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Antioxidant 21-aminosteroid "U-74389G" ameliorates the short-time effect of hypoxia-reoxygenation on the platelet count in rats

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Abstract: A i m: The aim of this experimental study was to examine the effect of the antioxidant drug "U-74389G", on rat model and particularly in a hypoxia-reoxygenation protocol. The beneficial effect or non-effectiveness of that molecule were studied hematologically using blood mean platelet count. Results were that U-74389G administration interacted or not with reoxygenation time decreased the platelet count by 6.12% ± 3.58% (p = 0.0857) and 12.83% ± 5.79% (p = 0.0303) respectively.
C o n c l u s i o n s: U-74389G administration interacted or not with reoxygenation time decreases the platelet count within short-term time of 2 hours by different significance levels.

Key words: hypoxia, U-74389G, reoxygenation, platelet count.

Introduction

Circulatory 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. The use of antioxidant substances has been a research subject for many years. How-

ever, even if important progress has been made, satisfactory answers have not been given yet to fundamental questions, such as, how much powerful should an antioxidant be, when should it be administered, and in which dosage. The particularly satisfactory action of the antioxidant U-74389G in tissue protection has been noted in several performed experiments. Since a careful literature search (PubMed — Medline) was conducted, it was realized that this certain antioxidant has been tried in HR experiments. However, just few relative reports were found, not covering completely this particular matter. Luo X *et al.* completely or partially attenuated lipid peroxidation (LPO) products and reduced lung DNA synthesis, consistent with a role for hydroxyl radicals (HO^-) or lipid hydroperoxides as second messengers in normal regulation of lung growth, by U74389G administration after exposure to 95% O_2 in 4–7-days old rats lungs and serum. Pulmonary O2 toxicity is thought to be a major contributor to the development of bronchopulmonary dysplasia of preterm infants and antioxidant interventions hold significant promise for therapy, although U74389G did not improve the survival rate [1]. Taherzadeh M *et al.* concluded that U74389G up-regulates CYP3A6 but inhibits its catalytic activity, prevents the hepatic malondialdehyde (MDA) enhancement and prevents CYP1A1/2 down-regulation and decrease in activity by a double mechanism: hindering the release of serum mediators and by averting intracellular events, effect possibly associated with its antioxidant activity in rabbits after induced inflammatory reaction [2]. Also, a lot of publications addressed trials of other similar molecules of aminosteroids (lazaroids) 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 is an antioxidant which prevents both arachidonic acid-induced and iron-dependent LPO [3]. It protects against ischemia reperfusion (IR) injury in animal heart, liver and kidney models. These membrane-associating antioxidants are particularly effective in preventing permeability changes in brain microvascular endothelial cells monolayers [4]. Nevertheless, the same authors proved that U-74389G rarely significantly altered other biochemical parameters in serum as depicted at table 1 in related IR injury experiments, at the same endpoints with these of the present experiment after ischemia removal in rats [5, 6].

Table 1. The U-74389G influence (±SD) on the levels of some biochemical parameters in serum[5] concerning reperfusion (rep) time.

Variable	1h rep	p-value	1.5h rep	p-value	2h rep	p-value	Interaction	p-value
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
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[6]	0% ± 1.75%	1.0000	–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

The authors were wondering whether the antioxidant capacity of U-74389G has further beneficial effect also at formed elements of blood and particularly in platelet count. If such an effect was proved, further benefit might be expected for situations related with platelet count. The aim of this experimental study was to examine the effect of the antioxidant drug "U-74389G" on rat model and particularly in an HR protocol. The beneficial effect or non-effectiveness of that molecule were studied by measuring blood mean platelet count.

