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The effect of erythropoietin on oophoritis during ischemia reperfusion injury in rats

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Abstract
Background: This experimental study examined the effect of erythropoietin on rat model and particularly in an ovarian ischemia reperfusion (IR) protocol. The effects of that molecule were studied pathologically using mean oophoritis (OI) lesions.
Materials and methods: 40 rats of mean weight 247.7 g were used in the study. OI lesions were evaluated at 60 min (groups A and C) and at 120 min (groups B and D) of reperfusion. Erythropoietin was administered only in groups C and D.
Results: Epo administration did not alter the OI scores. The increase in OI scores was 0.15 without lesions [-0.0158339 - 0.3158339] (p=0.0791). Reperfusion kept non-significantly increased the OI scores by 0.15 without lesions [-0.0158339 - 0.3158339] (P=0.0791). Together, the Epo administration and reperfusion kept significantly increased the OI scores by 0.1363636 without lesions [0.0421443 - 0.230583] (p=0.0057).
Conclusions: Epo administration particularly in concert with reperfusion kept significantly increased the OI score lesions in ovaries after experimental ischemia in rats.

Keywords: ischemia, erythropoietin, oophoritis, reperfusion

1. Introduction

Permanent or transient damage with serious implications on adjacent organs and certainly on patients' health may be due to tissue ischemia and reperfusion (IR). 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 IR experiments, not covering completely this particular matter. A meta-analysis of 18 published seric variables, 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 to 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 ovarian IR protocol. The kind of effects that molecule provokes, were studied by evaluating mean oophoritis (OI) lesions.

Table 1: The erythropoietin (Epo) influence (±SD) on the levels of some seric[1] variables concerning reperfusion (rep) time

Variable	1h rep	p-value	1.5h rep	p-value	2h rep	p-value	interaction of Epo and rep	p-value
White BC	+24.01%±13.38%	0.1012	+22.09%±9.11%	0.0351	+20.17%±12.94%	0.0902	+14.63%±5.40%	0.0080
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
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 protei	-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[2]	+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[3]	-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
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
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	+2.39%±10.96%	0.6131	+3.11%±10.01%	0.3210	+3.82%±11.86%	0.2897	+2.48%±6.25%	0.3696

2. Materials and methods

Animal preparation

This experimental study was licensed by Veterinary Address of East Attiki Prefecture under 3693/12-11-2010 & 14/10-1-2012 decisions. Everything needed for the study including consumables, equipment and substances, were a courtesy of Experimental Research Center of ELPEN Pharmaceuticals Co. Inc. S.A. at Pikermi, Attiki. Accepted standards of humane animal care were adopted for Albino female Wistar rats. Normal housing in laboratory 7 days before the experiment 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. Ischemia for 45 min followed by reperfusion for 60 min (group A). Ischemia for 45 min followed by reperfusion for 120 min (group B). Ischemia for 45 min followed by immediate Epo intravenous (IV) administration and reperfusion for 60 min (group C). Ischemia for 45 min followed by immediate Epo IV administration and reperfusion for 120 min (group D). The molecule Epo dosage was 10 mg/Kg body weight of animals.

The detailed preceded prenarcotic and general anesthesiologic techniques of animals are described in related references [1-3]. Oxygen supply, electrocardiogram and acidometry were continuously provided during whole experiment performance.

The protocol of IR was followed. Ischemia was caused by laparotomic forceps clamping inferior aorta over renal arteries for 45 min. Reperfusion was induced by removing the clamp and reestablishment of inferior aorta patency. The molecules were administered at the time of reperfusion, through catheterized inferior vena cava. The OI evaluations were performed at 60 min of reperfusion (for groups A and C) and at 120 min of reperfusion (for groups B and D). The mean weight of the forty (40) female Wistar albino rats used was 247.7 g [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 OI scores lesions. This suspicion was investigated. Also, detailed pathological[4] study and grading of OI findings was performed by scores, this is: 0 lesions were not found, 1 mild lesions were found, 2 moderate lesions were found and 3 serious lesions were found. The previous grading was transformed as follows: (0-0.499) without lesions, (0.5-1.499) mild lesions, (1.5-2.499) moderate lesions and (2.5-3) serious lesions damage, because the study concerns score ranges rather than point scores.

Model of ischemia-reperfusion injury

Control groups20 control rats (mean mass 252.5 g [Std. Dev: 39.31988 g]) experienced ischemia for 45 min followed by reperfusion.

Group A

Reperfusion lasted for 60 min (n=10 controls rats) mean mass 243 g [Std. Dev: 45.77724 g], mean without OI lesions score 0 [Std. Dev: 0] (Table 2).

