09	0.9904	+7.91±9.44	0.3549	+15.67±12.65	0.1509	+4.28±5.70	0.4430
LDH	+0.08±7.92	0.9904	+4.48±5.35	0.3549	+8.89±7.17	0.1509	+2.42±3.22	0.4430
Sodium	+0.72±0.74	0.3054	+0.21±0.63	0.7136	–0.29±1.09	0.7670	-0.11±0.38	0.7531
Potassium	–6.17±4.94	0.1540	–2.21±3.66	0.5134	+1.74±5.43	0.7299	+0.18±2.22	0.9338
Phosphorus	+1.92±5.25	0.6982	+3.95±3.35	0.2100	+5.98±4.81	0.2930	+2.45±2.01	0.2168
Magnesium[3]	+1±6.20	0.8596	–1.09±3.34	0.7248	–3.19±3.90	0.3729	–0.19±1.93	0.9197
Amylase[4]	+6.50±9.15	0.4161	+5.04±6.12	0.3831	+3.59±8.42	0.6649	+4.36±3.65	0.2258
Progesteron	–0.20±18.65	0.9904	–8.86±10.58	0.3549	–17.53±14.15	0.1509	–4.79±6.39	0.4430
Mean	+1.91±9.88	0.5997	+2.45±8.98	0.3835	+2.99±10.61	0.3685	+2.12±5.61	0.4282

rep, reperfusion; Epo, erythropoietin; BCC, blood cell counts; MCH, mean corpuscular hemoglobin; RbcDW, red blood cell distribution width; ALT, alanine aminotransferase; γGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; ACP, autologous conditioned plasma; CPK, creatine phosphokinase; LDH, lactate dehydrogenase.

reactive substances levels in IR rat small intestine and its mesentery samples treated by Ca^{++} carbonat than control ones. Yamagishi *et al.* found 1.33-fold higher post-IR coronary flow rate in 70% reduced fed male Wistar rats group than in *ad libitum* fed group.[8] Pollesello *et al.* attributed[9] a clinical benefit on acute heart failure progression by Ca^{++} sensitization of contractile proteins. Hale *et al.* noted that decreased[10] intracellular Ca^{++} overload reduced the frequency of angina attacks and myocardial stunning ten minutes before and during coronary IR in rabbits. Strömer *et al.* noted[11] 1.6-fold increase in intracellular Ca^{++} overload in left ventricular IR ($P<0.05$) of male Wistar rats *vs* control ones after 13 weeks. Nordlander *et al.* found[12] that vasoselective Ca^{++} antagonists that inhibits L-type Ca^{++} channels protect against IR injuries, reducing infarcts size by 40% in pigs. Riess *et al.* caused significant[13] reversible increases in mitochondrial Ca^{++} levels; preserved cardiac function and tissue viability and prevented hypercalcemia by cold perfusion (17 degrees C). Pang *et al.* explained the protection afforded to IR heart injury by limiting Ca^{++} overload, inhibiting influx of extracellular Ca^{++} through channels distinct from voltage-gated Ca^{++} channels into sarcoplasmic/endoplasmic reticulum Ca^{++} stores in neonatal and adult cardiomyocytes.[14] Volpe *et al.* recommended inhibitors of free radical production and scavengers for the management of[15] perinatal brain injury due to activation of a variety of accumulated Ca^{++}-mediated deleterious events. Ivanics *et al.* have shown[16] significantly increased Ca^{++} levels two hours after rat skeletal muscle IR without altering Ca^{++} homeostasis. Herzog *et al.* associated[17] the significantly diminished infarct size by 3.2-fold with pretreatment with Ca^{++}-channel blockers after left anterior descending myocardial (LADM) IR ($P=0.01$) in Yorkshire swine. Piana *et al.* improved[18] only minimally the dyskinetic ischemic region after LADM-IR to 1% for the IR-saline group ($P<0.05$) in pigs. Arteriolar endothelium-dependent responses Ca^{++} ionophore A23187 ($P<0.01$) were impaired after IR.

Table 2. Weight and calcium mean levels and standard deviation of groups.

Groups	Variable	Mean	Std. Dev
A	Weight Calcium	243 g 10.53 mg/dL	45.77724 g 0.3465705 mg/dL
B	Weight Calcium	262 g 10.69 mg/dL	31.10913 g 0.3984694 mg/dL
C	Weight Calcium	242.8 g 10.56 mg/dL	29.33636 g 0.1349897 mg/dL
D	Weight Calcium	243 g 10.54 mg/dL	32.84644 g 0.516828 mg/dL

Std. Dev, standard deviation.

