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

Abstract

Background: This experimental study examined the effect of the antioxidant drug "U-74389G", on a rat model and particularly in a generalized ischemia - reperfusion protocol. The effects of that molecule were studied biochemically using blood mean lactate dehydrogenase (LDH) levels.

Methods: 40 rats of mean weight 231.875 g were used in the study. LDH levels were measured at 60 min of reperfusion (groups A and C) and at 120 min of reperfusion (groups B and D). The drug U-74389G was administered only in groups C and D.

Results: U-74389G administration kept significantly increased the predicted LDH levels by 18.78%±4.52% (P= 0.0001). Reperfusion time non-significantly decreased the predicted LDH levels by 3.75%±5.46% (p=0.4103). However, U-74389G administration and reperfusion time together kept significantly increased the predicted LDH levels by 10.43%±2.82% (P= 0.0005).

Conclusion: U-74389G administration whether it interacted or not with reperfusion time kept significantly increased short – term the LDH levels.

Keywords: ischemia, U-74389G, lactate dehydrogenase, reperfusion

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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 U-74389G should be administered. 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 organs such as 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 23 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). Several publications addressed trials of other similar antioxidant molecules to which the studied molecule U-74389G belongs to.

The aim of this experimental study was to examine the effect of the antioxidant drug "U-74389G" on rat model and particularly in a generalized ischemia - reperfusion (IR) protocol. The effects of that molecule were studied by measuring blood mean lactate dehydrogenase (LDH) levels.

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. All consumables, equipment and substances, were 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. 7 days pre-experimental normal housing included *ad libitum* diet in laboratory. Prenarcosis of animals proceeded of continuous intra-experimental general anesthesia,[3–6] oxygen supply, electrocardiogram and acidometry. Post-experimental euthanasia did not permitted awakening and preservation of the animals. Rats were randomly delivered to four experimental groups by 10 animals in each one, using following protocols of IR: Ischemia for 45min followed by reperfusion for 60min (group A); ischemia for 45min followed by reperfusion for 120min (group B); ischemia for 45min followed by immediate U-74389G intravenous (IV) administration and reperfusion for 60min (group C); ischemia for 45min followed by immediate U-74389G IV administration and reperfusion for 120min (group D). The dose of U-74389G was 10 mg/Kg body mass of animals. Ischemia was caused by laparotomic clamping inferior aorta over renal arteries with forceps for 45min. Reperfusion was induced by removing the clamp and re-establishing inferior aorta patency. U-74389G was administered at the time of reperfusion; through catheterized inferior vena cava. The LDH 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 231.875g [Standard Deviation (SD): 36.59703g], with minimum weight 165g and maximum weight 320g. Rats' weight could be potentially a confusing factor, e.g. more obese rats to have higher LDH levels. This assumption was also investigated.

Control groups

20 control rats (mean mass 252.5 g [SD: 39.31988 g]) experienced ischemia for 45 min followed by reperfusion.

