Original Paper

Eur Surg Res 1995;27:69–76

Effect of Allopurinol, Folinic Acid, SOD and Cyclosporine A on Ischemic Liver Regeneration

V. Portugal
I. García-Alonso
P. Barceló
J. Méndez

Laboratory of Experimental Surgery, Department of Surgery, University of The Basque Country, Leioa, Spain

Key Words
Liver ischemia
Liver regeneration
Cyclosporine
Superoxide dismutase
Allopurinol
Folinic acid
Antioxidants

Abstract
Liver regeneration plays a key role in restoring the liver/body ratio after partial liver transplantation. However, hepatic ischemia hinders the proliferative response of the hepatocytes. In this study, different ways of improving the regenerating capacity of ischemic hepatocytes are tested. Following 70% hepatectomy and 15 min of normothermic liver ischemia, the percentage of regenerating hepatocytes and the regenerative gradient are assessed. Cyclosporine A (hepatotrophic agent), superoxide dismutase and folinic acid (antioxidants), administered during the ischemic period, have significantly increased these indices. The later drug has restored the regenerative response to the levels of normoperfused livers.

Introduction

Since Pringle described his maneuver in 1908 [1], liver ischemia has been commonly used during those surgical procedures which benefit from a bloodless surgical field. Nowadays, the main indications are hepatic injury, partial hepatectomies, metastasectomies, nontumoral hepatic resections, and liver transplantation [2]. In all these situations, liver regeneration is necessary to restore the liver/body ratio. In addition, the shortage of pediatric donors has impelled partial liver transplantation [3, 4]; and so, it happens to be a yet more important aim to control liver regeneration, as it is the only way to adapt body and liver sizes after transplantation [5]. On the other hand, normothermic liver ischemia impairs the normal liver function [6]. The widespread practice of liver surgery has increased the interest on the ischemia-reperfusion syndrome. The correct knowledge of the lesions it induces and of its pathogenic mechanisms (e.g. oxygen free radicals (OFR)

Received:
December 28, 1993
Accepted:
October 17, 1994

Dr. V. Portugal
Laboratory of Experimental Surgery
Faculty of Medicine
E-48940 Leioa (Spain)

0014–312X/95/
0272–0069$8.00/0

[7], no-reflow phenomenon [8]) will allow designing specific treatments, which in turn will improve postsurgical liver function.

Previously, we have probed that hepatic ischemia decreases the hepatocytes' DNA synthesis following partial hepatectomy [9]. In order to ameliorate this deleterious effect we have tried two different approaches. The first consists of increasing the intensity of the regenerative stimulus, by adding a hepatotrophic substance (cyclosporine A) [10], hoping that the hepatocytes will respond better to a greater stimulus. The other focuses on reducing the ischemic-reperfusion damage by administering antioxidant drugs: superoxide dismutase (a scavenger of OFR), allopurinol and folinic acid (two inhibitors of xanthine oxidase).

In the present work, we have studied the regenerative response of the liver following 15 min of normothermic ischemia plus 70% hepatectomy in the rat, associated or not to antioxidant or hepatotrophic therapies.

Methods

Male Sprague-Dawley rats weighing 250 g were used. The animals received an ordinary pellet diet (A04-Panlab) and water ad libitum prior and after the experiments. Surgery was performed between 9 and 11 a.m. to standardize for natural diurnal rhythms.

Under ether anesthesia, liver ischemia was induced by occluding the hepatic hilum with a small vascular clamp for 15 min. Moreover, the mesenteric artery and the celiac trunk were also clamped in order to avoid splanchnic congestion [11]. Once the ischemic period was finished, the abdomen was reopened and the vascular clamps removed. Restoration of blood flow was appreciated by the coloration of the liver and gut. When performed, 70% hepatectomy was carried out just prior to reperfusion, following the Higgins' method.

Liver regeneration was studied in 11 groups of 10 randomly assigned animals by quantifying the nuclear DNA content of hepatocytes (table 1). In three of the groups the response of normoperfused livers to different hepatotrophic stimuli (CsA administration, 70% hepatectomy and both) was assessed. In the other three groups, the effect of ischemia on the previous groups was evaluated. Finally, the effect of CsA and antioxidants on the regenerative response of the liver following hepatectomy plus ischemia was analyzed.

