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Medial thickening of hepatic artery branches in biliary atresia. A morphometric study

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Abstract

Background/Purpose: Medial layer hypertrophy of hepatic arterial branches may be associated with biliary atresia (BA) pathogenesis. This study aimed at evaluating medial layer thickness in hepatic arterial branches at portoenterostomy and liver transplantation.

Methods: The authors evaluated 1274 arterial branches both in BA cases and in control subjects involving a total of 1108 arterioles and 166 arteries. Arterial branch characteristics were morphometrically evaluated in 47 BA patients at the time of portoenterostomy. Controls were patients with intrahepatic cholestasis (n = 3), immature neonates (n = 7), and infants (n = 7) without liver disease. Progression of medial layer thickening between the time of portoenterostomy and transplantation was evaluated in 7 BA patients. Biliary atresia patients at the time of transplantation were compared with non–BA-transplanted patients (n = 4).

Results: The arterial medial layer of BA cases at portoenterostomy was thicker than that of infants without liver disease ($P = .03$). The arterial medial thickness increased during the interval between portoenterostomy and transplantation ($P = .05$). Arterioles and arteries with thickened medial layers were found in transplanted BA patients but not in patients transplanted for other liver diseases ($P = .05$ and $P = .01$). Thickening of the medial layer of the hepatic arteries was associated with focal distribution of interlobular bile ducts in portal spaces in BA ($P = .02$).

Conclusions: In BA, there is a progressive thickening of the arterial medial layer, suggestive of vascular remodeling, which is associated to the disappearance of interlobular bile ducts.

The cholangiopathy observed in biliary atresia (BA) is of unknown cause and remains despite portoenterostomy. Characteristically, over the longer term, BA resembles a "vanishing" bile duct disease and remains the commonest indication for liver transplantation in children.

Ho et al [4] have observed arterial wall hypertrophy in BA and considered this alteration a primary event in its pathogenesis. Arterial abnormality of this kind could

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affect liver function because of the intimate relationship between bile ducts and the developing arterial system [1]. Lack of arterial blood flow may initiate progressive cholestasis in experimental models [2] and can be responsible for the disappearance of interlobular bile ducts in some liver diseases [3]. However, Ho et al [4] studied only small arteries and arterioles, defining the vascular characteristics in relation to previously stated concepts of normality, their control patients having no liver disease. Furthermore, progression of the vascular wall thickening was not evaluated and lumen size was measured by estimating perimeter. Nowadays, new computer programs can automatically assess the true size of perimeter by morphometry.

This study aimed at (1) detecting and measuring by morphometry the arterial medial wall thickening in a large sample of BA patients, (2) evaluating the morphometric changes between portoenterostomy and liver transplantation, and (3) comparing transplanted BA cases with patients transplanted for other liver diseases. A further objective was to evaluate the relationship between medial thickening of hepatic arterial branches and the presence and distribution of interlobular bile ducts in BA.

1. Materials and methods

The study group consisted of infants with BA who were admitted to our institution. The diagnosis of BA was based on laparotomy findings, operative cholangiography, and the histology of the portal bile duct remnant. Arterial morphometry was evaluated initially in wedge liver biopsies obtained during portoenterostomy and in specimens of explanted livers obtained during liver transplantation. Controls were immature neonates and infants without liver disease, age-matched infants with intrahepatic neonatal cholestasis, and children who had received transplants for other indications.

Formalin-fixed paraffin-embedded liver specimens were studied. Two sections, each 5 μm thick were obtained and stained with human-specific antiactin (clone HHF-35, DAKO, Vila Real, Carpinteria, USA, dilution 1:400) and anti-CK19 (DAKO, Glostrup, Denmark, dilution 1:200). HHF-35 stains vascular smooth muscle and facilitates measurement of the medial layer; anti-CK19 aids in visualization of bile ducts. The immunohistochemical method used was the avidin-biotin-peroxidase complex method as previously described [5]. Secondary antibody was antimouse, biotinilated.

Hepatic arterial branches were quantified by morphometry. From each slide stained for HHF-35, 10 images were obtained from HHF-35–positive structures and recorded in a CD-ROM in the TIFF format for later measurement. Microscope magnification was $\times$200, and halogen lamp voltage was held at 7.0 V ($\pm$0.1%), by voltage stabilization being constantly monitored by a digital multimeter (Digital Multimeter, DT 830B, CE). Reading of the material stained for HHF-35 was carried out by a quantitative method proposed by Chalkley [6]. Morphometric measurements were carried out by use of the Scion-Image Computer Program (Scion Corporation) on every arterial vessel in each captured image. Values obtained in each measurement were registered, and the mean of each case was calculated. For perimeter measurement, black and white images were used so as to cause the medial layer to stand out against the background. The image exhibited on the monitor was calibrated, and correction of defects related with the histological technique was carried out. Endothelial cells were eliminated so that only the inner face of the medial layer was used to measure the perimeter. Perimeter was defined as the length of the contour of the inner face of the medial layer and was automatically assessed by the computer program, according to the formula: Perimeter = $0.98 * N_e + 1.406 * N_o - 0.091 * N_c$ where N_e = number of even Freeman codes; N_o = number of odd Freeman codes, and N_c = number of angles [7].

