RESEARCH ARTICLE

Received on: 14/11/2015
Published on:08/03/2016

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Conflict of Interest: None Declared !

The effect of the antioxidant drug "U-74389G" on white blood cells levels during hypoxia reoxygenation injury in rats.

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ABSTRACT

Aim: The aim of this experimental study was to examine the effect of U-74389G on rat model and particularly in a hypoxia – reoxygenation (HR) protocol. The effect of that molecule was studied hematologically using blood mean white blood cells levels.

Materials and methods: 40 rats of mean weight 231.875 g were used in the study. White blood cells levels were measured at 60 min (groups A and C) and at 120 min (groups B and D) of reoxygenation. U-74389G was administered only in groups C and D.

Results: Results were that U-74389G administration kept significantly increased the white blood cells level by 30.12%±10.87% (p=0.0050). Reoxygenation time also kept significantly increased the white blood cells level by 25.18%±11.64% (p=0.0300). However, U-74389G administration and reoxygenation time together kept significantly increased the white blood cells level by 23.64%±6.32% (p=0.0003).

Conclusions: Results of this study show that U-74389G administration, reoxygenation time and their interaction kept short-term significantly increased the white blood cells levels. The U-74389G administration which has antioxidant capacity through oxygen free radical scavenging, could not disrupt short-term the vicious cycle of HR and leukocytosis.

Keywords: hypoxia; reoxygenation; U-74389G; white blood cells

Introduction

Tissue hypoxia and reoxygenation (HR) remain of the main causes of permanent or transient damage with serious implications on adjacent organs and certainly on patients' health. Although important progress has been made regarding the usage of U-74389G 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 in which dosage. The particularly satisfactory action of U-74389G as antioxidant factor has been noted in several performed experiments. However, just few relative reports were found concerning U-74389G trial in HR experiments, not covering completely this particular matter. Furthermore, several publications addressed trials of other similar antioxidant molecules to which the studied molecule also belongs to. U-74389G or better 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-pregna-1,4,9(11)-triene-3,20-dione maleate salt[1] is an antioxidant which prevents both arachidonic acid-induced and iron-dependent lipid peroxidation. It protects against IR injury in animal heart, liver and kidney models. These membrane-associating antioxidants[2] are particularly effective in preventing permeability changes in brain microvascular endothelial cells

monolayers. A meta-analysis of 13 published seric variables, coming from the same experimental setting, tried to provide a numeric evaluation of the U-74389G efficacy at the same endpoints. (Table 1).

The aim of this experimental study was to examine the effect of the antioxidant drug "U-74389G" on rat model and particularly in a HR protocol. The question is whether U-74389G administration which has oxygen free radical scavenging properties, attenuates leukocyte adherence, suppresses proinflammatory gene up-regulation and is a promising new antioxidant drug for the treatment of vicious cycle IR injury. The beneficial effect or non-effectiveness of that molecule were studied by measuring the white blood cells (wbc) levels.

Materials and methods

Animal preparation

This experimental study was lisenced by Veterinary Address of East Attiki Prefecture under 3693/12-11-2010 & 14/10-1-2012 decisions. All settings needed for the study including consumables, equipment and substances used, were a courtesy of Exprerimental 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 continuous access to water and food. The experiment was acute, that means that awakening and preservation of the rodents was not following the experiment. 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 U-74389G intravenous (IV) administration and reoxygenation for 60 min (group C). Hypoxia for 45 min followed by immediate U-74389G IV administration and reoxygenation for 120 min (group D). The molecule U-74389G dosage was 10 mg/Kg body weight of animals.

At first, the animals were submitted into prenarcosis followed by general anesthesia. The detailed anesthesiologic technique is described in related references[3,4,5]. Oxygen supply, electrocardiogram and acidometry were continuously provided during whole experiment performance.

The protocol of HR was followed. Hypoxia was caused by forceps clamping inferior aorta over renal arteries for 45 min after laparotomic access had been achieved. Reoxygenation was induced by removing the clamp and reestablishment of inferior aorta patency. The molecules were administered at the time of reoxygenation, through inferior vena cava after catheterization had been achieved. The wbc 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). Forty (40) female Wistar albino rats were used of mean weight 231.875 g [Std. Dev: 36.59703 g], with min weight ≥ 165 g and max weight ≤ 320 g. Rats' weight could be potentially a confusing factor, e.g. fatter rats to have greater wbc levels. This suspicion was also investigated.

