



Ascorbic acid supplementation has a cytoprotective effect on secondary biliary cirrhosis: experimental study in young rats

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Abstract

Objective: To test whether ascorbic acid supplementation has any cytoprotective effect on a model of secondary biliary cirrhosis in young rats.

Methods: We studied 40 Wistar rats weaned at the 21st postnatal day. Each group of 10 was subjected to one of the following four treatments, until 49th postnatal day, when they suffered euthanasia: 1) LC-double ligature and resection of the common bile duct and daily administration of ascorbic acid [100 mg/g of body weight (bw)]; 2) LA-double ligature and resection of the common bile duct and daily administration of aqueous vehicle (1 mL/g bw); 3) SC-sham operation and daily administration of ascorbic acid (100 mg/g bw); 4) SA-double ligature and resection of the common bile duct and daily administration of aqueous vehicle (1 mL/g bw). The rats were weighed daily. On the 27th day after the operation they received an intra-peritoneal injection of 1.5 mg/g bw of sodium pentobarbital, and the pentobarbital sleeping time was measured. Blood was collected for serum alanine aminotransferase and aspartate aminotransferase activity measurements, serum albumin and globulin concentrations, and the liver was assessed for liver water and fat content. Data were submitted to two-way ANOVA and paired comparisons between groups were tested using the SNK method. Significance level was set at 0.05.

Results: Ascorbic acid supplementation attenuated the effects of cholestasis: decreased the pentobarbital sleeping time, serum globulin, and the liver fat content.

Conclusions: Our results corroborate the hypothesis that ascorbic acid supplementation has a cytoprotective effect in secondary biliary cirrhosis.

J Pediatr (Rio J). 2008;84(6):522-528: Biliary liver cirrhosis, biliary atresia, ascorbic acid, extra-hepatic cholestasis.

Introduction

Great interest in the cholestasis syndrome has been awakened due to the frequency of its incidence, both in clinical practice with children and with adults, and due to the large variety of causes identified up to the present moment.¹⁻³ In newborns and infants, the incidence is as much as 1 in 500 live births.⁴ Some diseases which cause cholestasis in newborns and infants evolve to chronic hepatic disease with bil-

iary cirrhosis, evolving faster than cholestatic diseases in adults.⁵ Biliary atresia is the most important cause initiating cholestasis in the first four months of life, as it always evolves to death if untreated and is a principal indication for liver transplant in infancy.^{3,6}

Ligature and common biliary duct resection in adult rats has been used as a model for obstructive cholestasis and secondary biliary cirrhosis. However, the execution of these pro-

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cedures in young rats would provide a closer model for biliary atresia, since the model using adult rats usually suffers interference from secondary bacterial infections,⁷⁻⁹ which does not occur in young rats. The young rat model has been applied by few authors,^{2,10} aside from members of our group (Hepatology Research Group at Faculdade de Medicina de Botucatu).

Oxidative stress is an intermediary mechanism in the production of the hepatic lesions of cholestasis.^{11,12} Considering that cholestasis leads to malabsorption of liposoluble vitamins, two of which (vitamin A and E) present antioxidant action, this malabsorption aggravates oxidative stress, thus increasing hepatic lesions.¹³ We should also consider that ascorbic acid has important physiological functions, such as action over the P450 cytochrome and antioxidant, and that ascorbic acid is hydrosoluble^{14,15} and easily absorbable in cholestasis.¹⁶ We proposed the study of a possible hepatoprotective effect of ascorbic acid supplementation in the biliary cirrhosis model by ligation and resection of the common bile duct (LRCBD) in young rats.

Methods

Experiment design

In order to test the principal hypothesis, we formulated auxiliary hypotheses:

- First hypothesis: cholestasis influences hepatic lesions, independent of ascorbic acid administration;
- Second hypothesis: ascorbic acid influences hepatic lesions, independent of the presence of cholestasis;
- Third hypothesis: ascorbic acid interferes in the effects of cholestasis on hepatic lesions.

