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Original article

The beneficial effect of a new anti-inflammatory compound with antioxidant properties (IA) and of U-74389G (21-Lazaroid) on intestinal recovery after acute mesenteric ischemia and reperfusion in rats

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SUMMARY

We investigated whether the administration of a novel anti-inflammatory compound with antioxidant properties (IA) and of the aminolazaroid U-74389G has a beneficial effect on the repair process of the intestinal mucosa after transient mesenteric ischemia. The administration took place in a clinical setting (i.e. after the onset of ischemia). Randomized, blinded trial in animal laboratory was performed. 54 male Wistar rats were studied in nine intervention groups. The rats weighing 300-350gr, were housed in plastic cages under standardized conditions (23°C, 60% relative humidity, 12hr light and 12hr dark cycles). Groups 1, 4 and 7 were the groups of sham-operated animals. Groups 2, 5 and 8 were the groups of ischemia. Finally, groups 3, 6 and 9 were the groups where the ischemia/reperfusion protocol was applied. Intestinal ischemia was produced in anesthetized rats by occluding the superior mesenteric artery (SMA) for 60 mins with a microvascular clamp. At the end of ischemia, normal saline, IA or U-74389G was administered intravenously and the clamp was removed allowing reperfusion (groups 3, 6 and 9 respectively). At 60 mins after reperfusion animals were sacrificed and a 10 cm section of terminal ileum was resected. Intestinal mucosa morphology and presence of polymorphonuclear leucocytes (PMN's) were determined by two blinded observers. All groups had intestinal mucosal injury following ischemia, but after 1 hour of reperfusion the mucosal damage was less in IA-treated rats compared with the control and U-74389G rats, which was statistically significant. Moreover, the number of PMN's in intestinal mucosa was significantly lower in IA group. IA and U-74389G did not prevent ischemic damage of the mucosa. However, they did accelerate the repair of the mucosa after reperfusion. IA acted at a statistically significant level while U-74389G did not. The mechanism of IA action must be through its potent antioxidant, free radical scavenging activity and PMN infiltration. The increased number of mucosa cells in mitosis and their role in mucosal repair should be studied further.

Key words: Intestine, Ischemia, Oxidative stress, Experimental colitis, Reperfusion, Aminolazaroid, U-74389G, Mucosal damage, Neutrophils, Lipid peroxidation, Anti-inflammatory agents

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INTRODUCTION

Despite effective surgical treatment, the mortality rate for patients with acute occlusive mesenteric ischemia remains high because of delays in diagnosis and definitive treatment.[1,2] In an attempt to improve survival after acute mesenteric ischemia, a number of experimental studies have tested several pharmacological agents and molecules that might attenuate reperfusion injury of the intestinal mucosa, in conjuction with surgical revascularization.[3-10] However, few of these studies administered the agent following ischemia and before reperfusion, simulating the likely use of a protective agent in the clinical setting.

U-74389G is part of Lazaroids family.[11] Lazaroids is a group of synthetic 21 aminosteroids which inhibit iron-dependant lipid peroxidation without glucocorticoid actions. They also suppress cytokine production, adhesion molecule expression and neutrophil activation and infiltration. Their action against ischemia/reperfusion was proved in CNS, heart, lungs, liver and kidney.[12,13] In case of intestine, results have been conflicting. The effect of U-74389G on the intestinal tract following ischemia and repefusion has never been reported.

The compound IA [5-(2-amino-ethylamino)-1-phenyl-2-pentanone] was designed and synthetized as an anti-inflammatory agent with basic character (fewer side effects than commonly used NSAID's). It has been shown to inhibit the non-enzymatic lipid peroxidation almost completely at 25μM (IC 50value 18μM). This compound is also a very potent scavenger of OH radicals since it was found to inhibit very significantly the oxidation of DMSO. It has also been shown that the IA interacts with the stable free radical DPPH, an ability which expresses the reducing activity of the compound and its ability to scavenge free radicals.[14] This compound also possesses significant anti-inflammatory activity by reducing the carraggenean - induced rat paw oedema.[15,16] IA is not commercially available. The aim of this study was to investigate the effect of this new agent as well as of U-74389G on the histological features.