Materials and methods

This experimental study was laid out at the Exprerimental Research Center of ELPEN Pharmaceuticals Co. Inc. S.A. at Pikermi, Attiki and by Veterinary Address of East Attiki Prefecture under 3693/12-11-2010 & 14/10-1-2012 licenses. The experimental setting needed for the study including of consumables, equipment and substances used, was a courtesy of that company. A brief description of the measures taken to insure compliance with established guidelines and accepted standards for humane use and care of laboratory Albino female Wistar rats, according to guides of Institution's Animal Care Committee, NIH and National Research Council, follows. They were housed in laboratory 7 days before the experiment, having easy access 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): Hypoxemia for 45 min followed by reoxygenation for 60 min (group A) or 120 min (group B). Hypoxemia for 45 min followed by immediate U-74389G intravenous (IV) administration and reoxygenation for 60 min (group C) or for 120 min (group D). The molecule U-74389G dose was 10 mg/kg body weight of animals.

The experiment started with animals submitted into prenarcosis followed by general anesthesia. Their electrocardiogram and acidometry were continuously monitored. Their inferior aorta flow was excluded by forceps. After exclusion, the protocol of HR was applied, exactly as described in experimental groups. The molecules were administered at the time of reoxygenation, through inferior vena cava after catheterization had been achieved. The pletelet count measurement was performed at 60 min of reoxygenation (groups A and C) and at 120 min of reoxygenation (groups B and D). The sampling was performed after vena cava venipuncture by filling a 2cc dimpled control stroke syringe with pre-set volume for withdrawal and sterile insulin needle. Then, the sample was transferred to vacuum blood collection tube (disposable vacutainer) of 2ml containing K^2EDTA, the "anticoagulant of choice in specimen collection and blood cell counting" according to both NCCLS and the International Council for Standardization in Hematology. Platelet count measurements were performed by Nihon Kohden celltac a MEK-6450 K automatic hematology analyzer with preset rat type and cyanide-free reagents.

The details of general anesthesia are described in related references [5, 6]. Continuous oxygen supply was administered during whole experiment performance. Hypoxemia was caused by clamping inferior aorta over renal arteries for 45 min after laparotomic access was achieved. Reoxygenation was induced by removing the clamp and reestablishment of inferior aorta patency. Forty (40) female Wistar albino rats were used of mean weight 231.87 g

[Standard Deviation (SD): 36.59 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 blood platelet count. This suspicion was investigated.

Control groups

20 control rats of mean weight 252.5 g [SD: 39.31 g] were subjected to hypoxemia for 45 min followed by reoxygenation (Table 2).

Table 2. Weight and platelet count (Plt) and Standard Deviation (SD) of groups.

Groups	Variable	Mean	SD
A	Weight	243 g	45.77 g
A	Plt	1003.20 10^3/μL	143.98 10^3/μL
B	Weight	262 g	31.10 g
B	Plt	956 10^3/μL	196.83 10^3/μL
C	Weight	212.50 gr	17.83 g
C	Plt	839.30 10^3/μL	211.34 10^3/μL
D	Weight	210 g	18.10 g
D	Plt	883.50 10^3/μL	80.63 10^3/μL

Group A

Reoxygenation which lasted 60 min concerned 10 controls rats

Group B

Reoxygenation which lasted 120 min concerned 10 controls rats

Lazaroid (L) group

20 rats of mean weight 211.25 g [SD: 17.53 g] suffered by hypoxemia for 45 min followed by reoxygenation in the beginning of which 10 mg U-74389G/kg body weight were IV administered (Table 2).

Group C

Reoxygenation which lasted 60 min concerned 10 L rats

Group D

Reoxygenation which lasted 120 min concerned 10 L rats

Results

Every rats' weight group initially was compared with each one from 3 remained groups applying statistical paired t-test (Table 3). Any emerging significant difference among platelet count levels was investigated whether owed in the above mentioned probable significant weight correlation. Every rats' platelet group initially was compared with other one from 3 remained groups applying statistical paired t-test (Table 3). The statistical software used was STATA 6.0. Applying generalized linear models (glm) with dependant variable the platelet count and independent variables the U-74389G administration or no, the reoxygenation time