Model of hypoxia reoxygenation injury

Control groups: 20 control rats of mean weight 252.5 g [Std. Dev: 39.31988 g] suffered by hypoxia for 45 min followed by reoxygenation.

Group A: Reoxygenation which lasted 60 min concerned 10 controls rats of mean weight 243 g [Std. Dev: 45.77724 g], mean wbc levels 3.81 $10^3/mm^3$ [Std. Dev: 0.7430418 $10^3/mm^3$] (Table 2).

Group B: Reoxygenation which lasted 120 min concerned 10 controls rats of mean weight 262 g [Std. Dev: 31.10913 g], mean wbc levels 4.5 $10^3/mm^3$ [Std. Dev: 1.417353 $10^3/mm^3$] (Table 2).

Lazaroid (L) group

20 rats of mean weight 211.25 g [Std. Dev: 17.53755 g] suffered by hypoxia for 45 min followed by reoxygenation in the beginning of which 10 mg U-74389G /kg body weight were IV administered.

Group C: Reoxygenation which lasted 60 min concerned 10 L rats of mean weight 212.5 g [Std. Dev: 17.83411 g], mean wbc levels 4.8 $10^3/mm^3$ [Std. Dev: 1.396026 $10^3/mm^3$] (Table 2).

Group D: Reoxygenation which lasted 120 min concerned 10 L rats of mean weight 210 g [Std. Dev: 18.10463 g], mean wbc levels 6.56 $10^3/mm^3$ [Std. Dev: 2.510511 $10^3/mm^3$] (Table 2).

Results

Every rats weight group initially was compared with each other from 3 remained groups applying statistical paired t-test. (Table 3). Any emerging significant difference among wbc levels will be investigated whether owed in the above probable mentioned significant weight correlations. Every rats wbc levels group initially was also compared with

each other from 3 remained groups applying statistical paired t-test (Table 3).

Table 1: The U-74389G influence (±SD) on the levels of some seric variables[3] concerning reperfusion (rep) time

Variable	1h rep	p-value	1.5h rep	p-value	2h rep	p-value	interaction of U-74389G and rep	p-value
RBC	+1.39%±0.71%	0.7161	+0.64%±0.32%	0.8106	-0.10%±0.05%	0.9762	+1.05%±0.53%	0.4911
Hemoglobin	+5.2%±2.8%	0.0925	+3.9%±2.1%	0.0604	+2.7%±3.2%	0.3544	+2.5%±1.3%	0.0423
Mean corpuscular hemoglobin	+1.77%±0.96%	0.0663	+2.40%±0.57%	0.0001	+3.03%+0.71%	0.0003	1.33%+0.36%	0.0005
Platelet-crit[4]	+3.80%±9.87%	0.6373	+9.23%±6.29%	0.1064	+14.66%±9.03%	0.0833	+6.72%±3.73%	0.0712
Glucose	-6.41%±3.50%	0.0663	-8.57%±2.06%	0.0001	-10.74%±2.52%	0.0003	-4.76%±1.28%	0.0005
Total protein	-5.48%±2.99%	0.0663	-7.34%±1.76%	0.0000	-9.20%±2.16%	0.0000	-4.08%±1.10%	0.0000
Alkaline phosphatase	+22.66%±12.37%	0.0663	+31.91%±7.69%	0.0001	+41.16%±9.65%	0.0003	+17.75%±4.79%	0.0005
Creatine[5] phosphokinas	+54.32%±13.75%	0.0012	+35.34%±17.20%	0.0260	+16.37%±30.24%	0.4951	+18.52%±9.44%	0.0770
Sodium	+1.22%±0.66%	0.0707	+0.17%±0.61%	0.7714	-0.87%±1.03%	0.3995	-0.32%±0.36%	0.3693
Chloride	-0.58%±0.77%	0.4533	-0.97%±0.53%	0.0879	-1.36%±0.76%	0.1113	-0.75%±0.38%	0.0159
Calcium	0%±1.75%	1	-0.14%±1.10%	0.8782	-0.28%±1.54%	0.8492	+0.14%±0.64%	0.8245
Phosphorus	-2.23%±5.51%	0.7966	-1.61%±3.32%	0.5789	-1%±4.48%	0.8129	-1.09%±2%	0.5771
Magnesium	+1.33%±3.59%	0.7033	-0.28%±2.75%	0.9171	-1.90%±5.28%	0.7161	+0.36%±4.58%	0.8228
Mean	+5.92%±16.16%	0.3643	+4.97%±13.48%	0.3259	+4.03%±13.50%	0.3691	+2.87%±7.33%	0.2532

