

Capillary blood flow as an index of the therapeutic effect of folinic acid in ischemia-reperfusion syndrome

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ABSTRACT

Objective: an intestinal reperfusion study with two aims: a) to assess the usefulness of intestinal capillary blood flow measurement by laser-Doppler for intestinal reperfusion studies; and b) to compare the effects of racemic and levo forms of folinic acid in treating the syndrome.

Experimental design: a murine model of intestinal ischemia by completely clamping the superior mesenteric artery for 90 minutes. A comparison was made of three treatment groups: saline, folinic acid, and levo-folinic acid. The following factors were analyzed: changes in biochemical parameters (levels of creatine kinase, lactic dehydrogenase, and alkaline phosphatase at 60 minutes, and at two and seven days after restoring blood flow), capillary flow in the jejunum and ileum by laser-Doppler (during ischemia and after the first hour of reperfusion), intestinal mucosa injury, and survival curve.

Results: laser-Doppler provided reliable data on how the different treatments affected capillary flow during intestinal reperfusion. Levo-folinic acid improved capillary flow in the ileum after 25 minutes of reperfusion, and also reduced mucosal injury in the same stretch of intestine by the seventh day post-reperfusion ($p < 0.05$). On the other hand, it produced an initial increase in serum enzymes during reperfusion, and did not modify survival.

Conclusions: the changes observed in intestinal capillary blood flow measurement by laser-Doppler have similarities with the effects of drugs on pathological mucosal changes. We could not prove that the levo form of folinic acid has a stronger protective effect versus racemic folinic acid in intestinal ischemia-reperfusion syndrome.

Key words: Ischemia-reperfusion. Intestine. Folinic acid. Laser-Doppler. Capillary blood flow.

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INTRODUCTION

Ischemia-reperfusion syndrome, particularly in the intestine, has been the subject of numerous studies in recent decades. Today, however, much remains to be learned, and treatments being tried require further improvement. In the years since McCord's first report on the involvement of oxygen free radicals in the pathophysiology of this process (1), new mediators of intestinal cell damage have been discovered (2-6). It is now generally accepted that polymorphonuclear leukocytes are the main agents of reperfusion injury, due to the lesions they cause in the vascular endothelium (7-9). This endothelial lesion worsens histological injury in reperused tissue and extends damage to other organs (kidney, liver, lung). For these reasons, the use of laser-Doppler to study the microcirculation during reperfusion of an ischemic organ (10) could prove to be a useful tool for evaluating treatments. The results would have to be compared with other parameters currently utilized: pathoanatomical disorders (11), serum enzyme levels (12), and survival of animals subjected to ischemia-reperfusion processes (13,14).

Since oxygen free radicals appear to be the initiators of this pathologic process, researchers have studied the capacity of different antioxidant drugs to reduce the damage caused by reperfusion. One of the most effective agents tested is folinic acid, the active form of folic acid. In our experiment, the intravenous administration of folinic acid during the minutes prior to the restoration of mesenteric blood flow following complete blockage of the superior mesenteric artery in rats reduced both mucosal injury extent (15) and mortality (16). A drug is now available that contains only the levo enantiomer, or biologically active form, of folinic acid. However, there are as yet no data on whether this new drug is more effective in managing reperfusion injury than the racemic compound tested earlier.

The work reported here was designed to assess the effectiveness of monitoring capillary blood flow by laser-Doppler as a new tool for the study of the reperfusion of

ischemic tissue. The flow meter data obtained are compared to the data provided by pathology, serum biochemistry, and survival studies. At the same time, these four parameters are used to compare the therapeutic usefulness of the levo and racemic forms of folinic acid in a murine model of intestinal ischemia-reperfusion syndrome.

METHODS

Animals and surgical procedures

The study was performed using 12-week-old male WAG/RijCrI rats. The animals were kept in individual cages with free access to water and food (Panlab A-04), with a constant 12-hour light-dark cycle. Spain's "National Guidelines for the care of animals kept for experimentation and other scientific purposes" (Royal Decree Law no. 223/88) were respected at all times.