Table 1. Experimental series

Control
CsA treated
70% hepatectomy
CsA + 70% hepatectomy
Hepatic ischemia
Hepatic ischemia + CsA
Hepatic ischemia + 70% hepatectomy
Hepatic ischemia + 70% hepatectomy + CsA
Hepatic ischemia + 70% hepatectomy + SOD
Hepatic ischemia + 70% hepatectomy + allopurinol
Hepatic ischemia + 70% hepatectomy folinic acid

Drugs Administration

Cyclosporine A (CsA; Sandimun®, Sandoz) has been administered intraperitoneally at doses of 20 mg/kg, the day before and 2 h prior to surgery. At these doses and via this route, CsA has previously been shown to be hepatotrophic [12]. The different antioxidant therapies have been administered through the left femoral vein by means of a continuous perfusion pump, 10 min prior to reperfusion. All the drugs were administered as a bolus, diluted into 2 cm^3 of saline (control: saline alone), at doses having a probed antioxidant effect: Superoxidodismutase (SOD) 6 mg/kg [13] (Ontosein-Orgoteina, Zambeletti), folinic acid 2.5 mg/kg [14] (Lederfolin, Lederle) and allopurinol 50 mg/kg [15] (dehydrated sodium salt, Sigma A-8003).

DNA Quantification

Rats were sacrificed 24 h after surgery and a fragment of liver was quickly removed and embedded in paraffin. On 5-μm histological sections stained in Schiff reagent, DNA was quantified in 100 hepatocytic nuclei by means of a microspectrocytophotometer (VMS-0.5, Carl Zeiss, Oberkochen, Germany) (λ = 560 nm). Then, frequency histograms were calculated for each animal, where DNA values were expressed in arbitrary units. Using a modification of Bartel's method, histograms were resolved into Gaussian curves, the left one corresponding to static cells and the other to regenerative hepatocytes. This method allowed us to assess the percentage of regenerating hepatocytes (%RH) of each animal, which fairly represents the intensity of the regenerative response in a certain cellu-

Table 2. Percentage of regenerating hepatocytes

Experimental series	Mean	SD	Median	Minimum	Maximum
Control	1.21	1.16	0.76	00.00	3.88
CsA treated	4.01	3.24	3.91	00.00	11.69
70% hepatectomy	22.29	9.69	20.82	10.66	35.51
CsA + 70% hepatectomy	44.46	12.56	42.09	26.72	71.00
Hepatic ischemia	0.75	1.48	00.00	00.00	4.77
Hepatic ischemia + CsA	3.43	2.37	2.88	00.00	7.59
Hepatic ischemia + 70% hepatectomy	8.85	9.38	7.56	00.00	33.29
Hepatic ischemia + 70% hepatectomy + CsA	13.16	5.54	11.68	7.89	20.94
Hepatic ischemia + 70% hepatectomy + SOD	15.66	14.26	11.40	0.72	34.04
Hepatic ischemia + 70% hepatectomy + allopurinol	6.41	4.99	5.01	0.67	15.61
Hepatic ischemia + 70% hepatectomy + folinic acid	20.10	11.28	27.89	13.84	47.91

Table 3. Regenerative gradient

Experimental series	Mean	SD	Median	Minimum	Maximum
Control	2.161	0.241	2.097	1.892	2.660
CsA treated	2.386	0.211	2.326	2.064	2.666
70% hepatectomy	1.607	0.125	1.624	1.389	1.773
CsA + 70% hepatectomy	1.891	0.149	1.857	1.675	2.193
Hepatic ischemia	2.166	0.382	2.188	1.720	2.568
Hepatic ischemia + CsA	2.040	0.259	2.054	1.621	2.321
Hepatic ischemia + 70% hepatectomy	1.739	0.332	1.761	1.302	2.244
Hepatic ischemia + 70% hepatectomy + CsA	2.287	0.368	2.260	1.845	2.893
Hepatic ischemia + 70% hepatectomy + SOD	2.166	0.407	2.099	1.799	3.131
Hepatic ischemia + 70% hepatectomy + allopurinol	2.096	0.227	2.026	1.853	2.459
Hepatic ischemia + 70% hepatectomy + folinic acid	1.955	0.234	1.890	1.602	2.378

lar population. In the same way the regenerative gradient (RG) of each animal was obtained from the ratio DNA content of regenerating hepatocytes to DNA content of static ones. This new parameter reflects the quickness of the regenerative process (DNA synthesis) and it tries to express the differences observed between curves with the same %RH [9].

