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Liver injury due to iron overload in thalassemia: histopathologic and ultrastructural studies

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The livers of 30 cases of thalassemia (19/β-thal/HbE, seven thal/HbH and four β-thal major) were studied histopathologically and electron microscopically, in an effort to define the morphologic alterations due to iron overload. The results of light and electron microscopy were similar in most cases. Iron accumulation and fibrosis were the common features found in these patients, except that thal/HbH exhibited lesser hepatic damage. The degrees of iron deposition and fibrosis were found to be higher in splenectomized and cirrhotic than non-splenectomized and non-cirrhotic patients. The subcellular changes were swollen mitochondria, with the presence of an electron dense matrix and ruptured mitochondrial membrane. Proliferation of smooth endoplasmic reticulum (ER) and dilated rough ER was observed. Increases in lysosomal hemosiderin in hepatocytes and in Kupffer cells were demonstrated. The possible ways by which the iron compounds or free radicals mediated membrane damage are mentioned. The pattern of liver cell damage is similar to that of viral hepatitis.

Keywords: liver injury, iron overload, thalassemia, histopathology, ultrastructure

Introduction

Thalassemia is recognized as a major health problem, and is widely distributed throughout Southeast Asia and the Mediterranean. It is the most common genetic disorder among the Thais. Iron overload in thalassemia results either from regular blood transfusions or from excessive gastrointestinal iron absorption (Weatheral 1981). Iron is accumulated and stored in the body, mainly in the form of hemosiderin and ferritin. Iron is well confined and controlled in normal conditions; however, if it is not protein-bound or effectively chelated it can be toxic. Unbound iron is toxic and produces progressive damage when this process occurs repeatedly (Wheby 1984). Iron toxicity produces wide-spread organ dysfunctions. Of these, the most prominent is the liver. The liver is a major participant in the metabolism of iron because it stores the metal and also synthesizes transferrin, an iron-binding protein necessary for the transport of iron. Transferrin is responsible for the movement of iron from its absorption in the small bowel to be stored in the liver and other tissues. An easily mobilized form of iron is stored as ferritin hepatocytes. In excess iron storage, ferritin apparently is degraded to hemosiderin (Craig 1990). Iron overload seems to cause cell damage (Trump *et al.* 1973, Richter 1978, Frigerio *et al.* 1984, Tavil *et al.* 1990). Cell injury is one of the most common cell reponses in disease. The molecular mechanisms responsible for cell injury are complex (Cotran *et al.* 1989). It is believed that tissues unable to eliminate excess oxygen free radicals are subject to serious damage. These radicals can injure a wide range of cellular components (Editorial 1986) and iron toxicity is thought to arise from the iron catalyzed formation of oxygen-activated products.

In this report the histopathological and ultrastructural findings of liver injury secondary to iron overload in 30 thalassemic patients are presented.

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Materials and methods

Twenty four surgical and six autopsy specimens from 30 patients, 19 with β-thalassemia/hemoglobin E (β-thal/HbE),

seven with thalassemia/hemoglobin H (thal/HbH) and four with β-thalassemia major (β-thal major) were studied. The subjects consisted of 10 males and 20 females, and ranged in age between 14 and 60 years at the time when liver biopsies or autopsies were performed. All patients received a routine hematologic examination. Thirteen cases were splenectomized and did not receive iron chelating agents, except for two cases of β-thal/HbE. Cirrhosis developed in eight patients (three β-thal/HbE, two thal/HbH and three β-thal major). Hematological and iron status were obtained by standard methods.

Histopathology

Formalin-fixed, paraffin-embedded sections were stained with hematoxylin & eosin, Perls' prussian blue reaction for iron, Masson trichome, reticulum and orcein for hepatitis B surface antigen (HBsAg). Histologic grading for stainable iron and fibrosis in the liver was performed.

Electron microscopy

The fresh tissues were immediately fixed in 4% glutaraldehyde in Millonig's phosphate buffer, post-fixed in 2% osmium tetroxide, dehydrated in a graded series of ethanol and embedded in epoxy resin. Semithin sections (1 μm thick) were cut on a LKB Ultrotome V ultramicrotome and stained with toluidine blue. Ultrathin sections (60–90 nm) of selected areas were stained with lead citrate and uranyl acetate, and examined in a Jeol JSM-100 SX electron microscope at 80 kV.