The animals were anesthetized with Nembutal® (40 mg/kg i.p.), and a median laparotomy was performed, dissecting the superior mesenteric artery at its base where it branches off the aorta. Mesenteric ischemia was induced by clamping the artery with two Yasargil clips for 90 minutes. Ten minutes before restoring mesenteric flow, the drugs (dissolved in 2 ml of saline) were administered through the inferior suprarenal vena cava (27G needle). Delivery speed (0.2 ml/min) was controlled using an infusion pump (Cordis Hyperion®). On completion of ischemia time, the clips were removed and checks were made for restored bpm in the straight mesenteric arteries.

Experimental groups and series

Three experiments were performed (Table I), using in each of them three groups of 10 animals. Group 1, which served as the control group, received only saline; group 2 was treated with folinic acid (Lederfolin® 2.5 mg/kg); and group 3 received levo-folinic acid (Isovorin® 2.5 mg/kg).

In the first experiment (series I) all animals were sub-

jected to the ischemia-reperfusion process, and the various treatments were administered; 48 hours later the animals were sacrificed to study mucosal injury and serum enzyme levels.

In the second experiment (series II) we studied the restoration of capillary blood flow in the intestine using three probes, which lightly rested on the antimesenteric serose layer of the jejunum, the ileum, and a middle stretch of the small intestine, connected to a laser-Doppler flow-meter (Oxford Optronix®). To ascertain capillary flow values in baseline conditions and during ischemia the capillary blood flow was monitored continuously for at least two minutes of stable measurement before placement of the clips (baseline measurement) and following the induction of ischemia. During reperfusion a continuous recording was maintained and average flow values were subsequently calculated during three ten-minute intervals: initial (0' to 10'), intermediate (25' to 35') and final (50' to 60'). These data were then used to calculate the rate of capillary blood flow recovery as compared to baseline values in each animal (Table II). On measurement completion animals were sacrificed.

The purpose of the third experiment (series III) was to study survival. To this end the animals were subjected to the same procedures as in the first series, but this time deaths were monitored: hourly during the first 12 hours, and daily until the seventh day, whereupon all remaining animals were sacrificed.

Table II. Calculation of the recovery rate for intestinal capillary flow

$$\% \text{ recovery} = \frac{\text{MF} - \text{Ischaemia}}{\text{Baseline} - \text{Ischaemia}} \times 100$$

- MF: mean flow at each interval (initial, intermediate and final) of reperfusion
- Ischaemia: flow during ischaemia
- Baseline: flow prior to ischaemia

Table I. Experimental series

	<i>Time of sacrifice</i>	<i>Studies performed</i>
Series I	Day 2 (48 h)	Injury (enzymes and mucosal injury) 48 h
Series II	60' reperfusion	Capillary flow recovery Injury (enzymes and mucosal injury) 60 minutes
Series III	Day 7	Survival Injury (enzymes and mucosal injury) day 7

Parameters studied

Following the sacrifice of animals three consecutive 1-cm long samples of intestine were taken from the terminal ileum and proximal jejunum, embedded in paraffin, and sectioned at 6-mm. Histological sections were stained with hematoxylin-eosin, and examined for degree of injury in each of the four quadrants of each sample, according to a scale based on Park et al. (11) (Table III).

To study serum enzyme levels 2 ml of blood were drawn at the time of sacrifice. In order to provide a control serum blood was also extracted in the second experiment prior to the placement of clips, after which 2 ml of

Table III. Histological damage scale (adapted from Parks et al.) (11)

0. No apparent damage (normal mucosa)
1. Development of a subepithelial space at the tip of the villi
2. Apical denuding of villi (< 20%)
3. Partial loss of villus epithelium (20-50%)
4. Major loss of villus epithelium (50-80%)
5. Total loss of villus epithelium (> 80%)
6. Flattening of villi
7. Destruction of villi

saline were administered. Enzyme levels (LDH, alkaline phosphatase, and creatine kinase) were determined using a spectrophotometer (Schimazu®).

The statistical analysis was performed using the Graph-Pad® software. Results have been noted as mean and standard deviation. For survival analyses the Kaplan-Meier test was used. The results of the three experimental groups were compared using an analysis of variance, and applying the Newman-Keuls test in cases where ANOVA yielded a $p < 0.05$.

RESULTS

Survival studies

Mortality due to ischemia-reperfusion syndrome occurred in all cases during the first 24 hours, with a 70% survival rate in the control group. Neither of the two test treatments induced significant modifications in this regard (Fig. 1). In fact, although the animals treated with racemic folinic acid had a 50% survival rate, this difference was not significant ($p = 0.58$).

