Original Research

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The effect of the antioxidant drug "U-74389G" on acid phosphatase levels during ischemia reperfusion injury in rats

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ABSTRACT

Introduction: This experimental study examined the effect of the antioxidant drug "U-74389G", on a rat model and particularly in an ischemia - reperfusion protocol. The effects of that molecule were studied biochemically using blood mean acid phosphatase (ACP) levels. **Methods:** 40 rats of mean weight 231.875 g were used in the study. ACP levels were measured at 60 min of reperfusion (groups A and C) and at 120 min of reperfusion (groups B and D). Administration of the drug U-74389G was predicted only for groups C and D. **Results:** U-74389G administration significantly decreased the ACP levels by 128.45% ±14.84% (p= 0.0000). Reperfusion time non-significantly decreased the ACP levels by 22.44% ± 25.86% (p= 0.2000). However, U-74389G administration and reperfusion time together significantly decreased the ACP levels by 74.45% ± 9.63% (p= 0.0000). **Conclusions:** U-74389G administration whether it interacted or not with reperfusion time, offers spectacular significant decreasing short – term effects on ACP levels.

KEY WORDS: Ischemia; U-74389G; Acid Phosphatase; Reperfusion

INTRODUCTION

Permanent or transient damage with serious implications on adjacent organs and systems may be due to tissue ischemia - reperfusion (IR). The use of U-74389G in IR has been a challenge for many years. However, although the progress was significant, several practical questions have not clarified. They include: a) how potent U-74389G should be b) when should it be administered and c) at what optimal dose. The promising effect of U-74389G in tissue protection has been noted in several IR studies. U-74389G or 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 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 [2]. A meta-analysis of 15 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) [3,4]. Several publications addressed trials of this antioxidant molecule. Flessas I et al improved the histological score in intestinal tissue after U-74389G administration in pigs [5]. Bimpis A et al neuroprotected the brain itself from strokes by U-74389G administration in a porcine model [6]. Tsaroucha AK et al attenuated the liver damage after U-74389G administration in a swine model [7]. Andreadou I et al protected the rat small intestine from oxidative damage after the administration of U-74389G [8].

U-74389G was administered to find out whether it is able to reverse IR short-term general inflammatory and bone damages. The determination of biochemical bone turnover markers (BBMT) allows a quantitative assessment of bone turnover. Biochemical markers are molecules produced by osteoblasts, osteoclasts or come from the type I collagen metabolism. They are classified as bone formation or bone resorption markers. Regarding the relationship of BBMT with bone mineral density, the correlation is more significant for the bone resorption markers than bone formation markers. The usefulness of BBMT concerns both the high risk individuals' for rapid detection of loss of bone mass, as well as following up patients under anti-

resorption treatment for the early therapeutic intervention assessment. Tartrate-resistant acid phosphatase (ACP or TRAP) levels as a bone resorption marker is useful for rapid bone mass loss detection as well as following up patients under anti-resorptive treatment. The effect of U-74389G administration was assessed on bone IR damage studied by changes on blood (ACP) levels. Tartrate-resistant acid phosphatase (TRAP or TRAPase), also called acid phosphatase 5, tartrate resistant (ACP5), is a glycosylated monomeric metalloprotein enzyme expressed in mammals. It has a molecular weight of approximately 35kDa, a basic isoelectric point (7.6-9.5) and optimal activity in acidic conditions. TRAP is synthesized as latent proenzyme and activated by proteolytic cleavage and reduction. It is differentiated from other mammalian acid phosphatases by its resistance to inhibition by tartrate, molecular weight and characteristic purple color. However, total TRAP was measured at this experiment and not the bone specific TRAPC-5b one. This means that the effect may be a tissue or serum inflammatory response and not only a bone response. The % exact origin of ACP was undetermined at present. Future designs will be more TRAPC-5b specific. TRAPC-5b is not the normal resorption marker measured, although it has been used mainly in cancer patients. It is more a measure of osteoclast number than resorptive activity. The responses seen are rapid and while they might show a potential increase in resorptive activity, there is unlikely to be significant bone loss ('damage') in the period of this study.

MATERIALS AND METHODS

Animal preparation

This basic experimental research was licensed by Veterinary Address of East Attiki Prefecture under 3693/12-11- 2010 & 14/10-1-2012 decisions. Every consumable, equipment and substance, was a grant of Experimental Research Centre of ELPEN Pharmaceuticals Co. Inc. S.A. at Pikermi, Attiki. Accepted standards of humane animal care were adopted for Albino female Wistar rats. Pre-experimental normal housing included ad libitum diet in laboratory for 7 days. Post-experimental awakening and preservation of animals was not permitted, even if euthanasia was needed. Rats 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 U-74389G intravenous (IV) administration and reperfusion for 60 min (group C); ischemia for 45 min followed by immediate U-74389G IV administration and reperfusion for 120 min (group D). The dose of U-74389G was 10 mg/Kg body mass of animals.

