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# Experimental study of iron effect on the liver function

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The effect of iron on the liver function was studied in rats. A total of 40 rats were divided into four groups. Group 1 was given iron; Group 2, carbon tetrachloride; Group 3, a combination of iron and carbon tetrachloride; and Group 4 was the control. The changes in liver function were evaluated by using hepatobiliary and liver scintigraphy as the index of hepatocyte function and reticuloendothelial system function, respectively. Determination of liver CT number and a histological study were made at the same time.

The administration of iron activated the reticuloendothelial system function per unit of liver weight. However, because of the decrease in liver weight, the total reticuloendothelial system function did not change at all. In the group given iron and carbon tetrachloride, liver cirrhosis and siderosis in the reticuloendothelial system occurred. Dysfunction in the reticuloendothelial system was more severe in this group than in the group given carbon tetrachloride only, but hepatocyte dysfunction was more mild. It is doubtful that the administration of iron after liver dysfunction had developed, which caused acceleration of fibrosis and reduction of liver blood flow, led to the enhancement of the reticuloendothelial system dysfunction.

**Key words:** Iron, Liver scintigraphy, Hepatobiliary scintigraphy, Rat

## INTRODUCTION

TISSUE DYSFUNCTION due to iron has been studied in relation to the causes of hemochromatosis. Many experiments have been carried out to determine the histological changes.[1–8] It is known that liver dysfunction is mild in hemochromatosis and that the hepatocyte function is especially well maintained.[9] On the other hand, it is reported that dysfunction in the reticuloendothelial system is found at an early stage,[10] and that dissociation between the hepatocyte function and the reticuloendothelial system function is useful for making an early diagnosis of this disease.[11] The dissociation of the two functions is an interesting phenomenon, but few studies have been made to compare the effect of iron on these two functions. Liver scintigraphy and hepatobiliary scintigraphy are well known evaluation methods for liver function, making possible an excellent separate evaluation of the reticuloendothelial system function and the hepatocyte function.[12] Using these methods, animal experiments were carried out on the effect of iron on the liver function.

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## MATERIALS AND METHODS

Wistar male rats (weight 200 g) were used. They were fed MF pellet chow (Oriental Yeast Industrial). The animals were divided into four groups. Group 1 was given iron; Group 2, carbon tetrachloride; Group 3, a combination of iron with carbon tetrachloride; and Group 4 was the control. As iron, iron oxide containing sugar (Fesin®) was injected into the tail vein once a week in a dose of 0.2 m*l* (4 mg as iron). Carbon tetrachloride mixed with olive oil at the ratio of 1 : 1 was sterilized with a 0.22 $\mu$m filter unit (Millex®, Millipore Corp., Bedford, MA), and then the mixture was injected intramuscularly into the thigh in a dose of 0.15 m*l*/100 g body weight twice a week. At 8 weeks and 16 weeks, all tests conducted in this experiment were performed on 5 animals from each of the above 4 groups.

At 8 and 16 weeks after the beginning of the experiment, hepatobiliary scintigraphy and liver

scintigraphy were taken as the indices of hepatocyte function and reticuloendothelial system function.

