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THE EFFECT OF ERYTHROPOIETIN ON ACID PHOSPHATASE LEVELS DURING ISCHEMIA REPERFUSION INJURY IN RATS

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Summary

Although the usage of erythropoietin (EPO) in ischemia reperfusion (IR) is known, the speed of administration, dose and time of suitable administration are not so well-known. The aim of this work was to study the effect of erythropoietin (EPO) on bone damage induced by ischemia and reperfusion studied by changes on blood acid phosphatase (ACP) levels in rats. ACP was measured at these time points: 60 min after reperfusion (groups A and C), and 120 min after reperfusion (groups B and D), A and B without but C and D with EPO administration. Results: EPO administration did not increase significantly the ACP levels. Reperfusion time did not increase significantly the ACP levels and the interaction of EPO administration and reperfusion time did not increase significantly the ACP levels. Conclusions are that EPO administration does not attenuate the osteoclastic bone action of ischemia within narrow context of 2 hours. Perhaps, a longer study may prove a reverse beneficial action for ACP values.

Keywords: erythropoietin, acid phosphatase, reperfusion

Resumen

EFECTO DE LA ERITROPOYETINA SOBRE LA FOSFATASA ACIDA DURANTE EL DAÑO PRODUCIDO POR LA ISQUEMIA REPERFUSIÓN EN RATAS

El proceso de isquemia reperfusión (IR) es una de las causas de daño tisular con implicancias en la salud de pacientes. Si bien el uso de eritropoyectina (EPO) es conocido, no lo es tanto la velocidad de administración, dosis y momento de administración adecuado. El objetivo de este trabajo fue estudiar el efecto de la administración de EPO sobre el daño óseo inducido por procesos isquémicos a través de la concentración sanguínea de fosfatasa ácida (ACP) durante un período de isquemia- reperfusión con y sin la administración de EPO. Se utilizaron 40 ratas en las que se produjo isquemia por 45 min y se midió la ACP 60 min luego de la reperfusión (grupos A y C) y a los 120 min luego de la reperfusión (grupos B y D). Los grupos A y B no recibieron EPO mientras que C y D recibieron EPO intravenosa. Los datos de ACP se analizaron

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aplicando modelos lineales y los coeficientes se consideraron significativos si p<0,05. La administración de EPO no afectó significativamente los niveles de ACP. El tiempo de reperfusión tampoco tuvo efecto sobre la ACP ni se detectó interacción entre EPO y tiempo de reperfusión. Se concluye que la administración de EPO no atenúa la acción osteoclástica del hueso de la isquemia en contexto de 2 horas. Tal vez, un tiempo de estudio más prolongado puede resultar una acción beneficiosa inversa para los valores de ACP.
Palabras clave: eritropoyetina, fosfatasa ácida, reperfusión

Introduction

Tissue ischemia and reperfusion (IR) remain the main causes of permanent or transient damage with serious implications on organs and certainly on patients' health. The use of erythropoietin (EPO) has been well-established for many years. However, although important progress has been made, satisfactory answers have not been given to questions, such as, what the rate of action is, when it should be administered, and administration doses. The effect of EPO on stem blood cells was demonstrated previously; however no information about rate of action has been reported. There have been publications reporting tests with other molecules of growth factor suppressor groups in which this particular molecule belongs.

In the present study, EPO was administered to find out whether it is able to reverse ischemic short-term general bone damages or not. The determination of biochemical bone turnover markers (BBMT) allows a quantitative assessment of bone turnover. Biochemical markers are molecules produced by osteoblasts, osteoclasts or come from the type I collagen metabolism. They are classified as bone formation or bone resorption markers. Regarding the relationship of BBMT with bone mineral density, the correlation is more significant for the bone resorption markers than bone formation markers. The usefulness of BBMT concerns both the high risk individuals' for rapid detection of loss of bone mass, as well as following up patients under anti-resorption treatment for the early therapeutic intervention assessment. Acid phosphatase (ACP) levels as a bone resorption marker is useful for rapid bone mass loss detection as well as following up patients under anti-resorptive treatment.

The aim of this work was to assess the effect of EPO administration on bone damage induced by ischemic processes studied by changes on blood (ACP) levels.

Materials and Methods

This experimental study was approved by the Scientific Committee of Ippokrateion General Hospital, Athens University, and the Veterinary Address of East Attiki Prefecture. The Institutional and National Guide for the Care and Use of Laboratory Animals was followed.

Experimental groups

This experimental study was laid out by Experimental Research Center of ELPEN Pharmaceuticals Co. Inc. S.A. at Pikermi, Attiki. Animals were managed in accordance with accepted standards of humane animal care. Female Wistar rats (247.7±34.9 g of body weight) were used. Rats were housed in the laboratory 7 days before experimentation with access to water and food. They were randomly assigned into the following experimental groups (n=10 per group):

Group A: ischemia for 45 min followed by reperfusion for 60 min.
Group B: ischemia for 45 min followed by reperfusion for 120 min.
Group C: ischemia for 45 min followed by immediate EPO administration and reperfusion for 60 min.
Group D: ischemia for 45 min followed by immediate EPO administration and reperfusion for 120 min.

