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The Immune System and Oxygen-Derived Free Radicals in Small Bowel Reperfusion Injury in the Rat

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ABSTRACT

Reperfusion syndromes involve many pathogenic pathways. This study tests whether free radicals and immune hypothesis constitute different steps of one same mechanism. Sprague-Dawley rats were subjected to two hours of superior mesenteric artery ischemia and reperfusion. Mortality rate, mean survival time, length of damaged intestine and mucosal damage index were assessed in five experimental groups: control, allopurinol, superoxide dismutase, cyclosporine A and allopurinol plus cyclosporine A. Each of these drugs administered individually improved two or three of the indices. However, the association of allopurinol and cyclosporine A improved all indices. The sequential intervention of lipid peroxidation induced by free radicals and immune cells is postulated.

Key words: Acute intestinal ischemia. Reperfusion. Immune system. Rats. Cyclosporine A. Free radicals. Superoxide dismutase. Allopurinol.

INTRODUCTION

The mechanisms underlying mucosal injury due to intestinal ischemia and reperfusion remain open to controversy. It has been established that significant damage occurs not only during ischemia but also following reoxygenation of the ischemic tissue (1,2), and superoxide radicals generated during reperfusion are thought to play an important role (3,4). Oxygen derived free radicals (ODFR) induce lipid peroxidation that leads to membrane degradation and eventually to cell death. ODFR may also react with nucleic acids and proteins causing genetic, metabolic and functional alterations (5,6).

Treatment of intestinal ischemia with drugs that to an extent minimize the action of toxic free radicals has provided good results. Two of these substances are well known: superoxide dismutase (SOD) - which reduces superoxide ion to hydrogen peroxide - and allopurinol (ALLO), which inhibits hypoxanthine reduction to xanthine (one of the possible origins of free radicals in ischemic tissue) (7). The good results obtained with these and other free radical scavengers have been accepted as indirect evidence for ODFR participation in intestinal reperfusion injury (3-4,6-7).

We have previously reported the excellent results obtained with cyclosporine A (CsA) in reperfusion syndrome following 120 min. of acute occlusion of the superior mesenteric artery; this allowed us to theorize on immune system participation in the reoxygenated intestine (8,9). Assuming that the immune response takes place following free radicals deterioration of cellular membranes, free radical scavengers and CsA should act synergically.

The purpose of this study was to determine whether scavengers and CsA show additive effects in an experimental intestinal ischemic model in the rat.

Groups	Alive	Dead	% mort.	X^2	p
Controls	5	15	75	-	-
ALLO	13	7	35	6.4	0.012
SOD	7	13	65	0.47	NS
CsA	13	7	35	6.4	0.012
ALLO-CsA	14	6	30	8.1	0.005

Table I. Mortality rates (48 h) corresponding to the different experimental groups and controls.

Groups	N	Mean	SD	U	p
Controls	15	11.86	4.70	-	-
ALLO	7	13.71	5.20	48	NS
SOD	13	12.00	4.60	96	NS
CsA	7	17.42	4.27	20	0.021
ALLO-CsA	6	16.83	3.86	19	0.044

Table II. Mean Survival Time corresponding to the different experimental groups and controls.

MATERIAL AND METHODS

Female Sprague-Dawley rats (Stabulary of the University of the Basque Country) weighing 200 g and housed in cages kept at a constant temperature (24-26°C) were used. The animals were acclimated to the conditions of our laboratory for 10 days before starting the experiments. They received an ordinary pellet diet (Panlab S.L. A-04, Spain) and water "ad libitum" prior to the experiments, which began between 9 and 11 a.m.

Surgical procedures

All animals were anesthetized with sodium pentobarbital (30 mg/kg intraperitoneally). Ether was used to supplement anesthesia as needed. The superior mesenteric artery (SMA) was exposed close to its origin from the aorta and occluded for 120 min. with an atraumatic vascular clamp (Yasargil-clip, Aesculap), as described in a previous study (9). The SMA pulse was present in all of the animals following removal of the clamp. All animals were sacrificed eight days after operation. The Draft Convention for the Protection of Animals used for Experimental Purposes was always followed.