Group B

Reperfusion lasted for 120 min (n=10 controls rats) mean mass 262 g [Std. Dev: 31.10913 g], mean without OI lesions score 0 [Std. Dev: 0] (Table 2).

Erythropoietin group

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

Group C

Reperfusion lasted for 60 min (n=10 Epo rats) mean mass 242.8 g [Std. Dev: 29.33636 g], mean without OI lesions score 0 [Std. Dev: 0] (Table 2).

Group D

Reperfusion lasted for 120 min (n=10 Epo rats) mean mass 243 g [Std. Dev: 32.84644 g], mean without OI lesions score 0.3 [Std. Dev: 0.4830459] (Table 2).

Statistical analysis

Rats of each group were compared by mass with each other by statistical paired t-test and by OI lesions scores by statistical Wilcoxon signed-rank test (Table 3). Any significant difference among OI scores, was investigated whether owed in potent significant weight correlations. The application of generalized linear models (glm) with dependant variable the OI scores and independent variables the Epo or no drug, the reperfusion time and both variables in combination was followed. Considering the masses of the animals as independent variable at glm, a non-significant association with OI lesions scores was revealed (p= 0.3691), suggesting that further investigation was not needed.

3. Results

The glm resulted in: Epo administration possessed a trend to keep none significantly increased the OI scores by 0.15 without lesions [-0.0158339 - 0.3158339] (p=0.0749). This finding was crepant with the results of Wilcoxon signed-rank test (p= 0.0833). Reperfusion time kept non-significantly increased the OI scores by 0.15 without lesions [-0.0158339 - 0.3158339] (P=0.0749), also in accordance with the Wilcoxon signed-rank test (P= 0.0833). However, Epo administration and reperfusion time in combination kept significantly increased the OI scores by 0.1363636 without lesions [0.0421443-0.230583] (p=0.0057). The above and table 3 are summed in tables 4 and 5.

Table 2: Weight and oophoritis (OI) score mean levels and Std. Dev. of groups

Groups	Variable	Mean	Std. Dev
A	Weight	243 g	45.77724 g
	OI	without lesions 0	0
B	Weight	262 g	31.10913 g
	OI	without lesions 0	0
C	Weight	242.8 g	29.33636 g
	OI	without lesions 0	0
D	Weight	243 g	32.84644 g
	OI	without lesions 0.3	0.4830459

Table 3: Statistical significance of mean values difference for groups (DG) after statistical paired t test application for weight and Wilcoxon signed-rank test for scores.

DG	Variable	Difference	p-value
A-B	Weight	-19 g	0.2423
	OI	without lesions 0	1.0000
A-C	Weight	0.2 g	0.9900
	OI	without lesions 0	1.0000
A-D	Weight	0 g	1.0000
	OI	without lesions -0.3	0.0833
B-C	Weight	19.2 g	0.2598
	OI	without lesions 0	1.0000
B-D	Weight	19 g	0.1011
	OI	without lesions -0.3	0.0833
C-D	Weight	-0.2 g	0.9883
	OI	without lesions -0.3	0.0833

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

Alteration	95% c. in. time	Reperfusion	p-values Wilcoxon	glm
without lesions 0	undefined	1h	1.0000	1.0000
without lesions 0.15	-0.0158339-0.3158339	1.5h	0.0833	0.0749
without lesions 0.3	-0.0209211-0.6209211	2h	0.0833	0.0652
without lesions 0.15	-0.0158339–.3158339	Reperfusion time	0.0833	0.0749
without lesions 0.1363636	0.0421443 – 0.230583	interaction		0.0057

Table 5: Concise presence of the restorating influence of erythropoietin in connection with reperfusion time.

Alteration	95% c. in.	Reperfusion time	p-values
without lesions 0	undefined	1h	1.0000
without lesions 0.15	-0.0158339 - 0.3158339	1.5h	0.0791
without lesions 0.3	-0.0209211 - 0.6209211	2h	0.0742
without lesions 0.15	-0.0158339 – 0.3158339	reperfusion time	0.0791
without lesions 0.1363636	0.0421443 – 0.230583	interaction	0.0057

4. Discussion

The following situations show the association between ischemia and oophoritis. Bayir Y *et al* effectively reversed [5] tissue damage induced by experimental IR in female rat ovaries, administering Nigella sativa. Chuang SM *et al* investigated [6] the effects of ovarian hormone depletion and estrogen administration on IR-induced bladder damage in female rabbits. Ovarian hormone depletion further increased the expression of these inflammatory markers. Ovariectomy and thus ovarian hormone deficiency significantly exacerbated the IR-induced oxidative damage. Puzianowska-Kuźnicka M attributed [7] the longer life expectancy of women than men in part to the function of estrogens. 17β-estradiol (E2) is produced mainly by the ovaries in premenopausal women.