Statistical Analysis

As the distribution of the results was not normal, comparison between the different experimental series was carried out using a nonparametric test: rank-sum test; differences with $p < 0.05$ were considered significant.

Results

Normoperfused Livers

A 'resting liver' does not imply null regeneration and a certain degree of hepatocytic proliferation may be found (%RH = 1.21, SD = 1.16; table 2) (RG = 2.15, SD = 0.24; table 3). This is the regenerative basal index. On the other hand, partial hepatectomy has induced an important regenerative response in the liver (%RH = 22.29%, SD = 9.69; RG = 1.61, SD = 0.12).

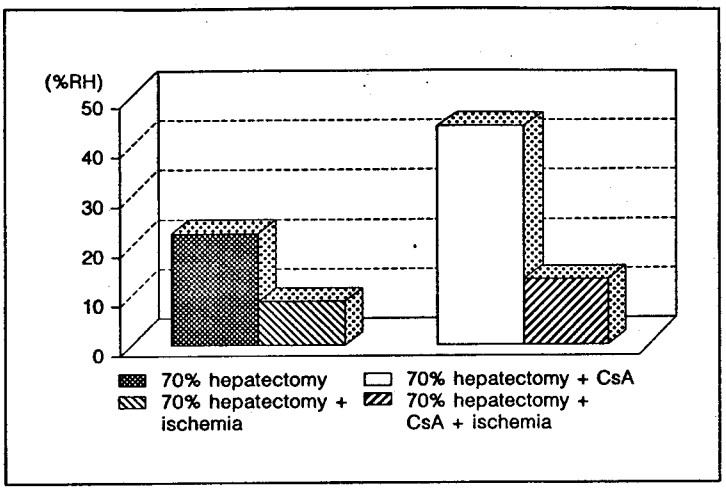

Fig. 1. Effect of normothermic hepatic ischemia (striped bars) upon liver regeneration. It significantly reduces the percentage of regenerating hepatocytes (%RH) following the regenerative stimuli (70% hepatectomy with or without CsA).

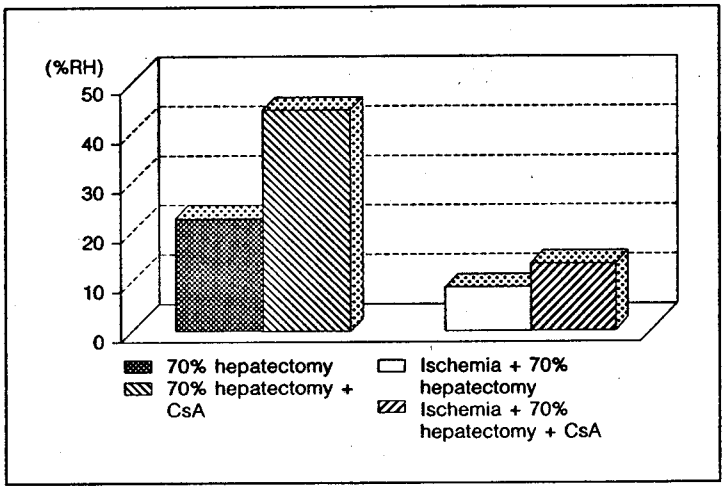

Fig. 2. Effect of CsA (striped bars) on liver regeneration. It significantly increases the percentage of regenerating hepatocytes (%RH) following 70% hepatectomy in both normoperfused and ischemic livers.

Ischemic Livers

Normothermic ischemia has decreased the regenerative basal index, though this diminution does not reach statistical relevance (%RH = 0.76, SD = 0.48, p = 0.082). However, in those animals subjected to 70% hepatectomy, 15 min of warm liver ischemia has significantly decreased the %RH (fig. 1) (%RH = 8.85%, SD = 4.38, $p < 0.005$), but without modifying the rhythm of DNA synthesis (1.74 vs. 1.61, p = 0.21).