Table 2: Weight and wbc levels and Std. Dev. of groups

Groups	Variable	Mean	Std. Dev
A	Weight	243 g	45.77724 g
	wbc	3.81 10^3/mm^3	0.7430418 10^3/mm^3
B	Weight	262 g	31.10913 g
	wbc	4.5 10^3/mm^3	1.417353 10^3/mm^3
C	Weight	212.5 g	17.83411 g
	wbc	4.8 10^3/mm^3	1.396026 10^3/mm^3
D	Weight	210 g	18.10463 g
	wbc	6.56 10^3/mm^3	2.510511 10^3/mm^3

Table 3: Statistical significance of mean values difference for groups (DG) after statistical paired t test application.

DG	Variable	Difference	p-value
A-B	Weight	-19 g	0.2423
	wbc	-0.69 10^3/mm^3	0.0765
A-C	Weight	30.5 g	0.0674
	wbc	-0.99 10^3/mm^3	0.1197
A-D	Weight	33 g	0.0574
	wbc	-2,75 10^3/mm^3	0.0058
B-C	Weight	49.5 g	0.0019
	wbc	-0.3 10^3/mm^3	0.7114
B-D	Weight	52 g	0.0004
	wbc	-2.06 10^3/mm^3	0.0148
C-D	Weight	2.5 g	0.7043
	wbc	-1.76 10^3/mm^3	0.1039

Table 4: The increasing influence of U-74389G in connection with reoxygenation time.

			p-values	
Increase	95% c. in.	Reoxygenation time	t-test	glm
0.99 10^3/mm^3	-0.0606712 10^3/mm^3 - 2.040671 10^3/mm^3	1h	0.1197	0.0632
1.525 10^3/mm^3	0.4595437 10^3/mm^3 - 2.690456 10^3/mm^3	1.5h	0.0032	0.0069
2.06 10^3/mm^3	0.2654907 10^3/mm^3 - 4.054509 10^3/mm^3	2h	0.0148	0.0277
1.275 10^3/mm^3	0.1189510^3/mm^3 - 2.43105 10^3/mm^3	reoxygenation time	0.0286	0.0315
1.197273 10^3/mm^3	0.5686708 10^3/mm^3 - 1.825875 10^3/mm^3	interaction		0.0003

Table 5: The (%) increasing influence of U-74389G in connection with reoxygenation time

Increase	±SD	Reoxygenation time	p-values
22.99%	±53.60%	1h	0.0914
30.12%	±10.87%	1.5h	0.0050
37.25%	±93.02%	2h	0.0212
25.18%	±11.64%	reoxygenation time	0.0300
23.64%	±6.32%	interaction	0.0003

Applying generalized linear models (glm) with dependant variable the white blood cells levels and independent variables the U-74389G administration or no, the reoxygenation time and their interaction, resulted in: U-74389G administration kept significantly increased the white blood cells level by 1.575 10^3/mm^3 [0.4595437 10^3/mm^3 - 2.690456 10^3/mm^3] (p=0.0069). This finding was in accordance with the results of paired t-test (p= 0.0032). Reoxygenation time also kept significantly increased the white blood cells level by 1.275 10^3/mm^3 [0.1189510^3/mm^3 - 2.43105 10^3/mm^3] (p= 0.0315), in accordance also with paired t-test (p=0.0286). However, U-74389G administration and reoxygenation time together kept significantly increased the white blood cells level by 1.197273 10^3/mm^3 [0.5686708 10^3/mm^3 - 1.825875 10^3/mm^3] (p=0.0003). Reviewing the above and table 3, the tables 4 and 5 sum up concerning the increasing influence of U-74389G in connection with reoxygenation time. Inserting the rats' weight also as an independent variable at generalized linear models analysis, a non-significant relation with wbc results in (p= 0.1724), so as to further investigation concerning weight is not needed.