A scintillation camera (GCA-301: Toshiba) equipped with pin-hole collimator was used. Digital images recorded as $64 \times 64$ matrices were stored on an on-line computer system (GMS-80A: Toshiba). The hepatobiliary scintigraphy was obtained by bolus injection into the tail vein of 1 mCi (37 MBq) of $^{99m}$Tc-EHIDA (Amersham Buckinghamshire, UK), and sequential images were stored at 5-second intervals for 30 minutes following injection. The region of interest (ROI) was set on the whole area of the liver except for the hepatobiliary system, and a time activity curve for the whole study was obtained. Then liver excretion rate (Ke value) (%/min) was determined by using least squares regression on the logarithm of the excretion phase of the curve. The liver scintigraphy was obtained by the injection of 1 mCi (37 MBq) of $^{99m}$Tc-Sn-colloid (Nihon Mediphysics, Osaka) and sequential images were stored at 2-second intervals for 10 minutes. ROI was set on the whole area of the liver and a time activity curve was obtained. The liver uptake rate (1/min) was determined as follows. Each point on the curve was subtracted from a plateau and the value was obtained by using least squares regression on the logarithm of the subtracted curve. Thirty minutes after injection, the animals were sacrificed, and blood was taken from the heart. Then liver, spleen and bone marrow were removed for weighing and the radioactivity of the organs was determined with a well-type scintillation counter (ACR-300: Aloka). Making a correction of the radionuclide attenuation time, the amount of $^{99m}$Tc-Sn-colloid uptake per gram tissue per $\mu$Ci of injected dose (cpm/g/$\mu$Ci) was calculated. The whole liver uptake of $^{99m}$Tc-Sn-colloid was obtained by multiplying it by the liver weight. The excised liver was fixed in 10% formalin, and stained with hematoxylin and eosin (H-E) stain, Azan stain, and Berlin blue stain to make histological studies. Serum total iron-binding capacity (TIBC) and unsatisfied iron-binding capacity (UIBC) were determined by the radioiron assay method (TIBC, UIBC Micro Test, Daiichi Radioisotope Laboratories, Tokyo) to calculate serum iron (SI).

CT examinations were carried out with a TCT-60 A-27 (Toshiba). The rat was fixed using bean bags. Liver attenuation coefficient (HU) was determined at 9 seconds of scan time and at the interval of every 5 mm in each 1 cm slice. ROI was carefully fixed with an 800-pixel area, avoiding the hepatic vein and main portal vein.

In consideration of the total dose of radionuclide, CT and hepatobiliary scintigraphy were carried out on one day, and two days later liver scintigraphy was performed.

The animals were anesthetized by intraperitoneal injection of pentobarbital sodium (Nembutal®, Abbott Laboratories, Chicago, IL) in a dose of 6 mg/100 g body weight.

All results are shown by mean$\pm$SD, and an examination of the significant difference between each group was performed by using the Student's *t*-test.

## RESULTS

Table 1 shows the $^{99m}$Tc-colloid uptake by the reticuloendothelial system in the liver, spleen and bone marrow of each group No significant interval change in the uptake of the liver, spleen and bone marrow was found in the control group. However, administration of iron for 16 weeks resulted in a significant increase ($P<0.05$) in the uptake to the liver. In the group receiving carbon tetrachloride, a significant increase ($P<0.05$) in the uptake to the spleen was observed, and administration for 16 weeks caused a significant increase of the uptake to the bone marrow ($P<0.01$), while uptake to the liver was significantly ($P<0.01$) decreased. In the groups given iron and carbon tetrachloride, a significant increase ($P<0.05$) in the uptake to the liver and bone marrow was observed at first, and then a remarkable decrease ($P<0.001$) was found 16 weeks after administration was initiated.

Table 2 shows the liver weight and total liver uptake of $^{99m}$Tc-Sn-colloid in each group. Liver weight was significantly ($P<0.05$) decreased after the administration of iron for 16 weeks. On the other hand, in the group given iron and carbon tetrachloride, a significant decrease in liver weight ($P<0.05$) was found at an early stage, but recovery to the normal was observed in the animals administered the combination for 16 weeks.

In the control group and the group receiving iron, no significant interval change was found. The administration of carbon tetrachloride for 16 weeks resulted in the significant decrease of the uptake ($P<0.05$). In the group given iron and carbon tetrachloride for 16 weeks a remarkable decrease ($P<0.001$) was found.

Table 3 shows the liver uptake rate of $^{99m}$Tc-Sn-colloid (1/min) and liver excretion rate (Ke value) of $^{99m}$Tc-EHIDA, and the CT number of the liver in each group. In the control group, the liver uptake rate increased significantly ($P<0.01$) with the passage of time. In the group given iron, a significant increase ($P<0.05$) was found at an early stage. The group given carbon tetrachloride for 16 weeks showed a significant decrease ($P<0.01$). In the group given iron and carbon tetrachloride, the administration for 16 weeks resulted in a remarkable decrease ($P<0.001$) in the rate.

