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Acute effect of the antioxidant drug U-74389G on hematocrit levels during hypoxia and reoxygenation injury in rats

Efeito agudo do fármaco antioxidante U-74389G sobre os níveis de hematócrito durante a lesão induzida por hipóxia e reoxigenação em ratos

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ABSTRACT

Aims: This experimental study evaluated the effect of the antioxidant drug U-74389G on hematocrit levels using a rat model of hypoxia and reoxygenation following an established protocol.

Methods: Forty rats with a mean weight of 231.875 g were employed in the study. Hematocrit levels were determined at 60 min (groups A and C) and at 120 min (groups B and D) after starting reoxygenation. Groups A and B received no drugs, whereas U-74389G was administered to rats for groups C and D.

Results: U-74389G administration significantly increased hematocrit levels by 4.73%±2.25% (p=0.0435). Reoxygenation time increased hematocrit levels non significantly by 3.96%±2.29% (p=0.1025). U-74389G administration combined with reoxygenation time significantly increased hematocrit levels by 3.16%±1.33% (p=0.0196).

Conclusions: U-74389G administration, whether it interacted or not with reoxygenation time, significantly increased hematocrit levels in the short term in a rat model of hypoxia and reoxygenation.

KEY WORDS: hypoxia; reoxygenation; hematocrit; U-74389G; models, animal.

RESUMO

Objetivos: Este estudo experimental avaliou o efeito da droga antioxidante U-74389G nos níveis de hematócrito, utilizando um modelo murino de hipóxia e reoxigenação, de acordo com um protocolo estabelecido.

Métodos: Quarenta ratos com um peso médio de 231,875 g foram utilizados no estudo. Os níveis de hematócrito foram determinados aos 60 min (grupos A e C) e aos 120 minutos (grupos B e D) após o início da reoxigenação. Os grupos A e B não receberam nenhuma droga, enquanto que a U-74389G foi administrada aos ratos dos Grupos C e D.

Resultados: A administração de U-74389G aumentou significativamente os níveis de hematócrito em 4,73%±2,25% (p=0,0435). O tempo de reoxigenação aumentou não significativamente os níveis de hematócrito em 3,96%±2,29% (p=0,1025). Aadministração de U-74389G combinada com o tempo de reoxigenação aumentou significativamente os níveis de hematócrito em 3,16%±1,33 (p=0,0196).

Conclusões: A administração de U-74389G, quer interagindo ou não com o tempo de reoxigenação, aumentou significativamente, no curto prazo, os níveis de hematócrito em um modelo murino de hipóxia e reoxigenação.

DESCRITORES: hipóxia; reoxigenação; hematócrito; U-74389G; modelos animais.

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Abbreviations: HR, hypoxia and reoxygenation; SD, standard deviation; IR, ischemia-reperfusion; C2C12, a *mouse myeloblast cell line*; γGT, gamma-glutamyl transferase; SOD, superoxide dismutase; GSH, glutathione; TNF_{α}, tumor necrosis factor alpha; IC, inhibitory concentration; ST, synaptic transmission; $t_{1/2}$, half-life; hct, hematocrit; IV, intravenous; L, lazaroid; NOS, nitric oxide synthase; ATP, adenosine triphosphate.