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
A-B	Plt	47.20 10^3/μL	0.2668
A-C	Weight	30.5 g	0.0674
A-C	Plt	163.90 10^3/μL	0.0716
A-D	Weight	33 g	0.0574
A-D	Plt	119.70 10^3/μL	0.0342
B-C	Weight	49.50 g	0.0019
B-C	Plt	116.70 10^3/μL	0.1997
B-D	Weight	52 g	0.0004
B-D	Plt	72.50 10^3/μL	0.2924
C-D	Weight	2.50 g	0.7043
C-D	Plt	–44.20 10^3/μL	0.5355

and their interaction, resulted in: U-74389G administration significantly decreased the platelet count by 118.20 10^3/μL (p = 0.0280) after glm appliance. This finding was in accordance with the results of paired t-test (p= 0.0327). Reoxygenation time non-significantly decreased the platelet count by 1.50 10^3/μL (p = 0.9784) after glm appliance, also in accordance with paired t-test (p = 0.9705). However, U-74389G administration and reoxygenation time together non-significantly decreased the platelet count by 56.43 10^3/μL (p = 0.0857) after glm appliance. Reviewing the above and table 3, tables 4 and 5 sum up concerning the decreasing influence of U-74389G in connection with reperfusion time. Inserting the rats' weight also as an independent variable at generalized linear models analysis, a non significant relation results in (p = 0.2724), so as to further investigation is not needed.

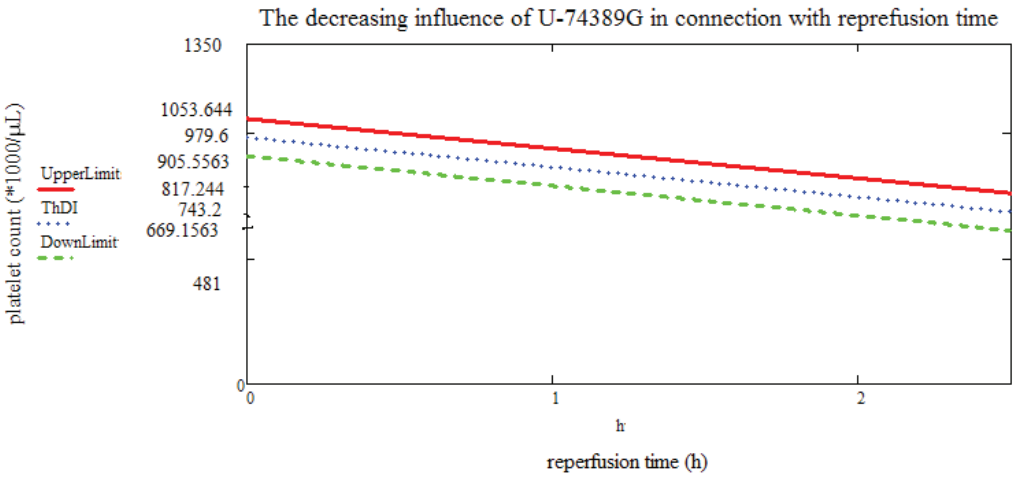

Table 4. The decreasing influence of U-74389G in connection with reperfusion time.

Decrease	95% c. in.	Reperfusion time	t-test (p-value)	glm (p-value)
163.90 $10^3/\mu L$	–333.79 $10^3/\mu L$ — 5.99 $10^3/\mu L$	1h	0.0716	0.0578
118.20 $10^3/\mu L$	–222.91 $10^3/\mu L$ —13.48 $10^3/\mu L$	1.5h	0.0327	0.0280
72.50 $10^3/\mu L$	–213.82 $10^3/\mu L$ — 68.82 $10^3/\mu L$	2h	0.2924	0.2954
1.50 $10^3/\mu L$	–113.17 $10^3/\mu L$ — 110.17 $10^3/\mu L$	reoxygenation time	0.9705	0.9784
56.43 $10^3/\mu L$	–121.17 $10^3/\mu L$ — 8.30 $10^3/\mu L$	interaction	–	0.0857

Table 5. The (%) decreasing influence of U-74389G in connection with reperfusion time.