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
WBCC	0.3544	0.0914	0.4199	0.0045	0.5609	0.0185	0.2973	0.0004
RBCC	0.021	0.7161	0.0096	0.8106	-0.0005	0.9762	0.0158	0.4911
Hematocrit	0.0858	0.0852	0.0698	0.0435	3.9244	0.2608	0.0449	0.0196
Hemoglobin	0.08	0.0925	0.06	0.0604	0.059	0.3544	0.038	0.0423
MCH	0.0273	0.0663	0.0297	0.0001	0.0374	0.0003	1.33%+0.36%	0.0005
MCHC4	0.0024	0.482	-0.0032	0.1124	-0.0028	0.1603	-0.0032	0.0655
RbcDW	-0.024	0.0667	-0.0269	0.0175	-0.0073	0.1383	-0.0115	0.679
Platelet count	-0.0839	0.0647	-0.0704	0.0303	-0.0005	0.2939	-0.0254	0.0857
Platelet-crit	0.1367	0.6373	0.1552	0.1064	0.2369	0.0833	0.1045	0.0712
PDW	0.0198	0.2368	0.0255	0.0314	0.0382	0.0807	0.0142	0.0396
Glucose	-0.0291	0.0663	-0.0651	0.0001	-0.0822	0.0003	-0.0348	0.0005
Creatinine5	-0.0725	0.0663	-0.1596	0.0001	-0.1997	0.0003	-0.0853	0.0005
Uric acid6	0.353	0.1614	0.2453	0.096	0.2211	0.3946	0.1042	0.3873
Total protein	-0.0249	0.0663	-0.0558	0	-0.0704	0	-0.0298	0
γGT	0.3793	0.2362	0.2171	0.6442	0.1439	0.7809	0.1023	0.8877
ALP	0.3503	0.0663	0.396	0.0001	0.5081	0.0003	0.2254	0.0005
ACP	-0.9159	0.0006	-1.1361	0	-1.2274	0	-0.6482	0
CPK	0.6807	0.0012	0.5254	0.026	0.4661	0.4951	0.2796	0.077
Sodium	0.0188	0.0707	0.0078	0.7714	0.0016	0.3995	0.0004	0.3693
Chloride	0.0019	0.4533	-0.0044	0.0879	-0.006	0.1113	-0.0037	0.0159
Calcium	0%+1.75%	1	0.0096	0.8782	0.0126	0.8492	0.0078	0.8245
Phosphorus	0.0328	0.7966	0.0171	0.5789	0.0348	0.8129	0.0091	0.5771
Magnesium	0.0492	0.7033	0.0247	0.9171	0.0338	0.7161	0.0494	0.8228
Mean	0.2852	0.2707	0.2885	0.2268	0.304	0.3011	0.1682	0.2107

Group A

Reperfusion lasted for 60 min (n=10 controls rats) mean mass 243g [SD: 45.77724g], mean LDH levels 1609.9 IU/L [SD: 834.4269 IU/L] (Table 2).

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

Groups	Variable	Mean	Std. Dev
A	Weight LDH	243g 1609.9IU/L	45.77724g 834.4269 IU/L
B	WeightLDH	262g 1622.6 IU/L	31.10913g 793.438 IU/L
C	WeightLDH	212.5g 2568.3 IU/L	17.83411g 622.0566 IU/L
D	WeightLDH	210g 2259.5 IU/L	18.10463g 524.1311 IU/L

Group B

Reperfusion lasted for 120 min (n=10 controls rats) mean mass 262 g [SD: 31.10913g], mean LDH levels 1622.6 IU/L [SD: 793.438 IU/L] (Table 2).

Lazaroid (L) group

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

Group C

Reperfusion lasted for 60min (n=10 L rats) mean mass 212.5g [SD: 17.83411g], mean LDH levels 2568.3 IU/L [SD: 622.0566 IU/L] (Table 2).

Group D

Reperfusion lasted for 120min (n=10 L rats) mean mass 210g [SD: 18.10463g], mean LDH levels 2259.5 IU/L [SD: 524.1311 IU/L] (Table 2).

Statistical analysis

The generalized linear model (GLM) is a flexible generalization of ordinary linear regression that allows for response variables that have error distribution models other than a normal distribution. The GLM generalizes linear regression by allowing the linear model to be related to the response variable via a link function and by allowing the magnitude of the variance of each measurement to be a function of its predicted value. It is proposed an iteratively reweighted least squares method for maximum likelihood estimation of the model parameters as remaining the most popular. Ordinary linear regression predicts the expected value of a given unknown quantity (the *response variable*, a random variable) as a linear combination of a set of observed values (*predictors*). This implies that a constant change in a predictor leads to a constant change in the response variable (i.e. a *linear-response model*). This is appropriate when the response variable has a normal distribution (intuitively, when a response variable can vary essentially indefinitely in either direction with no fixed "zero value", or more generally for any quantity that only varies by a relatively small amount, e.g. the rats weights). The investigation of a high LDH value may outcome to one of the 3 following explanations: it may be proportional to tissue damage size, or may be influenced by the U-74389G, or may be proportional to weight of the post-injury animal or tissue. Only the GLM testing associating the LDH levels with the weights can provide an answer to this question. If the response is statistically significant, then the variation of LDH levels is also due to the animals' weight. This variable must be eliminated and find other LDH levels which will reflect only the degree of the effect of the drug as the tissue damage size is the same for all animals. These new LDH levels are the predicted LDH levels. However, these assumptions are inappropriate for some types of response variables. For example, in cases where the response variable is expected to be always positive and varying over a narrow or wide range, constant input changes lead to geometrically varying, rather than constantly varying, output