Arteries were defined as vessels with external diameter >100μm with compact muscle walls expressing HHF-35. Arterioles were defined as similar vessels with smaller external diameters. Careful differentiation was made between arterial and venous branches, the latter being typified by the presence of abundant collagenous and elastic fibers interspersed in the medial layer. Vessels of doubtful characterization were not included.

The vascular components used to assemble the artery branch characteristics were:

Medial layer thickness (MT)—a mean of 4 values of medial thickness was obtained by a line drawn to the central axis and connecting 2 points, one at the outer and the second at the inner face of the medial layer.
Internal diameter (ID)—calculated from the perimeter obtained by morphometric technique. The formula of the internal diameter calculation was ID = perimeter/π.
External diameter (ED)—calculated by the formula ED = (2 $\times$ MT) + ID.

The artery branch characteristics used to evaluate medial thickening were:

RMED (relation of medial layer thickness/external diameter) $\times$ 10.
RIDED (relation of internal diameter/external diameter) $\times$ 10.

Medial thickening was considered as increased RMED values and/or decreased RIDED values.

The presence and distribution of interlobular bile ducts were determined on the histological specimens stained for CK19 of BA patients. This was done independently by 2 pathologists and the first author of this study, with subsequent consensus. Interlobular bile duct distribution

Table 1 Arterial branches vascular characterization according to the groups of BA at portoenterostomy and controls

Arterioles	BA (n = 47)	Controls			*P*
		Imneo (n = 7)	Infants (n = 7)	IHC (n = 3)	
RMED	1.7 ± 0.2	1.6 ± 0.3	1.6 ± 0.4	1.4 ± 0.01	.305
RIDED	6.7 ± 0.53	6.7 ± 0.8	6.5 ± 0.9	7.2 ± 0.01	.370
Arteries	n = 32	n = 6	n = 4	n = 3	*P*
RMED	1.0 ± 0.3[a]	0.8 ± 0.2	0.7 ± 0.3[a]	0.8 ± 0.1	.031
RIDED[b]	8.1 ± 0.5	8.4 ± 0.4	8.5 ± 0.7	8.4 ± 0.2	.151

Imneo indicates immature neonates; IHC, intrahepatic neonatal cholestasis; RMED, relation medial layer thickness/external diameter; RIDED, relation internal diameter/external diameter. Statistical method: analysis of variance.

[a] Means are different at $P < .05$ (Duncan's post hoc test).

[b] Number of BA cases = 27; number of immature neonates = 6; number of infants without liver disease = 3.

was considered focal if these structures were present in a maximum of 3 portal spaces.

1.1. Statistical analysis

Quantitative variables were expressed as mean ± SD and were compared using Student's *t* test or, where indicated, by 1-way analysis of variance followed by Duncan's procedure for multiple comparisons. $P < .05$ was accepted as significant. Microsoft Excel 2000 (Microsoft Corporation, Redmond, WA) and SPSS 10.0 (SPSS Inc, Chicago, IL) were used for data processing and statistical analysis.

1.2. Ethics

The Ethics Committee of the Research and Postgraduation Group of the Hospital de Clínicas de Porto Alegre approved the present study.

2. Results

The study group consisted of 47 infants with BA [mean age at portoenterostomy, 91 (range 24 to 251) days]. Of these, 7 BA cases had already undergone liver transplantation by the time of the study at mean age of 59 (range 28-120) months. The control group consisted of immature neonates (n = 7) without liver disease [mean gestational age, 29 (range 22-35) weeks], infants (n = 7) without liver disease [mean age at necropsy, 55 (range 1-150) days], and 3 infants with intrahepatic neonatal cholestasis [mean age at biopsy, 114 (range 45-198) days]. One of the patients with intrahepatic cholestasis had panhypopituitarism and the other 2 presented idiopathic neonatal hepatitis. The explanted livers of 4 patients transplanted for reasons other than BA [neonatal sclerosing cholangitis (n = 2), α_1-antitrypsin deficiency and autoimmune hepatitis] were also evaluated [mean age at transplantation, 80 (range 36-120) months]. We evaluated 823 arterioles and 120 arteries in the BA group and 285 arterioles and 46 arteries in the controls.