Restoration of capillary blood flow

In the ileum of non-treated animals, following the removal of the superior mesenteric artery clamp, capillary blood flow amounted to approximately 20% of the baseline value. During the next sixty minutes capillary flow decreased gradually to about 15% (Fig. 2).

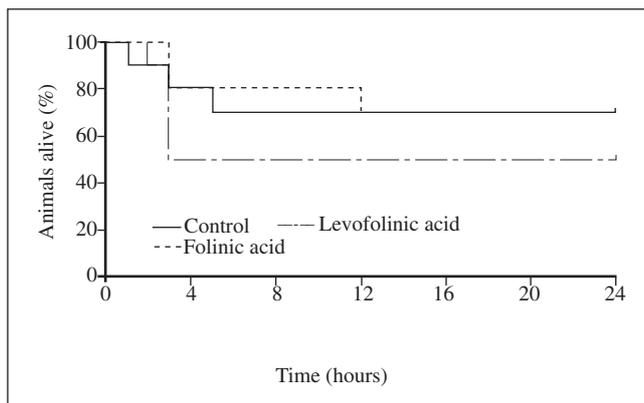


Fig. 1. Survival curve of the three experimental groups. Differences with the levofolinic-treated group were not statistically significant.

In the animals treated with racemic folinic acid blood flow values during the first moments of reperfusion reached 35%, but the difference was not statistically significant. During the first hour of reperfusion capillary blood flow decreased slightly, but always remained above the figures in the control group. Again, the difference was not statistically significant.

On the other hand, the animals treated with levofolinic acid did exhibit a different pattern of capillary blood flow in the ileum. While initial figures were similar to those of the group that received the racemic compound, values increased as reperfusion progressed and finally stood at 50% of baseline figures (intermediate interval, $p = 0.0279$; final, $p = 0.0362$).

Capillary blood flow during the reperfusion of the jejunum was very similar to that of the ileum. However, at this level differences were never statistically significant during the study (Fig. 3).

Assessment of mucosal injury in the intestine

Samples obtained at the end of reperfusion exhibited a serious degree of injury. According to the scale

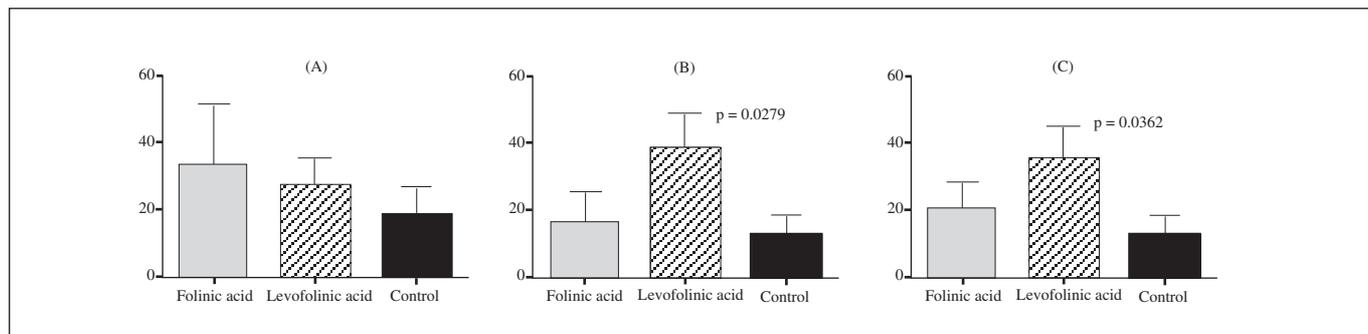


Fig. 2. Capillary blood flow during reperfusion of ileum: A 0'-10', B 25'-35', C 50'-60'. Results are expressed as a percentage of baseline flow. p values refer to comparison against the control group.