changes (e.g. the study times: 1 hour or 2 hours). Similarly, a model that predicts a probability of making a yes/no choice (a Bernoulli variable) is even less suitable as a linear-response model, since probabilities are bounded on both ends (they must be between 0 and 1, e.g. the drug or no administration). GLM cover all these situations by allowing for response variables that have arbitrary distributions (rather than simply normal distributions) and for an arbitrary function of the response variable (the *link function*) to vary linearly with the predicted values (rather than assuming that the response itself must vary linearly). Every weight and LDH level group was compared with each other by statistical standard t-tests (Table 3). Any significant difference among LDH levels, was investigated whether owed in any potent significant weight one. The application of GLM with dependant variable the LDH levels was followed. The 3 independent variables were the U-74389G or no drug administration, the reperfusion time and both variables in combination. Inserting the rats' weight also as an independent variable at GLM analysis, a very significant relation resulted in (p=0.0070), so as to further investigation was needed. The predicted LDH values, adjusted for rats' weight were calculated and are depicted at Table 4. Afterwards, every predicted LDH level group was compared with each other by statistical standard t-tests (Table 5). The application of GLM with dependant variable the predicted LDH levels was followed. The 3 independent variables were again the same as previously.

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

DG	Variable	Difference	p-value
A-B	Weight LDH	-19 g -12.7 IU/L	0.2423 0.9738
A-C	Weight LDH	30.5 g-958.4 IU/L	0.0674 0.0369
A-D	Weight LDH	33 g-649.6 IU/L	0.0574 0.0694
B-C	Weight LDH	49.5 g-945.7 IU/L	0.0019 0.0167
B-D	Weight LDH	52 g-636.9 IU/L	0.0004 0.0101
C-D	Weight LDH	2.5 g-308.8 IU/L	0.7043 0.3258

Table 4 Mean predicted LDH values adjusted for weight and Std. Dev. of groups

Groups	Mean	Std. Dev
A	1913.476 IU/L	418.0609 IU/L
B	1739.958 IU/L	284.1044 IU/L
C	2192.017 IU/L	162.8703 IU/L
D	2214.848 IU/L	165.3407 IU/L

Table 5 Statistical significance of mean predicted values difference for groups (DG) after statistical standard t test application

DG	Difference	p-value
A-B	173.5177 IU/L	0.2423
A-C	-278.5414 IU/L	0.0674
A-D	-301.3726 IU/L	0.0574
B-C	-452.0591 IU/L	0.0019
B-D	-474.8903 IU/L	0.0004
C-D	-22.83118 IU/L	0.7043

Results

The first GLM resulted in: U-74389G administration kept significantly increased the LDH levels by 797.65 IU/L [352.6114 IU/L - 1242.689 IU/L] (P= 0.0008). This finding was in accordance with the results of standard t-test (p= 0.0016). Reperfusion time non-significantly decreased the LDH levels by 148.05 IU/L [-662.1633 IU/L - 366.0633 IU/L] (P= 0.5634), also in accordance with standard t-test (p= 0.5383). However, U-74389G administration and reperfusion time together kept significantly increased the LDH levels by 378.9364 IU/L [93.47671 IU/L - 664.396 IU/L] (P= 0.0106). Reviewing the above and Table 3, the table 6 sums up concerning the alteration influence of U-74389G in connection with reperfusion time. The second GLM resulted in: U-74389G administration kept significantly increased the predicted LDH levels by 376.7159 IU/L [198.7321 IU/L - 554.6996 IU/L] (P= 0.0001). This finding was in accordance with the results of standard t-test (p= 0.0002). Reperfusion time non-significantly decreased the predicted LDH levels by 75.34327 IU/L [-290.6826 IU/L - 139.9961 IU/L] (P= 0.4831), also in accordance with standard t-test (p= 0.3375). However, U-74389G administration and reperfusion time together kept significantly increased the LDH levels by 209.6325 IU/L [98.52261 IU/L - 320.7424 IU /L] (P= 0.0005). Reviewing the above and Table 5,7&8 sums up concerning the alteration influence of U-74389G in connection with reperfusion time.