Results

Patients' history and hemoglobin levels are shown in Tables 1–3. The actual hemoglobin is the hemoglobin value before biopsy and autopsy. The basal hemoglobin is the hemoglobin value at steady state. The range of actual and basal hemoglobins was 4.7–9.9 and 4.6–10.9 g dl^{-1}, respectively. There was no significant difference between actual and basal hemoglobin levels in most of the cases. Since normal average hemoglobin levels are 13.4 ± 0.95 g dl^{-1} for Thai females and 15.3 ± 1.05 g dl^{-1} for Thai males, anemia was found in most of the patients. The hemoglobin levels did not depend on splenectomy or the frequency of blood transfusion.

The correlation coefficients (r) between iron depositions and fibrosis in the liver, serum iron (SI), transferrin iron saturation (TIS) and serum ferritin (SF) were obtained by the method of Spearman. A P value of 0.05–0.0025 represented the significant level, while a P value of less than or equal to 0.01 represented the highly significant level. The relationships between the degree of iron deposition and SI, TIS, SF are demonstrated in Figures 1–3.

In general histopathological findings, most of the livers were heavily loaded with iron. Cells in the periphery of the lobules were more heavily loaded and lesser at the central area of the lobules. With increasing iron storage, the iron was heavy loaded throughout the hepatocytes (Figure 4). Ballooning hepatocytes were frequently observed. Regenerative nodules were observed in two cases (β-thal/HbE and β-thal major). The nodules showed a low degree of iron deposition. Twelve cases (11 β-thal/HbE and one thal/HbH) were associated with focal necrosis. Foci of extramedullary

Table 1. History and hemoglobin levels of 19 β-thal/HbE disease patients

Patients	Age at biopsy (years)	Sex	Hemoglobin		Years after splenectomy	No. of blood transfusions (unit)
			actual (g dl^{-1})	basal (g dl^{-1})		
S.P	31	M	6.6	6.9	20	4
S.K	26	M	6.0	5.4	9	>15
B.S	18	F	5.5	6.1	14	5
V.T[b]	33	F	4.7	7.4	13	>10
S.I	40	F	8.5	7.9	—	2
P.C	24	F	8.8	6.1		2
C.M	41	M	6.8	5.8	—	0
S.P	18	F	6.5	4.6	15	>20
K.N	21	F	5.1	5.7	—	7
T.S	45	M	5.6	6.3	—	2
L.P	30	F	7.0	4.9	—	>30
N.I	26	F	5.7	6.7	18	0
CP.M	14	F	6.3	6.7	10	>30
B.R	34	F	6.7	6.4	—	0
C.A[a,b]	25	M	6.8	5.6	18	16
SI.K[a,b]	27	F	7.2	7.4	21	>10
C.K	36	M	7.4	7.3	—	0
SJ.I	29	F	6.2	7.6	19	4
V.P	32	F	7.9	6.5	—	17

[a]Two autopsies are included.
[b]Patients with cirrhosis.

Table 2. History and hemoglobin levels in seven thal/HbH disease patients

Patients	Age at biopsy (years)	Sex	Hemoglobin		Years after splenectomy	No. of blood transfusions (unit)
			actual ($g\,dl^{-1}$)	basal ($g\,dl^{-1}$)		
S.K	25	M	7.7	6.9		1
V.K[b]	19	F	8.6	5.6	16	2
S.M	52	F	8.8	8.6	—	0
S.C	50	F	8.4	8.7	—	0
S.J	60	M	9.6	9.9	—	0
D.T[a,b]	30	M	4.8	5.2	—	5
P.A	37	M	9.9	10.9	—	0

[a]One autopsy is included.
[b]Patients with cirrhosis.

Table 3. History and hemoglobin level in four β-thal major patients

Patients	Age at biopsy (years)	Sex	Hemoglobin		Years after splenectomy	No. of blood transfusions (unit)
			actual ($g\,dl^{-1}$)	basal ($g\,dl^{-1}$)		
A.P[b]	23	F	6.8	6.5	—	>50
A.S[b]	51	F	5.5	5.4	—	3
P.c	31	F	8.6	8.5	7	4
AC.S[a,b]	15	F	5.2	4.6	13	>20

[a]One autopsy is included.
[b]Patients with cirrhosis.