CsA-Treated Animals

CsA has induced the proliferation of hepatocytes in both normoperfused (4.01 vs. 1.21%, p 0.001) and ischemic livers (3.43 vs. 0.76%, $p < 0.005$), and it has also increased the number of hepatocytes responding to partial hepatectomy in both situations (fig. 2): normoperfusion (44.46 vs. 22.29%, $p < 0.001$) and warm liver ischemia (13.16 vs. 8.85%, $p < 0.05$). In all of the series, CsA administration has also improved the RG.

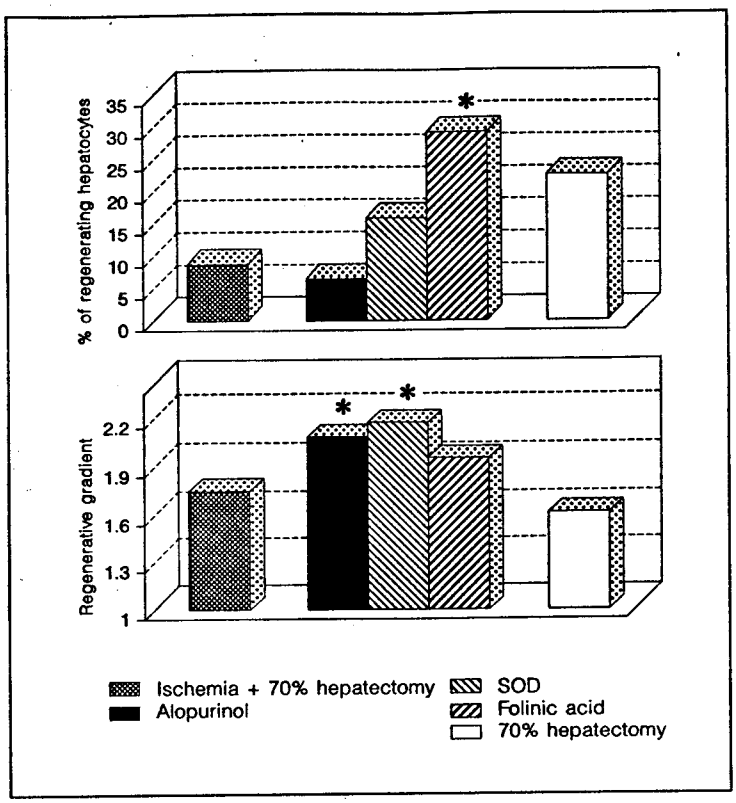

Fig. 3. Effect of antioxidant treatments (central bars) on the percentage of regenerating hepatocytes (top) and on the regenerative gradient (below). Folinic acid and SOD improve both the regenerative gradient and the percentage of regenerating hepatocytes. Allopurinol improves only the regenerative gradient. Statistically significant differences with nontreated animals are marked (*). The response to hepatectomy of normoperfused livers is represented on the right side of each graphic (white bar).

Antioxidant-Treated Animals

We have analyzed the effect of the different antioxidant treatments considering now the nontreated group (hepatic ischemia + 70% hepatectomy; fig. 3) as their 'control group'. Allopurinol has not increased the %RH (6.42 vs. 8.85, $p = 0.74$), but it has improved the RG (2.10 vs. 1.74, $p < 0.01$). On the other hand, SOD has improved both the RG (2.17 vs. 1.74, $p < 0.05$) and the %RH (15.66 vs. 8.85), although the effect on this latter parameter was not statistically significant ($p = 0.51$).

Finally, the folinic acid has highly increased the %RH (29.10 vs. 8.85, $p = 0.0005$), reaching a level of regeneration similar to that of normoperfused livers (29.10 vs. 22.29, $p = 0.15$).

Discussion

We have previously seen that hepatic regeneration decreases after liver ischemia. However, it is not clear which mechanisms are implicated. It is accepted that during the ischemia-reperfusion syndrome the storage of ATP, a molecule necessary for DNA synthesis, is depleted [16]. Moreover, oxygen free radicals originating after reperfusion directly induce DNA damage and cellular death [17], and through lipid peroxidation inhibit cellular division [18].