To test these hypotheses, the following variables were considered:

- Independent variables: the presence or absence of obstructive cholestasis (LRCBD or sham operation) and administration or non-administration of ascorbic acid (ascorbic acid or aqueous vehicle).
- Dependent variables: measurable consequences of hepatic lesions, namely: serum aspartate aminotransferase activity (AST, IU/L); alanine aminotransferase activity (ALT, IU/L); serum albumin concentration (g/dL); serum globulin concentration (g/dL); pentobarbital sleeping time test - PST (minutes); liver wet weight (g); liver water content (g/100 g); and liver fat content (g/100 g).

Experimental groups

Forty male Wistar rats, bred in the Pediatrics Department Experimental Laboratory of Faculdade de Medicina de Botucatu, were studied. On the 21st postnatal day (P21), rats weighing between 45 and 48 g were selected and randomly

placed in one of four groups of 10 rats. The groups and their respective treatment were:

LC group: LRCBD on P21 and euthanasia on the 49th postnatal day (P49). Daily administration of 1 mL/g of rat body weight of an aqueous solution of ascorbic acid in a concentration of 200 mg/mL (Roche®), by gavage.

LA group: LRCBD on P21 and euthanasia on P49. Daily administration of aqueous vehicle, by gavage, at a volume of 1 mL/g of rat body weight.

SC group: sham operation on P21 and euthanasia on P49. Daily administration of an aqueous solution of ascorbic acid (Roche®), by gavage, at the same volume stipulated for previous groups.

SA group: sham operation on P21 and euthanasia on P49. Daily administration of an aqueous solution of aqueous vehicle, by gavage, at the same volume stipulated for previous groups.

Execution of the experiment

The experimental procedure was approved by the Ethics Committee in Animal Experimentation (CEEA), Faculdade de Medicina de Botucatu, UNESP, protocol number 148, in accordance with the Ethical Principles of Animal Experimentation of the Brazilian College of Animal Experimentation (COBREA). All animal maintenance, anesthetic, surgical and euthanasia procedures were executed according to the recommendations contained in the Canadian Council of Animal Care (1984) guide and in the Guide for the Care and Use of Laboratory Animals National Research Council, USA.¹⁷

The LRCBD procedures were performed according to the technique previously described by Cameron & Oakley,⁷ adapted by Battochio et al.¹⁸ In the sham operation, an abdominal incision, exposure and dissection of the bile duct were performed. The suture thread was passed around the bile duct without proceeding with the ligation. Surgical closing was performed in the same manner as the ligation operation.

Previous work from the laboratory confirmed that this procedure caused liver fibrosis and ductular proliferation in the same pattern described by Cameron & Oakley of septal biliary fibrosis.⁷

Euthanasia

Euthanasia was performed on P49, 28 days after LRCBD and sham operation procedures. The rats were anesthetized with pentobarbital at a dose of 0.0017 mg/g of rat body weight and, after the abdominal incision, blood was collected by cardiac puncture for serum biochemical test. After euthanasia, the liver was weighed for posterior calculation of the water and fat content.

Observations during the experiment

On the 48th postnatal day (P48), all rats were subjected to PST. They were injected via intraperitoneal route with 0.0015 mg of pentobarbital per gram of rat body weight and the time during which placing reflex remained abolished was measured. The PST depends on the desintoxicating function of the liver.

Studies carried out after euthanasia

The serum activity of AST and ALT were determined by the optimized ultraviolet (UV) ray absorption method and expressed in international units (IU). The serum dosages of total albumin and globulin serum were determined by the colorimetric method and expressed in grams/deciliter (g/dL). Liver wet weight was confirmed on a Mettler analytic balance (model H35, maximum weight = 160 g), and the values expressed in grams.

The liver water content was determined by the gravimetric method. A fragment of liver was weighed on a Mettler analytic balance, dehydrated in a heater at 100 °C for 72 hours and weighed again, when water content weight was calculated in g/100 g of wet liver. To determine liver fat content, after drying, the liver was ground in a graal, wrapped in filter paper and the fat was extracted by ethylic ether, for a 12 hour period, in a Soxhlet extractor apparatus. The ethylic ether was evaporated at room temperature for 24 hours, dried in a heater at 100 °C for 12 hours and weighed again. From the weight difference, the liver fat content was calculated in g/100 g of wet liver.

Statistical analysis

Calculations were executed using the Microsoft Excel® and Sigma Stat version 2.0® programs.