**Table 1** Tc-99m-Sn-colloid uptake to the reticuloendothelial system (cpm/g/$\mu$Ci)

| | Control | | Fe | | $CCl_4$ | | Fe+$CCl_4$ | |
|---|---|---|---|---|---|---|---|---|
| | 8 W | 16 W | 8 W | 16 W | 8 W | 16 W | 8 W | 16 W |
| Liver | 8,826.1 ±685.3 | 8,390.6 ±1,617.4 | 8,151.5 ±1,243.9 | 12,083.5 ±2,360.1 | 7,680.1 ±1,303.8 | 5,311.3 ±378.1 | 18,833.5 ±4,696.4 | 3,375.2 ±465.7 |
| | | | $p<0.05$ | | | | | |
| | | | $p<0.01$ | | | | | |
| | | | $p<0.05$ | | | | | |
| Spleen | 6,571.9 ±122.9 | 6,613.5 ±1,799.4 | 5,884.5 ±1,300.3 | 7,946.8 ±1,791.2 | 8,196.4 ±827.3 | 8,057.3 ±1,491.1 | 10,722.0 ±5,204.8 | 5,218.8 ±805.1 |
| | | | $p<0.05$ | | | | | |
| Bone marrow | 249.7 ±80.0 | 285.7 ±90.9 | 176.4 ±42.2 | 340.9 ±75.3 | 161.6 ±87.8 | 1,377.6 ±431.7 | 1,504.8 ±806.2 | 229.3 ±85.5 |
| | | | $p<0.01$ | | | | | |
| | | | $p<0.05$ | | | | | |

**Table 2** Liver weight and total liver uptake of Tc-99m-Sn-colloid

| | Control | | Fe | | $CCl_4$ | | Fe+$CCl_4$ | |
|---|---|---|---|---|---|---|---|---|
| | 8 W | 16 W | 8 W | 16 W | 8 W | 16 W | 8 W | 16 W |
| Liver weight (g) | 20.64 ±5.05 | 22.50 ±4.32 | 23.73 ±2.18 | 15.81 ±1.18 | 20.60 ±3.35 | 22.89 ±3.92 | 9.86 ±2.61 | 25.67 ±2.24 |
| | | | $p<0.05$ | | | | | |
| | | | $p<0.05$ | | | | | |
| Total liver count (cpm/$\mu$Ci)$\times 10^4$ | 180.40 ±30.44 | 188.79 ±36.39 | 189.88 ±16.94 | 189.78 ±30.48 | 153.60 ±26.08 | 122.60 ±28.92 | 176.93 ±14.82 | 90.11 ±17.40 |
| | | | $p<0.05$ | | | | | |

In the control group, no significant interval change was found. The group given iron showed a significant increase ($P<0.05$) of the liver excretion rate after 16 weeks. In the group given carbon tetrachloride, longer administration resulted in a remarkable decrease in the rate ($P<0.001$). In the group given iron and carbon tetrachloride for 16 weeks, a remarkable decrease ($P<0.001$) was also found, but the rate was higher ($P<0.01$) compared with that in the group given carbon tetrachloride.

The control group did not show any significant change of liver CT number as time passed. In the group given iron, no significant difference from the control group was found. The group given carbon tetrachloride showed a remarkable decrease ($P<0.001$) at 8 weeks and an even larger decrease after 16 weeks. In the group given iron and carbon tetrachloride, a remarkable decrease ($P<0.001$) was found, but there was no significant difference between the groups examined at 8 and 16 weeks.

Table 4 shows TIBC, UIBC and serum iron in each group.

TIBC did not show any significant change in the control group with the passage of time. TIBC in the group given iron showed no significant change compared to that in the control group. In the group given carbon tetrachloride, the administration for 16 weeks resulted in a significant increase of TIBC