Discussion

LDH is found extensively in body tissues, such as blood cells, heart muscle and liver. Because it is released during tissue damage, it is a marker of common injuries like muscular failure and fatigue, tissue breakdown or turnover and hemolysis. It concerns all the ischemic tissues under the clapping level in the present experiment. A lot of clinical observations show how LDH levels are influenced in ischemic cases. Pisarenko et al.[7] combined enhanced functional recovery with an increase in LDH and LDH/pyruvate ratio levels leakage in early perfusate of isolated working rat IR hearts. Wang et al.[8] found remarkable decreases in both LDH levels release and myocardial infarction size in mouse transgenic IR hearts over expressing functional consequence of microRNAs miR-494, than wild-type ones. Yamagishi et al.[9] found the levels of LDH released into the IR coronary effluent inversely lower in 70% reduced fed Wistar rats than in *ad-libitum* fed rats. These results suggested that severe, short-term food restriction improves ischemic tolerance in rat hearts. Xu et al first determined[10] the cardiomyocyte shortening and then the roles of LDH release in culture medium in isolated IR myocytes of female rats. Ciminelli et al.[11] found released LDH levels in the IR coronary effluent similar in treadmill running trained rats than control ones. Hoeven et al.[12] found progressive organ; particularly kidney dysfunction and inflammatory responses most pronounced in hemodynamically unstable brain-dead donors studied by monitoring LDH levels in Wistar rats. Wang et al evaluated[13] the released LDH levels significantly less impaired in gene-targeted NHE1-null mutant (Nhe1-/-) mice IR hearts relative to wild-type ones, in absence of NHE1 inhibitor. Wang et al.[14] measured decreased release of skeletal muscle intracellular enzyme LDH in experimental groups protected by ischemic preconditioning (IP) in limb IR injury compared with the control ones. Baron et al.[15] assessed no significant influence of mycophenolic acid (MPA) on postischemic efflux rates of LDH levels in rat IR liver. Ko et al.[16] caused a LDH leakage to a smaller extent in isolated-perfused brief IR hearts of control and diabetic rats. Kume et al.[17] attenuated the IR liver damage by IP improving the restoration of hepatic function during reperfusion as explained by LDH release. Jaeschke et al.[18] assumed the intracellular generation of reactive oxygen species in IR hepatocytes injury of Fischer rats by LDH release.

Also, LDH levels may be influenced by U-74389G administration. Shopova VL et al.[19] considered that paraquat forms reactive oxygen species and increases the lipid peroxidation in the pulmonary cells. Paraquat dichloride was administered orally at 80mg/kg in Wistar rats.