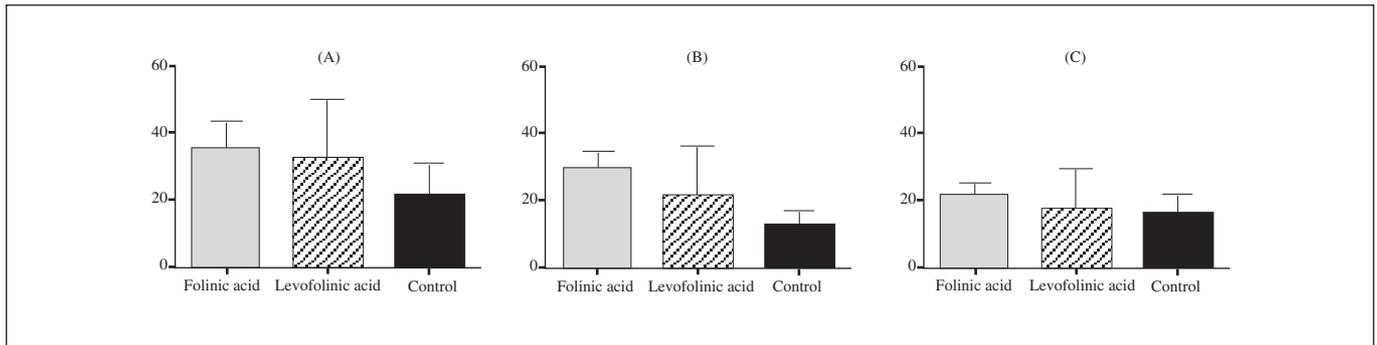


Fig. 3. Capillary blood flow during reperfusion of jejunum: A 0'-10', B 25'-35', C 50'-60'. Results are expressed as a percentage of baseline flow. p values refer to comparison against the control group.

shown in table II samples from the jejunum had a score of 5, and those from the ileum were close to 6. Significant improvement was not attained by either of the two treatments. In samples taken two days after the ischemic aggression injury extent was found to have decreased in all groups ($p < 0.01$) to below 3 (Fig. 4). Again, no differences were attributable to either of the two drugs, nor were there differences between the jejunum and ileum. According to the studies performed seven days after reperfusion, histological damage was similar to that observed at 48 hours post-reperfusion. The only exception was found in the ileum of animals

treated with levofolinic acid, where there was a significant improvement over the findings at 48 hours (2.6 vs. 1.5, $p < 0.05$).

Changes in serum enzymes

Lactate dehydrogenase

In samples taken from our animals prior to inducing ischemia we found serum LDH concentrations of around 300 UI/l. In samples taken after an hour of perfusion, how-