MATERIALS AND METHODS

Animal preparation. Fifty four (54) male Wistar rats weighing 300-350gr, fasted overnight but allowed free access to water, were anesthetized with ketamine hydrochloride (80mg/kg) and xylazine (16mg/kg) administered intramuscularly. A jugular venous cannula was inserted for fluid and drug administration.

Experimental protocol. Animals were divided into 9 experimental groups consisting of 6 rats each. (1) control-sham operated, (2) control-ischemia, (3) control-ischemia/reperfusion, (4) IA-sham operated, (5) IA ischemia, (6) IA ischemia/reperfusion, (7) U-74389G - sham operated, (8) U74389G - ischemia, (9) U74389G - ischemia/reperfusion. The dose of each agent was for IA 0,3 mmol/kg and U74389G 10mg/kg. Control agent was normal saline.

Through a midline abdominal incision using aseptic technique, the superior mesenteric artery was meticulously isolated at its origin, while the accompanying mesenteric vein remained intact. In groups (1), (4) and (7) the pharmacological agent was administered and the animal was sacrificed after a 60min period (5). In groups (2), (5) and (8) there was occlusion of the superior mesenteric artery (SMA) by application of an atraumatic microsurgical clamp for 60 minutes. Just before the end of this period substances were administered in respective groups. In groups (3), (6) and (9) we performed a 60 min period of ischemia and administration of the substances took place before the end of ischemia. Then the clamp was removed and a 60 min period of reperfusion followed. At the end of all instrumentations all animals were exsanguinated, tissue samples were obtained from the small intestine 10cm proximal to the ileoceacal area and sent for histopathological evaluation.

Histopathologic assessment. Small intestinal tissue specimens were rinsed promptly in cold saline solution and immediately fixed in 10% buffer formalin phosphate. The tissue was embedded in paraffin, sectioned transversely and stained with hematoxylin - eosine. A similarly prepared section was stained with Giemsa stain. Each slide was evaluated in blind fashion by two separate investigators.

Mucosal damage in each slide was graded on a six-tiered scale defined by Chiu et al as follows[6]:

Grade 0: Normal mucosa

Grade 1: Development of subepithelial (Gruegenhagen's) spaces near the tip of the villi with capillary congestion.

Grade 2: Extension of subepithelial space with moderate epithelial lifting from the lamina propria.

Grade 3: Significant epithelial lifting along the length of the villi with a few denuded villous tips.

Grade 4: Denuded villi with exposed lamina propria and dilated capillaries.

Grade 5: Disintegration of lamina propria with hemorrage and ulceration.

Polymorphonuclear leucocytes (PMNs) were counted per high power field of Giemsa stained n 20 separate areas of each slide immediately superior to the muscularis mucosae and the mean number of PMN's per high-power field was determined for each animal.

Statistical Analysis. All data are presented as mean±standard error (SE). Data were compared by one way analysis of variation with Bonferroni post hoc correction. Statistical significance was set at a value $p < 0,05$. For the PMN's statistical analysis the student t - test was used.

RESULTS

The histological results are summarized in Figure 1. Sham-operated groups (1, 4 and 7) did not show any mucosal damage, as expected.

The 60 minutes occlusion of SMA in groups (2), (5) and (8) induced moderate tissue damage and resulted in mucosa injury with no statistical difference when IA or U74389G was administered.

In groups (3), (6) and (9), where 60 mins of ischemia were followed by 60 mins of reperfusion, the mucosal damage increased considerably. However, the difference in tissue injury between the IA group and control was extremely statistically significant ($p<0,001$), (Table 1). The difference in tissue injury between the U-74389G and control was marginal and not statistically significant (Table 1). The difference between IA and U74389G is also statistically different in favor of IA (Table 1).