Now, the present work makes it clear that 15 min of normothermic liver ischemia hinder liver regeneration: the number of regenerating hepatocytes is reduced though their

rhythm of DNA synthesis (RG) is not affected. In short, a smaller number of hepatocytes regenerate, but they do it properly.

In order to improve hepatic regeneration after ischemic injury we have tried two different mechanisms: the administration of an hepatotrophic substance (CsA) and different antioxidant drugs.

As we had also previously observed, CsA induces hepatic regeneration in resting liver (both in normoperfused and ischemic ones [19]) and it increases the number of hepatocytes responding to the regenerative stimulus of partial hepatectomy. Different hypotheses try to clarify the pathway of this hepatotrophic effect. Francavilla et al. [20] suggest that some circulating substances are induced by CsA which may augment hepatic regenerative response. Our group hypothesized that the modifications induced on the immune response implies a disregulation of this regenerative process [19].

In this work, aimed to study whether such an hepatotrophic influence was to be found following ischemic liver injury, the beneficial effect of CsA on liver ischemia has been established. This result accords with the findings of Hayashi et al. [21] who showed the beneficial effect of CsA pretreatment in preventing ischemic damage to the liver in dogs. They postulate that CsA protects lysosomal membranes [22].

Our group has reported that CsA decreases the mucosal damage in a model of intestinal ischemia-reperfusion injury [13], postulating that it could hinder the activation of lymphocytes and macrophages that infiltrates the mucosa. In the same way, after liver ischemia-reperfusion, OFR derived from activated macrophages may cause damage to the hepatocytes, and Goldin and Keisari [23] have demonstrated that CsA could ameliorate the production of macrophage-derived OFR. And so the beneficial effect of CsA could be due to inhibition of Kupffer cells function, but the real interaction should be clarified in the future.

In short, CsA has partially reverted the deleterious effect of warm ischemia on liver regeneration. The question is whether the effect is only due to the hepatotrophic potential of the drug or its immunosuppressor property or an unclarified antiischemic effect.

Focusing now on the fact that OFR are implicated in ischemic-reperfusion injury to the liver, it could be expected that drugs preventing or decreasing this injury could improve the regenerative response of ischemic livers.

In our study SOD seems to be effective, but there were too large individual variations to extrapolate any reasonable conclusion. Similar results have been reported previously [24].

Different authors have communicated controversial results about the efficacy of allopurinol on postischemic hepatic functionalism [25, 26]. In our study, pre-reperfusion treatment with allopurinol has not improved the %RH, but it has accelerated the regenerative process (RG). At the doses used in our experiments allopurinol acts as an inhibitor of xanthine-oxidase system, but in other studies allopurinol using different doses shows other antioxidant effects [27]. The timing of administration has been carefully studied showing that allopurinol has to be inoculated prior to reperfusion in order to achieve its effect [25]. Our results have shown no beneficial effect of allopurinol on liver regeneration; however, more experiments are necessary to clarify its role in postischemic liver resuscitation.

A few years ago, Granger et al. [28] found that folic acid protected the gut against ischemia. We have also found very good results with this vitamin in a model of intestinal reperfusion injury [14]. This encouraged us to test it in this study about ischemic liver regen-

eration, and the results have been very impressive: the folinic acid has completely reverted the deleterious effect of ischemia. The antioxidant activity of folinic acid and folate analogues is probably due to their ability to inhibit xanthine oxidase [29].

However, such an intense effect and the failure of another xanthine oxidase inhibitor (allopurinol) in the same model, suggests an unsuspected hepatotrophic capacity of this drug. It should be necessary to clarify the hepatotrophic effect of this drug prior to becoming a real therapeutical tool in liver surgery.

In summary, CsA has proved to exert its hepatotrophic effect on ischemic livers. This drug and the folinic acid may be two therapeutic tools useful to minimize the noxious effect of temporary warm ischemia over the regenerative ability of the liver.