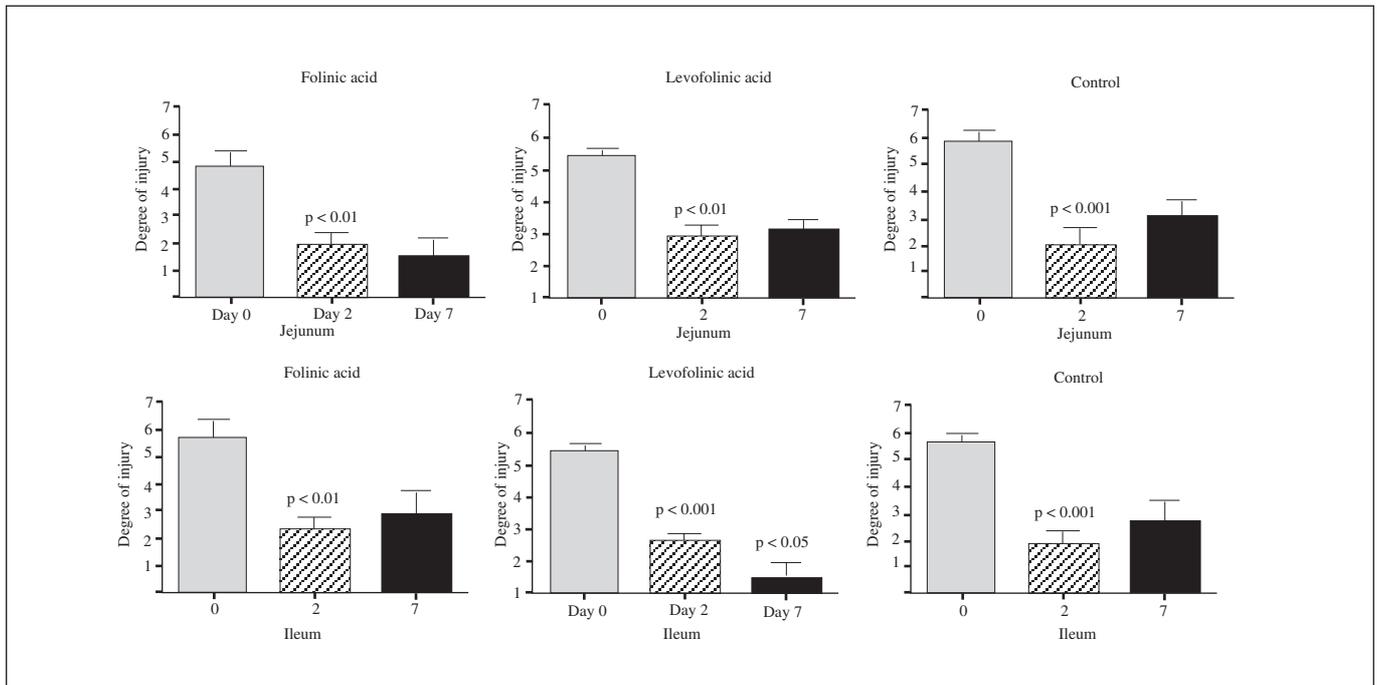


Fig. 4. Mucosal injury in jejunum and ileum assessed at one hour of reperfusion and on days 2 and 7 post-ischemia (p values refer to comparison against the column on the left side).

ever, these values had risen to around 750 UI/l both in treated animals and in the control group ($p < 0.01$). The next examination, performed at 48 hours, found normal values both in control animals and in those treated with the racemic compound. However, in animals treated with levofolonic acid values were still significantly elevated at 48 h (700 UI/l, $p < 0.05$). Finally, the analyses performed on the 7th day yielded normal values in all animals (Fig. 5).

Alkaline phosphatase

Serum alkaline phosphatase values at the end of the first hour of reperfusion were found to be within normal limits in all groups. After 48 hours a drastic change in these values ($p < 0.01$) was found in the control group, with a return to normal by the seventh day. In contrast, animals treated with both forms of folinic acid exhibited no change whatsoever (Fig. 5).

Creatine kinase

At the end of the first hour of reperfusion we found in all three experimental groups an important increase in serum CK values ($p < 0.05$). However, in animals treated with levofolonic acid the increase was much greater (124 ± 68 UI/l; $p < 0.05$) than in those treated with the racemic compound (50 ± 22 UI/l) or those in the control group (59 ± 23 UI/l). The analyses performed at 48 hours and on day 7 showed normal values in all cases.

DISCUSSION

Is microflow measurement by laser-Doppler a reliable aid in assessing the therapeutic effect of treatments applied in a model of intestinal ischemia-reperfusion syndrome? This was the first question to be answered by our experiment. In this regard, our measurements with laser-Doppler detected the positive effect of levofolonic acid limited to the ileum. However, the anatomopathological exploration also found a positive effect on the ileum only in animals treated with levofolonic acid (analyzed on day seven of the experiment). It could be said, therefore, that the sensitivity of laser-Doppler is similar to that of pathology but offering earlier results. Moreover, while our survival study failed to yield positive results, thus depriving this work of one of its projected comparisons, it is also true that laser-Doppler made it possible to detect a pharmacological effect which would otherwise have not been revealed by the survival analysis.

We have found no published reports on the effect of folinic acid on survival following intestinal ischemia. The only data we have are earlier results obtained by our own group using two models very similar to the present one. In both the drug used was Lederfolin (racemic folinic acid). In one experiment (15) where 200 g female Sprague-Dawley rats were used folic acid decreased mortality after 120 minutes of ischemia (56 vs. 25%, $p < 0.05$). In the other (17), despite reducing ischemia time to 60 minutes, the model's mortality rate was higher due to the fact that the hemodynamic monitoring techniques used increased the aggressiveness of the experiment. In this model folic acid

