

ORIGINAL RESEARCH

The Effect of Erythropoietin on Progesterone Levels during Ischemia Reperfusion Injury in Rats

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ABSTRACT

The aim of this experiment was to study the effects of erythropoietin on rat model, particularly in ischemia reperfusion protocol. The beneficial or other effects of that molecule were studied estimating the mean blood progesterone levels.

Materials and methods: Forty rats were used of mean weight 247.7 gm. Progesterone levels were measured 60 minutes after reperfusion for groups A and C and 120 minutes after reperfusion for groups B and D. Groups A and B without the drug but C and D with erythropoietin administration.

Results: That erythropoietin administration nonsignificantly increased the progesterone levels by 4.235501 nmol/l (-13.07804 nmol/l - 21.54904 nmol/l) ($p = 0.6233$). This finding was in accordance with the results of paired t-test ($p = 0.6724$). Reperfusion time nonsignificantly decreased the progesterone levels by -0.2034999 nmol/l (-17.5727 nmol/l - 17.1657 nmol/l) ($p = 0.9812$), also in accordance with paired t-test ($p = 0.9821$). However, erythropoietin administration and reperfusion time together nonsignificantly increased the progesterone levels by 1.713364 nmol/l (-8.74561 nmol/l - 12.17234 nmol/l) ($p = 0.7420$).

Conclusion: Results of this study indicate that Epo decreases the predicted progesterone levels by 4.7 to 8.8%. This decreasing effect although non-significant is reinforced along time. Perhaps, a longer study time than 2 hours may provide clearer and significant effect.

Keywords: Erythropoietin, Progesterone levels, Reperfusion.

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INTRODUCTION

Tissue ischemia and reperfusion (IR) remain one of the main causes of permanent or transient damage with serious implications on adjacent organs and certainly on patients' health. The use of erythropoietin (EPO) is a well established knowledge for many years. However despite important progress has been made, satisfactory answers have not been obtained yet to the fundamental questions, such as by what velocity this factor acts, when should it be administered, and in what dosage. The particularly satisfactory action of the erythropoietin in stem blood cells recovery has been noted in several performed experiments. Since a careful literature search (PubMed-Medline) was conducted, it was realized that this certain factor has been tried in experiments. However, just few relative reports were found, not covering completely this particular object of action velocity. A lot of publications are present on trials of other similar molecules of growth factors to which the studied molecule also belongs to.

The aim of this experimental study was to examine the effect of the drug EPO on rat model and particularly in an ovarian IR protocol. The beneficial effect or non-effectiveness of that molecule were studied by measuring blood progesterone levels.

MATERIALS AND METHODS

Animal Preparation

This experimental study was approved by Scientific committee of Ippokrateion General Hospital, Athens University and by Veterinary Address of East Attiki Prefecture under 3693/12-11-2010 and 14/10-1-2012 decisions. Institutional and national guide for the care and use of laboratory animals was followed. This experimental study was carried out at the Experimental Research Center of ELPEN Pharmaceuticals Co. Inc. SA at Pikermi, Attiki. All settings needed for the study including of consumables, equipment and substances used, were provided by them. Albino female Wistar rats were used in accordance with accepted standards of humane animal care. They spent 7 days in laboratory before experimentation with easy access

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to water and food. The experiment was acute, that is, the animal usage was completed by following experimental set of times without awakening and preservation of the rodents. They were randomly assigned to four experimental groups (10 animals in each group). Group A: Ischemia for 45 minutes followed by reperfusion for 60 minutes. Group B: Ischemia for 45 minutes followed by reperfusion for 120 minutes. Group C: Ischemia for 45 minutes followed immediately by EPO intravenous (IV) administration and reperfusion for 60 minutes. Group D: Ischemia for 45 minutes followed immediately by EPO IV administration and reperfusion for 120 minutes.

The molecule erythropoietin was administered in a dose of: 10 mg/kg body weight of the animal.

The experiment was beginning by preanesthesia and general anesthesia administration to the animals. Their electrocardiogram and acidometry were continuously monitored. The inferior aorta was prepared so as its blood flow could be excluded by forceps. After exclusion, the protocol of IR was applied, exactly as is described in experimental groups. The molecules were administered at the time of reperfusion, through inferior vena cava catheterization, which had been carried out after general anesthesia.