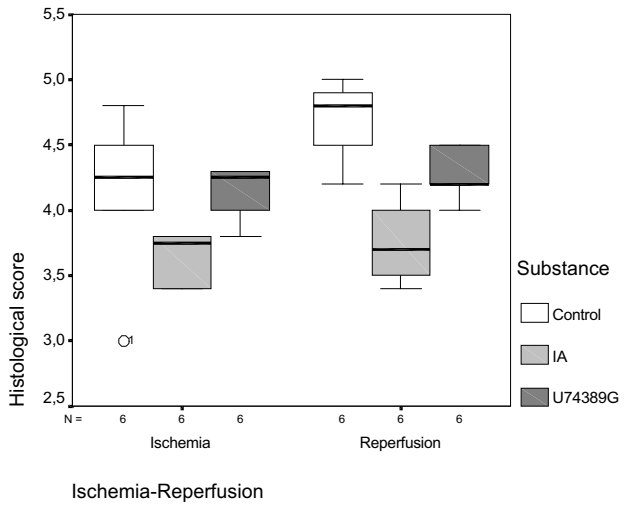

Figure 1. Histological grade per group when ischemia or ischemia/reperfusion was performed.

In histological sections of the groups in the study (ischemia/reperfusion) we observe a complete destruction of mucosa in the control group (Figure 3a). When U-74389 G was used there was a slightly lower grade of damage, with hemorragic infiltration and disruption of the mucosa architecture (Figures 3b,3c). When IA was the substance under study the mucosa remained less deranged with maintenance of the normal architecture to a significant degree (Figures 3d, 3e).

The number of PMNs per high-power field followed the same principals. The mean number of PMNs in the control group of ischemia/reperfusion was 4-5 per high-power field, while in U-74389G group it was 2-3 and in IA group only 1-2 per high-power field (Figure 2).

DISCUSSION

Intestinal ischemia followed by reperfusion causes tissue damage considerably in excess of that induced by ischemia alone (1). It has been shown that partial occlusion of the artery supplying blood to a segment of rat small intestine (hypoxia), followed by reperfusion, causes gross, histologically observable damage to the tissue and increases intestinal vascular permeability. Intravenous administration of superoxide dismutase (SOD), or oral administration of allopurinol (an inhibitor of xanthine oxidase) to animals before removal of the arterial occlusion, decreased damage.[17,18] Thus, regional ischemia in rats results in an accumulation of hypoxanthine in the tissue; the hypoxanthine disappears quickly in reperfusion and both lipid peroxidation and the formation of GSSG can be measured in the reperfused intestine.[19]

The hypoxanthine-xanthine oxidase system is probably not the only source of reactive oxygen species (ROS) to which reoxygenated intestine (and other tissues) are subjected in vivo.[20,21] Mitochondria damaged by ischemia

Table 1. Multiple comparisons between groups when ischemia/reperfusion was performed. Analysis with ANOVA and Bonferroni post hoc correction.

(I) Substance	(J) Substance	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	IA	.9500	.161	.000	.5160	1.3840
	U74389G	.4333	.161	.050	-7,0960E-04	.8674
IA	Control	-.9500	.161	.000	-1.3840	-.5160
	U74389G	-.5167	.161	.018	-.9507	-8,2624E-02
U74389G	Control	-.4333	.161	.050	-.8674	7.096E-04
	IA	.5167	.161	.018	8.262E-02	.9507

* The mean difference is significant at the .05 level

Groups	Number of PMN's Per high power field
3 (control)	4 – 5
6 (IA)	1 – 2
9 (U-74389G)	2 – 3
statistical analysis (t – test):	
- control vs IA	= statistically very significant
- control vs U-74389G	= statistically significant
- IA vs U-74389G	= no difference

Figure 2. Number of PMN's (groups 3, 6 and 9).

may 'leak' more electrons than usual from their electron transport chain, forming more O_2. Generation of ROS by activation of neutrophils entering (or already present within) reoxygenated intestine is another potential source.[19] Neutrophils can also synthesize and release arachidonic acid prodycts, mainly leukotriene (LT) B4 and Thromboxane (Tx) A2 which are potent chemo-attractants and induce adhesion of the neutrophil to the endothelium and activation of neutrophils to produce more oxygen radicals and proteolytic enzymes.[22,23] On the other hand, cytokines, particularly tumor necrosis factor (TNF) have been implicated in the pathogenesis of ischemia reperfusion injury, and activation of the endothelium to become adhesive for neutrophils can be induced by cytokines.[8]

The compound IA {5-(2-amino-ethylamino)-1-phenyl-2-pentanone} combined in one molecule both antioxidant and anti-inflammatory activities. It has been shown that this compound inhibits lipid peroxidation in a concentration less than that of vitamin E. Since lipid peroxidation is more likely to be an impotrant factor in the development of tissue injury during reperfusion than during the ischemic period[5], this very strong inhibition can be one of the ways of action of IA in protection during the acute phase of the mesenteric ischemia reperfusion.