Oophoritis is part of a complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants [8]. It is a protective response involving host cells, blood vessels, proteins and other mediators that is intended to eliminate the initial cause of cell injury, as well as the necrotic cells and tissues resulting from the original insult, and to initiate the process of repair. The classical signs of acute inflammation are pain, heat, redness, swelling, and loss of function. It is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. Inflammation is not a synonym for infection, even though the two are often correlated. Inflammation can even occur in absence of infection, although such types of inflammation are usually maladaptive (such as in atherosclerosis). Inflammation is a stereotyped response, and therefore it is considered as a mechanism of innate immunity. General chronic oophoritis might lead to a host of diseases, such as hay fever, rheumatoid arthritis and even cancer (as it happens e.g. on cholecystitis for gallbladder carcinoma). Oophoritis can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured ovarian tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of ovarian tissues from the inflammatory process. Acute inflammation is a short-term process, usually appearing within a few minutes or hours and ceasing upon the removal of the injurious stimulus [9]. It is characterized by the above mentioned five cardinal signs, from which loss of function has multiple causes [10]. These five signs appear [10] when acute inflammation occurs, whereas acute oophoritis may not result in the full set. Pain happens only where the appropriate sensory nerve endings exist in the inflammed area-e.g., acute oophoritis does not cause pain unless the inflammation access either the tunica albuginea or the medulla, which have pain-sensitive nerve endings. The process of acute inflammation is initiated by cells already present in ovarian tissues, mainly resident macrophages, histiocytes, and mastocytes. These cells present on their ovarian surfaces certain receptors named pattern recognition receptors (PRRs), which recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs). At the onset of an infection, burn,

or other injuries, these cells undergo activation (one of their PRRs recognize a PAMP) and release inflammatory mediators responsible for the above mentioned clinical signs of inflammation. Some of the released mediators such as bradykinin increase the sensitivity to pain (hyperalgesia, dolor). The mediator molecules also alter the blood vessels to permit the migration of leukocytes, mainly neutrophils and macrophages, outside of the blood vessels (extravasation) into the tissue. The neutrophils migrate along a chemotactic gradient created by the local cells to reach the site of injury. The loss of function (functio laesa) is probably the result of a neurological reflex in response to pain. In addition to cell-derived mediators, several acellular biochemical cascade systems consisting of preformed plasma proteins act in parallel to initiate and propagate the inflammatory response. These include the complement system activated by bacteria and coagulation and fibrinolysis systems activated by necrosis, e.g. a burn or a trauma. The acute OI response requires constant stimulation to be sustained. Hence, acute inflammation ceases once the stimulus has been removed. The plasma cascade systems which are activated during acute inflammation is the complement system, the kinin system, the coagulation system and the fibrinolysis system. Specific patterns of acute and chronic oophoritis are seen during particular situations that arise in ovaries, such as when inflammation occurs on an epithelial surface or medulla: granulomatous inflammation, serous inflammation, ulcerative inflammation, but mainly fibrinous or purulent inflammation. Fibrinous inflammation resulting in a large increase in vascular permeability allows fibrin to pass through the blood vessels. If an appropriate procoagulative stimulus is present, such as cancer cells, a fibrinous exudate is deposited. This is commonly seen in serous surfaces, where the conversion of fibrinous exudate into a scar can occur between serous membranes. The deposit sometimes forms a pseudomembrane sheet. During oophoritis, ovarian synechiae can be formed. During inflammation of the perimetrium, pelvic adhesions can be formed. Purulent inflammation resulting in large amount of pus, which consists of neutrophils, dead cells, and fluid. Infection by pyogenic bacteria such as staphylococci is characteristic of this kind of inflammation. Large, localised collections of pus enclosed by surrounding tissues are called abscesses. *Neisseria gonorrhoeae* and *Chlamydia trachomatis* were originally thought to be the only pathogens that caused acute salpingitis. Inflammatory abnormalities are a large group of disorders that underlie a vast variety of gynecologic diseases. The immune system is often involved with inflammatory disorders, demonstrated in either allergic reactions or immune system disorders resulting in abnormal inflammation. Non-immune diseases with etiological origins in inflammatory processes include cancer, and local ischemic disease. Examples of disorders associated with OI include: autoimmune diseases, autoinflammatory diseases, celiac disease, glomerulonephritis, hypersensitivities, pelvic inflammatory disease, reperfusion injury, rheumatoid arthritis, transplant rejection, vasculitis and interstitial cystitis.