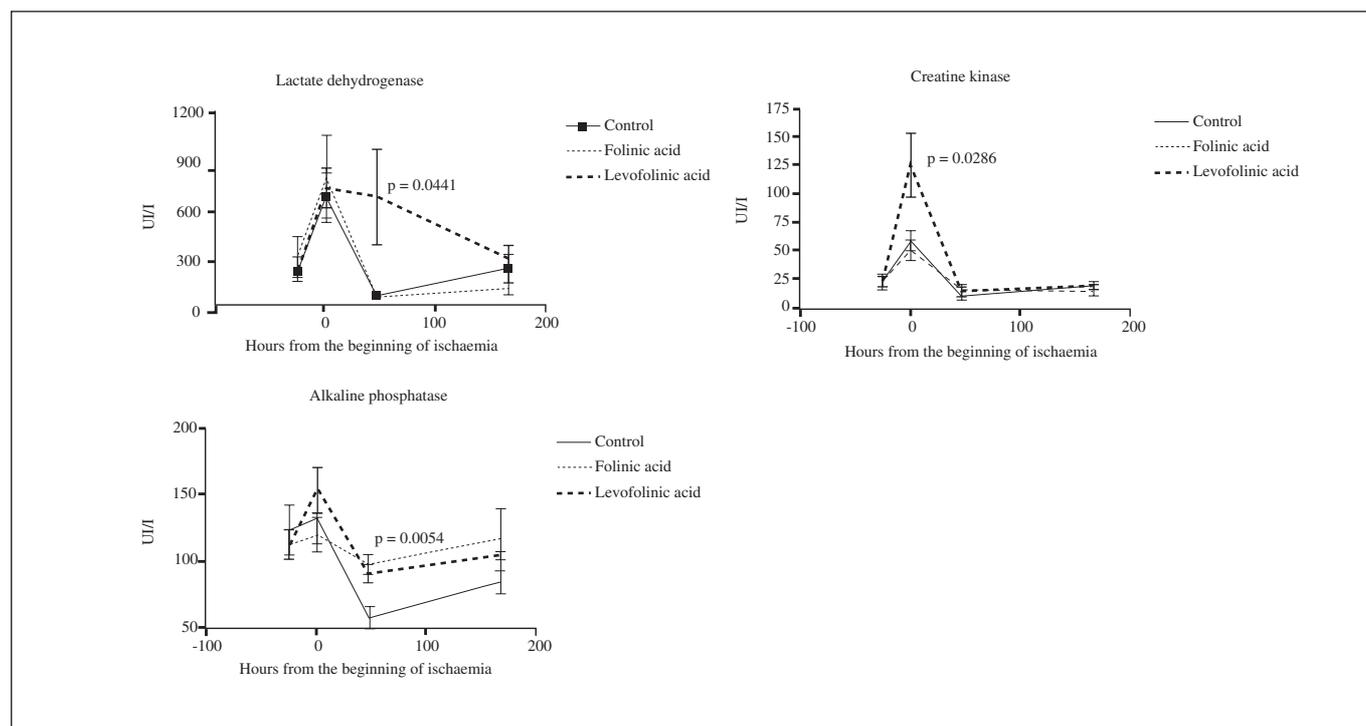


Fig. 5. Evolution of serum levels of enzymes studied. p values refer to comparison against the control group in that moment of the study.

reduced mortality from 83 to 50%, but this reduction was not statistically significant.

In contrast, in the present work with its intermediate ischemia time (90 minutes), mortality was slightly higher than in the 2-hour ischemia model. Again, this could be due to the aggressiveness of surgery, as the laparotomy remained open during the entire experiment to allow a constant monitoring of intestinal blood flow. However, as was the case in the second work cited above (17), the reduction in mortality attributable to Lederfolin was not statistically significant in the present work either (70 vs. 50%).

In view of these data it is even more surprising that the animals treated with levofolinic acid exhibited the same mortality rate as the control group. As we have found no reports on this subject in the literature, we are unable to provide an explanation to account for this finding. One possibility could be that the observed effect on mortality was not due to the folinic acid but to some property present in the excipient of the racemic compound (Lederfolin®), but new studies would be necessary in order to prove or disprove this hypothesis.

There are no studies analyzing the effect of folic acid on intestinal microcirculation, although there is a work published by Ulibarrena et al. (18) that analyzed the effect of folic acid on different hemodynamic parameters (macrocirculation). Specifically, it reports that animals treated with folinic acid exhibited a slight improvement in mean arterial blood pressure during the first moments of reperfusion, but that the effect did not last beyond fifteen minutes.

In our study, however, folinic acid exerted no detectable positive effect on the microcirculation of the intestinal areas studied at any time during reperfusion. Of course we did not expect that hemodynamic changes in the macrocirculation would be paralleled by intestinal microcirculation, as each circulation is regulated by different mechanisms and criteria.