It has been shown that catalase, an enzyme that catalyzes the reduction of hydrogen peroxide to oxygen and water, attenuates reperfusion-induced tissue permeability and mucosal damage. Similar results were obtained with dimethyl sulfoxide (DMSO), a hydroxyl radical scavenger. The protection provided by superoxide dismutase, catalase and DMSO suggests that the oxygen radical inducing tissue damage is the highly cytotoxic hydroxyl radical[1] IA is a very potent hydroxyl radical scavenger. It inhibited the formaldehyde production from 33mM of DMSO by 100% at 25mM, by 55% at 2.5mM, and by about 20% at 1mM, demonstrating a very potent hydroxyl radical scavenging activity under the experimental conditions applied.[14]

It has been shown, as mentioned above, that neutrophils can also synthesize and release inflammatory mediators products. On the other hand, it was shown in vitro that, due to the generation of lipid peroxides, oxygen free radicals indirectly stimulate arachidonic acid metabolism and lead to increased concentrations of prostaglandins, thromboxane and leukotrienes, which further contribute to permeability changes and micro- and macrocirculatory derangements. However, the enhanced prostaglandin metabolism seems to be independent of the generation of lipid peroxides by oxygen radicals. Superoxide dimutase and catalase treatment before reperfusion prevent the increase in conjugated dienes in intestinal tissue, but do not influence production of prostaglandins.[1] IA was proved a potent anti-inflammatory agent, since it reduces the carraggeenan-induced rat paw edema.

Our study examined the effects of a novel molecule (IA) using a procedure that simulates its potential clinical use: the drug is given after ischemic insult and just prior to reperfusion. The majority of papers studying intestinal ischemia in the past presented molecules or substances which were administered to subjects at varying time points before ischemia was performed. In a recent review article it was suggested that the previously used settings were not satisfactorily similar to real clinical conditions. Therefore, there was a place for studies where any substance with possible antioxidant effect be administered after ischemia had been performed, just before reperfusion took place.[24] That is a setting which could be characterized as almost clinical since in every day practice one does not have the opportunity to begin treatment before the onset of intestinal ischemia. Moreover, we used U-74389G a compound -member of the lazaroid family as a positive control. U074389G has been proved to be a potent antioxidant in other organs ischemia, but it had not been tested in intestinal ischemia.[25]

In our study, U-74389G attenuated the intestinal ischemia/reperfusion injury, though not in a statistically significant level. However, IA reduced the intestinal injury to an exceptionally statistically significant level. These results were consolidated by the number of PMNs per high-power field, which were in favour of IA when compared to U-74389G or control.

We do have to mention another finding regarding intestinal mucosa when IA was the drug in focus. There was an enormous augmentation in the number of mitoses in the cells of intestinal mucosa, a possible sign of