**Table 3** Liver uptake rate of Tc-99m-Sn-colloid, Liver excretion rate (Ke) of Tc-99m-EHIDA and CT number

| | Control | | Fe | | $CCl_4$ | | Fe+$CCl_4$ | |
|---|---|---|---|---|---|---|---|---|
| | 8 W | 16 W | 8 W | 16 W | 8 W | 16 W | 8 W | 16 W |
| Uptake rate (1/min) | 1.19 ±0.07 | 1.59 ±0.18 | 1.38 ±0.12 | 1.59 ±0.18 | 1.30 ±0.12 | 1.00 ±0.20 | 1.57 ±0.32 | 0.78 ±0.18 |
| | | $p<0.01$ | | | | | | |
| | | $p<0.05$ | | | | | | |
| | | | | | | $p<0.01$ | | |
| Ke (%/min) | 18.6 ±3.5 | 20.7 ±3.1 | 20.2 ±1.8 | 25.1 ±2.0 | 18.1 ±4.2 | 3.0 ±1.2 | 17.4 ±4.9 | 8.5 ±2.9 |
| | | | | $p<0.05$ | | | | $p<0.01$ |
| CT number (HU) | 70.8 ±4.2 | 70.7 ±3.3 | 69.9 ±3.3 | 68.8 ±3.3 | 56.2 ±5.2 | 38.4 ±5.4 | 53.9 ±4.7 | 53.7 ±2.4 |
| | | | | | | $p<0.001$ | | |

**Table 4** TIBC, UIBC and serum iron (SI) ($\mu$g/d*l*)

| | Control | | Fe | | $CCl_4$ | | Fe+$CCl_4$ | |
|---|---|---|---|---|---|---|---|---|
| | 8 W | 16 W | 8 W | 16 W | 8 W | 16 W | 8 W | 16 W |
| TIBC | 496.2 ±49.5 | 530.7 ±66.4 | 472.7 ±35.5 | 537.0 ±94.2 | 594.6 ±59.0 | 721.3 ±126.9 | 614.9 ±117.8 | 558.3 ±86.2 |
| | | | | | | $p<0.05$ | | |
| UIBC | 362.6 ±53.2 | 344.8 ±26.3 | 341.0 ±41.6 | 229.9 ±90.5 | 516.2 ±44.5 | 482.6 ±151.8 | 395.5 ±139.0 | 299.2 ±140.6 |
| | | | | | | $p<0.01$ | | |
| SI | 133.6 ±61.8 | 162.5 ±44.1 | 131.8 ±21.0 | 307.1 ±31.5 | 78.4 ±39.1 | 238.7 ±71.0 | 251.9 ±20.0 | 259.1 ±68.5 |
| | | | | $p<0.01$ | | $p<0.01$ | | |
| | | | | | | | | $p<0.05$ |

($P<0.05$). TIBC in the group given iron and carbon tetrachloride was not significantly different from that in the control group.

UIBC in the control group did not change significantly with time. UIBC in the group given iron and in the group given iron and carbon tetrachloride also did not show significant change with time, and were not significantly different from the control UIBC. However, the group given only carbon tetrachloride showed a significant increase of UIBC ($P<0.01$) at 8 weeks, after which it declined.

In the control group, serum iron did not significantly change with time. In the group given iron, 16-week administration resulted in a significant increase of serum iron ($P<0.01$). In the group given carbon tetrachloride, serum iron increased significantly with time ($P<0.01$) without any significant difference from that in the control group. In the group given iron and carbon tetrachloride, serum

**Table 5** Pathological change of the liver

| | | Fatty degeneration | Cell damage | Fibrosis | Iron deposit |
|---|---|---|---|---|---|
| Control | 8 W | (−) | (−) | (−) | (−) |
| | 16 W | (−) | (−) | (−) | (−) |
| Fe | 8 W | (−) | (−) | (−) | (+) |
| | 16 W | (−) | (+) | (−) | (++) |
| $CCl_4$ | 8 W | (++) | (+) | (−) | (−) |
| | 16 W | (++) | (++) | (+++) | (−) |
| Fe+$CCl_4$ | 8 W | (++) | (+) | (−) | (+) |
| | 16 W | (++) | (++) | (+++) | (++) |