References

1 Foster JH: History of liver surgery. Arch Surg 1991;126:381–389.

2 Kemeny MM: Hepatic resection: When, what kind and for which patients. J Surg Oncol 1991;(suppl 2): 54–58.

3 Badger IL, Czerniak A, Beath S, Tisone G, Deakin M, Sherlock DJ, Kelly DA, McMaster P, Buckels JAC: Hepatic transplantation in children using reduced size allografts. Br J Surg 1992;79:47–49.

4 Houssin D, Soubrane O, Boillot O, Dousset B, Ozier Y, Devictor D, Bernard O, Chapius Y: Orthotopic liver transplantation with a reduced size graft: An ideal compromise in pediatrics. Surgery 1992;111:532–542.

5 Francavilla A, Ove P, Polimeno L, Coetzee M, Makowka L, Barone M, Van Thiel DH, Starzl TE: Regulation of liver size and regeneration: Importance in liver transplantation. Transplant Proc 1988;20(1, suppl 1):494–497.

6 Blankestein JD, Terpstra O: Liver preservation: The past and the future. Hepatology 1991;13:1235–1250.

7 Marubayashi S, Dohi K, Ochi K, Kawasaki T: Role of free radicals in ischemic rat liver cell injury: Prevention of damage by tocopherol administration. Surgery 1986;99:184–192.

8 Koo A, Komatsu H, Tao G, Inoue M, Guth PH, Kaplowitz N: Contribution of no-reflow phenomenon to hepatic injury after ischemia-reperfusion: Evidence for a role for superoxide anion. Hepatology 1992;15: 507–514.

9 García-Alonso I, Portugal V, Iturburu I, L de Tejada I, Méndez J: Modifications induced by cyclosporin A on ischemic liver regeneration. Surg Res Commun 1990;9:227–233.

10 García-Alonso I, Méndez J, Barberá E: Cyclosporin modifies liver regeneration following partial hepatectomy. Surg Res Commun 1989;6:43–49.

11 Yamamoto S, Nitta N, Ozaki N, Morimoto T, Kamiyama Y, Yamaoka Y, Okamoue T, Ozawa K: Deleterious effect of splanchnic congestion on hepatic energy metabolism following repeated portal triad crossclamping in dogs. Circ Shock 1988; 26:193–201.

12 García-Alonso I, Tejada IL, Iturburu I, Ortiz J, Apecechea A, Méndez J: Cyclosporin A affects liver regeneration following partial hepatectomy. Eur Surg Res 1988;20(suppl 1): 168.

13 Bilbao J, García-Alonso I, Portugal V, Barceló P, Ortiz J, Méndez J: Antioxidant drugs as therapeutic agents in experimental intestinal reperfusion injury. Rev Esp Enf Digest 1991;80:237–241.

14 Bilbao J, García-Alonso I, Portugal V, Barceló P, Apecechea A, Méndez J: Efficacy of antioxidant therapy (folic acid and alphatocopherol) in intestinal lesions produced by reperfusion. Cirug Esp 1992;51:262–265.

15 Nauta RJ, Tsimoyiannis E, Uribe M, Walsh DB, Miller D, Butterfield A: Oxygen-derived free radicals in hepatic ischemia and reperfusion injury in the rat. Surg Gynecol Obstet 1990;171:120–124.

16 Metzger J, Lauterburg BH: Postischemic ATP levels predict hepatic function 24 hours following ischemia in the rat. Experientia 1988; 44:455–457.

17 Fridovich I: In Gilbert DL (ed): Oxygen and Living Processes. 1981.

18 Players T, Mills DJ, Horton AA: Age-dependent changes in rat liver microsomal and mitochondrial NADPH-dependent lipid peroxidation. Biochem Biophys Res Commun 1977:1397–1402.

19 Portugal V, García-Alonso I, Bilbao J, Barceló P, Múgica P, Méndez J: Hepatotrophic effect of cyclosporin A on ischemic rat liver. Rev Esp Enf Digest 1993;83:255–259.

20 Francavilla A, Barone M, Zeevi A, Scotti C, Carrieri G, Mazzaferro V, Prelich J, Todo S, Eiras G, Fung J, Porter KA: FK-506 as a growth control factor. Transplant Proc 1990;23 (1, suppl 1):90–92.