Experimental Groups

Five groups of 25 rats were used. In Group I (untreated controls) the SMA was occluded for 120 min. In Group II, two doses of 50 mg/kg allopurinol (zyloric, Galloso-Welcome) were administered orally 48 h prior to the experiment. In Group III, 3.5 mg/kg superoxide dismutase (Peroxinorm, Andrómaco) was given 30 min. before occlusion of the superior mesenteric artery i.p. and the same dose, i.v., just after restoration of blood flow. CsA was given i.p. (20 mg/kg) 24 h and 4 h before initiating mesenteric ischemia in Group IV. The drug was diluted in a 1/10 solution of ethanol 96° and Intralipid 10% (KaviBitrum). The association of ALLO and CsA was the treatment used in Group V.

Parameters evaluated

The mortality rate during the first 48 h was assessed in 20 animals of each group. Nevertheless, the animals were kept alive in our laboratory for a week. Mean survival time (MST) for the deceased animals was also recorded.

Five animals from each group were sacrificed 30 min. after reperfusion, and the gut (from pylorus to ileocecal valve) was rapidly removed and measured. The zones showing macroscopic lesions were then evaluated, and the percentage of intestinal length affected by ischemia was calculated. The mean value corresponding to each series was termed the Longitudinal Damage Index (LDI).

Two 1 cm fragments of distal ileon were excised and embedded in paraffin. Three histological sections taken at 4 mm intervals were cut and mounted on slides, followed by hematoxylin-eosin staining. The Mucosal Damage Index (MDI) for each animal was calculated as shown in Figure 1. The evaluation of MDI was performed consecutively by two independent observers in a blind code fashion. Due to the good correlation observed between both sets of data, their mean was accepted as the definitive value.

Statistical Analysis

Comparison of mean survival rates, LDI and MDI was made using the Kruskal-Wallis and U-Mann Whitney tests, while mortality rates were compared using the Fisher Exact test. Correlation between the measurements made by the two independent observers was performed with Kendall's Concordance test. Values of "p" under 0.05 were regarded as significant.

RESULTS

Mortality Rates

The control animals showed a mortality rate of 75% following 120 min. of intestinal ischemia (Table I). The treatment with ALLO or CsA significantly decreased the mortality rate to 35% (X^2; p=0.012) when compared with the controls. SOD also reduced mortality to 65%, but it was not statistically different from control values. When the asociation of ALLO-CsA was used, a highly significant reduction in mortality rates was observed when compared with the control group (X^2, p= 0.005).

When evaluating the survival period, a similar trend was observed (Table II). In the ALLO and SOD treated animals, MST was similar to that of the controls (13.71±5.2 h and 12.40±4.60 h vs 11.86±4.70 h). The animals treated only with CsA showed the longest MST (17.42±4.27 h). When ALLO was associated to CsA, MST was also significantly increased compared with the controls.

Longitudinal Damage Index

Statistical analyses (Kruskal-Wallis) predicted significant differences between the controls and treated