Table 1 shows the hepatic artery branch characterization in BA cases at portoenterostomy, in infants and immature neonates without liver disease, and in infants with intrahepatic cholestasis. (Table 1).

For arteries, RMED was higher in the BA group than in infants without liver disease (quote value, $P = .03$), but otherwise, there were no significant differences between BA and controls with regard to arterioles.

Table 2 compares the arterial branch characteristics at portoenterostomy and at liver transplantation in the BA patients who have undergone both procedures (Table 2).

Regarding arteries, the value of RIDED decreased between portoenterostomy and liver transplantation ($P = .05$). There were no significant differences regarding arterioles. But in 3 of 7 specimens of BA explanted livers, arterioles were found with extremely

Table 2 Progression of the hepatic arterial branch characteristics between portoenterostomy and liver transplantation on the BA group

Arterioles	BA		Difference	95% CI	*P*
	Portoenterostomy (n = 7)	Transplantation (n = 7)			
RMED	1.7 ± 0.1	1.9 ± 0.4	−0.2	−0.5 to 0.1	.191
RIDED	6.6 ± 0.4	6.2 ± 0.7	0.4	−0.2 to 1.0	.133
Arteries	n = 3	n = 3	Difference	95% CI	*P*
RMED	0.9 ± 0.2	1.1 ± 0.3	−0.2	−0.4 to 0.1	.091
RIDED	8.1 ± 0.4	7.8 ± 0.5	0.3	<0.1 to 0.6	.050

CI indicates confidence interval. Statistical method: Student's *t* test.

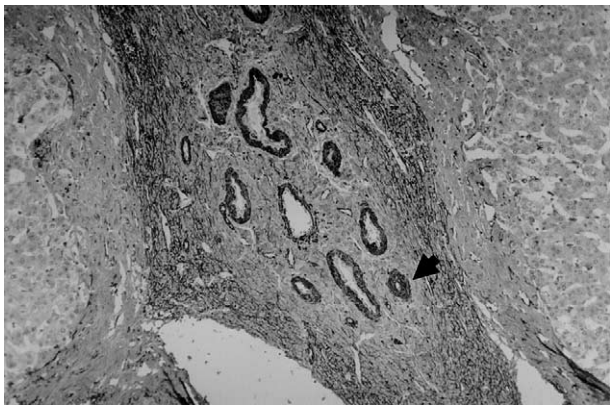

Fig. 1 Vascular branches in a patient with BA at the time of liver transplantation. Observe the presence of an arteriole with extremely thickened medial layer (arrow). Magnification ×40, immunohistochemistry with HHF-35.

thickened medial layers, some of them having their lumen almost occluded by concentric smooth muscle thickening (Fig. 1, arrow).

Table 3 presents data comparing BA cases which underwent liver transplantation with transplanted patients with other liver diseases (Table 3).

Regarding both arterioles and arteries, significant differences occurred in RMED (P = .05 and P = .01, respectively) and RIDED (P = .05 and P = .01, respectively). In both instances, BA patients presented higher RMED and lower RIDED values.

Table 4 presents the relationship between the distribution of interlobular bile ducts and the arterial characteristics in the BA group at portoenterostomy (Table 4).

Interlobular bile duct distribution was focal in 10 (21%) cases and diffuse in 34 (72%). In 3 other BA patients, no interlobular bile ducts were found (6%). The value of RMED was higher on the group with focal interlobular bile duct distribution (P = .02).

Table 4 Relation between hepatic arterial vascular characteristics and interlobular bile duct distribution in portal spaces in the BA group at portoenterostomy

Characteristic	Interlobular bile duct		P
	Focal (n = 5)	Diffuse (n = 25)	
RMED	1.2 ± 0.4	1.0 ± 0.2	.022
RIDED[a]	7.8 ± 0.8	8.1 ± 0.4	.234

Statistical method: Student's t test.

[a] Number of cases with diffuse distribution of interlobular bile ducts = 20.