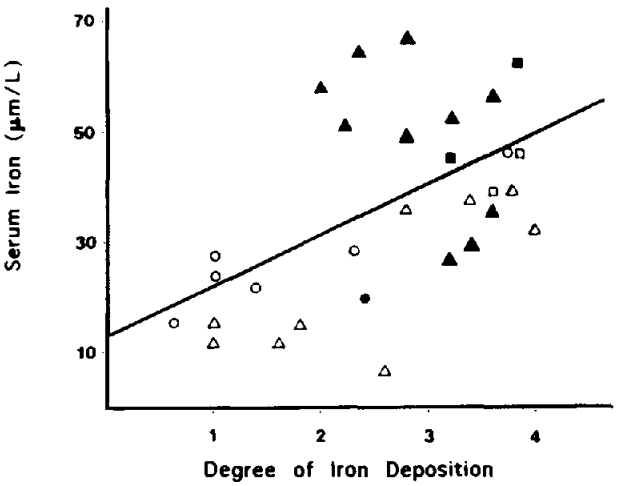


Figure 1. Correlation between serum iron ($\mu mol\,l^{-1}$) and degree of iron deposition in the liver of 30 thalassemic patients ($r=0.545$, $P<0.005$). β-thal/HbE (19, diamonds), thal/HbH (7, circles), β-thal major (4, squares). Filled symbols, splenectomized; open symbols, non-splenectomized.

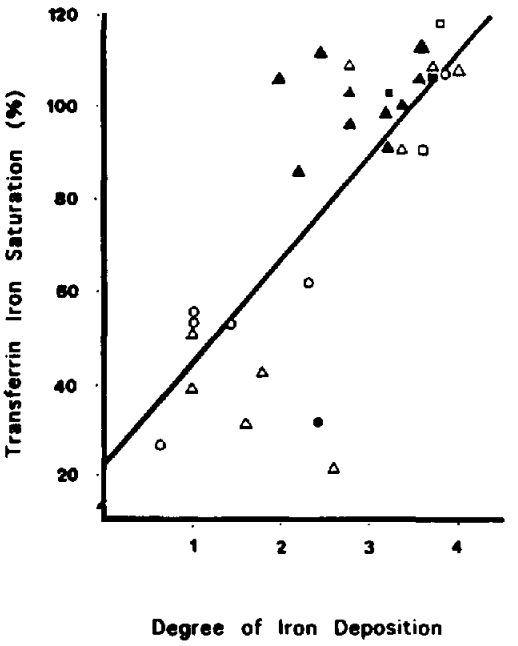


Figure 2. Correlation between transferrin iron saturation (%) and degree of iron deposition in the liver of 30 thalassemic patients ($r=0.771$, $P<0.0005$). β-thal/HbE (19, diamonds), thal/HbH (7, circles), β-thal major (4, squares). Filled symbols, splenectomized; open symbols, non-splenectomized.

hemopoiesis were demonstrated in 13 cases (11 β-thal/HbE, one thal/HbH and one β-thal major). No significant fatty change was seen in this study. Increased portal fibrosis was seen in all cases to varying degrees. The correlation between