Discussion

Ischemic[5] injury occurs whenever the oxygen or vascular supply is insufficient to meet the metabolic demands of the tissue. Ischemia and damage can result from arterial or venus occlusion or, most frequently, from the reduced blood flow. The effect of ischemia in wbc level is described by following various authors.

In nervous tissue, Ishikawa T. et al[7] concluded that wbc-platelets complex were significantly higher in atherothrombotic infarction than in lacunar infarction in acute phase of cerebral infarction. Jastrzębska M. et al[8] associated shorter survival times with higher number of wbc levels in acute ischemic stroke patients. Thornton P. et al[9] proposed IL-1a, as a critical molecule for the entry of wbc and transendothelial migration and infiltration support of neutrophils across a brain inflammed-mediated injury endothelium in rats. Carrera E. et al.[10] associated elevated wbc count by 1.1-fold (p = 0.003) with delayed cerebral ischemia within 48 h of onset in aneurysmal subarachnoid hemorrhage patients. Satas S. et al. observed[11] mean wbc level significantly lower by 27.45% after peaked at 12 h, in posthypoxic hypothermia (HT) pigs than at 6 h for the normothermia (NT) ones (p = 0.04). HT may protect other organs including brain. Zaremba J. et al.[12] supposed that invasion of leukocytes into the evolving brain infarct, seems to play a key role in the deterioration of brain ischemic impairment. The peripheral wbc counts were increased within first 24 hours of ischemic stroke. Tomita M. et al[13] involved microvascular derangement as a cause of secondary brain damage following cerebral ischemia. The inflammatory processes which appeared to be elicited by polymorphonuclear leukocytes (PMNL) in the brain ischemic region, may begin with adhesion of PMNLs to endothelial cells, followed by blood-brain barrier disruption, transudation/exudation, edema, necrosis, and scar formation. Further, macrophages may release

cytokines which stimulate healing processes, such as astroglial proliferation and revascularization, and release neurotoxins which could gradually kill surviving neurons. Corvin S. et al[14] shown neutrophil chemotaxis across the blood-brain barrier by vasodilation, rolling and adherence of leukocytes to endothelium of small venules, more effectively stimulated than firm attachment, pertinent in induction of microcirculatory disturbances during acute cerebral disorders such as ischemia or stroke in rats brain. Pozzilli C. et al[15] indicated active migration and tracking of labelled leukocytes in cerebral infarcted hemisphere.

In myocardium, Huang WH. et al counted[16] significantly increased wbc, oxygen radicals, malondialdehyde (MDA) and myeloperoxidase levels than their own baseline values, during myocardial IR injury in patients undergoing elective coronary artery bypass operation with cardiopulmonary bypass (CABOCB). Delgaudine M. et al found[17] decreased wbc levels in bone marrow (BM) but increased in peripheral blood (PB) after mice myocardial infarction (MI). Shahzad F. et al counted[18] significantly higher total wbc and neutrophils but significantly lower lymphocytes ($p<0.001$) counts in ischemic heart disease group than control one ($p<0.001$). Abacilar F. et al[19] showed changes in cardiovascular and pulmonary function by stimulated systemic inflammatory processes of TNF-a and wbc level, after CABOCB, in patients with high level of TNF-a > 20 pg/mL. Müller-Ehmsen J. et al[20] related a shorter ischemia time and reduced cell death with the reasons of reduced CD34+ cell count mobilization in peripheral blood of acute MI patients. Domański L.[21] assessed the intensity of free radical reactions during first days following acute MI by the increased level of MDA and leukocyte count. Kassirer M. et al[22] reflected the presence of an inflammatory atherosclerotic lesion with significantly ($P<0.002$) higher concentrations of CD11b/CD18 antigen on the surface of polymorphonuclear leukocytes and monocytes than control group in patients with ischemic heart disease. Martin SE. et al[23] produced a significant reduction in regional coronary blood flow and myocardial function, along with significantly increased arteriovenous difference of wbc after anaphylatoxin infusion, indicating intravascular trapping in the myocardium.