The detailed preceded prenarcotic and general anesthesiologic techniques are described in related references [3,4]. Oxygen supply, electrocardiogram and acidometry were continuously provided during whole experiment performance. The protocol of IR was followed.

Ischemia was caused by laparotomic clamping inferior aorta over renal arteries with forceps for 45 min. Reperfusion was induced by removing the clamp and reestablishment of inferior aorta patency. U-74389G was administered at the time of reperfusion; through catheterized inferior vena cava. The ACP levels were determined at 60th min of reperfusion (for A and C groups) and at 120th min of reperfusion (for B and D groups). Fourty female Wistar albino rats were used (mean weight 231.875 g [Std. Dev: 36.59703 g], with minimum weight 165 g and maximum weight 320 g. Rats' weight could be potentially a confusing factor, e.g. more obese rats to have higher ACP levels. This suspicion was also investigated.

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

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
MCH	+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 count [4]	-17.79%±9.40%	0.0647	-12.83%±5.79%	0.0303	-7.88%±7.83%	0.2939	-6.12%±3.58%	0.0857
Platelet-crit	+3.80%±9.87%	0.6373	+9.23%±6.29%	0.1064	+14.66%±9.03%	0.0833	+6.72%±3.73%	0.0712
PDW	+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
ALP	+22.66%±12.37%	0.0663	+31.91%±7.69%	0.0001	+41.16%±9.65%	0.0003	+17.75%±4.79%	0.0005
CPK	+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	+4.02%±16.18%	0.3358	+3.57%±13.31%	0.2865	+3.13%±12.87%	0.3449	+2.14%±7.18%	0.2278

Control groups

20 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 ACP levels 21.05 IU/L [Std. Dev: 6.805921 IU/L] (Table 2).

Group B

Reperfusion lasted for 120 min (n=10 controls rats) mean mass 262 g [Std. Dev: 31.10913 g], mean ACP levels 18.57 IU/L [Std. Dev: 6.874761 IU/L] (Table 2).

Lazaroid (L) group

20 L rats (mean mass 211.25 g [Std. Dev: 17.53755 g] experienced ischemia for 45 min followed by reperfusion in the beginning of which 10 mg U-74389G /kg body weight were IV administered.

Group C

Reperfusion lasted for 60 min (n=10 L rats) mean mass 212.5 g [Std. Dev: 17.83411 g], mean ACP levels 5.89 IU/L [Std. Dev: 4.798252 IU/L] (Table 2).

Group D

Reperfusion lasted for 120 min (n=10 L rats) mean mass 210 g [Std. Dev: 18.10463 g], mean ACP levels 3 IU/L [Std. Dev: 0.2708012 IU/L] (Table 2).

Statistical analysis

Weight and ACP levels of every group were compared with each other by statistical paired t-tests (Table 3). Any significant difference among ACP levels, was investigated whether owed in potent significant weight correlations. The application of generalized linear models (glm) with dependant variable the ACP levels and independent variables the U-74389G or no drug, the reperfusion time and both variables in combination was followed. Inserting the rats' weight also as an independent variable at generalized linear models analysis, a non significant relation resulted in (p=0.1695), so as to further investigation was not needed.

Table 2. Weight and ACP mean levels and Std. Dev. of groups

Groups	Variable	Mean	Std. Dev
A	Weight	243 g	45.77724 g
A	ACP	21.05 IU/L	6.805921 IU/L
B	Weight	262 g	31.10913 g
B	ACP	18.57 IU/L	6.874761 IU/L
C	Weight	212.5 g	17.83411 g
C	ACP	5.89 IU/L	4.798252 IU/L
D	Weight	210 g	18.10463 g
D	ACP	3 IU/L	0.2708012 IU/L

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	ACP	2.48 IU/L	0.1681
A-C	Weight	30.5 g	0.0674
A-C	ACP	15.16 IU/L	0.0012
A-D	Weight	33 g	0.0673
A-D	ACP	18.05 IU/L	0.0000
B-C	Weight	49.5 g	0.0019
B-C	ACP	12.68 IU/L	0.0010
B-D	Weight	52 g	0.0009
B-D	ACP	15.57 IU/L	0.0001
C-D	Weight	2.5 g	0.7390
C-D	ACP	2.89 IU/L	0.0913

RESULTS

The glm application resulted in: U-74389G administration significantly decreased the ACP levels by 15.365 IU/L [-18.84453 IU/L - -11.88547 IU/L] (P= 0.0000). This finding was in accordance with the results of paired t-test (p= 0.0000). Reperfusion time non-significantly decreased the ACP levels by 2.685 IU/L [-8.750513 IU/L - 3.380513 IU/L] (P= 0.3758), also in accordance with paired t-test (p= 0.0243). However, U-74389G administration and reperfusion time together significantly decreased the ACP levels by 8.906364 IU/L [-11.16608 IU/L - -6.646648 IU/L] (P= 0.0000). Reviewing the above and table 3, tables 4 and 5 sum up concerning the alteration influence of U-74389G in connection with reperfusion time.