The progesterone levels measurements were performed at 60 minutes after reperfusion for groups A and C and at 120 minutes after reperfusion for groups B and D.

Protocol of the Experiment

The experimental rats were given general anesthesia by initial intramuscular (IM) administration of 0.5 cc of a compound, constituting 0.25 cc xylazine, (25 cc, 20 mg/cc) and 0.25 cc ketamine hydrochloride (1000, 100 mg/cc, 10 cc). 0.03 cc butorphanol (10 mg/cc, 10 cc) anesthetic agent was administered subcutaneously (SC) before laparotomy. Continuous oxygen supply was administered during the whole experiment. Ischemia was caused by clamping inferior aorta over renal arteries for 45 minutes after laparotomic access. Reperfusion was achieved by removing the clamp and inferior aorta patency re-establishment. Forty (40) female Wistar albino rats were used of mean weight 247.7 gm (std. dev: 34.99172 gm), with minutes weight ≥ 165 gm and max weight ≤ 320 gm. Rats' weight could be potentially a confusing factor, e.g. fatter rats to have greater blood progesterone levels. This suspicion will be investigated.

MODEL OF ISCHEMIA-REPERFUSION INJURY

Control Groups

Twenty control rats of mean weight 252.5 gm (std. dev: 39.31988 gm) were subjected to ischemia for 45 minutes followed by reperfusion.

Group A: Ten controls rats of mean weight 243 gm (std. dev: 45.77724 gm), mean progesterone levels 36.94 nmol/l (std. dev: 35.57759 nmol/l) were subjected to 60 minutes reperfusion (Table 1).

Group B: Ten controls rats of mean weight 262 gm (std. dev: 31.10913 gm), mean progesterone levels 39.816 nmol/l (std. dev: 21.82161 nmol/l) were subjected to 120 minutes reperfusion (see Table 1).

Erythropoietin Group

Twenty rats of mean weight 242.9 gm (std. dev: 30.3105 gm) were subjected to ischemia for 45 minutes followed by reperfusion in the beginning of which 10 mg EPO/kg body weight were IV administered.

Group C: Ten EPO rats of mean weight 242.8 gm (std. dev: 29.33636 gm), mean progesterone levels 44.255 nmol/l (std. dev: 25.23905 nmol/l) were subjected to 60 minutes reperfusion (see Table 1).

Group D: Ten EPO rats of mean weight 243 gm (std. dev: 32.84644 gm), mean progesterone levels 40.972 nmol/l (std. dev: 26.43451 nmol/l) were subjected to 120 minutes reperfusion (see Table 1).

RESULTS

Every weight rats group initially was compared with other one from three remained groups applying statistical paired t-test (Table 2). Any emerging significant difference among progesterone levels, will be investigated whether owed in the above-mentioned significant weight correlations. Every progesterone rats group initially was compared with other one from three remainder groups applying statistical paired t-test (see Table 2). Applying generalized linear models (GLM) with dependant variable the progesterone levels and independent variables the EPO administration or no, the reperfusion time and their interaction, resulted in: Erythropoietin administration nonsignificantly increased the progesterone levels by 4.235501 nmol/l (-13.07804 nmol/l-21.54904 nmol/l) (p = 0.6233). This finding was in accordance with the results of paired t-test (p = 0.6724). Reperfusion time nonsignificantly decreased the progesterone levels by -0.2034999

Table 1: Weight and progesterone receptor (PR) mean levels and standard deviation of groups

Groups	Variable	Mean	Std. dev.
A	Weight	243 gm	45.77724 gm
	PR	36.94 nmol/l	35.57759 nmol/l
B	Weight	262 gm	31.10913 gm
	PR	39.816 nmol/l	21.82161 nmol/l
C	Weight	242.8 gm	29.33636 gm
	PR	44.255 nmol/l	25.23905 nmol/l
D	Weight	243 gm	32.84644 gm
	PR	40.972 nmol/l	26.43451 nmol/l

nmol/l (-17.5727 nmol/l – 17.1657 nmol/l) ($p = 0.9812$), also in accordance with paired t-test ($p = 0.9821$). However, EPO administration and reperfusion time together nonsignificantly increased the progesterone levels by 1.713364 nmol/l (-8.74561 nmol/l – 12.17234 nmol/l) ($p = 0.7420$).