IR rats were anesthetized by initial intramuscular (IM) administration of 0.25 ml xylazine, [20 mg/ml] and 0.25 ml ketamine hydrochloride [100 mg/ml]. Before laparotomy, 0.03 ml butorphanol [10 mg/ml] was administered subcutaneously (SC). Continuous oxygen supply was administered during the whole experiment. Ischemia was caused by clamping inferior aorta for 45 min after laparotomy. Reperfusion was achieved by removing clamp and inferior aorta patency re-establishment. The electrocardiogram and acidimetry were continuously monitored.

The EPO was intravenously (IV) administered at a dose of 10 mg/kg body weight, through inferior vena cava catheterization.

The ACP level measurement was performed at 60 min of reperfusion (groups A and C) and at 120 min of reperfusion (groups B and D).

Data are expressed as mean ± SD and differences were considered significant if $p<0.05$. STATA 6.0 software of Athens University (USA) was used for statistical analyses. ANOVA and generalized linear models were used to analyze weights and ACP levels.

Results

Weights and ACP levels of groups were not different (ANOVA, $p>0.05$). Applying generalized linear models (GLM) with ACP levels as dependent variable and EPO administration and reperfusion time as independent variables and their interaction, resulted: EPO administration significantly decreased the ACP levels by 7.1 IU/l after 60 min reperfusion ($p<0.05$) but not after 120 min of reperfusion. Reperfusion time did not increase significantly the ACP levels. The interaction of EPO administration and reperfusion time did not increase significantly the ACP levels (Table 1).

Table 1. Influence of erythropoietin on acid phosphatase (ACP) levels in connection with reperfusion time.

ACP decreasing influence	95% CI	Variable	p values
7.1 IU/l	-12.2 IU/l – -1.9 IU/l	60 min	0.0098
1.1 IU/l	-6 IU/l – 3.7 IU/l	120 min	0.6198
1.6 IU/l	-3.8 IU/l – 0.5 IU/l	interaction	0.1396

After adding the body weight of rats as independent variable at GLM, a very significant relation turned up on ACP levels ($p=0.0057$), further investigation will be needed. The predicted ACP values, adjusted for rats' weight were calculated and showed in Table 2.

Applying GLM with dependent variable, the predicted ACP levels and independent variables with or without EPO administration, the reperfusion time and their interaction, resulted: EPO administration did not increase significantly the predicted ACP levels. Reperfusion time did not increase significantly the predicted ACP levels. The interaction of EPO administration and reperfusion time did not increase significantly the predicted ACP levels (Table 3 and 4).

Table 2. Mean predicted ACP values adjusted by weight and SD of groups.

Groups	Mean±SD
A	18±2.5 IU/l
B	16.9±1.7 IU/l
C	18±1.6 IU/l
D	18±1.8 IU/l

Table 3. Innfluence of erythropoietin on acid phosphatase (ACP) levels in connection with reperfusion time.

ACP increasing influence	95% CI	Variable	p values
0 IU/l	-2 IU/L – 2 IU/l	60 min	0.9908
1 IU/l	-0.6 IU/L – 2.7 IU/l	120 min	0.2007
0.2 IU/l	-0.4 IU/L – 1 IU/l	interaction	0.4430

Table 4. Increase rate (%) influence of APO on ACP levels in connection with reperfusion time.

ACP increasing Influence (%±SD)	95% CI	Variable	p values
0.06±5.79	-2 IU/L – 2 IU/l	60 min	0.9904
6.16±4.97	-0.6 IU/L – 2.7 IU/l	120 min	0.1509
1.68±2.23	-0.4 IU/L – 1 IU/l	interaction	0.4430

Discussion

In this study the effect of EPO on bone damage induced by ischemia and reperfusion analyzed by changes on ACP levels was evaluated. EPO administration, reperfusion and the interaction did not increase significantly the ACP levels.

Many clinical situations can prove how ACP levels are influenced by ischemic cases. Otero JE et al [6] reported a 7-year-old boy with generalized arterial calcification of infancy (GACI), an autosomal recessive disorder that features hydroxyapatite deposition within arterial elastic fibers. He developed arterial calcification after 5 months of 1-hydroxyethylidene-bisphosphonate (EHDP) lifesaving therapy 200 mg/day orally during infancy. The surveillance for toxicity was crucial since brain isoform of tartrate-resistant ACP 5b (TRAP-5b) was elevated. Frederiks WM et al [7] investigated early ischemic damage in mitochondria of rats heart after ischemia. It was shown that ACP enzyme had not decreased until 240 min heart fragments incubation, but did not correlate with the irreversible stage of myocyte damage. Kikuchi T et al[8] examined the