However, treatment with levofolinic acid did result in a significant improvement in capillary perfusion in the terminal ileum during reperfusion of the ischemic intestine. We do not know whether this local improvement was accompanied by an improvement in macrocirculation hemodynamics.

Regarding mucosal injury the experiment conducted by Bilbao et al. (15) using lighter female rats subjected to 120 minutes of ischemia showed that folinic acid had a positive effect, since the Mucosal Injury Index dropped from 9.76 to 8.54 ($p < 0.05$). In our model, however, this drug failed to demonstrate any capacity to diminish damage to the intestinal mucosa following reperfusion. This may be due to the facts that, with a less aggressive model from the local point of view (rats with greater weight and shorter ischemia time) (13), the experiment fell outside the "therapeutic window" of folinic acid.

On the other hand, treatment with levofolinic acid had a positive effect at the mucosal level, as seen in the values obtained on the seventh day of follow-up. This result

is consistent with earlier work done by our group, which has always obtained good results following the administration of folic acid in intestinal ischemia models.

Our analysis of serum enzymes in the control group yielded results that are consistent with reports published by other authors. For example, in a rabbit model of 60 minutes ischemia, Caglayan et al. (12) observed after one hour of reperfusion that LDH and CK values had doubled, while PA remained normal. The fact that in our experiment enzyme concentrations –following a similar pattern– turned out to be somewhat higher might be due to differences in the animals used and/or mesenteric ischemia time.

In their turn Thompson et al. (19) found, in a different experimental model, an elevation of CK levels at 4 hours of reperfusion, and of LDH and PA at 24 hours. Our measurements at 48 hours show normal values for all three enzymes.

The analysis of how drugs had affected plasma levels for these enzymes revealed a lack of correspondence between these values and the other parameters measured in the experiment. According to our initial hypothesis, levofolinic acid should provide a more effective protection *versus* folinic acid, and therefore be associated with lower serum enzyme levels. We found, however, that it took at least 24 hours for LDH values to normalize, and that there was a significant increase in the initial elevation of CK.

With the information currently available it is not possible to provide a reliable explanation for these facts. Levofolinic acid did produce a situation that was somewhat different from that of the control and folinic acid treatment groups, but it is difficult to find a consistent explanation for the effects observed. A higher or longer enzyme elevation would seem to indicate a worsening of injury, but both our mucosal injury and capillary microcirculation studies demonstrate the positive effect of levofolinic acid regarding injury prevention. To reconcile such contradictory results we could perhaps surmise that, thanks to levofolinic acid treatment, a highly acute situation was transformed into a subacute condition with a better prognosis.

In any event there are many authors who feel that increased enzyme levels do not provide relevant information on the extent or reversibility of intestinal injury (15,19,20). If this were the case, the therapeutic effectiveness of these antioxidants should not be called into question by a lack of correspondence in enzyme levels.

In conclusion, the only parameter in which levofolinic acid has shown a stronger protective effect than the racemic compound was mucosal injury –and only when considering the terminal ileum, and values from the seventh day. Differences found in the other parameters are not statistically significant. On the other hand, in this experiment the highest values of capillary blood flow during the reperfusion of the ischemic intestine were found in those animals with the lowest mucosal injury on the

seventh day. New studies with different levels of mortality and anatomopathological injury are needed in order to establish clearly the "lesion window" where microflow measurement will prove most useful for studying treatments of intestinal reperfusion syndrome. However, the above results do show that microflow measurement is a promising tool and that its use in these studies is justified.