INTRODUCTION

Tissue hypoxia and reoxygenation (HR) may cause permanent or transient damage with serious implications for adjacent organs and systems. The use of U-74389G in HR has been a challenge for many years. However, although progress has been significant in this case, several practical questions have not been clarified, such as: a) how potent should U-74389G be?, b) when should it be administered?, and c) at what optimal dose should U-74389G be administered? The promising effect of U-74389G on tissue protection has been noted in several HR studies. U-74389G, also known as 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 lipid peroxidation [1]. It protects against ischemia-reperfusion (IR) injury in animal heart, liver, and kidney models. Membrane antioxidants are particularly effective in preventing permeability changes in brain microvascular endothelial cell monolayers [2]. Lazaroids (21-aminosteroids), a novel series of glucocorticoid compounds, are free radical scavengers. U-74389G is one of the 132 lazaroid compounds. It has a molecular weight of 726.90406 g/mol; it has a selective action on the vascular endothelium with vitamin E-like properties. It is mostly known for its neuroprotective and membrane-stabilizing properties. Although it accumulates in the cell membrane, thus protecting the vascular endothelium from peroxidative damage, it hardly penetrates the blood-brain barrier. More specifically, Hori et al. [3] showed its excellent effect on central nervous system trauma and ischemia. The degree of elevation of action potential thresholds and the rate of missing outer hair cells were significantly reduced, demonstrating that U-74389G has a protective effect on cisplatin-induced ototoxicity without glucocorticoid action. Schmid-Elsaesser et al. [4] showed significantly less neurological deficits postoperatively and significantly reduced cortical infarct volumes by the neuroprotective micro-vascularly acting 21-aminosteroid U-74389G. Passaquin et al. [5] elicited a beneficial effect of glucocorticoids on Duchenne muscular dystrophy, attributing it to a reduction of the pathological increase in Ca^{++} influx via an effect on the sarcolemma of C2C12 skeletal muscle cells. Van Klaveren et al. [6] supposed that direct inactivation of the membrane-bound γGT by hyperoxia is the most likely mechanism for the increased γGT, SOD and GSH levels in oxygen-exposed cells treated with U-74389G. Schmid-Elsaesser et al. [7] concluded that antioxidative compounds which cross the blood-brain barrier are more effective in focal cerebral ischemia than agents which predominantly act on the endothelium of cerebral microvessels. Lehmann et al. [8] decreased TNF_{α} release during endotoxemia permitting treatment of septic states. As an immunosuppressant, U-74389G may act through the activation of T-cells or by inhibiting the activation of helper cells. While immunosuppression primarily prevents rejection of transplanted organs, new applications involving mediation of the effects of interleukins and other cytokines have emerged. Lehmann et al. [9] attenuated leukocyte adherence and their rolling behavior in intestinal venules, which is found increased during endotoxemia. Horáková et al. [10] estimated the preventive effect of U74389G against lipid peroxidation at 160 IC50 μmol/l in oxidative stress. Heim et al. [11] totally prevented learning impairments, suggesting that lipid peroxidation may be responsible for learning deficiencies later in life. Vlkolinský [12] demonstrated protective activity in synaptic transmission (ST) recovery and in $t_{1/2}$ during hypoxia; the protective effect of U-74389G on population spike recovery and the possibility to delay early ST decay during hypoxia, which might indicate improved energetic state of neurons in the treated tissue. Durmaz et al. [13] showed antiproliferative properties in cancer cells by calculating an IC50 value at a 91 mM concentration. Kondziolka et al. [14] prevented regional brain edema permitting radiosurgery, without reducing the desired therapeutic effect. A meta-analysis of 14 published serum variables, from the same experimental setting, tried to provide a numerical evaluation of U-74389G efficacy at the same endpoints [15] (**Chart 1**). Several publications addressed trials of other similar antioxidant molecules to which U-74389G belongs.

The aim of this experimental study was to evaluate the effect of U-74389G on mean blood hematocrit (hct) levels using a rat model of HR.

Chart 1. U-74389G influence on the levels of some serum variables [15] concerning reperfusion time

Variable	1h rep Mean±SD	p-value	1.5h rep Mean±SD	p-value	2h rep Mean±SD	p-value	Interaction of U-74389G and rep Mean±SD	p-value
Red blood count	+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
Plateletcrit	+3.80%±9.87%	0.6373	+9.23%±6.29%	0.1064	+14.66%±9.03%	0.0833	+6.72%±3.73%	0.0712
Platelet distribution width	+1.1%±0.88%	0.2368	+1.79%±0.76%	0.0314	+2.49%±1.33%	0.0807	+0.96%±0.46%	0.0396
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 phosphokinase	+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	+5.57%±15.58%	0.3552	+4.74%±12.98%	0.3049	+3.92%±12.98%	0.3485	+2.73%±7.06%	0.2380

SD, standard deviation; rep, reperfusion,; h, hour.