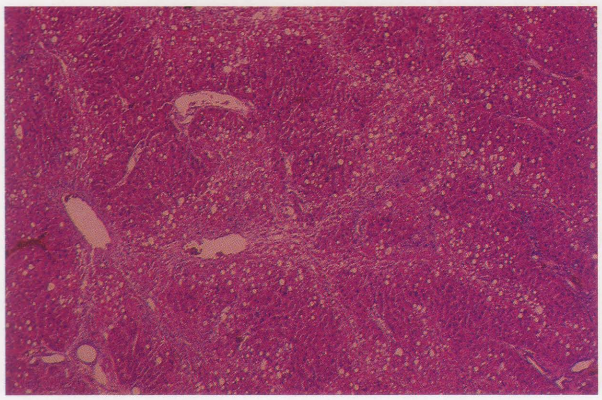

**Fig. 1** $CCl_4$ (16 W) group. Cirrhosis of liver with fatty change. fatty degeneration (++) liver cell damage (++) Hematoxylin & eosin stain ×40

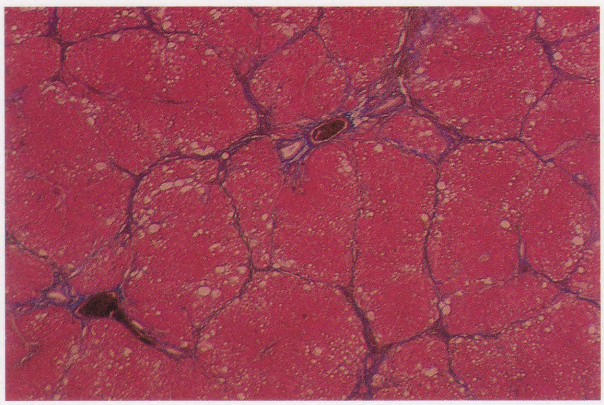

**Fig. 2** Fe+$CCl_4$ (16 W) group. Formation of typical pseudolobules. fibrosis (+++) Azan stain ×40

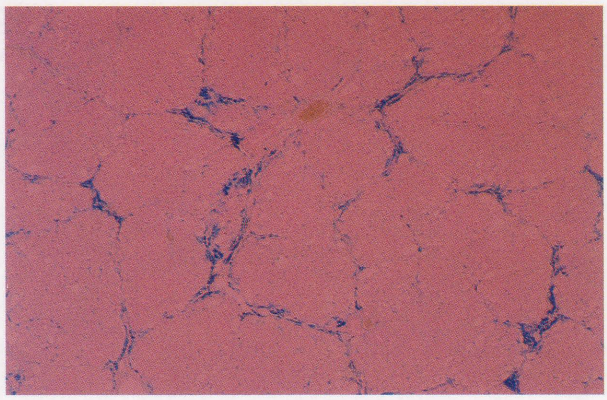

**Fig. 3** Fe+$CCl_4$ (16 W) group. Iron deposit in Kupffer cells, mainly in lobar periphery and fibrous tissue. iron deposit (++) Berlin blue stain ×40

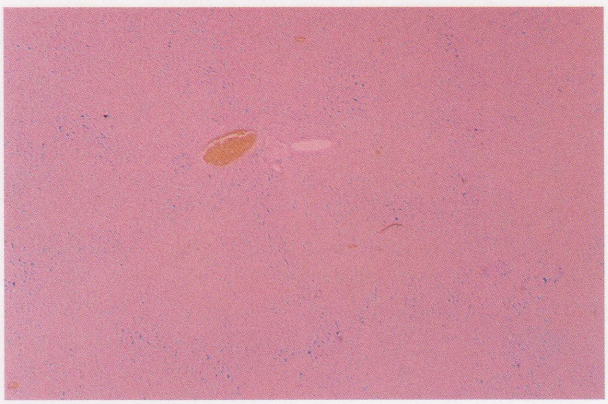

**Fig. 4** Fe (16 W) group. Iron deposit in Kupffer cells with tendency of more dense distribution in lobular periphery. iron deposit (++) Berlin blue stain ×40

iron increased significantly at an early stage ($P < 0.05$).

Table 5 shows the pathological changes of the liver, fatty degeneration of hepatocytes, hepatocyte damage, fibrosis and iron deposit. The changes were expressed by 4 grades; (−), (+), (++), and (+++).