In lungs, Chen CF. et al[24] resulted in elevated nitric oxide ($P<0.05$), hydroxyl radicals ($P<0.01$), wbc levels ($P<0.001$), respiratory work [decreases in FRC and lung compliance] and lung/body weights ratio in pancreas IR injury rat group. Davis KA, et al[25] caused a three- to fourfold increase in bronchoalveolar lavage (BAL) wbc counts, in both the injured blunt right chest trauma (pulmonary contusion) and the noninjured (left) lungs, influencing the trauma-induced destruction of the alveolar capillary membrane of the contralateral uninjured lung in Mongrel pigs. Welbourn R. et al[26] caused higher plasma leukotriene B4 levels ($p<0.01$), reduction in circulating wbc levels ($p<0.01$), PMNs sequestered in the hind limbs ($p<0.05$) and PMNs sequestered in lungs, by a value higher than controls by lower torso IR in rabbits.

In digestive system, Zanoni FL. et al[27] assessed 86% of intestinal IR cases presenting positive cultures for E. coli, 57% positive hemocultures, induced neutrophilia, 2-fold number of rolling, 5-fold adherent and 11-fold migrated leukocytes; accompanied by 2-fold intercellular adhesion molecule-1 in the mesenteric microcirculation than sham group 24 hours later in Wistar rats. Fan CL et al[28] counted increased wbc levels in patients with ischemic hepatitis B related cirrhotic liver than control group ($P<0.05$). Lu Y. et al[29] observed the total number of wbc levels decreased 1 hour after gut ischemia, but increased after gut reperfusion in Wistar rats. Leukocytes mRNAs expression of inflammatory (TNFa) and antiinflammatory (IL-10, IL-4 and γ-interferon) cytokines were upregulated at 1 hour after gut ischemia and down-regulated distinctly at 2 hours following reperfusion. Champagne BJ et al[30] documented elevated immature wbc levels in patients underwent bowel resection because of grade III colon ischemia. Conner WC. et al[31] documented elevated number of circulating neutrophils, increased oxidative burst potential at 2 h, maximum at 6 h, and normal at 24 h after IR than sham operated ones in Sprague-Dawley rats. Massberg S. et al[32] analyzed leukocyte-endothelial cell interaction after their restriction only to postcapillary venules of the intestinal submucosa in vivo, besides capillary perfusion, which are well-known sequelae of mesenteric IR. Under physiological conditions only a few wbc were found rolling along or firmly adherent to the microvascular

endothelium. Zhang P. counted[33] increased circulating wbc levels after partial liver ischemic injury in rat models.

In kidneys, Molitoris BA.[34] noted the endothelial cells key role and position between the epithelial cells and wbc. Kidneys interact and respond to signals from both cell types. Microvascular endothelial cells within the kidney mediate wbc attachment, migration into the interstitium, microvascular flow rates and permeability. Low regeneration potential and endothelial-mesenchymal transformation lead to fibrosis and subsequent microvascular dropout. Ojteg G. et al[35] suggested the adhesiveness of wbc of renal capillaries due to oxygen free radicals during 45 min medullary vasa recta IR.

In muscular tissue, Anderson SI. et al documented[36] impaired functional hyperemia and the wbc adherence as a circulating signal for postcapillary endothelial swelling in chronic IR rat extensor digitorum longus muscles following increased activity (7 days). They carried on increasing further along increased activity when activated cells were inserting also into arterial blood. Sabido F. et al[37] correlated the basis for tissue injury during ischemia with depletion of tissue oxygen and energy substrates. Irreversible cellular changes occur after 4-6 h of skeletal muscle ischemia. Following acute arterial occlusion, the restoration of blood flow heralds the onset of biochemical events, forming the basis of what is known as the reperfusion syndrome. Both endothelial and wbc have the biochemical machinery and capacity to generate molecular signals, to express adhesion proteins, to produce toxic metabolic by-products and to explain many of the reactions. Menger MD et al[38] associated prolonged periods of striated muscle and skin IR with activation, accumulation and microvascular adherence of wbc, formation of reactive oxygen metabolites and release of potent mediators (leukotrienes, platelet-activating factor) with the consequence of increased microvascular permeability due to the loss of endothelial integrity, interstitial edema and cell damage in hamster mouse models. Menger MD. et al[39] described the post-IR damage in skeletal muscle microcirculation, by microvascular accumulation of leukocytes which adhere to postcapillary venules endothelium increasing vessel permeability with swelling of endothelial cells and interstitial edema. Reperfusion with consecutive reentry of molecular oxygen into the microvasculature provokes the formation of oxygen-radicals and the accumulation of leukocytes adhering to the endothelium of postcapillary venules. The activated wbc release additional oxygen-radicals and deliver aggressive mediators, such as proteases, cytokines and eicosanoids, which have chemotactic influence on leukocytes, resulting in a vicious circle. Walker PM[40] resulted in prolongation of the reperfusion injury due to sequestration of wbc in skeletal muscle. This subsequently resulted in damage to remote organs, including lung, heart, and kidneys. Rubin BA. et al[41] suggested that a significant early and prolonged sequestration of wbc mediated injury and early complement cascade activation occurs during the first 40 minutes of reperfusion, reducing muscle necrosis ($p<0.0025$) in canine gracilis muscle model. Yokota J. et al[42] implicated leukocyte-generated oxygen free radical as mediators of reperfusion-associated cellular membrane injury in infrarenal aorta hindlimb skeletal muscles IR of Sprague-Dawley rats .