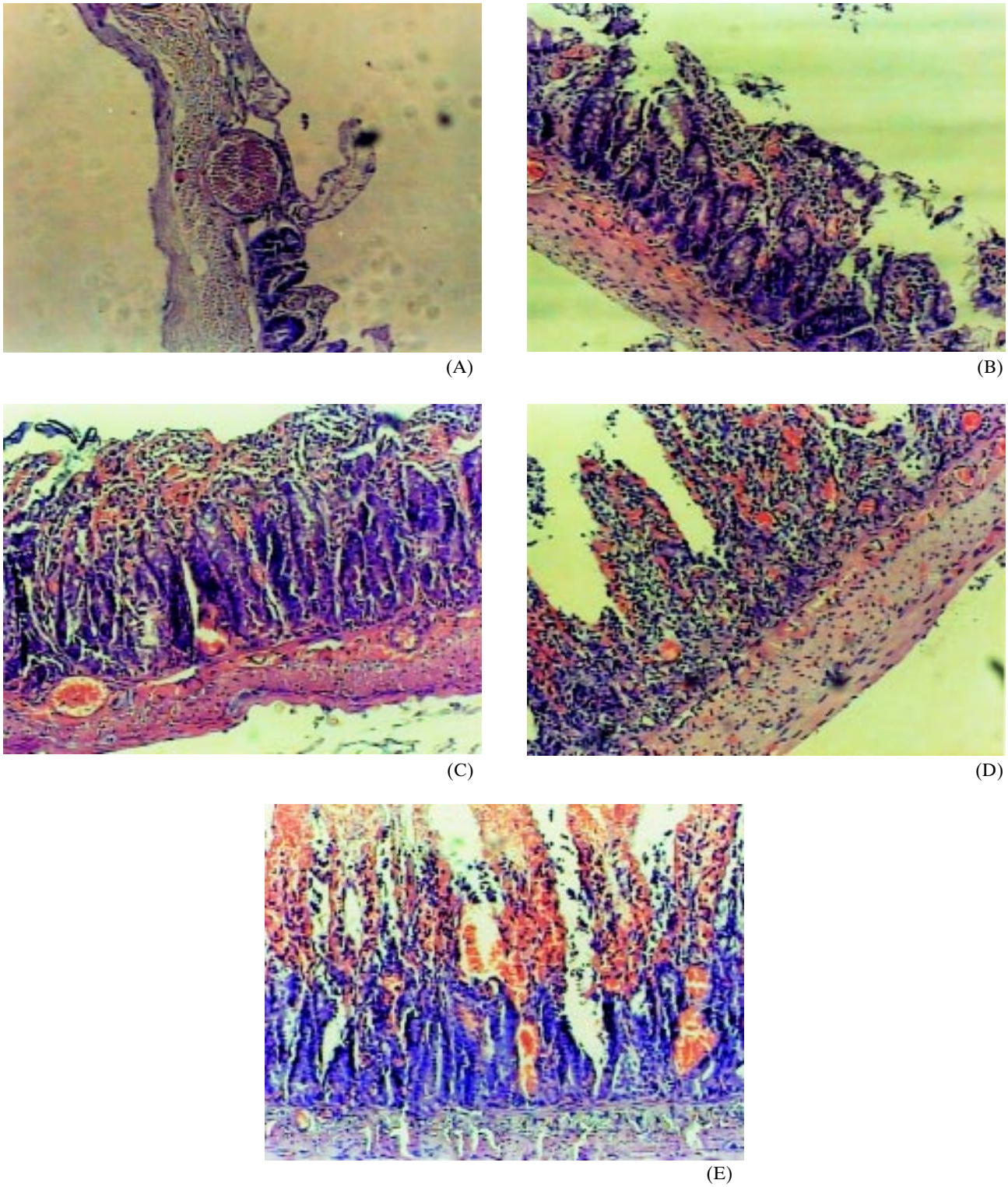

Figure 3. Low power photomicrographs of full thickness sections of small intestine in (a) Control - ischemia/reperfusion, (b, c) IA treated ischemia/ reperfusion and (d, e) U74389G treated ischemia/reperfusion. Note the marked mucosal injury when control only was used. U74389G use was associated with less damage while IA attenuated mucosal damage at most.

cell regeneration and damage control or mucosal restoration, a point which should be more efficiently focused on in a future study.

In conclusion, several mechanisms seem to be instrumental in the development of postischemic lesions of the gut. First oxygen radicals, generated initially by the hypoxanthine-xanthine oxidase system, are the "molecular triggers". Second, inflammatory mediators mostly generated by activation of phospholipase A2 constitute the "enzymatic trigger". Both these pathways lead to the accumulation and activation of neutrophils in intestinal tissue. These cells seem to be largely responsible for the creation of severe mucosal lesions. Compound IA combines in one molecule both antioxidant and anti-inflammatory properties. Thus, it seems that it can 'target' both the "molecular" and "enzymatic" triggers. This beneficial effect of the compound IA on intestinal viability after acute mesenteric ischemia and reperfusion needs further investigation in order to elucidate the exact mechanism of action of this novel anti-inflammatory antioxidant agent with basic character. In addition, we believe that the administration of the antioxidant in research should take place after ischemia is performed and right before reperfusion. It is our belief that this is closer to real clinical conditions.

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