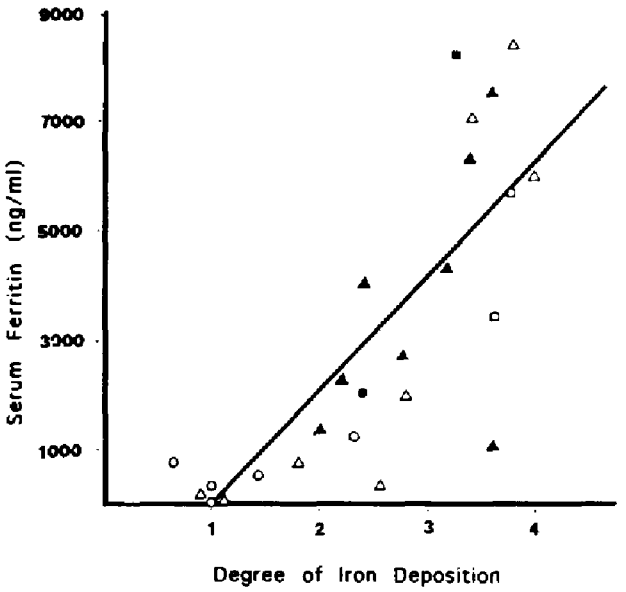


Figure 3. Correlation between serum ferritin (ng ml^{-1}) and degree of iron deposition in the liver of 25 thalassemic patients ($r=0.785$, $P<0.0005$). β-thal/HbE (16, diamonds), thal/HbH (6, circles), β-thal major (3, squares). Filled symbols, splenectomized; open symbols, non-splenectomized.

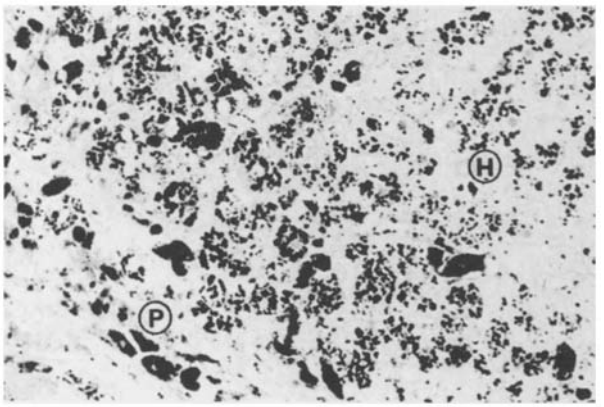


Figure 4. Heavy deposits of iron in hepatocytes (H) and macrophages in the portal area (P). × 130.

iron deposition and degree of fibrosis was significant (Figure 5). Shikata's orcein stain for HBsAg was negative in all cases. Ferro-acidophilic degeneration (FAD) or the acidophilic degeneration commonly seen in viral hepatitis but with heavy deposits of iron and ferro-acidophilic body (FAB) was seen in 50% of the cases.

Under electron microscopy, the ultrastructural changes of all cases showed identical results, regardless of the types of hemoglobin. Alterations of the hepatocyte membrane were sometimes observed. Facing the spaces of Disse and bile canaliculi was a reduction and stunting of the microvilli (Figure 6). Most of the nuclei exhibited unremarkable changes, except pyknosis seen in one case (Figure 7). Intranuclear cytoplasmic pseudoinclusions were demonstrated in five cases (three β-thal/HbE and two β-thal major). Mitochondria looked strikingly abnormal. Swollen mitochondria and irregularities of cristae were seen in eight cases of β-thal/HbE, five cases of thal/HbH and in all cases of β-thal major. Giant mitochondria were also observed in varying degrees. An electron dense matrix was demonstrated in one case of β-thal/HbE, three cases of thal/HbH and two cases of β-thal major (Figure 8). There was a variation in the amount of endoplasmic reticulum (ER). Dilatation of rough ER (RER) and proliferation of smooth ER (SER) were observed in some cases. Numerous glycogen particles were found in most of the patients Lysosomal hemosiderin appeared in all 30 cases. Marked hemosiderin in lysosomes

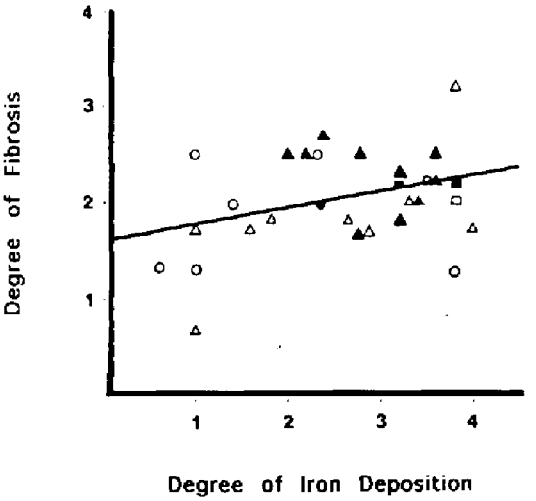


Figure 5. Correlation between degrees of fibrosis and iron deposition in the liver of 30 thalassemic patients ($r=0.352$, $P<0.05$). β-thal/HbE (19, diamonds), thal/HbH (7, circles), β-thal major (4, squares). Filled symbols, splenectomized; open symbols, non-splenectomized.