Regarding lower extremities, Fei YF. et al[43] evaluated a higher wbc and neutrophils count in amputated patients with diabetic foot than non-amputation patients ($P<0.05$). Karahalil B. et al[44] showed that even short-time IR-induced DNA damage can be investigated in human peripheral leucocytes. After ischemia, the genotoxic damage detected in human peripheral leucocytes locally in the reperfused tissue results in numerical, morphologic and biochemical alterations of all circulating wbc in the human organism. It leads particularly to the release of substantial amounts of oxygen radicals and other reactive agents. Simultaneously, local ischemia in the reperfused tissue is extended to the whole body systemically through these activated inflammatory cells and, possibly, results in secondary detectable tissue damage in endothelial cells of the systemic circulation inducing prolonged DNA damage even in early reperfusion period. Danielsson P. et al[45] counted decreased wbc, granulocyte levels and endothelial marker sICAM-1, 4 weeks after revascularization than before, most evident in the subgroup of critical limb ischemia (CLI) patients with ulcer and gangrene following operation. Danielsson P. et al[46] downregulated the wbc levels 2 hours following endovascular procedures, more

evident in group of peripheral arterial occlusive disease (PAOD) patients with CLI than both patients with intermittent claudication and before intervention, relating endovascular restenosis with intimal hyperplasia, a process depending on inflammatory mechanism. Willy C. et al[47] said that IR-injury is initiated by leukocyte accumulation, early measurable local and systemic activation of circulating PMN-granulocytes induction, adhesion to vascular endothelium as well as oxygen free radicals, playing a pivotal role in pathogenesis. Ischemia of the upper limb significantly increased the CD 11b-expression ($p<0.05$) and the CD 18-expression ($p<0.05$). Furthermore there was a spontaneous release of free radical oxygen ($p<0.01$). Nash GB. et al[48] documented that flow abnormalities may reflect activation of wbc by factors released in ischemic legs. A vicious cycle of wbc trapping, activation and tissue damage was engaged. Paterson IS. et al[49] led to neutrophil activation, manifested by intracellular H_2O_2 production ($p<0.01$), an event mediated by increased plasma thromboxane A2 & B2 ($p<0.01$) in lower-torso IR rats. Klausner JM. et al[50] led to a rise in plasma thromboxane (Tx) B2 levels ($p<0.05$), in lymph TxB2 concentration ($p<0.05$), in microvascular permeability but to circulating wbc level decrease and documented increased vascular permeability during reperfusion in hind limb tourniquet IR dogs.