Descriptive statistics: measurements of central tendency (means and medians) and measurements of dispersion (standard deviation, variation coefficient, amplitude variation, minimum and maximum values) of the variables of each experimental group were calculated.

Comparative statistics: 1) The results of each variable for each experimental group were submitted for sample testing for normality and equality of variances. 2) If these conditions were satisfied, variance analysis for two factors was applied: the first factor, administration or non-administration of ascorbic acid, analyzed for two levels: administration of ascorbic acid and administration of aqueous vehicle; the second factor, presence or absence of cholestasis, analyzed for two levels: LRCBD (cholestasis) and sham operation (absence of cholestasis). This test analyzed the effects associated with each factor on the dependent variables, independently of the effect of the other, as well as the interaction of both factors. 3) When significant interaction between these factors ($p \leq 0.05$) was found, multiple paired comparisons by the

Student-Newman-Keuls (SNK) method were performed, identifying the groups which presented significant differences between them (p of $\alpha \leq 0.05$). 4) When the samples did meet item 1 criteria, their values were replaced with the rank order of these values - to satisfy the criteria of normality and equality of variances.

Results

Cholestasis significantly increased PST ($p \leq 0.001$) and ascorbic acid interfered by attenuating this effect ($p \leq 0.001$).

Cholestasis significantly increased the serum level of AST, independently of the effect of ascorbic acid ($p \leq 0.001$), and ascorbic acid did not interfere in the effect of cholestasis ($p \geq 0.873$).

Cholestasis significantly increased the serum level of ALT, independently of the effect of ascorbic acid ($p \leq 0.001$), and ascorbic acid did not interfere in the effect of cholestasis ($p \geq 0.126$).

In relation to wet liver weight, no significant difference occurred between the LRCBD and sham operation groups ($p \geq 0.355$), nor on the effect of ascorbic acid ($p \geq 0.001$).

Cholestasis significantly increased the liver water content, independently of the effect of ascorbic acid ($p \leq 0.001$), and ascorbic acid did not interfere in the effect of cholestasis ($p \geq 0.517$).

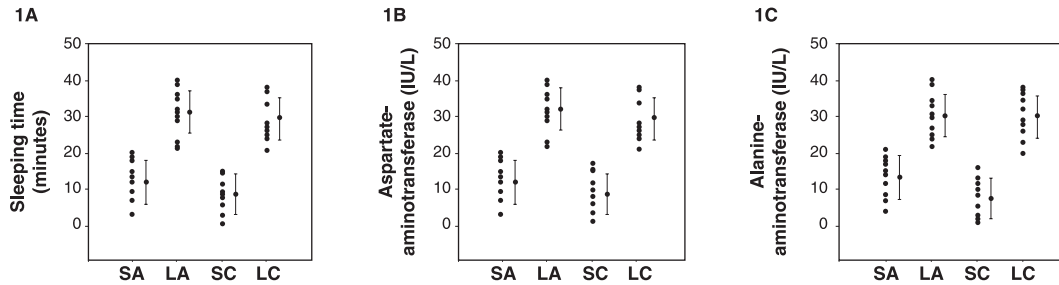
Cholestasis significantly increased the liver fat content, independently of the effect of ascorbic acid ($p \leq 0.001$), and ascorbic acid interfered in the effect of cholestasis ($p = 0.006$).

Cholestasis significantly reduced the serum levels of albumin, independently of the effect of ascorbic acid ($p \leq 0.001$), and ascorbic acid did not interfere in the effect of cholestasis ($p \geq 0.476$).

Cholestasis significantly increased the serum levels of globulin, independently of the effect of ascorbic acid ($p \leq 0.001$), and ascorbic acid interfered attenuating this effect ($p \leq 0.012$).

Discussion

Cholestasis prolonged PST, though in part, and ascorbic acid attenuated this effect of cholestasis (Figure 1 and Table 1). PST has been used to evaluate the functional efficiency of hepatic cells in many experimental models, including cholestatic disease of the liver and hepatic lesions by other mechanisms.¹⁹⁻²¹ A deficiency in ascorbic acid may lead to a decrease in hepatic P450 cytochrome and, thus, affect the hepatic activity of enzymatic drug metabolism.¹⁴ Hepatocytic lesions in cholestasis induced by the ligation of the bile duct may lead to a decrease in the P450 cytochrome isoenzymes.²² The results shown here suggest that ascorbic acid supplementation stimulates the P450 cytochrome or maintains its function by protecting hepatocytes.