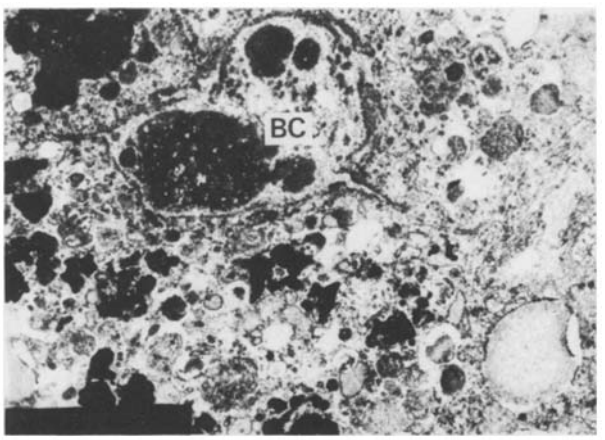


Figure 6. Markedly dilated bile canaliculus (BC) with loss of microvilli. × 5880.

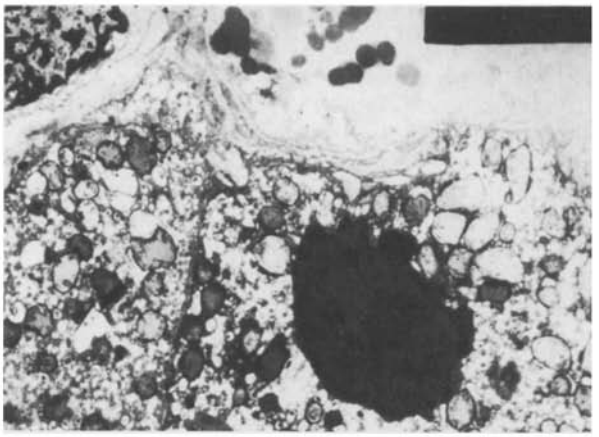


Figure 7. A pyknotic nucleus is observed in the hepatocyte. The cytoplasm of the hepatocytes is filled by increased numbers of the vesicular form of SER and dilated mitochondria. × 1890.

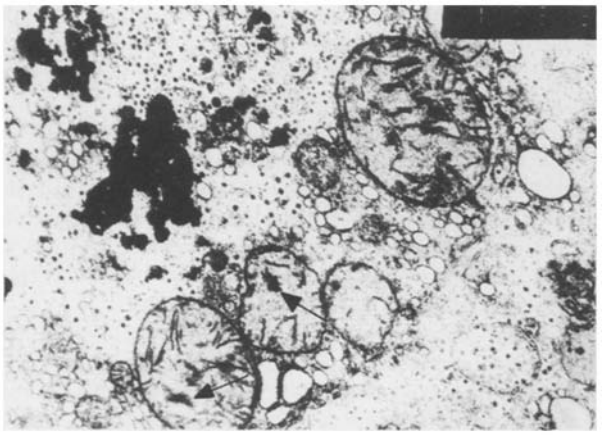
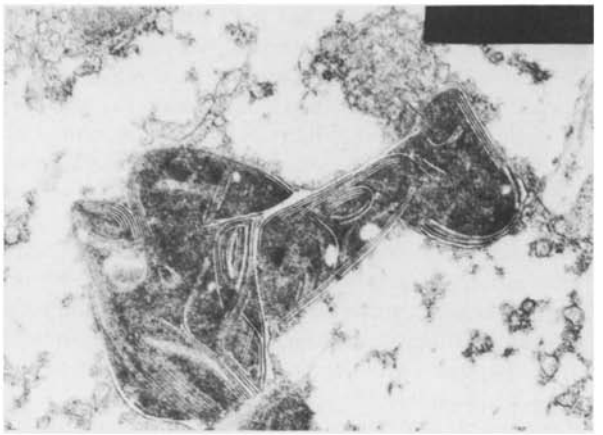
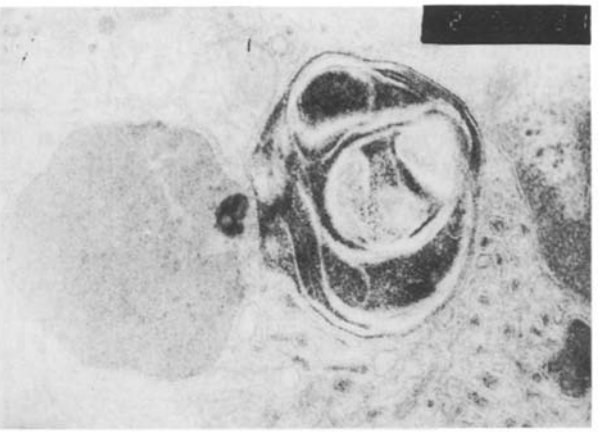