In vascular system, Danielsson P. et al[51] associated elevated interleukin (IL)-6 levels with increased cardiovascular morbidity and identified a common polymorphism in the promoter region of the IL-6 gene in peripheral arterial disease (PAD) patients diabetes mellitus (+/- DM). Also, the effect of CLI was significantly ($p < 0.05$) associated with elevated levels of wbc count, hsCRP, proinflammatory cytokines (IL-6, TNFalpha-R1-2), endothelial (sICAM, sVCAM) and wbc (CD11b gran) markers. Activation of cytokines and WBC was related with Fontaine stage of PAD. Fusman R. et al[52] showed that the state of leukocyte adhesiveness/aggregation is slightly increased in documented ischemic heart or brain vasculopathy with concomitant diabetes. Korthuis RJ et al[53] characterized activated wbc capable to direct a powerful cytotoxic arsenal at parenchymal cells following their extravasation into the tissues leading to the identification of the factors responsible for initiating leukosequestration and activation in such disorders and finally venous disease in chronic venous insufficiency (CVI). Leukocyte infiltration is a prominent feature in IR and activated neutrophils play a causative role in the reperfusion component of tissue injury via the targeted release of reactivate oxygen metabolites and hydrolytic enzymes. Willam C. et al[54] considered the binding of leukocyte β-integrins to endothelial adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) in human dermal microvascular and umbilical vein endothelial cells (EC) exposed to different oxygen tensions, for infiltration and extravasation of wbc. An increase in oxygen availability stimulates ICAM-1 and VCAM-1 expression on micro- and macrovascular EC, which may contribute to adhesion and transmigration of different leukocyte populations in IR injuries. Ciuffetti G. et al[55] confirmed significantly increased the WBC count and altered the mononuclear leukocytes filterability in stage II PAD patients than healthy control ones. Arber N. et al[56] concluded that an increase in adhesived/aggregated wbc can be detected in the circulating pool during major stress. Ciuffetti G. et al[57] associated calf pain with an increase in monocyte filterability ($P<0.01$) in PAD patients and ST depression with impaired granulocyte filterability ($P<0.04$) in stable angina pectoris (SAP) patients than control ones. Romanus M. et al[58] found extensive wbc sticking to vessel walls indicating endothelial damage in pressed hamster cheek pouch single layer of epithelium tissue.

U-74389G may also influence the wbc level. Andreadou I., et al. found[59] lower polymorphonuclear leukocytes number at terminal ileum intestinal mucosa 1h after superior mesenteric artery IR in IV U-74389G administration group of rats. Lehmann C. et al. observed[60] a four-fold increase in leukocytes count firmly adherent in induced-endotoxemic Wistar rats distal small intestine submucosal post-capillary and collecting venules. U-74389G administration 60 min after endotoxin challenge (1.5 mg/ kg) and 30 min before IR (3 mg/kg) significantly attenuated the sticking leukocytes count in collecting venules ($p < 0.05$). Administration of U-74389G, which has radical scavenging properties, attenuates leukocyte adherence increased by endotoxemia in selected populations of intestinal venules. Thus, 21-

aminosteroids may have an impact in treatment of endotoxin-induced intestinal injury. Kunihara T. et al., found[61] significantly lower plasma IL-8, IL-1ra and spinal IL-8 levels in U-74389G treated cerebral ischemic group (3 mg/kg) than those in control group ($p < 0.05$), in infrarenal aortic IR rabbits, attenuating the ischemic endothelial cell injury or activation of leukocytes. However, plasma TNFα levels peaked at 5 minutes after declamping and spinal IL-1ra and TNFα levels were not significantly different. Fukuma K. et al. protected[62] against lipopolysaccharide-induced mice liver injury in vivo 48 hours after lipopolysaccharide injection as indicated by the decreased hepatic lipid peroxidation, TNFα and inducible nitric oxide synthase mRNA formation, hepatic enzyme release, neutrophil infiltration and inhibitory effects on NFκ B activation in liver by U-74389G administration. Lazaroids U-74389G treatment can protect against endotoxin-induced hepatic injury by suppressing proinflammatory gene up-regulation, can increase the survival rates and is a promising new antioxidant drug for the treatment of endotoxin shock. Buttgereit F. et al. resulted[63] that lazaroids rather exert their tissue protective effects in the injured CNS, from in vitro effects of U-74389G on the mitogen-induced cytokine production in human peripheral blood mononuclear cells, which is known to be very sensitive and perhaps the most clinically relevant parameter reflecting immunomodulation.