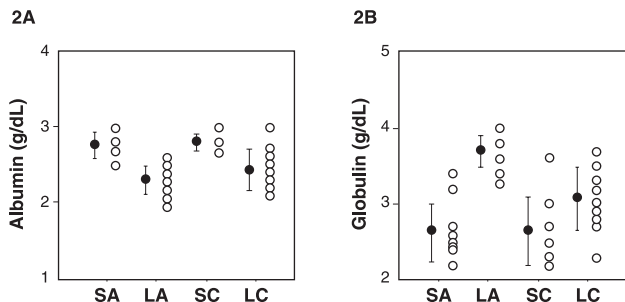
LA = ligature and resection of the common bile duct with vehicle; LC = ligature and resection of the common bile duct with ascorbic acid; SA = sham operation with vehicle; SC = sham operation with ascorbic acid.

Figure 1 - Means, standard deviations and individual values: pentobarbital sleeping time (1A), aspartate aminotransferase (1B) and alanine aminotransferase (1C)

Table 1 - Means, medians and standard deviations of the variables of each experimental group

Variable	SA	SC	LA	LC
Pentobarbital sleeping time (minutes) - rank order				
Mean (SD)	87 (17.55)	93.8 (14.42)	221.7 (30.77)	137.33 (28.05)
Median	88	95	212.5	137.66
Serum level of aspartate aminotransferase (UI/L)				
Mean (SD)	50.7 (10.14)	45.6 (4.69)	250.26 (54.44)	231.44 (41.40)
Median	47.5	45.5	246	226.22
Serum level of alanine aminotransferase (UI/L)				
Mean (SD)	53.6 (10.42)	42.6 (8.27)	120.2 (28.03)	116.11 (25.75)
Median	53	42	119.5	121.55
Liver weight (g) - rank order				
Mean (SD)	8.25 (1.19)	8.69 (1.25)	8.93 (2.13)	9.19 (2.88)
Median	8.29	8.67	8.31	9.89
Liver water content (g/100 g) - rank order				
Mean (SD)	71.44 (1.27)	71.73 (0.84)	76.47 (2.83)	75.476 (3.57)
Median	71.61	71.64	76.23	76.442
Liver fat content (g/100 g) - rank order				
Mean (SD)	1.05 (0.54)	1.09 (0.28)	6.61 (5.74)	1.01 (0.59)
Median	0.98	1.04	3.78	1.02
Serum level of albumin (g/dL)				
Mean (SD)	2.76 (0.17)	2.8 (0.115)	2.3 (0.19)	2.43 (0.27)
Median	2.8	2.8	2.25	2.36
Serum level of globulin (g/dL)				
Mean (SD)	2.510	2.350	3.550	3.156
Median	2.400	2.200	3.700	3.178

LA = ligature and resection of the common bile duct with vehicle; LC = ligature and resection of the common bile duct with ascorbic acid; SA = sham operation with vehicle; SC = sham operation with ascorbic acid; SD = standard deviation.



LA = ligation and resection of the common bile duct with vehicle; LC = ligation and resection of the common bile duct with ascorbic acid; SA = sham operation with vehicle; SC = sham operation with ascorbic acid.

Figure 2 - Means, standard deviations and individual values: albumin (2A) and globulin (2B)

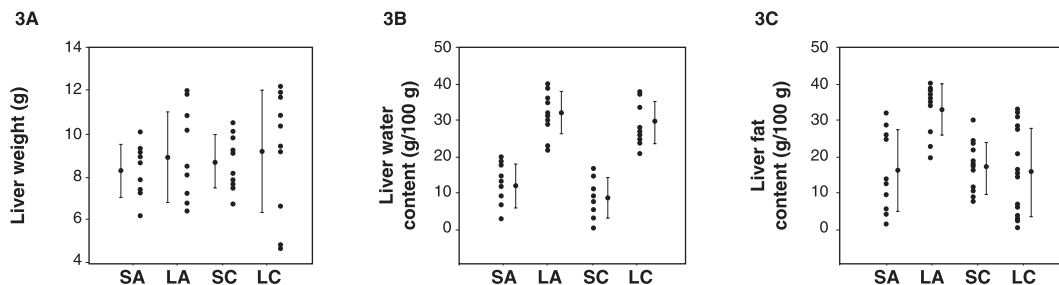


Figure 3 - Means, standard deviations and individual values: liver weight (3A), liver water content (3B) and liver fat content (3C)