Fatty degeneration was graded by fat area percentage of a view (×40); (−) as no fat, (+) as less than 10%, (++) as 10–30% and (+++) as more than 30%. Hepatocyte damage was estimated by cell dysplasia. It was graded by degree and incidence of cellular enlargement, nuclear pleomorphism and multinucleation; (−) as no change, (+) as mild, (++) as moderate and (+++) as severe change.[13] Fibrosis was graded by the criteria of Shiraishi et al[8]; (−) as no fibrosis, (+) as thin fibrous band, (++) as thick fibrous band and (+++) as pseudolobules. Iron deposit was graded by the criteria of Shiraishi et al[8]; (−) as no deposit, (+) as scattered, (++) as massive and (+++) as diffuse deposit.

The group given carbon tetrachloride for 16 weeks showed drug-induced fatty cirrhosis of the liver; the histological image stained with H-E stain is shown in Fig. 1. The group given iron and carbon tetrachloride showed liver cirrhosis and remarkable iron deposit in the Kupffer cells and interstitium. Figure 2 shows this image stained with Azan stain, and Fig. 3 shows the image with the Berlin blue stain. Iron deposit in the group given only iron is different from that in the group given iron and carbon tetrachloride. In the former group, iron deposit was found in the Kupffer cells with the tendency of a little more dense distribution in the margin of the lobules. In the latter, iron tended to deposit more thickly in the Kupffer cells and interstitium around the pseudolobules. Figure 4 shows the image with the Berlin blue stain at 16 weeks in the group given iron only.

## DISCUSSION

The idea that the deposit of a large amount of iron in organs over a long period of time will lead to some histological disorders was based on the findings that iron deposit occurs prior to histological disorders in hemochromatosis.[14] Thereafter, many experiments proved that iron itself is to some extent correlated with histological disorders.[15] However, as other factors such as malnutrition are suspected,[16–18] many questions remain unanswered about the above theory.

It is well known that hepatocyte function is relatively well maintained in hemochromatosis with light hepatocyte necrosis and inflammatory cellular infiltration,[9] but little has been studied about the function of the reticuloendothelial system. Based on the findings of liver scintigraphy, Alele, et al[10] reported the early decline of the reticuloendothelial system function in hemochromatosis and thought that it was due to the saturation of Kupffer cells with iron. Based on the findings of liver scintigraphy and hepatobiliary scintigraphy, Knopf, et al[11] also reported that in patients with the early stage of hemochromatosis the reticuloendothelial function is remarkably damaged in comparison with the hepatocyte function. On the other hand, it was said that iron depositing excessively in the reticuloendothelial system causes only slight dysfunction.[15] In our experiment, in the group given administered iron for 16 weeks, the deposit of a large amount of iron was found in the Kupffer cells, but the function of the reticuloendothelial system per unit weight of the liver was enhanced significantly compared with that in control group. It seems that saturation of the Kupffer cells with iron does not lead to a decrease of the function. MacDonald et al[19] reported after a carbon particle clearance study that the function of the reticuloendothelial system declined when iron was injected intravenously 4 hours before the study, but when iron was injected intramuscularly 1 week before, the function was enhanced. In general, it is said that the function of the reticuloendothelial system is enhanced when the system continuously absorbs a small amount of any foreign substance. Colloidal iron can not be considered a foreign substance, but the dose of iron in our experiment might have activated the system. In comparison with the control group, the group given iron showed a significant decrease in body weight and liver weight, but the whole liver uptake of $^{99m}$Tc-Sn-colloid, which expresses the reticuloendothelial system function of the whole liver, did not differ from that in the control group, which is an interesting observation. The decrease in liver weight may be an effect of the iron itself. Though iron deposits on the intestinal wall might lead to nutritional absorption disorders, this theory has not yet been proved by histological investigation. It is considered that the decrease in liver weight caused the decline of the function of the reticuloendothelial system in the whole liver, so the reticuloendothelial system function per unit weight might have been enhanced in order to compensate for the decline.