role of lysosomal enzymes in ischemic retinal pigment of epithelial cells of albino rabbits for ACP. Five days after posterior ciliary arteries PCA-cut, retinal pigment of epithelial cells in the ischemic region were disorganized by increased enzymatic digestion. In the border of ischemic retinal pigment of epithelial cells region, a lot of fragmented outer segments were phagocytosed 1 to 7 days after PCA-cut. At this time phagosomes appeared much more frequently than in normal retina. A strong ACP activity was encountered in the phagosomes of macrophages, derived from retinal pigment epithelial cells, and seemed to play the major role in scavenging destructed retinal elements. Robinson JW et al [9] subjected short loops of dog small intestine into IR. The release of lysosomal enzymes after ischemia was studied by gauging the levels of ACP in the ischemic loop and in the neighboring control one. Immediately after ischemia, considerable structural damage was observed in the intestinal mucosa, with desquamation of the villous tips, edema, vascular stasis, and hemorrhagic infiltration in the lamina propria. A significant release of lysosomal ACP enzyme into the venous blood was found.

Also, in some clinical cases, ACP levels can be influenced by EPO. Kim CD et al[10] evaluated the usefulness of bone marrow immunoscintigraphy in chronic renal failure patients under EPO plus either dialysis or conservative treatment. According to the presence of bone marrow expansion, which may represent marrow fibrosis, the most distinguishing characteristic of osteitis fibrosa (form of renal osteodystrophy), the patients were divided into two groups: Group I (36.84%) with bone marrow expansion and Group II (63.16%) with normal marrow distribution. There was no significant difference in TRAP between the two groups. Robinson AS et al [11] have found that the extractable levels of two endoplasmic reticulum-resident proteins involved in correct folding of efficiently secreted proteins -heavy chain binding protein and protein disulfide isomerase- are significantly reduced by prolonged constitutive overexpression of human EPO, or Schizosaccharomyces pombe ACP. Okumura N et al [12] micromanipulated single human hematopoietic progenitor cells individually to secondary culture. Produced erythroid bursts, were stained with various stainings among which ACP was also positive, sequentially expressing respective cell surface antigen. Sułowicz W et al [13] showed a significant increase of peripheral blood neutrophils ACP enzyme activity after 32 weeks of the recombinant human EPO treatment due to severe anemia in maintenance hemodialysed patients compared with baseline values prior therapy. Zakahrov IuM et al modeled [14] erythropoiesis in rats to study the functional condition of central macrophages of the erythroblastic islets in the bone marrow by determining the total activity of ACP content of lysosomes. The properties of the surface charges of the erythroblastic islet cells and certainly the intensity of erythropoiesis were judged according to the number of ACP. Sadahira Y et al [15] cultured erythroblastic islets composed of surrounding erythroid cells and central stromal macrophages (M phi) from mice spleens in the presence of EPO. One day later, erythropoietic activity on the M phi surface was continued. Some EI showed synchronized expansion of erythroblasts and others showed differentiation to reticulocytes. Two days later, about 50% of the EI still showed erythropoietic activity and most erythroblasts differentiated to the orthochromatic stage. Kurihara N et al [16] cultured double replated mice spleen blast cells into wells in presence of EPO. Multinucleated cells appeared from day 14 of culture and approximately 100 giant cells per well were scored on day 21 of culture. The large cells were revealed containing many nuclei in their cytoplasm, which is characteristic of bone-resorbing cells or osteoclasts. These cells showed a TRAP activity. In the presence of r interleukin-6 plus 1,25$(OH)_2D_3$, formation

of TRAP-positive multinucleated cells was lower than the support of rIL-3. Reynolds CW et al [17] found the most common of disorders referred as "chronic T γ-lymphoproliferative disease" (T γ-LPD) generally characterized by ACP positive lymphocytes. Fauser AA et al [18] prepared for further cytological examination the growth of mixed human colonies that contain more than one lineage of hemopoietic differentiation supported by EPO, in which erythroblasts also were identified by positive reaction for ACP. Levi EB et al [19] determined the activity of ACP in either anemic or polycytemic rats kidney tissue as being neither with significant changes nor a controlling factor in the renal EPO production.

Unfortunately, examples from other tissues were used lacking pure bone tissue examples. So, the above clinical situations show that ACP levels are elevated upon GACI, ischemic heart myocyte mitochondrial damage in rats, ischemic retinal pigment epithelial cells region and ischemic intestinal mucosa. Also, ACP levels were elevated by EPO treatment in all next clinical cases: in Schizosaccharomyces pombe constitutively co-expression, in 2 cases of erythroid bursts in anemic hemodialysed patients or anemic rat kidney tissue, in 4 cases of erythropoiesis in either normal human or rats or polycytemic rats, one composed of characteristic osteoclasts in mice spleen, in chronic T γ-lymphoproliferative disease, except osteitis fibrosa (form of renal osteodystrophy) where ACP levels remained comparable with control patients. However, EPO stimulates everywhere the osteoclastic marker ACP. These statements agree with the present experimental results concerning exclusively of bone tissue of more valid data: the fitted ones for rat's weight .

Conclusion

It was concluded that EPO administration does not attenuate the osteoclastic bone action of ischemia within narrow context of 2 hours. Perhaps, a longer study time may prove a reverse beneficial action for ACP values.

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