Furthermore, Squadrito F. et al reduced[64] enhanced leukopenia by U-74389G administration (30 mg/kg, IV) in rats subjected to total superior mesenteric artery and coeliac trunk IR for 45 min. Along, they increased survival rate by 43.33%, survival time by 3.13-fold, lowered the serum levels of TNFα less than 267 ± 13 U/ml and the plasma MDA levels less than 57 ± 7 nmol/ ml, the myeloperoxidase activity by 90.2% in ileum and lungs and restored the reduced responsiveness to 10 nM-10 μM ACh of aortic rings, revealing the antishock and endothelial protective actions of U-74389G.

HIV primarily infects human immune subclasses of wbc such as CD4+ T cells, macrophages and dendritic cells. It directly and indirectly destroys CD4+ T cells. If U-74389G administration increases the wbc levels, it unavoidably increases also the CD4+ T cells, macrophages and dendritic cells levels, enforcing so the immune system. Nevertheless, this has to be proved in tailored clinical trials testing U-74389G administration in HIV patients.

Any tissue or organ ischemia is a stimulus which provokes inflammation. As such, inflammation results in leukocytosis. Removing ischemia theoretically would restore inflammation and concomitantly leukocytosis. However, literature mentions that even during reperfusion phase, a reperfusion syndrome occurs which seamlessly carries on the vicious cycle of leukocytosis. All authors agree that mRNAs expression of inflammatory (TNFa) and antiinflammatory (IL-10) cytokines were upregulated still 1 hour after ischemia removal. The combination of increased macromolecular permeability and adhesiveness of capillaries for wbc are due to oxygen free radicals. Reperfusion with consecutive reentry of molecular oxygen into microvasculature, provokes the formation of oxygen-radicals and accumulation of leukocytes adhering to endothelium of postcapillary venules. During reperfusion, activated neutrophils play a causative role via the targeted release of reactivate oxygen metabolites, hydrolytic enzymes, additional oxygen-radicals and aggressive mediators delivery, such as proteases, cytokines and eicosanoids, which have chemotactic influence on wbc, resulting in a vicious cycle during reperfusion phase of tissue injury. Leukocyte-generated oxygen free radical are implicated as mediators of reperfusion-associated cellular membrane injury in tissues IR. Simultaneously, local ischemia at reperfused tissues is extended to the whole body systemically through these activated inflammatory cells and, possibly, results in secondary detectable tissue damage in endothelial cells of the systemic circulation inducing prolonged DNA damage even in early reperfusion period. A vicious cycle of wbc trapping, activation and tissue damage is engaged. The question is whether U-74389G administration which has oxygen free radical scavenging properties, attenuates leukocyte adherence, suppresses proinflammatory gene up-regulation and is a promising new antioxidant drug for the treatment of IR injury. Table 5 shows that the wbc levels were sped up elevated during this short-term U-74389G reperfusion. So, the antioxidant capacity of U-74389G is impossible to short-term restorate leukocytosis at this certain dose. Perhaps a greater dose or study time than 2 hours may provide more

spectacular results. The results also in table 1, agree with these findings. Certainly, they show that the antioxidant capacity of U-74389G is not only impossible to restorate short-term the alkaline phosphatase levels, but also has anabolic capacities for RBC count. However, the antioxidant capacity of U-74389G generally protects short-term the sodium, chloride, calcium and phosphorus levels deviation at this certain dose. A second raised question is whether the antioxidant capacity of U-74389G offers any short-term qualitative advantage to the function of rised wbc level, possibly beneficial for immunodeficient leucopenic situations as HIV.

Conclusions

U-74389G administration, reoxygenation time and their interaction kept short-term significantly increased the wbc levels. The U-74389G administration which has oxygen free radical scavenging properties cannot short-term disrupt the vicious cycle of IR and leukocytosis. Perhaps a greater dose or study time than 2 hours may provide more efficient results. Along, any short-term qualitative advantage offered to the function of rised wbc level, worths to be investigated.

Acknowledgment

This study was funded by Scholarship by the Experimental Research Center ELPEN Pharmaceuticals (E.R.C.E), Athens, Greece. The research facilities for this project were provided by the aforementioned institution.

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Cite this article as:

C. Tsompos, C. Panoulis, K Toutouzas, G. Zografos, A. Papalois. The effect of the antioxidant drug "U-74389G" on white blood cells levels during hypoxia reoxygenation injury in rats. Asian Journal of Pharmacology and Toxicology, 04(13), 2016, 22-32.