Animal experiments have revealed that administration of iron alone never causes liver cirrhosis,[1–8] which was also found in our experiments. However, it is known that when iron is administered after the development of liver dysfunction, fibrosis of the liver is accelerated and increases the incidence of liver cirrhosis.[4,8] It has been proved that absorption of iron into the liver is increased in the rats with liver dysfunction[20] induced by carbon tetrachloride. As mentioned above, it is evident that iron accelerates the development of fibrosis of the liver. Though many problems of the mechanism remain unsolved, it is thought that iron-laden Kupffer cells and iron deposited in the interstitium may accelerate the production of connective tissue.[4,8] Recently, the increase of prolyl hydroxylase, whose coenzyme is iron under the condition of an excess of iron, is thought to be one of the factors that accelerate fibrosis.[21] In our experiment, fibrosis was found in the group given iron and carbon tetrachloride for 16 weeks, which showed the histological change of liver cirrhosis. In this group, function of the reticuloendothelial system in both unit weight and total liver decreased more significantly than in the group with the administration of carbon tetrachloride alone for 16 weeks. This might have been caused mainly by the decrease in effective blood flow due to portal hypertension and shunt formation in the liver as the result of fibrosis.[4] The effect of the circulation disorder in the sinusoid caused by the enlarged, iron-laden Kupffer cells is also considered one of the causes. Severe fibrosis was found in the group given carbon tetrachloride for 16 weeks. However, it is doubtful that the difference in the reticuloendothelial system dysfunction of these two groups can be explained only by the severity of fibrosis. Liver blood flow was estimated using the index of liver uptake rate of $^{99m}$Tc-Sn-colloid. Liver uptake rate decreased greatly in both groups without any significant difference between them. As hepatic removal coefficients of $^{99m}$Tc-Sn-colloid were not determined in both groups, the liver uptake rate does not necessarily reflect the liver blood flow. However, it is thought that there may be no significant difference in the liver blood flow in both groups. It cannot be denied that there may be some other iron-correlated factors which affect the Kupffer cells and do not affect the blood flow.

In the group given iron and carbon tetrachloride for a long period, no obvious iron deposit was

found in the hepatocytes but liver cirrhosis and siderosis were found in the reticuloendothelial system. So the liver dysfunction in this condition was not the same as that in hemochromatosis. It is interesting that in this group hepatocyte dysfunction was mild compared with that in the group given carbon tetrachloride for 16 weeks. Kent et al[4] reported that iron dextran has a mild effect to protect hepatocytes, because rats given carbon tetrachloride and iron dextran intramuscularly showed less increase of serum GPT than those given only carbon tetrachloride. Though it is doubtful that the activity of serum GPT directly reflects hepatocyte function, this result seems to be quite similar to our results. It is said that sugar protects hepatocyte function. The iron preparations used in our experiment contained sugar, which might have prevented hepatocyte dysfunction. In the group given iron, the liver excretion rate was higher than in the control group. The effect of the iron itself cannot be denied, but it can also be considered that the sugar in the iron preparations activated the hepatocyte function.

In the group given iron for 16 weeks serum iron increased but without the decrease of UIBC or the increase of the saturation of transferrin. This finding may suggest that iron deposits only in the Kupffer cells, and not in the hepatocytes. A similar tendency could be found in the group given carbon tetrachloride for 16 weeks.

CT is a noninvasive method widely used in clinical medicine[22–24] to determine the amount of iron content in the liver. In the group given iron for 16 weeks, the CT number did not increase, which was thought to be due to a lower amount of iron deposit than exists in hemochromatosis in man. In the group given iron and carbon tetrachloride for 16 weeks the liver CT number decreased, which was thought to be an effect of fatty degeneration.

As mentioned above, the histological changes in an experimental model given iron and carbon tetrachloride for 16 weeks are different from those seen in hemochromatosis in man. However, the changes can be considered very similar to the initial phase of secondary hemochromatosis, which is a modified condition of liver cirrhosis caused by the excessive administration of iron. At least with regard to the dysfunction of the reticuloendothelial system, changes very similar to hemochromatosis might be obtained. The reticuloendothelial system prevents iron deposit in the organs resulting from the ingestion of excessive iron, which seems to be one of the factors leading to the development of secondary hemochromatosis.


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