Cholestasis increased the serum levels of AST and ALT, independently of the effect of ascorbic acid, and ascorbic acid did not interfere in this effect of cholestasis. Therefore, probably there was no effect on the velocity of hepatocytic necrosis (Figure 1 and Table 1).

Cholestasis reduced the serum levels of albumin, independently of the effect of ascorbic acid, and ascorbic acid did not interfere in this effect of cholestasis (Figure 2 and Table 1). Thus, ascorbic acid seems not to interfere in the mechanisms of hypoalbuminemia in cholestasis: increased capillary permeability; increased plasmatic volume; lack of compensatory albumin synthesis; reduced hepatocyte mass or lack of elements for albumin synthesis, resulting from secondary mal-nutrition due to the malabsorption present in cholestasis.¹⁹

Cholestasis increased the serum levels of globulin (independently of the effect of ascorbic acid), and ascorbic acid attenuated this effect (Figure 2 and Table 1). This effect probably occurs over immunoglobulins, which are a part of the globulins. Keraan et al.²³ showed that in rats with a porta-caval shunt an increase in globulins occurs at the expense of immunoglobulins. This increase is most likely caused by the passage of endotoxins directly to the systemic circulation due

to an increase in intrahepatic portal circulation resistance, diverting them from the liver, and thus, reducing the depuration of antigens and immunocomplexes by the liver. This increase in antigens in the systemic circulation would increase immunoglobulin production by the B lymphocytes of the peripheral immune system.²⁴ Other possible antigens are released by the destruction of hepatocytes.^{25,26} Ascorbic acid could interfere with one or more of these factors which cause hyperglobulinemia.

It was not possible to demonstrate the effect of cholestasis, nor of ascorbic acid, on liver wet weight (Figure 3 and Table 1); ductal hyperplasia and fibrosis may compensate for the reduction in hepatocytes in the phase in which these rats were studied.

Cholestasis increased the liver water content, independently of the effect of ascorbic acid, a fact which could reflect an intracellular water increase (Figure 3 and Table 1). According to Takahashi,²⁷ experimental cholestasis in rats causes: dysfunction of the mitochondria; reduced phosphorylation capacity; reduced ATP production; functional damage of ATPase dependent on Na⁺ and K⁺, and increased hepatocyte water content. Ascorbic acid did not interfere in this effect of cholestasis.

Cholestasis increased the liver fat content (Figure 3 and Table 1), possibly due to lipid transport deficiency. With the reduction of apolipoprotein A1, due to the presence of cholestasis, abnormal catabolism and reduction of very low density lipoproteins (VLDL) can occur.²⁸ The liberation of VLDL triglycerides through the activity of lipoprotein lipase is affected in such a way that triglyceride fatty acids are not adequately transported to the adipose cells,² so fat accumulation occurs in the liver. Ascorbic acid attenuated this cholestatic effect. Some studies relate ascorbic acid and serum levels of apolipoprotein A1.²⁹ Ascorbic acid supplementation probably attenuated the effects of cholestasis, normalizing or increasing apolipoprotein A1 production and, consequently, of VLDL and lipoprotein lipase, normalizing the transport of triglycerides to the tissues and reducing the percentage of fat in the liver.

Conclusions

Studying the experimental model of secondary biliary cirrhosis in young rats, we observed that ascorbic acid appears to present the following beneficial effects in secondary biliary cirrhosis: hepatoprotective effect, since it stimulates the des-intoxication function of the liver, as shown by its effect on the sleeping time test induced by pentobarbital; attenuates hyperglobulinemia resulting from cholestasis and attenuates hepatic fat deposit caused by cholestasis. The hepatoprotective effect was not corroborated by the study of aminotransferases.

Ascorbic acid supplementation could be of significant use in the treatment of children with cholestatic chronic hepatic disease.

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