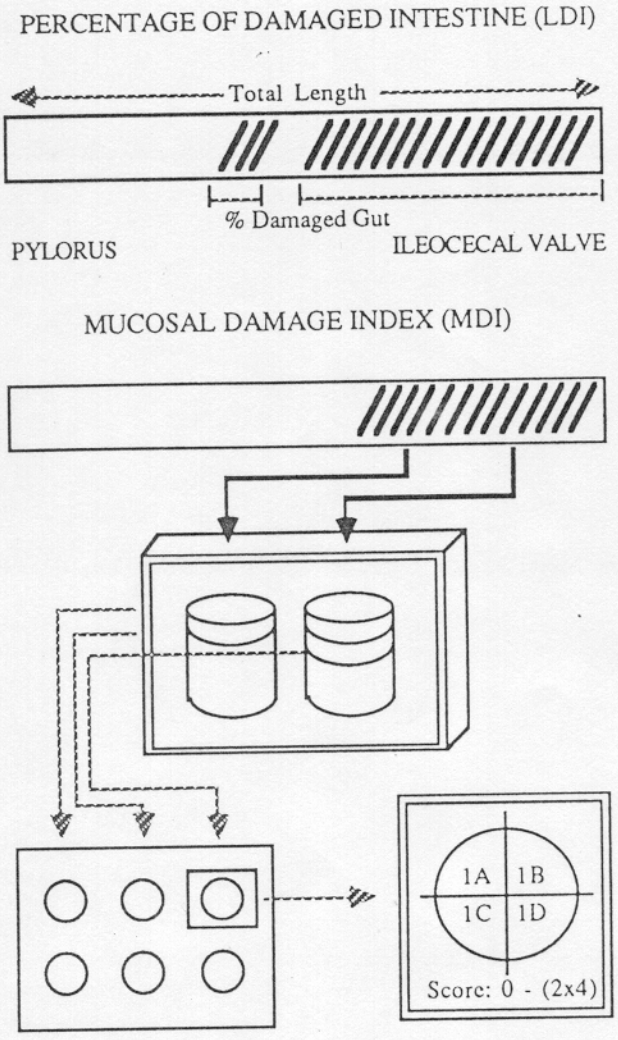

Fig. 1. Histological assessment of intestinal injury was carried out using a 0-2 grading scale for each quarant. Grade 0 represents a lesion with minimal tissue damage. Grade 1 involves moderate damage of the bowel mucosa, whereas Grade 2 indicates severe mucosal damage. The MDI was obtained by adding the grade of mucosal dmage of the 24 quadrants evaluated in each slide and dividing by 6 (total number of sections). Thus, MDI values range from 0 to 8.

animals. The results in Table III clearly demostrate that all treatments diminished the length of damaged intestine. However, CsA alone did not improve this index when compared with the control animals. The results indicate that the LDI of SOD treated animals was greatly reduced (U, p=0.007).

Mucosal Damage Index

As previously described, the MDI was calculated separately by two investigators in a blind manner with no knowledge of the treatment protocol employed. The two sets of values obtained showed a good correlation (r=0.87, W=0.95, p<0.001).

Two hours of intestinal ischemia resulted in an MDI of 4.08 h in the control group. In all the treated groups MDI was significantly reduced, as can be appreciated in Table IV. (Fig. 2)

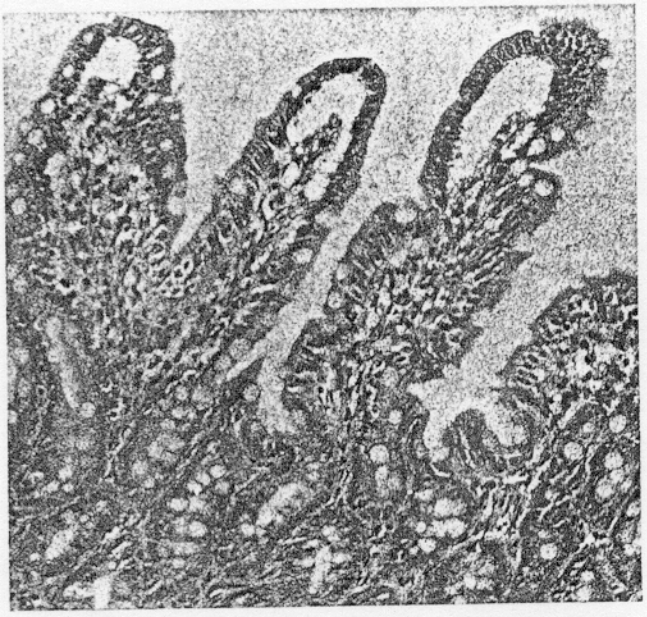

Fig. 2. A good preservation in the animals treated with allopurinol can be seen after 120 min. mesenteric artery ischemia. Grünhagen spaces at the top of the villi are present. HE, x100.

When comparing the four parameters of the treated groups between each other, no statistical differences were found.

DISCUSSION

The harmful effect of complete interruption of the blood flow to an organ has long been a topic of interest. It has been generally accepted that reperfusion of ischemic organs increases the damage previously induced by ischemia (10,11). Many mechanisms have been proposed to explain the origin of the lesions ocurring in intestinal ischemia-reperfusion models.

One of the most important hypothesis is the participation of oxygen-derived free radicals. It has been shown that ischemic injury in the heart (12), kidney (13), liver (14) and intestine (3-4,7), is largely caused by superoxide radicals generated during the reperfusion period.