Figure 8. Swelling mitochondira with electron dense matrix (arrows). × 10 500.



(a)



(b)

Figure 9. Lysosomal hemosiderin showing multiple arrays of ferritin (**a**). Molecules of ferritin are clear seen in unstained section (**b**). × 21 000.

was observed in 11 and three cases of β-thal/HbE and thal/HbH, respectively, and in all cases of β-thal major. In addition, ferritin was associated with lysosomal hemosiderin in half of the cases (Figures 9 and 10). In all cases, the Kupffer cells were variably loaded with hemosiderin. Collagen was noted in great numbers, either intrahepatocyte, inter-hepatocyte, in the space of Disse or in the sinusoidal space (Figure 11).

Discussion

Liver biopsy was considered to be the most reliable criterion for the evaluation of both parenchymal iron overload and tissue damage (Editorial 1968, Milder *et al.* 1980). The role of excess iron in producing the pathologic changes of acute

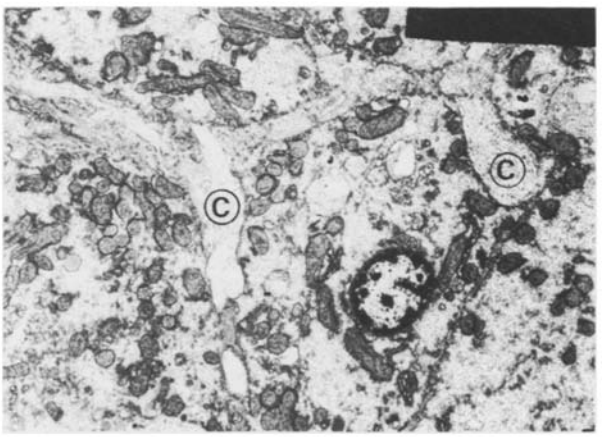


Figure 10. Excessive collagen fibers (C) are demonstrated between the hepatocytes. × 3150.

iron toxicity is well-known, but the pathogenesis of tissue damage due to chronic iron overload has been disputed.

Clinical evidence for hepatotoxicity resulting from iron overload has been elucidated by many investigators (Witzleben & Wyatt 1961, Barry *et al.* 1974, Risdon *et al.* 1975, Sonakul *et al.* 1978, Cohen *et al.* 1984, Niederau *et al.* 1985, Bassett *et al.* 1986). It appears from clinical studies that there is a relationship between both hepatic iron concentration and cellular distribution and the development of iron-induced hepatotoxicity (Tavill & Bacon 1990). In this study the direct measurement of liver iron concentration was lacking. However, the iron status (SI, TIS, SF) showed a close correlation with degree of both iron deposition and fibrosis in the tissue.