Reviewing the above and Table 2, Table 3 sums up concerning the increasing influence of EPO in connection with reperfusion time. Inserting the rats weight as independent variable at glm, a very significant relation turns on progesterone levels ($p = 0.0008$), so as to further investigation needs. The predicted progesterone values adjusted for rats weight were calculated and are depicted at Table 4. The differences between predicted mean progesterone values as calculated by paired t-tests are depicted at Table 5. Applying GLM with dependant variable the predicted progesterone levels and independent variables the EPO administration or no, the reperfusion time and their interaction, resulted in: erythropoietin administration nonsignificantly decreased the predicted progesterone levels by 3.753567 nmol/l (-12.5406 nmol/l – 5.033463 nmol/l) ($p = 0.3926$). This finding was in accordance with the results of paired t-test ($p = 0.3172$). Reperfusion time nonsignificantly increased the predicted progesterone levels by 3.753566 nmol/l (-5.033464 nmol/l – 12.5406 nmol/l) ($p = 0.3926$), also in accordance with paired t-test ($p = 0.3517$). However, EPO administration and reperfusion time together nonsignificantly decreased the predicted progesterone levels by 2.033182 nmol/l (-7.342022 nmol/l – 3.275657 nmol/l) ($p = 0.4430$). Reviewing the above and Tables 5, 6 and 7 sum up concerning the decreasing influence of EPO in connection with reperfusion time.

Table 2: Statistical significance of mean values difference for groups (DG) after statistical paired t-test application

DG	Variable	Difference	p-value
A-B	Weight	-19 gm	0.2423
	PR	-2.876 nmol/l	0.8466
A-C	Weight	0.2 gm	0.9900
	PR	-7.315 nmol/l	0.6312
A-D	Weight	0	1.0000
	PR	-4.032 nmol/l	0.7611
B-C	Weight	19.2 gm	0.2598
	PR	-4.439 nmol/l	0.7123
B-D	Weight	19 gm	0.1011
	PR	-1.156 nmol/l	0.9353
C-D	Weight	-0.2 gm	0.9883
	PR	3.283 nmol/l	0.7783

Table 3: The increasing influence of erythropoietin in connection with reperfusion time

Increase	95% confidence interval	Reperfusion time	p-value	
			t-test	GLM
7.315 nmol/l	-21.66535 – 36.29535 nmol/l	1 hour	0.6312	0.6024
4.2355 nmol/l	-13.07804 – 21.54904 nmol/l	1.5 hours	0.6724	0.6233
1.156 nmol/l	-21.61713 – 23.92913 nmol/l	2 hours	0.9353	0.9162

DISCUSSION

Ulbrich C et al have¹ shown that progesterone (P) applied directly during the first 48 hours after ischemic stroke reduced the infarct volume by more than 70% revealing neuroprotection after 2 weeks. Besides anti-inflammatory and anti-apoptotic actions, angiogenesis in the damaged area appears to be initially affected early after ischemia. Cutler SM et al exacerbated² ischemia suddenly withdrawing progesterone (PW) by repeated dosing after traumatic brain injury (TBI) and stroke in adult male Sprague-Dawley rats. All progesterone treatments resulted in improved molecular recovery. Although progesterone treatment decreases inflammation and apoptosis, only a tapered and not an acute one withdrawal of the hormone further enhances short-term recovery after TBI. Sbarouni E et al did³ not attenuate short-term ischemic myocardium protection by the addition of a progestin compound in IR hypercholesterolemic oophorectomized female rabbits. Giannattasio C et al found⁴ alterations in radial artery distensibility during the natural menstrual cycle in premenopausal healthy women. The arterial stiffening of the luteal phase depends on vascular smooth muscle contraction due to an endothelial impairment due to an increase in progesterone hormone levels among other phenomena.