Decrease	± SD	Reperfusion time	p-values
17.79%	± 9.40%	1h	0.0647
12.83%	± 5.79%	1.5h	0.0303
7.88%	± 7.83%	2h	0.2939
0.16%	± 6.07%	reoxygenation time	0.9744
6.12%	± 3.58%	interaction	0.0857

Discussion

The following situations show how hypoxia can influence the platelet count. Chen *et al.* measured decreased number of platelet count within first 3 days but gradually increasing ones after the first postoperative week in IR sheep. Granulocyte-platelet and monocyte-platelet aggregates reached the peak at postoperative day 2 [7]. Lessiani *et al.* reported persistent platelet activation, in vivo indexes of residual thromboxane biosynthesis, oxidative stress and platelet-derived inflammation in critical limb IR patients [8]. Lev *et al.* found that statins which prevent cardiovascular disease and concomitantly tissue hypoxia, exert favorable effects on platelet count during IR [9]. Vilahur *et al.* associated short-term pigs myocardial ischemia with higher ($p < 0.001$) local recruitment and deposition of renders platelets more susceptible to activation [10]. Elkind characterized patients at increased risk of ischemic events by diffuse immunologically mediated activation of platelets [11]. Schuerholz *et al.* measured significantly higher platelet count compared to baseline values only in short-term IR after kidney transplantation [12]. Nemeth *et al.* measured higher platelet count in short-term IR groups of outbred rats [13]. Götz *et al.* found significantly reduced platelet count in coronary effluent and demasked intracoronary transient formation of micro aggregates between PMN and platelets after global heart ischemia in pigs [14]. Witczak *et al.* administered more platelets transfusions in chronic renal failure patients underwent cardiovascular operation ($p < 0.02$) [15]. Scheinichen *et al.* found significantly decreased GPIIb/IIIa expression on circulating platelets in kidney transplantation preservation group compared with healthy volunteers. A significantly reduced P-selectin expression was found in the long-term preservation group compared with the short-term one [16]. Gresele *et al.* increased platelet

activation in vivo in acute short-term hyperglycemia [17]. Verstraete *et al.* found that platelet aggregation is due to activation of the platelet glycoprotein (GP IIb/IIIa) receptor on platelet surface [18]. Hayreh *et al.* produced vasospasm followed by transient, complete occlusion or impaired blood flow in the central retinal artery and/or PCA developing ischemic disorders in retina and optic nerve of atherosclerotic monkeys when platelets were aggregated [19]. Zhu *et al.* found significantly higher ω-3 fatty acid levels in platelets of rats fed 8 weeks by fish oil, compared with control ones ($p < 0.05$) also correlated with infarcts size reduction [20]. Sokolov *et al.* showed depressed platelets antiaggregant and latent disseminated intravascular platelets microcoagulation that tended to progress under emotional mental stress in coronary ischemia patients [21].

Unpleasantly, situations showing how U-74389G can influence the platelet count do not exist. However, Pratt MF studied the effects of U-74389G on the pathophysiology of random skin flap necrosis or survival in pig model. Demonstrated mechanisms of skin flap failure included the alteration of platelet function with resultant accumulation of damaging oxygen-free radicals ($O^{=}$). Random skin flap survival was improved significantly with U-74389G administration [22]. Of course, the action mechanism of U-74389G can be linked by its $O^{=}$ concentration decline. Olas B *et al.* inhibited ADP-induced platelet aggregation by GS-Pt complex in pig blood platelets. GS-Pt complex as a major metabolite from loss of both protein –SH and the thiol groups of GSH, in platelet cytosol, was found to induce the very active platelet LPO, measured as $O^{=}$ generation [23]. Fan *et al.* protected myocardial cell membrane, reduced attacks of angina pectoris and improved myocardial ischemia reducing plasma LPO due to the accelerating clearance of $O^{=}$ by the increased superoxide dismutase (SOD) and sulfhydryl group which inhibit platelet aggregation by TXA2/PGI2 ratio regulation in plasma [24]. Késmárky *et al.* associated coronary stenosis, endothelial injury and IR caused by the balloon inflation and deflation with markedly increased plasma fibrinogen concentration, with elevated plasma, whole blood at 90 s^{-1} and corrected blood viscosities, with increased superoxide production of leukocytes that can effectively scavenge superoxide radicals, with increased spontaneous platelet aggregation, with elevated blood reactive oxygen species (ROS) and OH^{-} levels (all $p < 0.05$) during already the hospital phase, in patients undergoing percutaneous transluminal coronary angioplasty (PTCA) [25]. Wang *et al.* and Chen *et al.* claimed that a potent $O^{=}$ scavenger improved the histopathologic outcomes and attenuated the spinal cord tissue IR injury in New Zealand White rabbits [26, 27].