METHODS

Preparation of the animals

This experimental research was approved by Veterinary Address of East Attiki Prefecture (protocol 3693/12-11-2010 & 14/10-1-2012). Accepted standards of humane animal care were adopted for female albino Wistar rats. The animals received food *ad libitum* in the laboratory seven days before the experiment. Post-experimental awakening and preservation of animals was not permitted, even if euthanasia was not required. Rats were randomly allocated to four experimental groups with 10 animals each, using the following HR protocols: 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); and hypoxia for 45 min followed by immediate U-74389G IV administration and reoxygenation for 120 min (group D). The dose of U-74389G was 10 mg/kg of body mass.

The detailed prenarcosis and general anesthesia techniques are described in the related reference [15]. Oxygen supply, electrocardiogram, and acidimetry were continuously provided during the whole experiment. The HR protocol was strictly followed.

Hypoxia was induced by clamping of the inferior aorta over the renal arteries using a forceps for 45 min. Reoxygenation was achieved by removing the clamp and re-establishing inferior aorta patency. After interruption of the blood flow, the HR protocol was applied to each experimental group, as described above. U-74389G was administered at the time of reoxygenation by an inferior vena cava catheter. The hct levels were determined at 60 min of reoxygenation (for groups A and C) and at 120 min (for groups B and D).

Forty female albino Wistar rats were used (mean weight of 231.875±36.59703 g, with minimum weight of 165 g and maximum weight of 320 g). Weight could be a potential confounding factor (e.g., more obese rats could have higher hct levels) and therefore, this assumption was also investigated.

Control groups

Twenty control rats (mean weight±S.D: 252.5± 39.31988 g) experienced hypoxia for 45 min followed by reoxygenation. **Group A**: Reoxygenation lasted 60 min (n=10 control rats), mean weight±S.D: 243±45.77724 g, mean hct level±S.D: 41.22± 2.674904%. **Group B**: Reoxygenation lasted 120 min (n=10 control rats), mean weight±S.D: 262± 31.10913 g, mean hct level±S.D: 43.26±3.640268% (**Table 1**).

Table 1. Weight and hematocrit mean levels and standard deviation of groups.

Groups	Variable	Mean	Standard deviation
A	Weight Hematocrit	243 g 41.22%	45.77724 g 2.674904%
B	Weight Hematocrit	262 g 43.26%	31.10913 g 3.640268%
C	Weight Hematocrit	212.5 g 43.59%	17.83411 g 2.638792%
D	Weight Hematocrit	210 g 44.98%	18.10463 g 2.623314%

Table 2. Statistical significance of the difference in mean values for groups after the paired t test.

Difference for groups	Variable	Difference	p-value
A-B	Weight	-19 g	0.2423
	Hematocrit	-2.04%	0.2496
A-C	Weight	30.5 g	0.0674
	Hematocrit	-2.37%	0.1089
A-D	Weight	33 g	0.0574
	Hematocrit	-3.759999%	0.0154
B-C	Weight	49.5 g	0.0019
	Hematocrit	-0.3300007%	0.8352
B-D	Weight	52 g	0.0004
	Hematocrit	-1.72%	0.2806
C-D	Weight	2.5 g	0.7043
	Hematocrit	-1.389999%	0.3497

U-74389G (Lazaroid) groups

Twenty lazaroid-treated rats (L rats) (mean weight±S.D: 211.25±17.53755 g) experienced hypoxia for 45 min followed by reoxygenation, at the beginning of which 10 mg of U-74389 g/kg of body weight was administered intravenously. **Group C**: Reoxygenation lasted 60 min (n=10 L rats), mean weight±S.D: 212.5±17.83411 g, mean hct level± S.D: 43.59±2.638792%. **Group D**: Reoxygenation lasted 120 min (n=10 L rats), mean weight±S.D: 210±18.10463 g, mean hct level ± S.D: 44.98± 2.623314% (**Table 1**).