Ovarian tissue transplantation is used to preserve fertility in patients undergoing chemoradiotherapy. However, IR injury and the production of free radicals occurring during revascularization of the transplanted tissue are the major limitations. The effect of antioxidant drugs in reducing the oxidative stress and improving ovary survival following transplantation is investigated. It is hypothesized that less hypoxemia and less erythropoiesis would occur in the L than the F phase of the cycle at altitude, because the ovarian steroid hormones, progesterone and estrogen, have higher blood levels in the luteal (L) than in the follicular (F) phase of the menstrual cycle since their known effects on ventilation and hematopoiesis. The efficiency of female sex hormone treatment could be related either to the central effects of progesterone and estrogen and/or to the impact of these hormones on erythropoiesis at the kidney/bone marrow level. Steroid metabolites, derived from the *in vivo* biotransformation of progesterone, are capable in other systems of inducing the synthesis of δ -aminolevulinic acid synthetase, the limiting enzyme in the heme biosynthetic pathway.

Table 4: Predicted PR mean levels and standard deviation of groups

Groups	Mean	Std. dev.
A	38.65807 nmol/l	17.89875 nmol/l
B	46.087 nmol/l	12.16356 nmol/l
C	38.57987 nmol/l	11.47042 nmol/l
D	38.65806 nmol/l	12.84285 nmol/l

Mahmoodi M et al found⁵ the mean total volume of ovary, cortex, medulla and the number of follicles significantly increased in the grafts +EPO group ($p < 0.01$) than control one. Trošt N et al analyzed⁶ the correlation between the expression of genes for EPO and PGR cell lines also treated with recombinant human EPO (rHuEPO). rHuEPO treatment only influenced the hormone-independent cell lines. Trost N et al cultured⁷ breast carcinoma model MDA-MB-231 cells with or without rHuEpo for 24 hours or 9 weeks. MDA-MB-231 cells are almost irresponsive to long-term rHuEpo, supposedly due to the mutated PR(-) status which may predict tumor response on rHuEpo treatment. Furth PA et al found⁸ that progesterone and EPO signaling pathways regulate signal transducer and activator of transcription 5 (STAT5) expression and activity in normal development and cancer respectively. Linnertz R et al investigated⁹ various receptor agonists including progesterone and EPO evoked a voltage-gated Na⁺ and Ca²⁺ channel-dependent release of glutamate in retinal glial cells of the rat. Volgger B et al associated¹⁰ EpoR levels positively expression with progesterone receptor positivity status and with decreased locoregional disease control in breast cancer patients. Klotz RK et al revealed¹¹ polyglobulia and a Leydig cell tumor in the hilus and stroma of the left ovary in a 58-year-old woman whose basal laboratory tests were normal for levels of 17alpha-hydroxy-progesterone and EPO. Kwon BK et al reviewed¹² the available neuroprotective therapies including EPO and progesterone administered for acute SCI. Larsson AM et al suggested¹³ that EPOR expression in premenopausal women breast cancer affects tumor behavior since high expression of EPOR is related to an impaired tamoxifen response in ER(+)/PR(+) tumors and to improved survival in untreated patients. Pelekanou V et al reported¹⁴ that EPO and membrane-associated receptors for progesterone (mPR) were expressed in 80 and 94% of samples in breast cancer specimens respectively. Significant correlations between EPO-hypoxia-induced mPR-Her2

Table 5: Statistical significance of mean values difference for groups (DG) after statistical paired t-test application