Examination of the liver by light and electron microscopy revealed identical morphologic alterations in all cases, regardless of the genotypes, except milder hepatic damage in thal/HbH. Concerning the ages of the patients, as described by Iancu *et al.* (1977a), our subjects were at advanced stage in 24 cases and at end stage with advanced cirrhosis in six. At these stages, a marked or more marked degrees of iron should be found. Greater hepatic iron was located at the periphery than at the central area of the lobule. This is in agreement with previous reports (Bowdler & Huehns 1963, Block 1966, Bhamarapravati *et al.* 1967, Masera *et al.* 1976, Okon *et al.* 1976, Edwards *et al.* 1980). This pattern was similar to that of primary hemochromatosis. It seems likely that the iron is derived from gastrointestinal absorption. In addition, these subjects had received minimal or no blood transfusion. In splenectomized patients, the liver exhibited a high degree of iron deposition. Wasi & Pravatmuang (1979) first reported the elevation of serum iron after splenectomy. They suggested that after splenectomy the iron was shifted over to the liver. It is clear that the effect of iron loading is reflected in widespread organ dysfunction, one of which is hepatic cirrhosis (Weatherall & Clegg 1981).

In this study, increased hepatic periportal fibrosis was found to varying degrees—more severe in splenectomy and cirrhosis. It was surprising that in most of the patients in thal/HbH disease, the degree of fibrosis was higher than degree of iron deposition. The average age of the patients in this group was 39 years, whereas in the β-thal/HbE and β-thal major group the averages were 29 and 30 years, respectively. The severity of fibrosis might be correlated with both iron concentration and with age. This is similar to previous studies (Risdon *et al.* 1975, Okon *et al.* 1976). However, Iancu *et al.* (1977a) and Jean *et al.* (1984) stated that marked fibrosis was only age related.

Twenty three cases (15 β-thal/HbE, four thal/HbH and four β-thal major) showed marked fibrosis. Of these, iron-laden macrophages were noted in 14 cases. Increased fibrosis with iron loading is related not only to iron in hepatocytes but possibly due to extracellular iron deposition. Iron-laden macrophages might stimulate fiber formation and later condense to form connective tissue septa (Kent *et al.* 1964, Orfel *et al.* 1968, Tanikawa 1976). As the iron pigment in the tissues becomes heavier, the excess iron might have a primary effect on stimulating collagen synthesis (Chojkier *et al.* 1988), leading to fibrosis without any evidence of tissue injury (Iancu *et al.* 1977b, Weintraub *et al.* 1985).

A number of studies had demonstrated possible ways in which iron might produce cellular injury with progression to fibrosis and cirrhosis. Increased lipid peroxidation is an important expression of both acute and chronic toxicity (Hershko 1989). Iron might promote free radical mediated lipid peroxidation (Powell *et al.* 1980). Extensive peroxidation of lipids in biological membranes leads to increased membrane rigidity with rupture. Patients with thalassemia in this series, regardless of genotype, showed lysosomal hemosiderin with and without ferritin molecules in varying numbers in all cases. Under the above theory, lysosomal membranes might appear to be abnormally fragile. Some researchers stated that cytological changes in iron toxicity closely resemble those seen in vitamin E deficiency (Goldberg & Smith 1958). In thalassemia major with iron load, excess lipid peroxidation in erythrocytes is associated with reduced levels of vitamin E (Rachmilewitz *et al.* 1976). Apart from secondary lysosomes, this mechanism possibly effects other membrane-associated cellular structures. Swollen mitochondria were observed in 50% of cases, some of which contained an electron dense matrix. The nature of the matrix is not exactly known. Lipid, lipid–protein complexes, calcium or even hemosiderin should be considered. Reissmann & Coleman (1955) presented that mitochondrial damage was associated with the development of lactic acidosis following acute intestinal iron intoxication, suggesting toxicity to Kreb's cycle enzymes. Moreover, Hanstein *et al.* (1975) and Bacon *et al.* (1985) performed the experiments in iron loaded rats, and they suggested a cause and effect relationship between iron-induced lipid peroxidation and impaired mitochondrial function. Other morphologic alterations resulting from cell injury were liver cell ballooning with distortion and blunting of microvilli seen in some cases. Myelin-like figures derived from plasma and other organelle membranes were noted. Dilatation of RER represented a common and important acute reaction to injury. As in carbon tetrachloride poisoning, dilated RER were observed 1 h after exposure to the toxin. Proliferation and vacuolation of SER represented changes after toxic injuries.

The presence of FAD and FAB, with negative orcein stain, and no viral particles were found in this study. We suggest that the morphologic alterations were similar to those changes seen in viral hepatitis in many aspects.

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