Analysis

Rats from each group were compared with each other according to weight and hct level using paired t-tests (**Table 2**). Any significant difference between hct levels was investigated whether due to probable significant weight correlations. The application of generalized linear models with dependent variable the hct levels, and independent variables the U-74389G or no drug, the reoxygenation time and both variables in combination was followed. It yielded the following results: U-74389G administration significantly increased hct levels by 2.045% [0.1336849%-3.956315%] (P=0.0367). This finding is consistent with the results of the paired t-test (p=0.0503). Reoxygenation time increased hct levels by 1.714999% [-0.2310073%-3.661006%] (P=0.0824), which is nonsignificant and also in line with the paired t-test (P=0.1226). However, U-74389G administration and reoxygenation time together significantly increased hct levels by 1.368182% [0.2321644%-2.504199%] (P=0.0196). **Table 2**, **3** and **4** show the increasing influence of U-74389G in connection with reoxygenation time. Using the weights of rats as an independent variable in the generalized linear model, a nonsignificant relation was obtained (p=0.4276).

Table 3. Absolute values for increasing influence of U-74389G in connection with reperfusion time.

Increase	95%CI	Reperfusion time	t-test	glm
2.37%	-0.1263274%-4.866328%	1 h	0.1089	0.0615
2.045%	0.1336849%-3.956315%	1.5 h	0.0503	0.0367
1.72%	-1.26104%-4.701039%	2 h	0.2806	0.2411
1.714999%	-0.2310073%-3.661006%	reoxygenation time	0.1226	0.0824
1.368182%	0.2321644%-2.504199%	interaction		0.0196

CI, confidence interval; glm, generalized linear model; h, hour.

Table 4. Percentage increasing influence of U-74389G in connection with reperfusion time

Increase	Standard deviation	Reperfusion time	p-value
+5.58%	±3%	1h	0.0852
+4.73%	±2.25%	1.5h	0.0435
+3.89%	±3.44%	2h	0.2608
+3.96%	±2.29%	reoxygenation time	0.1025
+3.16%	±1.33	interaction	0.0196