DG	Difference	p-value
A-B	-7.428935 nmol/l	0.2423
A-C	0.0781984 nmol/l	0.9900
A-D	0	1.0000
B-C	7.507133 nmol/l	0.2598
B-D	7.428936 nmol/l	0.1011
C-D	-0.078197 nmol/l	0.9883

were found. Schouten JW considered¹⁵ EPO and progesterone antagonists promising in experimental traumatic brain injury. Wang KK et al examined¹⁶ progesterone and EPO as neuroprotection targets after traumatic brain injury. Fu ES et al included¹⁷ progesterone and EPO in acute spinal cord injury treatment. Ogawa A et al revealed¹⁸ that EPO bioactivity was significantly increased transiently by 5-fold by treatment with progesterone of human amniotic epithelial cells. Acs G et al observed¹⁹ increased Epo and EpoR expression over the course of the cycle in benign endometrial tissue samples, with the highest levels in the late secretory phase. Epo expression in benign endometrial samples showed a negative correlation with PR expression. Hypoxia-inducible autocrine Epo signaling in endometrial carcinoma may contribute to tumor progression and increased aggressiveness. Nordstrom JL designed²⁰ a geneplasmid based therapy, muscle-specific GeneSwitch system that provides regulated EPO expression dependent on orally administered mifepristone for the treatment of anemia in rats. Spitz IM found²¹ antiproliferative effects on the endometrium of many progesterone antagonists and receptor modulators, enabling controlled expression of specific genes (e.g. EPO) as inducer and thus having application in the treatment of endometriosis. Reeves JT et al did not relate²² blood level of EPO with the levels of progesterone at 4,300 m altitude in women taken for 11 days. Chien CC et al increased²³ the hemoglobin level within a month with a complex hormone therapy with progesterone in a 50-year-old uremic HD maintenance woman who developed severe anemia resistant to treatment with EPO 4 months prior to watermelon stomach demonstration. Wang RY et al suggested²⁴ that the mechanism of the secretion of EPO by kidneys in female rats during aging is ovarian Steroid hormones independent since ovariectomy increased rat plasma EPO concentration which might be inhibited by p ($p < 0.01$) replacement. Favier R et al decreased²⁵ EPO

Table 6: The decreasing influence of erythropoietin in connection with reperfusion time

Decrease	95% confidence interval	Reperfusion time	p-value	
			t-test	GLM
0.0781784 nmol/l	-14.20189 – 14.04549 nmol/l	1 hour	0.9900	0.9908
3.7535672 nmol/l	-12.5406 – 5.033463 nmol/l	1.5 hours	0.3172	0.3926
7.428936 nmol/l	-19.18079 – 4.322922 nmol/l	2 hours	0.1011	0.2007

Table 7: The decreasing influence (%) of erythropoietin in connection with reperfusion time

Decrease	Standard deviation	Reperfusion time	p-value
0.20%	±18.65%	1 hour	0.9904
8.86%	±10.58%	1.5 hours	0.3549
4.79%	±6.39%	Interaction	0.4430
17.53%	±14.15%	2 hours	0.1509

levels and the degree of polycythemia by ovarian steroids including progesterone in young male rats born and living at high altitude (3600 m) than control untreated animals for 6 weeks. Makinoda S et al did²⁶ not see changes in EPO throughout the menstrual cycle in normal healthy female volunteers who recorded basal body temperature every day. Morra L et al blocked²⁷ burst forming unit-erythroid (BFU-E) growth, even at largely suboptimal concentrations of burst-promoting activity (BPA) by preincubation of BFU-E with equimolar progesterone. Lavrijssen KL et al preferentially²⁸ stimulated adult hemoglobin synthesis but not fetal one by high concentrations of progesterone, or those cells which have a high capacity to synthesize adult hemoglobins are less sensitive to toxic concentrations of the hormone, in the erythropoietic calf liver cells. Singer JW et al examined²⁹ 5 β -H pregnane as active for colony growth enhancement. Golde DW et al observed³⁰ maximal 13-day mouse fetal livers colony formation with 0.5 U/ml of sheep EPO and inhibition by progesterone 10⁻⁶ M. Erythroid progenitor cells can modulate the response to EPO in cultures *in vitro*. Mizoguchi H et al have shown³¹ that steroid action is independent of EPO and it is suggested that they may play a physiologic role in the regulation of human erythropoiesis.

CONCLUSION

Original and predicted progesterone results are opposite. Predicted results, as more reliable, must be believed. Epo decreases the progesterone levels by 4.7 to 8.8%. This decreasing effect although nonsignificant is reinforced along time. Perhaps, a longer study time than 2 hours may provide clearer and significant effect.

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