Peire *et al.* determined that thrombus growth is a reflection of platelet recruitment. $O^{=}$ scavengers and thromboxane synthase inhibitors reduce thrombus growth [28]. Taylor *et al.* found $O^{=}$ reaction products (MDA) significantly increased by 22.22%, also red cell superoxide dismutase increased by 33.57%, plasma thiol reduced by 3.82%, significantly higher levels of collagen-induced by 67.44% and spontaneous whole blood platelet aggregation increased by 24.32% (all $p < 0.05$) in renal transplant patients compared with controls. Renal transplant patients are subject to oxidative cell damage, and may be at increased risk of vascular thrombosis [29]. Chiu *et al.* showed the benefit for neurons subjected to $O^{=}$ damage after an effective scavenging of them by SOD in rat brain astrocytes [30]. Poelstra *et al.* proposed this sequence of events; $O^{=}$ produced by activated neutrophils reduce glomerular ADPase activity - this highly sensitive enzyme for $O^{=}$ — leading to facilitation of thrombus

formation in an O_{2-} dependent manner in rat experimental glomerulonephritis models [31]. Nakamura *et al.* prevented platelet hyperaggregation activities by decreasing $O^{=}$ mediation on diabetic neuropathy [32]. Bednar *et al.* significantly reduced platelet aggregation by the administration of tPA although autologous clot embolization resulted in a trend toward significantly rising approximately 2.5-fold neutrophil count and acute activation (aggregation, $O^{=}$) from baseline ($p < 0.001$) in New Zealand white rabbit model [33].

Emrecan *et al.* improved blood flow and histologic scores according to the presence of tubular necrosis and atrophy, regenerative atypia and hydropic degeneration by inhibition of $O^{=}$ production which decreases neutrophil activation and aggregation in rabbit renal model [34]. Chan *et al.* associated platelet activation and dysfunction with $O^{=}$ generation and matrix metalloproteinase (MMP) –9 activation. At 4hr of reoxygenation, platelet count were decreased but no animal had a platelet count $< 100 \times 10^9$/L. Platelet aggregation was significantly reduced with a rightward shift of concentration-response curve, along with decreased plasma MMP-9 activity in newborn piglets [35]. Praticó *et al.* associated collagen-induced whole-blood aggregation with human platelet aggregation and activation accompanied by an increase in OH^- levels formation by 6.51-fold, also by TxB2 formation approximately 22.5-fold and protein kinase C (PKC) translocation from the cytosol to the cell membrane, promoting atherosclerosis and coronary artery disease [36].

Conclusions

U-74389G administration interacted or not with reoxygenation time decreases the platelet count within short-term time of 2 hours by different significance levels, exerting a potent beneficial effect on a range of diseases such as vascular thrombosis, myocardial cell membrane, attacks of angina pectoris, myocardial ischemia, atherosclerosis and coronary artery disease, percutaneous transluminal coronary angioplasty, spinal cord tissue and brain injuries, diabetic neuropathy, skin flap and renal transplants, glomerulonephritis, bronchopulmonary dysplasia, inflammations, even in mental stress. Further human clinical trials will make this effect clearer.

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