DISCUSSION

ACP is considered a reliable index not only for special tissue function but also for general metabolism. Its production is influenced by ischemia and particularly by certain mode, as the next references show. Otero JE et al reported a 7-year-old boy with generalized arterial calcification (AC) of infancy (GACI), an autosomal recessive disorder that features hydroxyapatite deposition within arterial elastic fibers [9]. He developed AC after 5 months of (1-hydroxyethylidene-bisphosphonate) EHDP lifesaving therapy 200 mg/day orally during infancy. The surveillance for toxicity was crucial since brain isoform of tartrate-resistant acid phosphatase 5b (TRAP-5b) was elevated. Frederiks WM et al investigated early heart IR damage in rats [10]. Mitochondria can be seen as a sign of irreversible cell damage. It was shown that ACP enzyme was neither decreased up to 240 min heart fragments incubation, nor was correlated with the irreversible stage of damage of myocytes. Kikuchi T et al examined the role of lysosomal enzymes for ACP in IR retinal pigment epithelial cells (RPE) of albino rabbits [11]. Five days after posterior ciliary arteries (PCA)-cut, RPE in the ischemic region were disorganized by increased enzymatic digestion. In border of ischemic RPE region, a lot of fragmented outer segments were phagocytosed 1 to 7 days after PCA-cut. At this time, phagosomes appeared much more frequently than in normal retina. A strong ACP activity was encountered in the phagosomes of macrophages, derived from RPE and seemed to play the major role in scavenging destructed retinal elements. Robinson JW et al subjected short loops of dog small intestine into one hour IR [12]. The release of lysosomal enzymes after ischemia was studied by gauging the ACP levels in the ischemic loop and in the neighboring control one. Immediately after ischemia, considerable structural damage was observed in the intestinal mucosa, with desquamation of the villous tips, edema, vascular stasis, and hemorrhagic infiltration in the lamina propria. A significant release of lysosomal ACP enzyme into the venous blood was noted.

Shopova VL et al estimated the protective effects of the lazaroid U-74389G possessing antilipid peroxidation activity and membrane-stabilizing effect in male Wistar rats [13]. Paraquat dichloride which forms reactive oxygen species and increases the lipid peroxidation in the pulmonary cells, was administered orally at 80mg/kg. The lazaroid U-74389G was injected intraperitoneally twice - 2h before receiving the paraquat with 10mg/kg and four hours later with 5mg/kg. Isolated application of paraquat increased ACP enzyme activity content in bronchoalveolar lavage fluid (BALF). The combined treatment with paraquat and U-74389G

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

Decrease	95% c. in.	Reperfusion time	t-test	glm
15.16 IU/L	-20.6924 IU - -9.627599 IU/L	1h	0.0012	0.0000
15.365 IU/L	-18.84453 IU/L - -11.88547 IU/L	1.5h	0.0000	0.0000
15.57 IU/L	-20.14093 IU/L - -10.99907 IU/L	2h	0.0000	0.0000
2.685 IU/L	-8.750513 IU/L - 3.380513 IU/L	reperfusion time	0.0243	0.3758
8.906364 IU/L	-11.16608 IU/L - -6.646648 IU/L	interaction		0.0000

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

Decrease	±SD	Reperfusion time	p-values
112.54%	±20.95%	1h	0.0006
128.45%	±14.84%	1.5h	0.0000
144.36%	±21.62%	2h	0.0000
22.44%	±25.86%	reperfusion time	0.2000
74.45%	± 9.63%	interaction	0.0000

significantly elevated the enzyme activity of ACP less than the unique application of paraquat. It is concluded that the lazaroid U-74389G reduces the pneumotoxic effects of paraquat, estimated by sensitive ACP enzyme activity in BALF. The protective effect of U-74389G is well-expressed until day 3 after the treatment.

It is repeated that ACP levels is more a measure of osteoclast number than resorptive activity. The responses seen are rapid and while they might show a potential increase in resorptive activity, there is unlikely to be significant bone loss ('damage') in the period of this study. Because of this, it was also important to include along some measure of bone mass such as bone mineral density (BMD) in future studies. As the experiment got statistically very significant, the next design should include in vitro osteocyte bone cell confirmation and histopathological IR assessment.

CONCLUSION

U-74389G administration whether it interacted or not with reperfusion time, offers impressive significant decreasing short – term effects on ACP levels. This has huge impacts at inflammatory response but a BMD and pathologic osteoclast co-evaluation is needed for bones response.

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