Table 3. Statistical significance of mean values difference for groups after statistical standard t-test application.

DG	Variable	Difference	P-value
A-B	Weight Calcium	–19 g –0.16 mg/dL	0.2423 0.1999
A-C	Weight Calcium	0.2 g –0.03 mg/dL	0.9900 0.8114
A-D	Weight Calcium	0 g –0.01 mg/dL	1.0000 0.9580
B-C	Weight Calcium	19.2 g 0.13 mg/dL	0.2598 0.4082
B-D	Weight Calcium	19 g 0.15 mg/dL	0.1011 0.3434
C-D	Weight Calcium	–0.2 g 0.02 mg/dL	0.9883 0.9095

DG, difference for groups.

Table 4. The alteration influence of erythropoietin in connection with reperfusion time.

Alteration (mg/dL)	95% CI (mg/dL)	Reperfusion time	P-values	
			t-test	glm
–0.03	–0.2171003-0.2771003	1 h	0.8114	0.8016
0.06	–0.2969947-0.1769949	1.5 h	0.5409	0.6113
0.15	–0.5835689-0.283569	2 h	0.3434	0.4767
–0.07	–0.1666988-0.3066988	Reperfusion time	0.5034	0.5529
0.0363636	–0.1792719-0.1065446	Interaction	-	0.6095

CI, confidence interval; glm, generalized linear models.

Table 5. The (%) alteration influence of erythropoietin in connection with reperfusion time.

Alteration (%)	±Std. Dev (%)	Reperfusion time	P-values
+0.28	±1.19	1 h	0.8065
–0.56	±1.13	1.5 h	0.5761
–1.41	±2.08	2 h	0.4100
+0.65	±1.12	Reperfusion time	0.5281
–0.34	±0.68	Interaction	0.6095

Std. Dev, standard deviation.

Also the majority of the following examples concern the influence of Ca^{++} levels fluctuation on Epo and a minority only the influence of Epo fluctuation on Ca^{++} levels. Kojima *et al.* suggested[19] that home hemodialysis improved patients' survival reducing Epo-stimulating agent and Ca^{++}-phosphate production. Muravyov *et al.* treated[20] adult solid cancer anemic patients (hemoglobin<100 g/L) initially with β-Epoetin 10,000 IU subcutaneously for 4 weeks. The drop of red blood cell aggregation (RBCA) was about 34% ($P<0.01$); the similar when RBCA suspensions were incubated with Ca^{++} ionophore (A23187) responded to the crosstalk between the Ca^{++} regulatory mechanism. Casino *et al.* suggested[21] a systematic monthly analysis of serum Ca^{++} levels with the following guideline based targets 8.4-9.5 mg/dL. Furthermore, they investigated all common causes associated with inadequate response to epoetin treatment in HD patients. Capelli *et al.* could not prove whether[22] Ca^{++} levels and higher erythropoietic stimulating agents (α-epoetin) dose levels usage were associated with higher mortality rates in HD patients. Fujishiro *et al.* elucidated that long-term response of mammals adaptation[23] to hypoxia is the increase in Epo production. Tozawa *et al.* related the 1st and 3rd most prescribed drug types with Ca^{++} metabolism (88%) and Epo (60%) respectively; being 2.66-fold more than mean medications prescription[24] number in ambulatory general practice patients and associated positively with short-term mortality by 1.14-fold ($P=0.007$) in HD patients. Ortega *et al.* showed[25] the need for higher Epo doses in predialysis patients using Ca^{++} levels. Pre-dialysis inflammation predicts poorer response to Epo. Sezer *et al.* decreased Epo dose[26] after change of treatment to 6 months continuous ambulatory peritoneal dialysis measuring Ca^{++} levels in HD patients. Taylor *et al.* noted[27] no significant influence on short-term Epo therapy with a Ca^{++}-channel blocker in HD patients. Kokot *et al.* reported[28] exacerbation of secondary Ca^{++} deposits in HD uremic patients treated with long-term rhEpo. Schiffl caused[29] a 1.5-fold rise in platelet cytosolic Ca^{++} concentration ($P<0.05$), in vascular smooth muscle cells and in cellular Ca^{++} influx after 12 weeks of rHuEpo treatment in HD patients.

Conclusions

Epo administration whether it interacted or not with reoxygenation time had non-significant decreasing short-term effects on calcium levels. Perhaps, a longer study time than 2 h or a higher Epo dose may reveal more significant effects; taken into consideration managing hypocalcemic or hypercalcemic clinical situations in elderly.

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