The lazaroid U-74389G was injected intraperitoneally twice - with 10mg/kg and 5mg/kg respectively. Isolated application of paraquat increased enzyme activities of LDH content in bronchoalveolar lavage fluid (BALF). The combined treatment with paraquat and U-74389G significantly elevated the enzyme activities of LDH less than the separate administration of paraquat. It is concluded that the lazaroid U-74389G reduces the pneumotoxic effects of paraquat, estimated by biochemical markers in BALF until day 3 after the treatment. Vignes et al.[20] suggested a partial neuroprotective effect of free radical scavengers since lipid peroxidation is a key cellular event in neuronal injury, and its inhibition with lazaroids could help to reduce brain ischemic lesions[20] by a LDH assay in rats. The excessive production of free radicals by oxidative stress is engaged in a large variety of diseases. Lazaroids (U-74389G), added to cultures, at different concentrations (10-7-5 M), caused a reduction in cortical neuronal death by 34.5 %. Alhan E et al.[21] induced acute necrotizing pancreatitis in rats resulted in a significant increase in serum LDH levels in BALF. U-74389G may be used in the treatment of lung injury during acute pancreatitis. Monte et al.[22] impaired significantly glutamate clearance when astrocytes were preincubated with 1-methyl-4-phenylpyridinium MPP (+). Astrocytes are the site of the toxic (MPP (+)) metabolite production. This effect became more pronounced by prolonging the incubation in the presence of MPP (+). Indeed, the lazaroid antioxidant U-74389G, was not capable of restoring glutamate net uptake. These results indicate that, by acting as a mitochondrial poison, MPP (+) impairs energy metabolism of astrocytes and significantly reduces their ability to maintain low levels of extracellular glutamate. Fukuma K et al.[23] compared the efficacy of U-74389G in endotoxin-induced liver injury. Lipopolysaccharide (Escherichia coli, 30 mg/kg given intraperitoneally [IP]) and U-74389G (3mg/kg IP) were administered simultaneously in male IR mice. U-74389G treatment significantly increased survival rates 48 hours after lipopolysaccharide injection and decreased hepatic enzyme release. These findings suggest that U-74389G can suppress proinflammatory gene up-regulation and ameliorate endotoxin shock. Ishizaki N et al.[24] significantly improved dogs survival 3-fold compared with the control ones after lazaroids administration IV at 5mg/kg before IR. Elevation of liver enzymes after reperfusion was markedly attenuated in treated groups. Structural abnormalities were markedly ameliorated showing less neutrophil infiltration in post-IR liver. Warm IR liver injury was attenuated with lazaroid compounds. Campo GM et al.[25] reduced the increased plasma LDH levels by 33.03% and 53.29% after 15 and 30 mg/kg U-74389G administration respectively (P< .001) in rats myocardial IR. Finally, U-74389G enhanced the survival rate at the end of the experiment (from 40 to 87%). The drug may have potential cardioprotective use in acute myocardial infarction. Stanimirovic DB et al.[26] retarded LDH release and membrane 'leakiness' from oxidant-treated cells by the steroid antioxidants U-74389G (5-20μM) in rat cerebromicrovascular endothelial cells. Despite the bibliographic efficacies of U-74389G in LDH levels recession, it is noticed that the distance between the LDH levels of animals treated by U-74389G is significant with that of normal levels and restoration of values has not been achieved at the present experiment. The weakness of values restoration may be due to either short U-74389G action study time or low U-74389G dosage.[27]

Table 6 The alteration influence of U-74389G in connection with reperfusion time

p-values Increase	95% c. in.	Reperfusion Time	t-test	glm
+958.4 IU/L	266.9372 IU/L - 1649.863 IU/L	1h	0.0369	0.0093
+797.65 IU/L	352.6114 IU/L - 1242.689 IU/L	1.5h	0.0016	0.0008
+636.9 IU/L	5.134471 IU/L - 1268.666 IU/L	2h	0.0101	0.0484
-148.05 IU/L	-662.1633 IU/L - 366.0633 IU/L	reperfusion time	0.5383	0.5634
+378.9364 IU/L	93.47671 IU/L - 664.396 IU/L	interaction	-	0.0106

Table 7 The predicted restore influence of U-74389G in connection with reperfusion time

p-values Increase	95% c. in	Reperfusion Time	t-test	glm
+278.5414 IU/L	-19.53909IU/L-576.6219 IU/L	1h	0.0674	0.0653
+376.71585 IU/L	198.7321IU/L-554.6996 IU/L	1.5h	0.0002	0.0001
+474.8903 IU/L	256.5027IU/L-693.2779 IU/L	2h	0.0004	0.0002
-75.34327 IU/L	-290.6826IU/L-139.9961 IU/L	Reperfusion Time	0.3375	0.4831
+209.6325 IU/L	98.52261IU/L-320.7424 IU /L	interaction	-	0.0005

Table 8 The (%) predicted restore influence of U-74389G in connection with reperfusion time

Increase	±SD	Reperfusion Time	p-values
13.56%	±7.40%	1h	0.0663
18.78%	±4.52%	1.5h	0.0001
24.01%	±5.63%	2h	0.0003
-3.75%	±5.46%	Reperfusion Time	0.4103
10.43%	±2.82%	interaction	0.0005

Conclusion

U-74389G administration whether it interacted or not with reperfusion time kept significantly increased short – term the LDH levels. Perhaps, a longer study time or a higher drug dose may reveal the citing restore capacity.

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Conflicts of interest

The authors declare there is no conflict of interests.

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