3. Discussion

Our findings show that the arterial medial layers in BA patients at the time of portoenterostomy were thicker than those found in infants without liver disease. No significant differences were found for arterioles. These results are different from those found by Ho et al [4]. Those authors described hypertrophy in small arteries and arterioles. In our study, a greater number of patients were evaluated; large muscular arteries, as well as small arterial vessels, were examined, and a morphometric study was undertaken without previously defined concepts of normality comparing BA patients with others, both those free from any liver disease and those having different types of liver disease. Perhaps the difference in the method of perimeter evaluation is responsible for the disagreement between that study and ours. Ho et al [4] determined lumen size by an estimated perimeter. However, small arteries in BA are often affected by flattening and collapse, and the luminal wall is commonly folded and irregular. The automatic morphometric measurement of the perimeter carried out by us is the most suitable way to evaluate the lumen size so as to correct the errors that arise in the evaluation of a folded or irregular lumen by the use of an estimated perimeter. This study further revealed the increase of the arterial medial layer thickening between the time of portoenterostomy and liver transplantation. Furthermore, 3 of 7 BA patients showed arterioles with extremely thickened medial layer and an almost obstructed lumen at the time of transplantation. Therefore, medial thickening that was restricted to larger vessels at portoenterostomy seemed to extend toward the periphery of the arterial tree. The arterial wall characteristics from BA explanted livers were different from those of transplanted patients with other liver diseases, suggesting that the arterial wall thickening may be specific of BA. In this study, luminal dilatation was neither found for

Table 3 Arterial branch characteristics according to the groups which underwent liver transplantation because of BA or other liver diseases

Arterioles	Liver transplantation		Difference	95% CI	P
	BA (n = 7)	Other diseases (n = 4)			
RMED	1.9 ± 0.4	1.5 ± 0.2	0.4	<0.1 to 0.9	.050
RIDED	6.2 ± 0.7	7.1 ± 0.4	−0.9	−1.8 to <0.1	.050
Arteries	n = 6	n = 4	Difference	95% CI	P
RMED	1.1 ± 0.2	0.6 ± 0.2	0.4	0.1 to 0.8	.016
RIDED	7.9 ± 0.4	8.8 ± 0.5	−0.9	−1.6 to −0.2	.016

Statistical method: Student's t test.

arterioles nor for arteries, but we did not evaluate arteries at the porta hepatis, where luminal dilatation has been ultrasonographically observed by Ho et al [4].

The arterial medial layer thickening we observed might be related to the pathogenesis of BA or might be an effect of the disease or just an associated event. However, hepatic artery branches nourish bile ducts, veins, and even nerves, and an arteriopathy of this kind may have profound influences in the liver morphology and functioning. This arteriopathy may explain the findings of Kardoff et al [8] and Broide et al [9] who detected increased arterial resistance in the hepatic arteries of BA patients with worse postportoenterostomy prognosis.

Medial layer thickening is associated to the vascular remodeling model and is the product of medial cell proliferation in response to aggressions to the vascular endothelium [10]. The process has been studied with regard to lung vessels in pulmonary hypertension [11]. Hemodynamic factors such as shear stress may trigger this proliferation [12]. The arterial medial thickening in BA might thus be related to hemodynamic factors. The presence of precocious and intense fibrosis leads to arterial collapse that, in the presence of sustained arterial blood flow, may alter shear stress, consequently injuring the vascular endothelium. Medial layer thickening might also be caused by prolonged vasoconstriction in response to the transmission of the increased portal pressure along the peribiliary plexus through the anastomoses between this plexus and terminal arterioles [13]. But why then did it not occur in the control patients with other chronic liver diseases? Maybe the precocious occurrence of portal hypertension explains the distinct arterial behavior in BA [14]. *Muscularization* of portal venous branches because of portal hypertension in BA can be detected around 10 months postportoenterostomy [15]. However, the mean age of the BA patients in our study was 91 ± 45 days, suggesting that the arterial wall thickening in BA was an early event. Besides hemodynamic factors, thickening of the arterial medial layer in BA might be linked to genetic factors associated with vascular remodeling and development [16], the action cytokines [17,18], chronic hepatic hypoxemia [19], or even a direct viral lesion to the endothelium [20].

Furthermore, thicker arterial medial layers were associated to focal distribution of interlobular bile ducts. This finding suggests that the arterial medial layer thickening found in BA might contribute to the disappearance of bile ducts, which may be related to ischemia or hypoxemia [21].

In summary, we evaluated by morphometry 1274 hepatic arterial branches in 47 BA patients and controls. In BA patients at portoenterostomy, arteries with medial layers significantly thicker than those of infants without liver disease were found. The thicker medial layers seemed to be associated with the disappearance of interlobular bile ducts. Between portoenterostomy and liver transplantation, the arterial medial thickening in the BA patients increased, and at transplantation, arterioles were found with extremely thickened medial layer suggesting an extension of the lesion toward the arterial system periphery. Medial layer thickening did not occur in the transplanted non-BA patients evaluated in this study. Further studies are required to characterize this arteriopathy and to investigate its causes.

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