DISCUSSION

Hypoxia influences hct levels in several ways. Marie I et al. [16] reduced hct levels to 35% by submitting patients with digital ischemic necrosis complications from hypothenar hammer syndrome to hemodilution therapy. Tang et al. [17] remarked that hct did not change on day 5 of their study compared with the placebo group (p=0.014) concerning the reduction of myocardial injury in experimental ischemia of acute coronary syndrome patients. Nemeth et al. [18] measured hct in cell suspensions daily for one week in order to find out the effect of 1-hour unilateral hind-limb IR in a follow-up experiment with rats. The relative cell transit time increased significantly on postoperative days 1 and 2 in the IR group compared with the control ones. Cappell [19] suggested short-term nasogastric intubation of acute gastrointestinal bleeding patients with an acute hct decline after recent myocardial ischemia, who may be particularly susceptible to further myocardial ischemia or cardiac arrhythmias following anxiety or discomfort during intubation. Götz et al. [20] administered human blood cells as bolus (1 min) during reperfusion (intracoronary hematocrit 7%) after global ischemia, significantly reducing post-ischemic recovery of pump cardiac function in pig hearts. External heart work served as parameter of function ($p<0.05$). A specifically blood-cell induced loss of myocardial pump function and coronary regulation has been demonstrated after short-term ischemia. The effect seems to be attenuated in the presence of erythrocytes compared with control devices. Arepally et al. [21] transfused patients who underwent catheter-directed thrombolysis to treat arterial and bypass graft occlusions using a low-dose protocol, in which the transfusion rate was 15% and major complications accounted for 10%. The combined low/high-dose protocol had a transfusion rate of 46% and a rate of major complications of 13%. The overall success rate and major complication rates were 86% and 11%, respectively, obtaining high efficacy in the relief of ischemia. The frequency of transfusions was 37% (mean, 2.8 U). Ostwald et al. [22] determined the effect on ocular blood flow and the electroretinogram of either nitric oxide synthase (NOS) inhibition or adenosine receptor blockade, or the combination of both, after 1 h of unilateral ocular IR in cats, controlling hct in each experiment. The other eye in each animal served as a non-ischemic control. Five minutes after IR, blood flows in retina and choroid were measured. Electroretinographic studies were carried out on IR. NOS inhibition and adenosine receptor blockade significantly reduced basal blood flow when combined with adenosine receptor blockade. Retinal hyperemia reappeared when either adenosine or adenosine receptor blockade and NOS inhibition were combined. Donaldson et al. found that short-term falls in temperature produced significant increases in hct and mortality from ischemic heart disease and cerebrovascular disease. The cold exposures of normal life are sufficient to induce significant and prolonged hemoconcentration, which may explain why deaths from arterial disease are more prevalent in winter [23]. Cole et al. observed a dose-dependent decrease in ischemic brain injury in rats due to a decline in hct (hemodilution). They performed daily neurological examinations and analyzed the brains after 72 h for infarct volume (mm^3). Neurological outcome was improved along with decreased infarct volumes in both groups ($p<0.05$) [24]. Comes et al. [25] concluded that isovolemic hemodilution caused by reduced hematocrit is a single therapeutic method, but it is not an option in the case of acute ischemic stroke. Palmon et al. found that early hyperemia was attenuated and delayed hypoperfusion was augmented during reperfusion as a result of low perfusion pressure in diabetic dogs. Cerebral perfusion pressure was kept constant during global incomplete cerebral ischemia, but was lower throughout reperfusion in diabetic dogs. During ischemia, cerebral blood flow was reduced similarly among groups of hyperglycemic, normoglycemic, and diabetic dogs. However, during the final 8 min of ischemia, mean arterial blood pressure decreased more sharply in diabetic than in hyperglycemic and normoglycemic groups and remained lower throughout 3 h of reperfusion [26]. Arend et al. concluded that a significant decrease in hct values within 24 h altered hemorheologic state and may contribute to the protective effect in acute myocardial ischemia patients with acute chest pain ($P<0.05$) [27]. Wong et al. found that the recovery of adenosine triphosphate (ATP) after 25 min of hepatic arterial IR was much slower compared to portal venous reperfusion only in the recuperative process of hepatocytes in the IR liver. The ability of liver cells to restore their tissue energy phosphates is related to the viability of the liver [28]. Aleksandrov [29] noted no substantial disturbances in microvascular hct level in the rat intestinal mesentery after 1 min of IR in the superior mesenteric artery, whereas 10 min of IR was followed by a decrease of flow velocity in microvessels and development of thrombosis at the site of arterial occlusion [29]. Pozin et al. [30] showed that the sequelae of a 3-5-minute reversible myocardial IR might be associated with

hct shifts after 24-120 hours in dogs with reversible coronary disorders. Röhnert et al. [31] investigated changes in serum hct during the liver preservation, after their successful auxiliary transplantation, which were decisively important for avoiding irreversible ischemic damage in dogs.

This study addressed the decreasing role of hypoxia in hematocrit levels. Furthermore, the assumption is whether U-74389G among and besides its above-mentioned protective effects on ototoxicity, central nervous system, Duchenne muscular dystrophy, inactivation of membrane-bound enzymes, septic states, endotoxemia, learning impairments and antiproliferative properties, also has any possible effects on acute hematopoiesis. The results of this study are very encouraging concerning hematocrit. There appears to be a current growing interest in the identification of such a capacity that could promptly elevate hematocrit levels, particularly in critically ill patients, until formal hematopoietic agents act or until blood transfusions occur. In conclusion, U-74389G administration, whether it interacted or not with reoxygenation time, significantly increased hct levels in the short term.

NOTES

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Conflicts of interest disclosure

Apostolos Papalois is the director of the Experimental & Research Center of ELPEN Pharmaceuticals. The other authors reported no conflicts of interest.

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