Hypoxia depresses the energy charge in all tissues. The reduction in ATP is accompanied by a rise in ADP and AMP. This reduction in intracellular energy modifies metabolic functions and affects cell viability. If hypoxia persists, the AMP is metabolized by dephosphorylation to adenosine which rapidly diffuses extracellularly. There, it is further degraded into inosine and hypoxanthine (6). As hypoxanthine needs molecular oxygen to be metabolized into xanthine and uric acid, this reaction is arrested during hypoxia. Consequently, hypoxia leads to an accumulation of hypoxanthine in the tissue (7). Usually, the metabolism of hypoxanthine is catalyzed by the NAD-reducing enzyme XD. However, it has been shown in rats that intestinal XD is rapidly converted into XO during ischemia (15). Conversion of hypoxanthine to

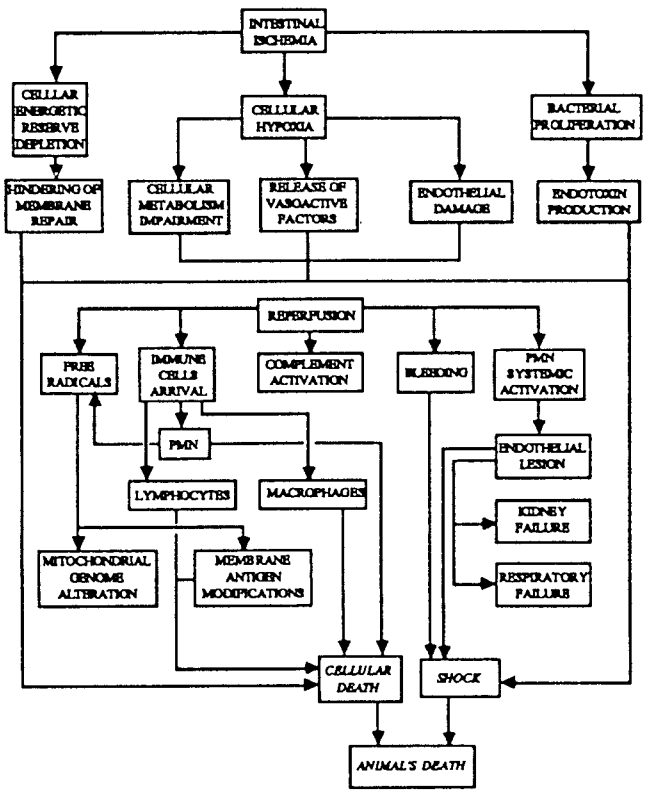

Fig. 3. Hypothetical mechanisms involved in the genesis of mucosal lesions in reperfused bowel.

xanthine catalyzed by XO after reperfusion generates superoxide radicals.

The cytotoxic effects of superoxide radicals presumably results from the peroxidation of lipid components of cellular and mitochondrial membranes (3,5).

An increased neutrophil infiltration of ischemic intestinal mucosa has also been reported. Such an infiltration might be regarded as a further source of free radicals in the ischemic small bowel because the activation of neutrophils results in the production and release of potentially toxic oxygen metabolites. The oxygen radicals generated by this system recruit and activate more PMNs, thus amplifying the postischemic reperfusion injury (16,17).

Neutrophil infiltration not only increases free radicals production in the ischemic mucosa, but also enhances the production of other potent cytotoxic agents such as PAF (Platelet Activating Factor) and TNF (Tumor Necrosis Factor) (18).

Many reports indicate that the activation of neutrophils is not only a local process, but that it also takes place in the whole organism following reperfusion of a single organ (16). These activated neutrophils not only increase their production of hydrogen peroxide but also increase their adhesion to endothelial cells. This fact

Groups	Mean	SD	U	p
Controls	44.4	3.7	-	-
ALLO	35.2	3.7	0.5	0.015
SOD	29.4	2.7	0	0.007
CsA	41.4	5.7	8.5	NS
ALLO-CsA	35.4	2.9	1	1

Table III. Percentage length of damaged intestine corresponding to the different experimental groups and controls.

Groups	Mean	SD	U	p
Controls	4.08	0.50	-	-
ALLO	3.18	0.49	1.5	0.031
SOD	2.74	0.60	0.5	0.015
CsA	2.92	0.60	1.5	0.031
ALLO-CsA	2.5	0.99	1.5	0.031

Table III. Mucosal Damage Index corresponding to the different experimental groups and controls.

is related to the damage that occurs in the lungs and kidneys after reperfusion. When the mortality rate occurring during bowel reperfusion is evaluated, we must also take into account the pulmonary and renal lesions.