Rigor BM Sr classified [12] pelvic cancer causes at several types of pain, i.e., visceral, neuropathic, and somatic pain. Visceral pain is the result of spasms of smooth muscles of hallow viscus; distortion of capsule of solid organs; inflammation; chemical irritation; traction or twisting of mesentery; and ischemia, or necrosis, and encroachment of pelvis and presacral tumors.

The inflammatory response must be actively terminated when no longer needed to prevent unnecessary "bystander" damage to ovarian tissues. Failure to do so results in chronic oophoritis, and cellular destruction. Resolution of inflammation occurs by different mechanisms in different tissues. Mechanisms that serve to terminate inflammation include: short half-life of inflammatory mediators in vivo [13], production and release of transforming growth factor (TGF)β from macrophages [14, 15, 16], production and release of interleukin 10 (IL-10) [17], production of anti-inflammatory lipoxins [18], downregulation of pro-inflammatory molecules, such as leukotrienes, upregulation of anti-inflammatory molecules such as the interleukin 1 receptor antagonist or the soluble tumor necrosis factor receptor (TNFR), apoptosis of pro-inflammatory cells [19], desensitization of receptors, increased survival of cells in regions of inflammation due to their interaction with the extracellular matrix (ECM) [20, 21], downregulation of receptor activity by high concentrations of ligands, cleavage of chemokines by matrix metalloproteinases (MMPs) might lead to production of anti-inflammatory factors [22], production of resolvins, protectins or maresins. Emerging evidence now suggests that an active, coordinated program of acute inflammation resolution initiates in the first few hours after an inflammatory response begins. After entering tissues, granulocytes promote the switch of arachidonic acid–derived prostaglandins and leukotrienes to lipoxins, which initiate the termination sequence. Neutrophil recruitment thus ceases and programmed death by apoptosis is engaged. These events coincide with the biosynthesis, from omega-3 polyunsaturated fatty acids, of resolvins and protectins, which critically shorten the period of neutrophil infiltration by initiating apoptosis. As a consequence, apoptotic neutrophils undergo phagocytosis by macrophages, leading to neutrophil clearance and release of anti-inflammatory and reparative cytokines such as transforming growth factor-β1. The anti-inflammatory program ends with the departure of macrophages through the lymphatics [23]. The outcome in a particular circumstance will be determined by the layer in which the injury has occurred and the injurious agent that is causing it. The possible outcomes to oophoritis are: resolution, fibrosis, abscess formation or chronic inflammation. Thus, if an antinflammatory capacity of Epo can be proved concerning OI, a detailed investigation at the molecular level oughts to be clarified. Thus, inflammation is associated with Epo in different tissues. Erbayraktar S *et al* established[24] that Epo is a member of the cytokine superfamily, with significant homology to mediators of growth and inflammation. Results from studies have shown that Epo and its receptor are widely expressed in embryonic and adult tissues, including the central nervous system, gut, kidney, muscle (eg, smooth, skeletal, and heart), uterus, retina, pancreas, gonads, and lung.

The following situations show the association between Epo and ischemic ovaries. Mahmoodi M *et al* found [25] that Epo reduced IR injury and free radical production, increasing follicle survival and function in transplanted ovarian tissue. Sayyah-Melli M *et al* determined [26] that rEpo was effective in reducing the oxidative damage of ovarian torsion in operated patients, 18-35 years old, with signs and symptoms of ovarian torsion. Karaca M *et al* evaluated [27] the Epo administration as effective in reversing tissue damage induced by IR in ovaries of adult female rats. Suzuki H *et al* demonstrated [28] that administration of asialo Epo could effectively enhance the

survival of the follicles of transplanted cryopreserved ovaries in frozen-thawed canine ovarian xenotransplantation. However, David RB *et al* did not detect [29] expression of Epo mRNA in porcine ovaries. Kristiansson B *et al* concluded [30] that females with carbohydrate-deficient glycoprotein syndrome type I have primary ovarian failure, but the syndrome does not affect the terminal charged carbohydrate portion in Epo. Hyttinen JM *et al* generated [31] a transgenic calf from in vitro produced bovine embryos microinjected with a gene construct consisting of genomic sequences encoding human Epo. Kamiński M claimed[31] that apoptosis regulates the atrophy of completely developed organs, e.g. thymus, and the hormonal restructuring of ovaries and others but on the other hand, the development of apoptosis is arrested by so called "survival factors" as Epo.

5. Conclusion

Epo administration particularλy in concert with reperfusion keeps significantly increased yet the OI score lesions in ovaries after experimental ischemia in rats. Perhaps, a longer study time than 2 hours or a hihger Epo dosage may reveal more efficient restorating effects at histologic level.

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