REFERENCES

1. McCord JM., Roy RS. Pathophysiology of superoxide: Roles in inflammation and ischemia. *Canad. J. Physiol Pharmacol* 1982; 60: 1346-51.
2. Kubes P, Ibbotson G, Russell J, Wallace JL, Granger DN. Role of platelet-activating factor in ischemia/reperfusion-induced leukocyte adherence. *Am J Physiol*. 1990; 259: 300-5.
3. Carey C, Siegfried MR, Ma XL, Weyrich AS, Lefer AM. Antishock and endothelial protective actions of a NO donor in mesenteric ischemia and reperfusion. *Circ Shock*. 1992; 38: 209-16.
4. Williams JP, Pechet TT, Weiser MR, Reid R, Kobzik L, Moore FD Jr, Carroll MC, Hechtman HB. Intestinal reperfusion injury is mediated by IgM and complement. *J Appl Physiol*. 1999; 86: 938-42.
5. Farber A, Connors JP, Friedlander RM, Wagner RJ, Powell RJ, Cronenwett JL. A specific inhibitor of apoptosis decreases tissue injury after intestinal ischemia-reperfusion in mice. *J Vasc Surg*. 1999; 30: 752-60.
6. Rosario HS, Waldo SW, Becker SA, Schmid-Schonbein GW. Pancreatic trypsin increases matrix metalloproteinase-9 accumulation and activation during acute intestinal ischemia-reperfusion in the rat. *Am J Pathol*. 2004; 164: 1707-16.
7. Granger DN, Benoit JN, Suzuki M, Grisham MB. Leukocyte adherence to venular endothelium during ischemia-reperfusion. *Am J Physiol* 1989; 257: 683-8.
8. Granger DN. Ischemia-reperfusion: mechanisms of microvascular dysfunction and the influence of risk factors for cardiovascular disease. *Microcirculation* 1999; 6: 167-78.
9. Goicoechea I, Fernández R, Otero B, Caramés J, Bilbao JE, García-Alonso I. Pretreatment with antioxidants decreases damage due to reperfusion of isolated intestines in the absence of leukocytes. *Eur Surg Res* 2003; 35: 293.
10. García-Alonso I, Ruiz de Gordejuela AG, Pérez-Agote J, J.J. Méndez JJ. Effects of antioxidant drugs on intestinal wall microflow in ischaemia-reperfusion syndrome. *Eur Surg Res* 2000; 32(S1): 96.
11. Park PO, Wallander J, Tufeson G, Haglund U. Cold ischemic and reperfusion injury in a model of small bowel transplantation in the rat. *Eur Surg Res* 1991; 23: 1-8.
12. Caglayan F, Caglayan O, Gunel E, Elcuman Y, Cakmak M. Intestinal ischemia-reperfusion and plasma enzyme levels. *Pediatr Surg Int* 2002; 18: 255-7.
13. Ortiz J, García-Alonso I, Portugal V, Méndez JJ. Influencia del peso corporal y del período de isquemia mesentérica aguda en la mortalidad de ratas sometidas a revascularización intestinal. *Rev Esp Enf Ap Digest* 1990; 77: 263-7.
14. Ulibarrena MA, García-Alonso I, Portugal V, García Redondo B, Méndez JJ. Monitorización hemodinámica en un modelo de shock por reperusión intestinal en rata. *Rev Esp Enf Digest* 1998; 90: 94-9.
15. Bilbao J, García-Alonso I, Portugal V, Barceló P, Apecechea A, Méndez JJ. Eficacia de tratamientos antioxidantes (ácido fólico y alfa-tocoferol) en las lesiones intestinales inducidas por reperusión. *Cir Esp* 1992; 51: 262-5.
16. Bilbao J, García-Alonso I, Portugal V, Barceló P, Ortiz J, Méndez JJ. Utilidad terapéutica de fármacos antioxidantes en el síndrome de reperusión intestinal experimental. *Rev Esp Enf Digest* 1991; 80: 237-41.
17. Ulibarrena MA, Conty JL, Emparan C, Portugal V, Bilbao J, García-Alonso I. Acción de los inhibidores de los radicales libres del oxígeno sobre la mortalidad causada por el síndrome de isquemia-reperusión intestinal. *Rev Port Med Intensiva* 1993; 2: 127.
18. Ulibarrena MA, Conty JL, Emparan C, Portugal V, Bilbao J, García-Alonso I. Acción de los inhibidores de los radicales libres del oxígeno sobre la tensión arterial de la rata en el síndrome de isquemia-reperusión intestinal. *Rev Port Med Intensiva* 1993; 2: 127.
19. Thompson JB, Bragg LE, West WW. Serum enzyme levels during intestinal ischemia. *Ann Surg* 1990; 211: 369-73.
20. Tanaka J, Malchesky PS, Omokawa S. Effects of prostaglandin I₂, superoxide dismutase, and catalase on ischemia-reperfusion injury in liver transplantation. *ASAIO Trans* 1990; 36: 600-3.