Our previous experiments showed certain evidence supporting the involvement of the immune system in the pathogenesis of reperfusion injury. Initially, we thought that NK cells could be the mediators of cytotoxicity. This idea concorded with the precocity of the cellular response. However, the disappointing results obtained with the use of cyclophosphamide (9), led us to explore other possibilities. Assuming the free radicals hypothesis, we suggested that cell membrane injury, mediated by lipid peroxides, could result in a modification of some membrane antigens. Taking into account that the process described parallels ageing phenomena, these "new" antigens might be recognized by memory lymphocytes, and then induce an antigen mediated response. In accordance with the above mentioned hypothesis, we suggested that free radical scavengers and CsA probably act synergically (9).

To prove this theory, we have selected two drugs which act at different stages in the metabolic pathways of free radicals. SOD is an enzyme that catalyzes the conversion of superoxide into hydrogen peroxide and molecular oxygen, which are less toxic. On the other hand, ALLO is a competitive inhibitor of XO, and should therefore decrease the production of free radicals. Thus, it would be expected that both drugs diminish the effects of reperfusion on the small bowel.

In this experiment, SOD did not significantly decrease mortality rate. However, it improved both lesional indices. This means that SOD is beneficial, but with a very short-term effect. In fact, most of the experiments with this enzyme have been performed during short periods of ischemia involving isolated segments of

jejunum, and with ligature of the renal vascular pedicles because of its rapid elimination by urine (19).

Another possible explanation of the lower efficacy of SOD on mortality and MST might reside in its inability to prevent the neutrophil activitation which is supposed to be responsible for the systemic alterations in reperfusion syndromes (16).

Animals treated with ALLO improved three of the registered parameters: Mortality rate, LDI and MDI. Therefore, this drug was chosen instead of SOD as the most effective to hinder free radical production in our experimental model; thus, a series including both treatments, CsA and ALLO, was performed to check our initial hypothesis.

As previously described (8,9), CsA accounts for a reduction of the mortality rate and MDI, while LDI was not decreased. And so, while CsA has failed to decrease the length of damaged intestine, ALLO has improved this particular index. On the other hand, allopurinol failed to increase MST, whereas CsA did.

The protective effects of CsA and ALLO could be attributed to their ability to prevent not only lipid peroxidation, but also neutrophil activation. Further support for involvement of neutrophils in bowel injury is provided by the observation that depletion of circulating neutrophils with antisera or prevention of adherence to endothelium by monoclonal antibodies provides significant protection against reperfusion induced injury (16). These data suggest that XO derived oxidants attract and activate granulocytes or other immune cells which exacerbate ischemic induced tissue injury. Neutrophils are only one of the components of the inflammatory response, and other immune cells are surely involved.

Though it has not been possible to establish statistically significant differences between results achieved with either of the two treatments (ALLO, CsA) when compared with ALLO+CsA, it may be observed that the combination of the two drugs improved all of the parameters analyzed, while each drug alone only improved three of them. Therefore, it can be concluded that the association of ALLO and CsA is more effective than each of them used individually.

This result led us to update our physiopathological hypothesis. Lipidic peroxidation could produce changes in cell membranes causing either a massive flow of Ca^{2+} in the intracellular medium (which produces cell death) or modifications in membrane antigens. Immune cells such as NK could produce nonantigen mediated cell lysis. Other lymphocytes with immunological memory could recognize these antigens, since surface antigen changes resemble those occurring in ageing or other biological ischemic processes, and produce cell death by specific cytotoxic immune reactions. However, all of the proposed mechanisms in ischemia-reperfusion syndrome may be a consequence of these initial stages. Free radicals would contribute to greater cytolysis and attraction of more cytotoxic cells, thereby amplifying the injurious response.

In any case, other mechanisms previously proposed to explain the genesis of mucosal lesions found in reperfused intestines must not be forgotten (20) (Fig. 3). Moreover, it seems very likely that mucosal lesions are not the main cause of animal death, as suggested in Fig 3. Shock induced by multiple mechanisms could play a very significant role. Therefore, clinical management of shock must not be left aside.

Further studies on immune system cells involved in this syndrome would surely help to advance our understanding of the physiopathology of this entity. The clinical applications of these experimental trials has yet to be proven, largely because there is no general agreement on the efficacy of treatments among the different investigators; in spite of this, it should be pointed out that preoperative treatment of patients undergoing intestinal ischemia with ALLO or CsA might reduce or even prevent complications that result from the reperfusion state.

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